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Circulating uncarboxylated matrix Gla protein, a marker of vitamin K status, as a risk factor of cardiovascular disease

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ABSTRACT

Objectives: Vitamin K plays a pivotal role in the synthesis of Matrix Gla protein (MGP), a calcification inhibitor in vascular tissue. Vascular calcification has become an important predictor of cardiovascular disease. The aim of the current study was to examine the potential association of circulating desphospho-carboxylated and -uncarboxylated MGP (dp-cMGP and dp-ucMGP), reflecting vitamin K status, with the incidence of cardiovascular events and disease (CVD) in older individuals.
Study design: The study was conducted in 577 community-dwelling older men and women of the Longitudinal Aging Study Amsterdam (LASA), aged >55 year, who were free of cardiovascular disease at baseline. Multivariate Cox proportional hazards models were used to analyze the data.
Main outcome measures: Incidence of CVD.
Results: After a mean follow-up of 5.6 ± 1.2 year, we identified 40 incident cases of CVD. After adjustment for classical confounders and vitamin D status, we observed a more than 2-fold significantly higher risk of CVD for the highest tertile of dp-ucMGP with a HR of 2.69 (95% CI, 1.09–6.62) as compared with the lowest tertile. Plasma dp-cMGP was not associated with the risk of CVD.
Conclusions: Vitamin K insufficiency, as assessed by high plasma dp-ucMGP concentrations is associated with an increased risk for cardiovascular disease independent of classical risk factors and vitamin D status. Larger epidemiological studies on dp-ucMGP and CVD incidence are needed followed by clinical trials to test whether vitamin K-rich diets will lead to a decreased risk for cardiovascular events.

1. Introduction

Vitamin K is an essential cofactor for the maturation of several proteins. Its role has first been established in the synthesis of a number of blood clotting factors. In addition, it is also known to play a pivotal role in the synthesis of the vascular calcification inhibitor matrix Gla protein (MGP). Vitamin K occurs in two natural forms in the diet: menaquinones (vitamin K_2) occurring in meat, eggs and fermented foods like cheese and curds [1,2] and phylloquinone (vitamin K_1) which is mainly found in leafy green vegetables and in some plant oils [3]. The function of all forms of vitamin K is similar: they serve as a cofactor for γ-glutamate carboxylase, an endoplasmic enzyme that converts specific glutamate residues into γ-carboxyglutamate (Gla). These Gla-residues are strong calcium-binding groups and are essential for the function of all Gla-proteins studied thus far. Particularly vitamin K_2 intake has been shown to be inversely associated with coronary artery calcification and the risk of coronary heart disease (CHD) [4–7]. Each incremental 10 μg increase of dietary menaquinone (MK-4 through MK-9) intake was reported to be associated with 9% cardiovascular risk reduction [5]. However, these studies assessed vitamin K intake with Food Frequency Questionnaires, a technique which has well known limitations. Biomarkers of vitamin K status, such as the γ-glutamate carboxylation of vitamin K-dependent proteins, are more robust measures and above all reflect vitamin K status in the tissues in which these proteins are formed.

Abbreviations: Gla, γ-carboxyglutamate; MGP, matrix Gla protein; dp-cMGP, desphospho-carboxylated MGP; dp-ucMGP, desphospho-uncarboxylated MGP; CVD, cardiovascular events and disease; LASA, Longitudinal Aging Study Amsterdam.

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Besides vitamin K-dependent carboxylation, MGP may undergo another post-translational modification: serine phosphorylation. Like Gla, also phosphoserine residues are strong calcium-binding groups in MGP. Remarkably, neither glutamate carboxylation nor serine phosphorylation is exerted in all MGP molecules. Hence four different MGP species may be found in the circulation only one of which (desphospho-uncarboxylated MGP, dp-ucMGP) is expected to have low affinity for calcium. Therefore, it is assumed that dp-ucMGP is set free in the circulation independent of prevalent calcium salt precipitates in the vasculature. Comparison of different MGP assays showed that the dp-ucMGP assay was particularly suited to assess vascular vitamin K status [8]. Supplemental menaquinone-7 (MK-7) at a daily dose of 10, 20, 45, 90, 180 or 360 mg increased the carboxylation degree of dp-ucMGP [9]. Moreover, in various patient groups an association was found between circulating dp-ucMGP and cardiovascular morbidity and mortality [10–12]. Therefore, we hypothesized that also in healthy individuals vascular vitamin K insufficiency as reflected by high circulating dp-ucMGP concentrations, is associated with clinically manifest endpoints including a higher incidence of CVD. Because one study reported an opposite association for the carboxylated form as well [13], we measured both dp-ucMGP and dp-cMGP.

2. Methods

2.1. Subjects

Data for this study were collected within the framework of the Longitudinal Aging Study Amsterdam (LASA), an ongoing cohort study of predictors and consequences of changes in physical, cognitive, emotional, and social functioning in older people in The Netherlands. The design of this study, including methods for sampling and data collection has been detailed elsewhere [14]. In summary, a random sample, stratified according to age, gender, degree of urbanization, and expected 5-years mortality, was drawn from the population registers of 11 municipalities in three geographical areas in The Netherlands [15]. In 2002/2003, ten years after the baseline measurement of the first LASA cohort, a new cohort was started with adults aged between 55 and 65 years at that time. This new population sample was selected using the same procedures and sampling frame as the initial LASA cohort. In total, 1002 men and women were enrolled for the baseline examination in 2002/2003. In 2008/2009, the second follow-up cycle of data collection took place and was used for the current study.

Participants, who did not provide a blood sample or whose sample was insufficient ($n = 306$), who had prevalent CVD at baseline ($n = 70$), who used a vitamin K antagonist (ATC-code B01AA; $n = 5$), or who had unknown follow-up ($n = 44$) were excluded. In the final analyses 577 participants were included, who all provided written informed consent. The study was approved by the Ethics Committee of VU University Medical Center Amsterdam.

2.2. Vitamin K status

Morning blood samples were collected in 2002 and 2003 when subjects were in a non-fasting state and in a sitting position. Citrated plasma samples were stored at −80 °C until analyses. Circulating dp-ucMGP and dp-cMGP were measured as described by Cranenburg [8]. Briefly, dp-ucMGP was assessed with a sandwich (dual antibody) ELISA, with the capture antibody directed against the non-phosphorylated MGP sequence 3–15 (mAb-dpMGP; VitaK BV, Maastricht, the Netherlands) and the detecting antibody directed against the uncarboxylated MGP sequence 35–49 (mAbucMGP; VitaK BV). The interassay coefficient of variation was 9.9%. The method for dp-cMGP measurement was also a sandwich ELISA, in which mAb-dpMGP again served as a capture antibody. The detecting antibody was directed against the carboxylated MGP sequence 35–54 (mAb-cMGP; VitaK BV) [8] and the interassay coefficient of variation was 11.5%.

2.3. Cardiovascular disease monitoring

In the present study, we examined risk of fatal and nonfatal cardiovascular disease events: coronary heart disease (CHD), peripheral arterial disease (PAD) and cerebrovascular disease (CVA). CHD was defined as myocardial infarction or angina pectoris or coronary arterial bypass grafting. The development of CHD, PAD, and CVA was based on self-reported (symptoms of) CVD at the second (2005/2006) and third (2008/2009) cycle of monitoring of participants, or based on death certificates that were available through June 1, 2007. Symptoms of angina pectoris were considered pain or a heavy or unpleasant feeling in the chest during exertion and disappearing within 10 min when standing still or after taking Nitroglycerin. Symptoms of PAD were present when pain developed in one or both calves during walking which disappeared when standing still [16,17].

Endpoints were all first cardiovascular events, either fatal or non-fatal. Onset of CHD, PAD and/or CVA, jointly described as cardiovascular events, was established at 3-year intervals by interviews at the respondents' homes. For myocardial infarction or CVA the actual year of an event was included. For other events time was defined as halfway the interval of the study cycle at which the event was first reported. Whenever multiple events occurred, the first diagnosis was taken as an endpoint [17].

Survival was calculated as the years from date of blood sampling until date of death or the end of follow-up, whichever came first [17]. Using death certificates from the Dutch Central Office of Statistics (CBS, The Netherlands), all ischemic cardiovascular deaths during follow-up were defined as International Classification of Disease, 10th Revision (ICD-10) codes I20-I25. Peripheral arterial deaths were defined as ICD-10 codes I70-I79, and cerebrovascular deaths as I60-I69.

2.4. Potential confounders

Data on age and sex were derived from the population registries at baseline. Self-reported lifestyle variables included smoking (never, former, current), alcohol consumption (Garretsen alcohol index: none, light, moderate, excessive) [18], and physical activity in the past 2 week using the LASA Physical Activity Questionnaire (LAPAQ) [19]. The following physical activities were included: walking outdoors, bicycling, light and heavy household activities, and a maximum of two sports activities. The total physical activity score was calculated as time spent on physical activity in minutes per day. This variable was divided in tertiles for analyses, with the first tertile representing the lowest activity and the third tertile the highest activity [17]. The level of education was assessed by asking the respondent for the highest education level completed and answers were categorized into three groups. The first group comprised all individuals who had education ranging from incomplete elementary education to lower vocational training. The second group had education ranging from general intermediate to general secondary; and the final group consisted of respondents who completed more than general secondary. Health status variables included presence of diabetes mellitus (DM) (based on self-report and medication use), hypertension (self-reported), serum total cholesterol, serum albumin, serum 25(OH)D concentration, body mass index (BMI) [weight (kg)/height (m)^2]. Total cholesterol was measured with a Hitachi 747 analyzer using enzymatic colorimetric assays (Roche diagnostics, Mannheim,

Germany). Serum albumin (as indicator for nutritional status) was measured in blood samples in three different laboratories in The Netherlands. The results were converted by use of a validated formula to make the data comparable [20]. Serum 25(OH)D concentration was determined using a competitive binding protein assay (Nichols Institute Diagnostics Inc, San Juan Capistrano, California).

2.5. Statistical analyses

Baseline differences between tertiles of dp-ucMGP or included and excluded participants were tested using one-way analyses of variance for normally distributed variables, Kruskal–Wallis test for skewed variables, and Pearson's χ^2-test for categorical data. The relationship between dp-ucMGP or dp-cMGP and baseline variables was tested using Spearman rank order correlation coefficient. Using Cox proportional hazards model, univariate analysis (Model 1) was performed to identify the crude relation between vitamin K status and CVD. The second model was adjusted for baseline age and sex. Model 3 included classical confounders (age, sex, smoking, alcohol consumption, physical activity, education, DM, hypertension, serum total cholesterol concentrations, BMI, serum albumin concentrations) and serum 25(OH)D. For clarity reasons and to be able to examine the shape of the association, the HR for fatal and nonfatal CVD was shown per 100 pmol/l increase in dp-ucMGP or dp-cMGP, and dp-ucMGP or dp-cMGP divided into tertiles using Visual Binning in SPSS. Statistical significance was considered present at the two-sided P value of 0.05. Analyses were performed using the SPSS 15.0 statistical package.

3. Results

The baseline sample consisted of 577 older persons (255 men, 322 women) without prevalent CVD. The mean (SD) age of all respondents was 59.9 (2.9) year and 55.8% was female. The median (interquartile range) of plasma dp-ucMGP and dp-cMGP, was 335 (229–457) pmol/l and 1708 (1426–2052) pmol/l, respectively. Excluded participants with known plasma dp-ucMGP ($n = 119$) were significantly older [60.6 (3.1) year; $P = 0.013$], and had higher plasma dp-ucMGP [500.3 (272.0–614.0) pmol/l; $P < 0.001$]. They were more often men ($P = 0.025$). Excluded participants with unknown plasma dp-ucMGP and dp-cMGP ($n = 306$) were older, although this difference was not significant [60.2 (3.0) year; $P = 0.068$].

Dp-ucMGP was correlated with dp-cMGP ($r = 0.51$, $P < 0.001$) and BMI ($r = 0.26$, $P < 0.001$). Dp-cMGP showed low correlation coefficients with BMI ($r = 0.12$, $P = 0.003$), age ($r = 0.13$, $P = 0.002$), and serum albumin concentration ($r = -0.08$, $P = 0.048$). Table 1 shows the baseline characteristics of the total study population, stratified by tertiles of dp-ucMGP concentration.

After a mean follow-up of 5.6 ± 1.2 year a total of 35 non-fatal and 5 fatal cardiovascular events had occurred, 28 of which were CHD, 4 PAD and 8 CVA. Participants in the middle and highest tertile of dp-ucMGP had a higher risk of CVD compared with persons with plasma dp-ucMGP concentrations in the lowest tertile with a HR of 2.22 (95% CI, 0.90–5.44; $P = 0.082$) and 2.71 (95% CI, 1.13–6.49; $P = 0.025$), respectively. After adjustment for classical confounders and vitamin D status, model 3 still showed a significant, more than 2-fold higher risk of CVD for the highest tertile of dp-ucMGP with a HR of 2.69 (95% CI, 1.09–6.62, $P = 0.032$) compared with the lowest tertile (Table 2). The association between dp-ucMGP and CVD was mainly due to the association between dp-ucMGP and CHD with a fully adjusted HR of 2.73 (95% CI 0.97–7.64; $P = 0.057$) for the highest compared to the lowest tertile of dp-ucMGP.

Plasma dp-cMGP was not associated with the risk of CVD (Table 3).

4. Discussion

This study shows that vascular vitamin K insufficiency, as reflected by higher plasma concentrations of dp-ucMGP, was significantly linearly associated with a higher incidence of first cardiovascular events. We did not observe an association between dp-cMGP and CVD in healthy individuals. This is in accordance with case–control comparisons or studies in specific disease populations [8,10,11] which showed an increased amount of dp-ucMGP among patients characterized by soft-tissue calcification. Moreover, our findings are consistent with other epidemiological studies that included vitamin K status by estimating the vitamin K intake [4–6] in healthy elderly.

It is generally assumed that the potential role of vitamin K in cardiovascular health is mediated through its co-factor activity in the carboxylation of vitamin K-dependent proteins. Indeed, carboxylation is critical for MGP function as arterial calcification inhibitor [21]. An obvious explanation is that vitamin K-insufficiency leads to CVD through vascular calcification. Previously a cross-sectional analyses in 200 healthy women showed a borderline significant ($P = 0.06$) association between higher circulating dp-ucMGP levels and high CAC [22]. However, Shea et al. [23] did not find an association between the 3-year change in dp-ucMGP and the 3-year change in coronary artery calcification in older individuals who received 500 μg/d of phylloquinone. Neither they found an association between the baseline dp-ucMGP and the 3-year change in coronary artery calcification in the control group [23]. The lack of effect by phylloquinone intervention is consistent with population-based evidence from the Rotterdam cohort and the Prospect cohort, showing that only menaquinone and not phylloquinone intake was associated with cardiovascular benefits [5,6]. But the high dose of phylloquinone given by Shea et al. [23] did induce a significant decrease of dp-ucMGP and the question remains why this did not have a clinical effect. One explanation is that the intervention period was too short. The population-based studies, such as the study of Dalmeijer et al. [22], reflect dietary habits that generally persist lifelong. Secondly, it has been demonstrated that independent of carboxylation, menaquinone (and not phylloquinone) may directly affect gene expression by binding to the steroid and xenobiotic receptor [24,25]. Moreover, vitamin K was reported to protect against oxidative stress [26], and consequently modulate inflammation [27], but the relative efficacy of phylloquinone and menaquinone in these processes has not yet been evaluated. Our study identified dp-ucMGP as an independent risk factor for CVD in the apparently healthy population. Dp-cMGP was not found to be a sensitive marker in this respect.

Our results are in accordance with results of case–control studies showing a higher disease severity and/or mortality with higher concentrations of dp-ucMGP [10–12] but not with lower concentrations of dp-cMGP [11,12]. It has been suggested that MGP carboxylation precedes and even facilitates phosphorylation; this would explain why circulating dp-ucMGP and dp-cMGP are not inversely correlated. Unfortunately, there are currently no assays available to measure total phosphorylated MGP or even phosphorylated carboxylated or uncarboxylated MGP [8].

To our knowledge, the current study is unique in the measurement of circulating MGP levels, as marker of vitamin K status, in a relatively large group of healthy elderly people. As compared to vitamin K intake, dp-ucMGP is a more robust and valid marker for vitamin K status. Strengths of the present study include the nationally representative and large sample and the long follow-up which enabled us to study clinically manifest CVD endpoints, which are more informative then intermediate endpoints.

A limitation of the present study was that only a single measurement of vitamin K status was available, hampering correction for changes over time. However, in 70 healthy subjects aged 55–64,

Table 1
Baseline characteristics of the study population stratified by tertiles of plasma dp-ucMGP concentration.

	Dp-ucMGP			
	<266 pmol/l	266–400 pmol/l	>400 pmol/l	P*
No. of subjects	194	191	192	
Age (year)[a]	59.7 (2.9)	59.9 (2.9)	60.1 (2.9)	0.398
No. of female[b]	105 (54.1)	101 (52.9)	116 (60.4)	0.281
Smoking[b]				0.678
Never	52 (26.8)	47 (24.6)	52 (27.1)	
Past	91 (46.9)	90 (47.1)	98 (51.0)	
Current	51 (26.3)	54 (28.3)	42 (21.9)	
Alcohol use[b]				0.685
None	7 (3.6)	13 (6.8)	14 (7.3)	
Light	100 (51.5)	86 (45.0)	92 (47.9)	
Moderate	69 (35.6)	71 (37.2)	68 (35.4)	
Excessive	18 (9.3)	21 (11.0)	18 (9.4)	
Physical activity (min/d)[b]				0.063
Tertile 1	75 (38.7)	62 (32.5)	58 (30.2)	
Tertile 2	50 (25.8)	56 (29.3)	74 (38.5)	
Tertile 3	69 (35.6)	73 (38.2)	60 (31.3)	
Education[b]				0.232
Primary education or less	73 (37.6)	90 (47.1)	75 (39.1)	
Secondary education	70 (36.1)	66 (34.6)	74 (38.5)	
Tertiary education	51 (26.3)	35 (18.3)	43 (22.4)	
Prevalent diseases[b]				
Diabetes	5 (2.6)	7 (3.7)	11 (5.7)	0.275
Hypertension	33.0 (17.0)	40.0 (20.9)	45.0 (23.4)	0.288
Serum total cholesterol (mmol/l)[a]	5.9 (0.9)	5.9 (1.0)	5.9 (1.0)	0.948
BMI (kg/m^2)[a]	26.1 (3.5)	26.9 (4.0)	28.4 (4.3)	**0.000**
Serum albumin (g/l)[a]	41.3 (3.1)	41.2 (2.7)	40.8 (3.1)	0.207
Serum 25(OH)D (<25 nmol/l)[b]	58.6 (22.4)	55.6 (19.8)	58.5 (20.0)	0.263

Abbreviation: 25(OH)D, 25-hydroxyvitamin D.
* P = P-value (in bold, P < 0.05).
[a] Values are mean (s.d.).
[b] Values are number (%).

Table 2
HR for fatal and nonfatal CVD by plasma dp-ucMGP per 100 pmol/l increase and when divided into tertiles.

	Dp-ucMGP (per 100 pmol/l increase)	dp-ucMGP (pmol/l)		
		<266	266–400	>400
No. of subjects	577	194	191	192
No. of events	40	7	15	18
Model 1[a]	1.12 (1.02–1.24)	Reference	2.22 (0.90–5.44)	2.71 (1.13–6.49)
Model 2[b]	1.13 (1.02–1.24)	Reference	2.19 (0.89–5.38)	2.72 (1.13–6.52)
Model 3[c]	1.14 (1.02–1.26)	Reference	1.99 (0.80–4.90)	2.69 (1.09–6.62)

[a] Model 1 = univariate model.
[b] Model 2 = adjusted for age and sex at baseline.
[c] Model 3 = adjusted for age, sex, smoking, alcohol consumption, physical activity, education, DM, hypertension, BMI, serum albumin, total cholesterol concentrations, and 25(OH)D.

a significant correlation ($r = 0.84$; $P < 0.05$) was found between plasma dp-ucMGP concentration at baseline and after 3 years follow-up (personal communication with Cees Vermeer). Concentrations of dp-ucMGP were within the normal range [8,22,23]. The outcome variable was partly based on self-reporting; this was also the case for some of the confounders. No dietary intake risk factors were included, total cholesterol rather than LDL and HLD cholesterol was included. Therefore, we cannot exclude the possibility of confounding by imperfectly measured or unmeasured factors.

Table 3
HR for fatal and nonfatal CVD by plasma dp-cMGP concentration per 100 pmol/l increase and when divided into tertiles.

	Dp-cMGP (per 100 pmol/l increase)	dp-cMGP (pmol/l)		
		<1531	1531–1918	>1918
No. of subjects	577	195	190	192
No. of events	40	12	11	17
Model 1[a]	1.03 (0.99–1.07)	Reference	0.94 (0.41–2.12)	1.50 (0.72–3.14)
Model 2[b]	1.03 (0.98–1.07)	Reference	0.90 (0.40–2.05)	1.45 (0.69–3.07)
Model 3[c]	1.02 (0.97–1.07)	Reference	0.84 (0.36–1.95)	1.19 (0.55–2.58)

[a] Model 1 = univariate model.
[b] Model 2 = adjusted for age and sex at baseline.
[c] Model 3 = adjusted for age, sex, smoking, alcohol consumption, physical activity, education, DM, hypertension, BMI, serum albumin, total cholesterol concentrations, and 25(OH)D.

In conclusion, plasma dp-ucMGP, an indicator of vascular vitamin K insufficiency, is associated with an increased risk of CVD, independent of classical risk factors and vitamin D status. Larger epidemiological studies on dp-ucMGP and CVD incidence are needed followed by clinical trials to test whether vitamin K-rich diets will lead to a decreased risk for cardiovascular events.

Ethical approval

Data for this study were collected within the framework of the Longitudinal Aging Study Amsterdam (LASA), an ongoing cohort study of predictors and consequences of changes in physical, cognitive, emotional, and social functioning in older people in The Netherlands. The design of this study, including methods for sampling and data collection has been detailed by Huisman et al. [14].

Contributors

The authors' responsibilities were as follows: this research was planned by EGHMvdH, PL, and NMvS; designed by DJHD, and PL. The analyses of MGP were conducted by CV and EM. EGHMvdH performed statistical analyses and wrote the manuscript. All authors evaluated the manuscript and contributed their comments. MdH and NMvS had primary responsibility for final content.

Competing interest

Cees Vermeer is CEO of VitaK b.v., The Netherlands. Ellen G. H. M. van den Heuvel is besides senior researcher at the VU University Medical Center, also senior scientist at FrieslandCampina, a dairy industry. All other authors did not report personal or financial conflicts of interest.

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