# Is matrix Gla protein the link between vitamin K and cardiovascular disease risk?

Gerdien Dalmeijer

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Julius Center for Heath Sciences and Primary Care, University Medical Center Utrecht.

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# Is matrix Gla protein the link between vitamin K and cardiovascular disease risk?

Is matrix Gla eiwit de link tussen vitamine K en het risico op hart- en vaatziekten?

(met een samenvatting in het Nederlands)

Proefschrift

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Geertje Wilhelmina Dalmeijer

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te De Bilt

Promotor: Prof. dr. ir. Y.T. van der Schouw

Co-promotor: Dr. ir. J.W.J. Beulens

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# TABLE OF CONTENTS

# Chapter 1

General introduction

## General introduction

*Vitamin K*

Vitamin K is a fat-soluble vitamin that occurs in two biologically active forms; phylloquinone (vitamin $K_1$) and menaquinones (vitamin $K_2$). Vitamin K is a group name for a number of related compounds that all share the functional 2-methyl-1,4 naphthoquinone ring structure, with an aliphatic side chain composed of a number of repeating isoprenoid residues at the 3-position. All differences between various forms of K vitamins originate from differences in the length and saturation degree of the side chain (1). Phylloquinone is a single compound with a side chain of 4 isoprenoid residues, 3 of which are saturated. Menaquinones commonly found in nature have side chains of varying length between 4-13 isoprenoid residues, most of which are unsaturated (2). Menaquinones are generally denoted as menaquinone-n, (MK-n) were n stands for the number of isoprenoid residues. The most abundant menaquinones in the human diet are the short chain MK-4 and the long chain MK-7 till MK-10. The major dietary source of vitamin K is phylloquinone, found in leafy green vegetables and vegetable oils (3;4). Menaquinones generally are of microbial origin. Important dietary sources are fermented food like cheese, curd and natto (a traditional Japanese food composed of fermented soy beans) (5). Estimates of intake of phylloquinone and menaquinones in the Netherlands suggest that up to 12% of total intake of K vitamins is provided by menaquinones (6). In the Netherlands the mean intake of phylloquinone and menaquinones is approximately 230 µg/d and 30 µg/d, respectively (7;8)

Vitamin K is required through its role as a cofactor for the enzyme gamma-glutamyl carboxylase. This enzyme converts certain protein-bound glutamate residues into gammacarboxyglutamate, generally known as Gla (9). After the discovery of Gla residues in prothrombin, the subsequently identified proteins containing these residues were those involve in blood coagulation; Factor VII, IX, and X as well as the protein C, S, and Z (10). The first extra-hepatic sites where Gla proteins have been identified and characterized are in bone and cartilage. Bone Gla protein, also known as osteocalcin, was identified in the early 1970s (11;12). Matrix Gla protein (MGP) was identified in the early 1980s (13). Vitamin K antagonists (also known as oral anticoagulants) block the recycling of vitamin K by inhibition of the enzyme (vitamin K-epoxide reductase) VKOR, resulting in vitamin K deficiency. Treatment with vitamin

K antagonists will therefore result in the synthesis of uncarboxylated vitamin K-dependent proteins (14).

*Matrix Gla protein*

Matrix Gla protein is a potent inhibitor of arterial calcification (15), and for its function depending on the presence of vitamin K. The importance of MGP for vascular health was demonstrated in MGP-deficient animals, who all died of massive arterial calcification within 6-8 weeks after birth (16). However, human studies investigating the association between circulating MGP concentrations and vascular calcification showed inconsistent results (15;17). Inability of the MGP assays to discriminate between different MGP species might explain these inconsistencies. MGP exists as various species, which differ in their state of phosphorylation and/or carboxylation: phosphorylated (pMGP), non-phosphorylated (desphospho-MGP, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP) (Figure 1). Development of assays to measure the different circulating MGP species enabled the investigation thereof in the circulation (18).

| *Carboxylation:* | *Phosphorylation:* | | | | |
|---|---|---|---|---|---|
| | Desphospho MGP (dpMGP) | | Phosphorylated MGP (pMGP) | | |
| Uncarboxylated MGP (ucMGP) | dp-ucMGP | + | p-ucMGP | → | Total uncarboxylated MGP (t-ucMGP) |
| | + | | + | | |
| Carboxylated MGP (cMGP) | dp-cMGP | + | p-cMGP | → | Total carboxylated MGP (t-cMGP) |
| | ↓ Total desphospho MGP (t-dpMGP) | | ↓ Total phosphorylated MGP (t-pMGP) | | |

**Figure 1:** Schematic overview of MGP phosphorylation and carboxylation

*Cardiovascular disease*

Cardiovascular disease (CVD) is the primary cause of death in most parts of the world and a major determinant of chronic disability (19). Large follow-up studies have established coronary artery calcification (CAC) as a strong and independent risk factor for future CVD events (20). Through carboxylation of MGP vitamin K is

thought to reduce vascular calcification and risk of CVD. Animal studies indeed showed that warfarin treatment resulted in vascular calcification, which was reversed by vitamin K supplementation (21;22). Increased vascular calcification is indeed also found in human on vitamin K antagonists (23;24). Subsequent studies investigated the association between vitamin K intake and vascular calcification or CVD risk (7;8;25-30). Although observational studies did not observe an association between phylloquinone intake and CAC or CVD risk (7;8;25-28), two intervention studies found improved vascular elasticity and reduced progression of coronary calcification after phylloquinone supplementation (29;30). We are not aware of intervention studies that investigated the effect of menaquinones on vascular calcification. However, observational studies have shown that a high intake of menaquinones is associated with reduced CAC and CVD risk (7;8;26). It is thought that these effects of vitamin K on vascular calcification or CVD risk are due to increased carboxylation of matrix Gla protein (31).

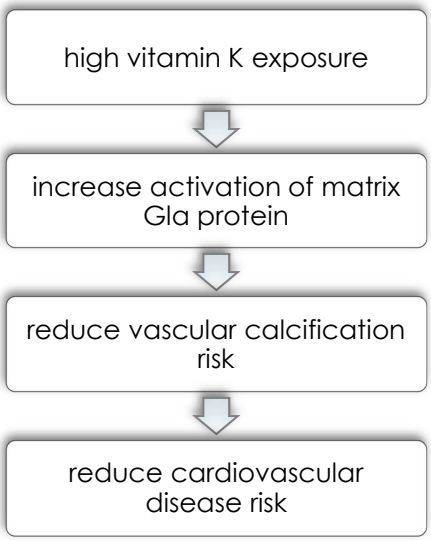


**Figure 2:** Schematic overview of the hypotheses how high vitamin K exposure can reduce cardiovascular disease risk

*Objectives of this thesis*

The overall aim of this thesis is to investigate the role of MGP carboxylation in the association of high vitamin K intake with reduced vascular calcification and CVD risk. Our hypothesis is that high vitamin K exposure will increase the carboxylation of MGP. Increased MGP carboxylation will reduce vascular calcification and will lead to less cardiovascular disease risk (figure 2).

*Outline of this thesis*

In **chapter 2** a review summarizes the literature on vitamin K intake in relation to vascular calcification and cardiovascular disease risk.

The first part of this thesis focuses on the association of exposure to vitamin K with circulating species of matrix Gla protein, vascular calcification and CVD risk. In **chapter 3,** the relation of phylloquinone concentration with vascular calcification risk in women is described, using data from a prospective study among 573 postmenopausal women. **Chapter 4** reports the relation between dairy intake, which is an important dietary source of menaquinones, and the risk of coronary heart disease or stroke, using data from a prospective cohort among 40.011 Dutch men and women. **Chapter 5** reports on the result of a randomized placebo controlled trial of dietary supplementation with menaquinone-7 on circulating MGP species.

The second part of this thesis focuses on the association between the circulating matrix Gla protein species and vascular calcification or CVD risk. **Chapter 6** describes a cross-sectional study which investigates the association of circulating MGP species with vitamin K exposure and coronary artery calcification. In **chapter 7,** a prospective study reports the relation of MGP species with vascular calcification. **Chapter 8** reports the relation of circulating MGP species and risk of cardiovascular events in 518 type 2 diabetes patients, using data from a prospective cohort. **Chapter 9** describes a case-cohort study which investigates the association of circulating desphosho-uncarboxylated MGP with risk of CHD and stroke.

In **chapter 10**, the main findings of this thesis are discussed and **chapter 11** gives a summary of the main findings described in this thesis.

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# Chapter 2

# Vitamin K and cardiovascular disease risk

Based on:

Vitamin K, coronary calcification and risk of cardiovascular disease

*Published in Bioactive food as dietary interventions for cardiovascular disease. 2013; 229-242*

GW Dalmeijer

YT van der Schouw

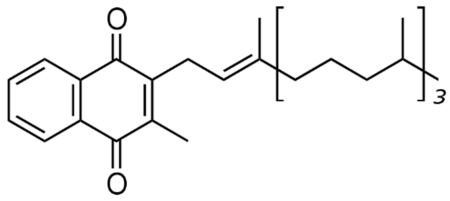
JWJ Beulens

**Abstract**

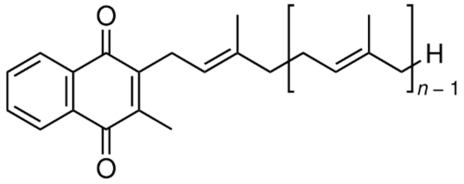
Vitamin K is mainly known for its function in blood coagulation, but recently its function in vascular calcification became apparent. Vitamin K is a fat-soluble vitamin present in vegetables as phylloquinone and as menaquinones in animal products. Vitamin K acts as a co-factor in the carboxylation of clotting factors, but also of proteins in the vessel wall, matrix Gla-protein (MGP). MGP is a potent inhibitor of vascular calcification. Vitamin K can thus reduce vascular calcification and eventually cardiovascular disease by carboxylation of MGP. Here we describe the evidence for the relations between vitamin K, MGP, calcifications and risk of cardiovascular diseases.

**Vitamin K**

Vitamin K was discovered in the early 1930s by the nutritional biochemist Hendrik Dam as an anti-haemorrhagic factor in chickens (1). Vitamin K is a fat-soluble vitamin that occurs in two biologically active forms; phylloquinone (vitamin $K_1$) and menaquinones (vitamin $K_2$) with side chains based on a number of repeating prenyl units; this number being given as a suffix i.e., menaquinone-n, (MK-n). Vitamin K is a group name for a number of related compounds, which have a 2-methylated-1,4-naphthoquinone ring structure in common, and vary in the aliphatic side chain attached at the 3-position (figure 1). It is generally accepted that the naphthoquinone ring is the functional group, and therefore the mechanism of action is similar for all K-vitamins. However, side chain-related differences may be expected with respect to intestinal absorption, transport, tissue distribution, and bio-availability. These differences are caused by the different hydrophobicity of the various side chains, and by the different food matrices in which they occur (2-6).




Phylloquinone




Menaquinone

**Figure 1:** phylloquinone and menaquinone

The major dietary source of vitamin K is the plant form, phylloquinone, which is widely distributed in foods. Leafy green vegetables and vegetable oils (soybean, cottonseed, canola and olives) are the largest contributors to dietary intakes (7-9). Dietary relevant menaquinones range from MK-4 through MK-10. The richest dietary sources of menaquinones are fermented foods. For that reason, there is a geographic distribution in menaquinone intake. In the Western diet the richest

dietary sources of menaquinones are cheeses and curd (MK-8 and MK-9), whereas in Japan natto, fermented soy beans, (MK-7) is the richest dietary sources of menaquinones (10). Many of the longer-chain menaquinones are also produced by intestinal bacteria and historically these forms were believed to be an important source of vitamin K. However, their contribution to vitamin K status is now considered insignificant, their low bioactivity may be due to their location in bacterial membranes and consequent poor absorption form the gut (11).

It has been demonstrated that the bioavailability of vitamin K is dependent upon the nature of the food matrix (12). It was found that phylloquinone absorption from vegetables is very poor, namely 5-10% without concomitant fat intake and 10-15 % if taken together with fat, whereas menaquinones absorption from dairy products and natto was much better, probably almost complete. Another difference between phylloquinone and the long chain menaquinones (not MK-4) is the half-life time. Phylloquinone has a disappearance curve with an apparent half-life time of 1.5 h, whereas menaquinones have more complex disappearance curves with a very long half-life time (13).

The adequate intake for vitamin K is established at 90 µg/d for women and 120 µg/d for men, based on median intakes from food as estimated from NHANES III (1998-1994) (14). These recommendations are set to meet requirements for haemostasis. The liver as main site for clotting factor synthesis, however, efficiently extracts vitamin K from the circulation, which could lead to insufficient amount to cover requirements of extra-hepatic tissues, including the vascular wall (15). However, this distribution could differ for phylloquinone and menaquinones.

Phylloquinone is predominantly transported with the triacylglycerol-rich fraction, which is mainly cleared by the liver (16). Phylloquinone is therefore cleared very effectively from circulation by the liver to function as a cofactor for proteins in blood coagulation (17). Accumulation and utilization of phylloquinone in extra-hepatic tissues such as the vessel wall is therefore lower than in hepatic tissues. Menaquinones on the other hand, are found in both triacylglycerol-rich lipoprotein and low-density lipoprotein, mainly though these low-density lipoprotein menaquinones are transported to extra-hepatic tissues. This is confirmed by higher accumulation and utilization of menaquinone-4 than phylloquinone in extra-hepatic tissues such as the vessel wall (18).

As a cofactor to the carboxylase that generates gamma-carboxyglutamic acid, vitamin K undergoes a cycle of oxidation and reduction that allows reuse (see figure 2). Vitamin K-epoxide reductase (VKOR) ensures the reutilization of vitamin K after it has been oxidized in the carboxylase reaction. On a molecular level, VKOR reduces vitamin K epoxide (KO) in two steps: first to vitamin K quinine (K), and subsequently to vitamin K hydroquinone ($KH_2$), the latter being the active cofactor of gamma-glutamyl carboxylase (GGCX), this process is visualised in figure 2. Anticoagulants such as warfarin block the reduction of vitamin K oxide to vitamin K, explaining their antagonistic effects on this cycle. Polymorphisms of VKORC1 and GGCX affect the necessary warfarin dose in coagulation (19;20). Such SNP's could therefore also influence coronary calcification.

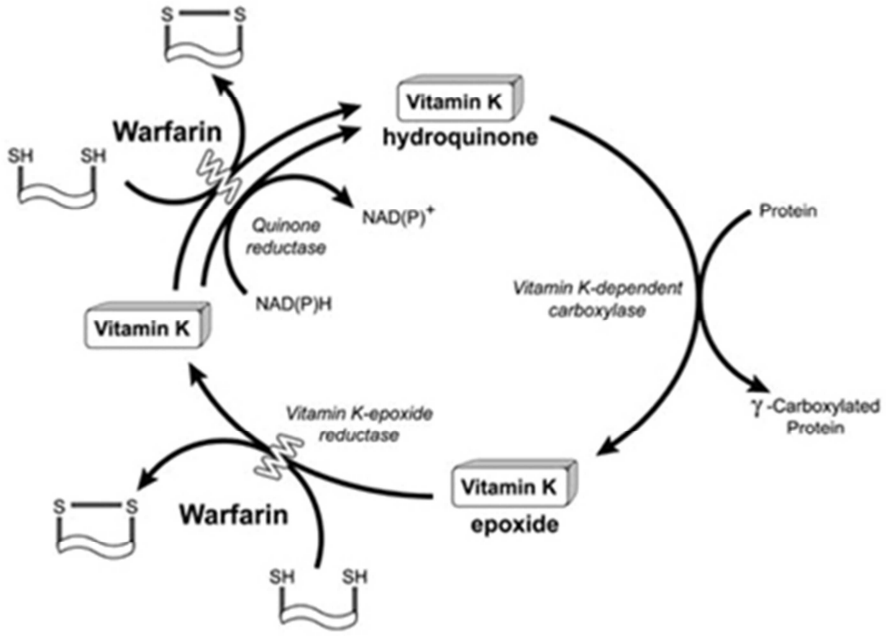



**Figure 2:** The vitamin K cycle

Vitamin K functions as a cofactor for the enzyme gamma-glutamyl carboxylase, catalysing the gamma-glutamyl carboxylation of certain glutamic acid residues (Gla) in proteins (21). These vitamin K-dependent proteins include the hepatic-coagulation factors prothrombin, Factor VII, IX, and X (21). Vitamin K also functions as a cofactor for extra-hepatic Gla-proteins in bone, such as osteocalcin, and the vessel wall, such as matrix Gla-protein (MGP). MGP is a powerful inhibitor of vascular calcification (22). Coronary calcification is a strong, independent predictor of coronary events (23). MGP knock-out mice develop severe vascular calcification (24). In humans, MGP loss-of function mutations also increase coronary calcification

(17) and impaired carboxylation of MGP is associated with vascular calcification (15). The anticoagulant warfarin, a vitamin K antagonist, indeed inhibits carboxylation of coagulation factors, but also of MGP and increases vascular calcification in humans and rats (22;25;26). These effects are prevented in warfarin-treated rats by vitamin K rich diets (18;27). Vitamin K could therefore reduce coronary calcification and eventually CVD through carboxylation of MGP (see figure 2).

To further support this hypothesis, few studies investigated whether VKORC1 SNP's associated with reduced formation of the active vitamin K form increased CVD risk. Two of these studies conclude that VKORC1 may serve as a novel genetic marker for the risk of CVD (28;29), while Watzka et al. (30) did not find an association between VKORC1 genotype and CVD risk. Similarly, the first human studies investigating the association between plasma MGP and CVD also showed inconsistent results (15;31-35). However, this is probably due to the measurement of total plasma MGP instead of carboxylated or uncarboxylated MGP.

In the next part, we will review the evidence on the relations between vitamin K intake and arterial calcification, CHD and stroke risk.

**Vitamin K and arterial calcification**

Whereas the genetic and biochemical studies establish that inadequate carboxylation of MGP results in abnormal calcification, there are limited observational data linking an inadequate intake of vitamin K with vascular calcification (table 1).

One study investigated the association between vitamin K intake and aortic atherosclerosis. In this nested case-control study postmenopausal women (n=113) with aortic atherosclerosis reported 42.9 µg/day (95% Confidence Interval (CI): -6.6; 92.5) lower vitamin K intake than women without atherosclerosis, after adjustment for age. In the age group 60-69 years the vitamin K intake was 74.8 µg/day (95% CI: 135.1; 14.6) lower in the women with calcification, while there was no significant difference in the 70-79 age-group (36).

*Phylloquinone*

Four cross-sectional studies investigated the association between phylloquinone intake and calcification, but none of these found an association. In the Rotterdam cohort study (37) the mean intake of phylloquinone was similar across categories of aortic calcification (249.2, 249.0 and 245.0 µg/day for mild, moderate, and severe stages, respectively). Cross-sectional analyses from this study showed that the intake of phylloquinone was not significantly associated with moderate or severe aortic calcification (37). Villines et al. (38) studied 807 consecutive active-duty US Army personnel, 39-45 year old, without known CHD and showed that the mean intake of phylloquinone was similar in subjects with (114.2 ± 78.7 µg/day) and without (119.2 ± 80.3 µg/day) CAC. After full adjustment there was no association between phylloquinone intake and the presence of CAC. Maas et al. (39) carried out a study among 1689 women, aged 49-70 years. The prevalence of breast arterial calcification (BAC) was similar (11%) across quartiles of phylloquinone intake. However, the clinical significance of breast artery calcification for future coronary disease is controversial, because its association with prevalent coronary heart disease is weak (40). In a study among 564 post-menopausal women, Beulens et al.(41) found that phylloquinone intake was not associated with CAC with a relative risk (RR) of 1.17 (95%CI: 0.96;1.42; $p_{trend}$=0.11) of the highest versus the lowest quartile.

Two randomized trials investigated the association between phylloquinone intake and calcification. Braam et al. (42) assessed the effect of 3-years of daily phylloquinone (1 mg/day), minerals, and vitamin D supplementation on vascular health in 181 postmenopausal women. They measure the vessel wall characteristics of the common carotid artery with ultrasound. The investigators concluded that a supplement containing phylloquinone, minerals and vitamin D had a beneficial effect on the elastic properties of the arterial vessel wall. However, due to the design of the study, it was not possible to distinguish whether these effects are due to phylloquinone alone or to the combination of phylloquinone, minerals and vitamin D. Shea et al. (43) performed a randomized trial in 388 healthy older adults; 200 received a multivitamin with additionally 500 µg/d phylloquinone, and 188 received a multivitamin alone. There was no difference in CAC progression between the two groups. However, in a subgroup-analysis of subjects who were ≥ 85% adherent to supplementation, there was less CAC progression in the phylloquinone group than in the control group (P=0.03). Furthermore, when restricting to subjects with pre-existing CAC, those who received phylloquinone supplements had 6% less progression than did those who received the multivitamin alone (P=0.04).

Overall, the two intervention studies with phylloquinone supplementation found small beneficial effects of phylloquinone intake on the arterial vessel wall, while none of the observational studies observed an association between phylloquinone intake and calcification. This discrepancy could be explained by the relative high doses of phylloquinone in the intervention studies compared to phylloquinone intake in the observational studies. Doses of 1 mg/day and 0.5 mg/day were used in the trials, while the mean intake of phylloquinone in the intervention studies varied between 0.1-0.25 mg/day. It also could be explained by the better absorption of phylloquinone from supplements compared with phylloquinone from food products (12). The randomized trials, however, do provide evidence that high doses of phylloquinone can indeed reduce coronary calcification.

*Menaquinones*

Three of the four previously mentioned cross-sectional studies also investigated associations of menaquinones intake and coronary calcification (37;39;41). In the Rotterdam study, Geleijnse et al. (37) found that menaquinones intake was lower in subjects with severe aortic calcification (25.6 µg/day) than in subjects with moderate

or mild calcification (28.6 and 28.8 µg/d, respectively; P=0.001). For severe calcification a strong inverse relationship with menaquinones intake was found with ORs for severe calcification of 0.71 (95%CI: 0.50, 1.00) and 0.48 (95%CI; 0.32, 071) in the mid and upper tertiles of menaquinones intake respectively, compared to the lower tertile. Maas et al. (39) showed that the prevalence of BAC was less common in the highest (9%) quartile of menaquinones intake, compared to the lowest quartile (13%). This study showed a similar association as Geleijnse et al. (37) with an OR of 0.7 (95%CI: 0.5; 1.1), although it did not reach significance. Finally, Beulens et al. (41) showed that high menaquinones intake was associated with reduced CAC with a relative risk of 0.80 (95% CI:0.65; 0.98; $p_{trend}$ =0.03) of the highest versus the lowest quartile. In conclusion, high menaquinones intake was associated with a modestly reduced risk of arterial calcification in two of the tree cross-sectional studies (table 1) (37;39;41). We are not aware of any randomized trials that investigated the association of menaquinones intake and risk of arterial calcification.

**Tabel 1:** Vitamin K and arterial calcification

| *Study* | *Study design* | *Participants* | *Measurement* | *Exposure/intervention* | *Endpoint* | *Result** |
|---|---|---|---|---|---|---|
| 36 | nested CCS | 34 women with aortic calcification<br>79 without aortic calcification | FFQ | vitamin K intake:<br>cases: 189.9 ± 15.5 µg/d<br>controls: 243.6 ± 15.3 µg/d | aortic athero-sclerosis | phylloquinone ↓ |
| 38 | CSS | 807 participants, (39-45 y) | BDQ | phylloquinone intake: 115.2 ± 79.0 µg/d | CAC | phylloquinone ↔ |
| 39 | CSS | 1689 women,<br>(49-70 y) | FFQ | phylloquinone intake:<br>216.6 µg/d (BAC+), 210.7 µg/d (BAC-)<br>menaquinone intake:<br>26.9 µg/d (BAC+), 29.4 µg/d (BAC-) | BAC | phylloquinone ↔<br>menaquinone ↔ |
| 41 | CSS | 564 post-menopausal women, (49-70 y) | FFQ | phylloquinone intake: 217.0 ± 92.3 µg/d<br>menaquinone intake: 31.6 ± 12.3 µg/d | CAC | phylloquinone ↔<br>menaquinone ↓ |
| 37 | CSS | 4473 men and women<br>(≥ 55 y) | FFQ | phylloquinone intake:<br>men; 257.1 ± 116.1 µg/d,<br>women; 244.3 ± 131.9 µg/d<br>menaquinone intake:<br>men; 30.8 ± 18.0 µg/d<br>women; 27.0 ± 15.1 µg/d | CAC | phylloquinone ↔<br>menaquinone ↓ |
| 42 | RCT | 181 women<br>(50-60 y) | | 3 years<br>group 1: placebo<br>group 2: minerals, vitamin D<br>group 3: minerals, vitamin D, 1 mg/d phylloquinone | Elastic properties | phylloquinone ↓ |
| 43 | RCT | 388 adults<br>(60-80 y) | | 3 years<br>group 1: multivitamin<br>group 2 ; multivitamin with 500 µg/d phylloquinone | CAC | phylloquinone ↓ |

*↑ High phylloquinone/ menaquinone intake was associated with increased calcification
↓ high phylloquinone /menaquinone intake was associated with reduced calcification
↔ there was no association between phylloquinone/menaquinone intake and calcification
CAC; coronary artery calcification, CCS; case control study, CSS; cross-sectional study, BAC; breast artery calcification: FFQ food frequency questionnaire, RCT; randomized controlled trial, BDQ; Block Dietary Questionnaire

**Vitamin K intake and risk of coronary heart disease**

*Phylloquinone*

To date, four prospective cohort studies investigated the relation between phylloquinone intake and risk of coronary heart disease. Erkkilä et al. (44;45) investigated the association between phylloquinone intake and CVD risk in two cohorts. In the Nurses' Health study (44), over 72,000 female nurses, aged 38-65y were followed for 16 years. After adjustment for CHD risk factors, diet and lifestyle, high phylloquinone intake was associated with a decreased risk of CHD with a relative risk (RR) of 0.84 (95%CI: 0.71; 1.00) for the highest versus lowest quartile. In a subsequent study among 40,087 men who participated in the Health Professionals's Follow-up Study, though, a significantly reduced risk was only observed in the age-adjusted model (RR: 0.79; 95%CI: 0.69; 0.91) (45). After adjustment for CHD risk factors, diet and lifestyle, the association attenuated to non-significant (RR: 0.91; 95%CI: 0.77; 1.06). The authors therefore concluded that phylloquinone intake may just be a marker for a heart-healthy diet instead of biologically linked to CHD.

Similarly, in the Rotterdam cohort, Geleijnse et al. (37) showed that after adjustment for CHD risk factors, diet and lifestyle, high phylloquinone intake was not associated with a decreased risk of CHD with a RR of 0.89 (95%CI: 0.63;1.25) for the highest versus the lowest tertile. Finally, in the Prospect-EPIC cohort, phylloquinone intake was not associated with risk of CHD (HR: 1.00; 95%CI: 1.00; 1.02) after adjustment for CHD risk factors, diet and lifestyle (46).

In conclusion, high phylloquinone intake has not been associated with a reduced risk of CHD in four cohort studies once the statistically analysis is adjusted for CHD risk factors, dietary and lifestyle factors associated with coronary heart disease (table 2).

*Menaquinones*

Two of the previously mentioned cohort studies also investigated the relation of menaquinones intake with risk of CHD. In the Rotterdam cohort (37), the RR of incident CHD was reduced in the upper tertile of menaquinones intake compared to the lower tertile (0.59 (95%CI: 0.40; 0.86). Similarly, the risk of CHD mortality was reduced in the upper tertile of menaquinone intake compared to the lowest tertile (0.43; 95%CI: 0.24; 0.77). In the prospect-EPIC cohort (46), the investigators also observed an inverse association between menaquinone intake and risk of CHD with

a hazard ratio (HR) of 0.91 (95%CI: 0.85; 1.00, p-value 0.08) per 10 µg/day menaquinones intake. This association was mainly due to menaquinone subtypes MK-7, MK-8 and MK-9.

The two cohort studies (37;46) which examined the effects of menaquinones intake on the incidence of CHD both reported a significantly reduced risk of CHD with higher menaquinone intake (table 2). These findings suggest that an adequate intake of menaquinone could be important for CHD prevention.

**Vitamin K intake and risk of stroke**

Two cohort studies, Nurses' Health study (44) and the Health Professionals study (45), investigated the association between phylloquinone intake and the incidence of stroke (table 3). Neither study found significant association between phylloquinone and incidence of stroke. The association between menaquinones intake and incidence of stroke has not been investigated to date.

**Table 2:** Vitamin K intake and risk of coronary heart disease

| *Study* | *Study design* | *Participants* | *Measurement* | *Exposure/intervention* | *Endpoint* | *Result** |
|---|---|---|---|---|---|---|
| 44 | cohort study | 72,874 females, (38-65 y) | semi quantitative FFQ. | phylloquinone intake: 184 ± 106 µg/d. | CHD | phylloquinone ↔ |
| 45 | cohort study | 40,087 males, (40-75 y) | semi quantitative FFQ. | phylloquinone intake: 165 µg/d | CHD | phylloquinone ↔ |
| 37 | cohort study | 4,807 adults, (≥ 55 y) | FFQ | phylloquinone intake: men; 257.1 ± 116.1 µg/d women; 244.3 ± 131.9 µg/d menaquinone intake: men; 30.8 ± 18.0 µg/d women; 27.0 ± 15.1 µg/d | CHD | phylloquinone ↔ menaquinone ↓ |
| 46 | cohort study | 16,057 women (49-70 y) | FFQ | phylloquinone intake: 211.7 ± 100.3 µg/d menaquinone intake: 29.1 ± 12.8 µg/d | CHD | phylloquinone ↔ menaquinone ↓ |

*****↑ high phylloquinone/ menaquinone intake was associated with increased CHD risk
↓ high phylloquinone/ menaquinone intake was associated with reduced CHD risk
↔ there was no association between phylloquinone/menaquinone intake and CHD risk

**Table 3:** Vitamin K intake and risk of stroke

| *Study* | *Study design* | *Participants* | *Measurement* | *Exposure/intervention* | *Endpoint* | *Result* |
|---|---|---|---|---|---|---|
| 44 | cohort study | 72,874 women (38-65 y) | semi quantitative FFQ | phylloquinone intake: 184 ± 106 µg/d. | stroke | phylloquinone ↔ |
| 45 | cohort study | 40,087 males, (40-75 y) | semi quantitative FFQ. | phylloquinone intake: 165 µg/d | stroke | phylloquinone ↔ |

*****↑ high phylloquinone intake was associated with increased stroke risk
↓ high phylloquinone intake was associated with reduced stroke risk
↔ there was no association between phylloquinone intake and stroke risk

**Conclusion**

Although animal experiments and other basic studies show compelling evidence linking vitamin K intake to a reduced coronary calcification and risk of CVD, the evidence from human observational and interventions studies are scarce and inconsistent. Observational studies have shown no associations between the intake of phylloquinone and arterial calcification, incidence of CHD or stroke. Nevertheless, results of intervention studies look promising as they showed improved vascular elasticity and reduced progression of coronary calcification after phylloquinone supplementation. The relative high doses of phylloquinone in the intervention studies or better absorption of phylloquinone supplements could explain these differences (12). For menaquinones intake, observational studies consistently show inverse associations of menaquinones intake with arterial calcification and CHD risk. These findings suggest that an adequate intake of menaquinones could be important for arterial calcification reduction and CHD prevention. However, these results should be confirmed by randomized controlled trials of menaquinones supplementation and coronary calcification.

Although the data from animal experiments and certain human observational and intervention studies are promising, the exact role of phylloquinone and menaquinones in the etiology of coronary calcification and cardiovascular disease in human remains to be established. Studies using biomarkers of vitamin K intake and status in relation to cardiovascular diseases and randomized controlled trials on vitamin K intake and coronary calcification are needed to further establish these relations

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# Part I

# Chapter 3

# Phylloquinone and vascular calcification risk

Based on:

Phylloquinone concentrations and the risk of vascular calcification in healthy women

*Submitted*

GW Dalmeijer

YT van der Schouw

SL Booth

PA de Jong

JWJ Beulens

**Abstract**

**Background:** Observational studies did not show an association between phylloquinone intake and coronary artery calcification (CAC). However, intervention studies show improved vascular elasticity and reduced progression of CAC in response to phylloquinone supplementation.

**Objective:** To investigate the association of phylloquinone concentrations with CAC and vascular calcification.

**Design:** In a prospective cohort of 508 postmenopausal women, plasma phylloquinone concentrations were measured by high-pressure liquid chromatography. Calcification was measured in the aortic valve, mitral valve, aortic arch, and coronary arteries by multi-detector computed tomography. CAC was present if Agatston score was >0 and calcification score was calculated as the sum of the calcified areas. Multivariate adjusted prevalence ratios (PR) and odds ratios (OR) were estimated using Poisson regression and multinomial logistic regression.

**Results:** After 8.5 years follow-up, 22% of the women had no calcification while 5% had calcification in all measured areas. Detectable phylloquinone concentrations (> 0 nmol/l) were associated with increased CAC compared to non-detectable phylloquinone concentrations (0 nmol/l) with a PR of 1.34 (95%CI: 1.01-1.77). When dividing women with detectable phylloquinone concentrations in low-detectable (>0-0.70 nmol/l) and high-detectable (>0.70 nmol/l) phylloquinone concentrations versus non-detectable phylloquinone concentrations, both were associated with increased CAC with a PR of 1.32 (95%CI: 0.99-1.76) and 1.36 (95%CI: 1.02-1.81). Phylloquinone concentrations were not associated with the number of calcified areas with an $OR_{(\geq 3 \text{ areas vs no calcifications})}$ of 1.60 (95%CI: 0.65-3.99 p=0.31).

**Conclusion:** Detectable phylloquinone concentrations are not associated with reduced vascular calcification, but seemed to be associated with an increased prevalence of CAC.

## Introduction

Vitamin K is a fat-soluble vitamin that occurs in two biologically active forms; vitamin $K_1$ (phylloquinone) and vitamin $K_2$ (menaquinone). Phylloquinone is present in green, leafy vegetables and certain vegetable oils (1), while menaquinone is mostly found in animal products like meat and fermented foods like cheese and buttermilk (2). Vitamin K acts as a cofactor for the enzyme gamma-glutamyl carboxylase, catalyzing the carboxylation of specific glutamic acid residues (Glu) to form gamma-carboxyglutamate (Gla) in vitamin K-dependent proteins. Matrix Gla protein (MGP) is a vitamin K-dependent protein and an inhibitor of vascular calcification (3). It has been proposed that vitamin K could therefore reduce coronary artery calcification (CAC) and risk of cardiovascular disease (CVD) through the carboxylation of MGP.

Results from observational studies investigating relations of vitamin K intake with CAC and risk of CVD are, however, inconsistent. For intake of menaquinones, observational studies consistently showed that high intakes are associated with reduced arterial calcification and CVD risk (4-6). However, for phylloquinone intake, observational studies do not show associations with CAC or incidence of CVD (4-9). Nevertheless, intervention studies showed improved vascular elasticity and reduced progression of coronary calcification following phylloquinone supplementation (10;11). This discrepancy could be explained by dose-differences between observational studies (mean phylloquinone intake of 115-257 µg/day) and intervention studies (supplements with 500 µg/day or 1 mg/day). Another explanation could be the low relative validity of phylloquinone intake as estimated by food frequency questionnaires (FFQ) (6). Circulating plasma phylloquinone concentrations may be a better measure for phylloquinone intake than phylloquinone intake measured by FFQ. Our hypothesis is that high phylloquinone concentrations is associated with less calcification. We therefore investigated the association of plasma phylloquinone concentrations with CAC and vascular calcification among 508 postmenopausal healthy women.

## Subjects and methods

*Study population*

We used data from a sample of 508 postmenopausal healthy women as detailed previously (12). In brief, these women were selected from participants of the PROSPECT cohort study, one of the two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition (EPIC). In PROSPECT, a total of 17,357 healthy breast cancer screening participants, aged 49-70 years, living in Utrecht and surrounding areas, were enrolled between 1993 and 1997. Between October 2002 and April 2004, 1,996 women were randomly selected from all 5,844 post-menopausal participants of the PROSPECT study who did not use contraceptives of hormone therapy. Of the 1,000 women who agreed to participate, a random selection of 573 underwent a multislice CT examination at a second visit between January and December 2004. There were no blood samples available for 56 women, for 8 women had no available calcification data and one woman used vitamin K antagonists. These 65 women were therefore excluded, leaving 508 women available for inclusion in the analyses. The study was approved by the Institutional Review Boards of the University Medical Center Utrecht and Tufts University, and written informed consent was obtained from all participants.

*Baseline measurements*

At baseline in 1993-1997, all participants filled in a general questionnaire containing questions on demographic characteristics, presence of chronic diseases, and risk factors for chronic diseases, such as hypertension, reproductive history, family history, smoking habits, drinking of alcohol and physical activity (13).

Systolic and diastolic blood pressure were measured twice using the left arm with the participants in sitting position after 10 min of rest with an automated and calibrated oscillomat (Bosch & Son, Jungingen, Germany) and the average value was used. Body height was measured to the nearest 0.5 cm with a wall mounted stadiometer (Lameris, Utrecht, The Netherlands). Body weight was measured in light indoor clothing without shoes to the nearest 0.5 kg with a floor scale (Seca, Atlanta, GA, USA). Body mass index (BMI) was calculated as weight divided by height squared ($kg/m^2$). Non fasting blood samples were taken to prepare citrated plasma and serum, which were subsampled and stored under liquid nitrogen at -196°C.

*Vascular calcification measurements*

In 2004, the participants underwent a multi-detector computed tomography (MDCT, Mx 8000 IDT 16, Philips Medical systems, Best, The Netherlands) as previously described (14). The amount of calcium in the coronary arteries was quantified on a separate workstation with software for calcium scoring (Heartbeat-CS, EBW, Philips Medical System, Best, The Netherlands). The Agatston (15) calcium score was obtained by multiplying the area by weighting factor that is dependent on the peak signal anywhere in the lesion. The score of individual lesions were added to obtain the Agatston calcium score for the entire coronary tree. Reproducibility was assessed by having 199 scans read by two independent observers and by having 58 women undergo a second scan within 3 months. In this study the inter-reader reproducibility and the inter-scan reproducibility were excellent with intra-class correlation coefficients greater than 0.95 (14).

The MDCT-scans were visually scored with regard to thoracic aortic, aortic valve and mitral valve calcification by a certificated radiologist with 10 years of experience in chest CT. The radiologist was blinded for participant characteristics and outcome status. Calcification of the heart valves was anatomically subdivided into calcification of the aortic valve leaflets (AVL) and the mitral valve leaflets (MVL). AVL and MLV calcification was graded as absent, mild (one leaflet affected), severe (2 or three leaflets affected). Aortic calcification was graded as absent, mild (≤3 foci), moderate (4–5 foci or 1 calcification extending over ≥3 slices) and severe (>5 foci or 1 calcification extending over ≥3 slices).

*Phylloquinone concentrations*

Plasma phylloquinoneconcentrations were measured by high-pressure liquid chromatography (16). Low and high control specimens had average values of 1.2 and 4.8 nmol/l with intra- plus inter-assay (total) coefficients of variation (CVs) of 14.5 and 8.2%, respectively.

*Data analyses*

Characteristics of the study population are presented as the mean (±SD). To handle missing data we used multiple imputations. We assumed that the missing data were at random. We generated 10 imputed datasets and used Rubin's rules to combine the estimates of the parameters (17).

Calcification was measured in the coronary arteries (continuous), aortic valve (none, mild and severe), mitral valve (none, mild and severe) and thoracic aorta (none, mild, moderate and severe) by multi-detector computed tomography. To combine these calcification scores we dichotomized each of the four areas into present or absent. Because of the continuous measurement of CAC we categorized this as calcification absent if Agatston score was <0 and present if Agatston score was ≥ 0. The total calcification score was calculated as the sum of all calcified areas (grades 0-4).

We first analyzed the association between phylloquinone concentrations and presence of CAC. We dichotomized phylloquinone concentrations in non-detectable phylloquinone concentrations (0 nmol/L) and detectable phylloquinone concentrations (> 0 nmol/L). In addition, women with a detectable phylloquinone concentrations were divided in two equal groups categorized as low-detectable (<0-0.70 nmol/L) and high-detectable (>0.70 nmol/L). Because of the high prevalence of CAC, an odds ratio will overestimate the effect size (18). We therefore used a modified Poisson regression model (18) to estimate prevalence ratios (PR) and 95% confidence interval (CI) of CAC, with non-detectable phylloquinone concentrations as the reference category. To test for linear trend, phylloquinone concentrations of each woman was replaced by median values of the phylloquinone status group (non-detectable, low-detectable, high-detectable) and included in the model as a continuous covariate.

Next, we analyzed the total calcification score. Due to the categorical outcome (0-4), we used multinomial logistic regression to estimate odds ratios (OR). The groups with one, two or ≥ three calcification areas were compared with the group without calcification in any area. In addition, we analyzed this association also for the areas aortic valve, mitral valve and thoracic aorta separately.

In model 1 the PRs or ORs were adjusted for follow-up time. In model 2 we additionally adjusted for age, smoking (pack years) and BMI. To explore the robustness of our associations, we adjusted for non-HDL cholesterol and hypertension in sensitivity analyses.

A two-tailed p-value of <0.05 was considered to be statistically significant. Statistical analyses were conducted using IBM SPSS (version 20 for Windows).

**Results**

Baseline characteristics of the study population dichotomized by detectable and non-detectable phylloquinone concentrations are presented in table 1. The mean (± SD) follow-up time was 8.5 (±1.3) years. Of these women, 42% had CAC, 22% had aortic valve calcification, 11% had mitral valve calcification and 62 % had aortic calcification. No calcification measures were available at baseline. Women with detectable phylloquinone concentrations (> 0 nmol/l) at baseline were more likely to be older and had a higher BMI, cholesterol ratio and phylloquinone intake but were less likely to be smokers. No significant differences were noted in baseline blood pressure, total cholesterol levels and energy or menaquinone intake between the two groups.

**Table 1:** Baseline characteristics of study participants by non-detectable and detectable plasma phylloquinone (vitamin $K_1$) concentrations, nmol/L data is presented as mean (±S.D.)

| | *N* | *Phylloquinone = 0 Mean (±S.D.) N=89* | *Phylloquinone >0 Mean (±S.D.) N=419* |
|---|---|---|---|
| Phylloquinone (nmol/L)[1] | 508 | 0.0 ± 0.0 | 1.08 ± 1.03 |
| Follow up time[1] | 508 | 8.2 ± 1.2 | 8.5 ± 1.3 |
| Age (years)[1] | 508 | 56.0 ± 4.6 | 57.5 ± 5.3 |
| BMI (kg/m$^2$)[1] | 508 | 24.3 ± 3.9 | 25.9 ± 4.0 |
| Waist hip ratio (cm)[1] | 506 | 0.77 ± 0.05 | 0.78 ± 0.05 |
| Smoking (pack years)[1] | 493 | 8.0 ± 11.5 | 5.5 ± 8.3 |
| | | | |
| Systolic blood pressure (mmHg) | 508 | 128 ± 20.9 | 131 ± 18.4 |
| Diastolic blood pressure (mmHg) | 508 | 78.7 ± 10.1 | 77.9 ± 9.3 |
| Total cholesterol (mmol/l) | 485 | 6.0 ± 1.0 | 6.2 ± 1.0 |
| HDL cholesterol (mmol/l)[1] | 484 | 1.64 ± 0.47 | 1.51 ± 0.40 |
| Cholesterol ratio[1] | 484 | 3.99 ± 1.45 | 4.41 ± 1.36 |
| | | | |
| Energy (kcal) | 507 | 1737 ± 426 | 1688 ± 372 |
| Vitamin C (mg/d)[2] | 507 | 122 ± 43 | 124 ± 48 |
| Vitamin K (µg/day)[1,2] | 507 | 241 ± 94 | 265 ± 97 |
| Phylloquinone (µg/day)[1,2] | 507 | 204 ± 94 | 230 ± 95 |
| Menaquinone (µg/day)[2] | 507 | 35 ± 13.8 | 35 ± 13.7 |

BMI, Body mass index; HDL, high density lipoprotein

[1]P<0.05 between non-detectable and detectable phylloquinone concentrations

[2] energy-adjusted intake

Detectable phylloquinone concentrations were associated with more CAC at follow-up compared to those with non-detectable phylloquinone concentrations with a prevalence ratio of 1.42 (95%CI: 1.7-1.87, p=0.01) in crude analyses. This association attenuated to a prevalence ratio of 1.34 (95%CI: 1.01-1.77, p=0.04) after adjustment

for follow-up time, age, smoking and BMI. Adding non-HDL-ratio and hypertension into the model did not materially change the results (prevalence ratio: 1.33; 95%CI: 1.01-1.75, p=0.04).

Table 2 shows the association of detectable phylloquinone concentrations divided in two equal groups versus non-detectable phylloquinone with follow-up CAC. The low detectable phylloquinone group was associated with increased CAC with a prevalence ratio of 1.32 (95%CI: 0.99-1.76, p=0.06), and the high detectable phylloquinone group with a prevalence ratio of 1.36 (95%CI: 1.02-1.81, p=0.04) (p for trend 0.07) after full adjustment. Similar, but slightly attenuated results were observed additionally adjusting for non-HDL cholesterol and hypertension, with prevalence ratios of 1.30 (95%CI: 0.98-1.71, p=0.09) and 1.29 (95%CI: 0.97-1.73, p=0.07) (p for trend 0.13) for the two detectable phylloquinone concentration groups, respectively.

Baseline phylloquinone concentrations were not associated with the number of calcified areas at follow-up with an OR (≥3 areas vs no calcification) of 1.60 (95%CI: 0.65-3.99 p=0.31) after adjustment. This association attenuated to an OR ( ≥3 areas vs no calcification ) of 1.46 (95%CI: 0.57-3.77, p=0.43) after including non-HDL cholesterol and hypertension into the model (table 3). Phylloquinone concentrations were not associated with aortic valve, mitral valve and thoracic aorta calcification (data not shown).

**Table 2:** Relative risks (95%CI) of phylloquinone concentrations with coronary artery calcification among 508 post-menopausal women

| | *Phylloquinone 0 nmol/L* | *Phylloquinone >0-0.70 nmol/L* | *Phylloquinone >0.70 nmol/L* | *P for trend* |
|---|---|---|---|---|
| n | 89 | 205 | 219 | |
| Crude | 1 | 1.37 (1.02-1.84) | 1.46 (1.09-1.95) | 0.02 |
| Model 1[1] | 1 | 1.35 (1.00-1.81) | 1.43 (1.07; 1.92) | 0.02 |
| Model 2[2] | 1 | 1.32 (0.99-1.76) | 1.36 (1.02-1.81) | 0.07 |

[1]Adjusted for follow-up time

[2]Adjusted for age, smoking habits, BMI, follow-up time

**Table 3:** Odds Ratios (95%-CI) of phylloquinone status (> 0 nmol/L) with calcification among 508 post-menopausal women

| | *calcified areas* | | | |
|---|---|---|---|---|
| | *0* | *1* | *2* | *≥3* |
| | | *OR (95%CI)* | *OR (95%CI)* | *OR (95%CI)* |
| n | 112 | 160 | 154 | 82 |
| Crude | 1 | 0.97 (0.54-1.75) | 1.83 (0.95-3.51) | 1.96 (0.88-4.37) |
| Model 1[1] | 1 | 0.95 (0.52-1.71) | 1.71 (0.89-3.30) | 1.73 (0.77-3.91) |
| Model 2[2] | 1 | 1.01 (0.54-1.88) | 1.70 (0.82-3.54) | 1.60 (0.65-3.99) |

[1]Adjusted for follow-up time

[2]Adjusted for age, smoke habits, BMI, follow-up time

**Discussion**

In contrast with our hypothesis, we could not detect that detectable phylloquinone concentrations were associated with a reduced risk of vascular calcification. Instead, detectable concentrations seemed to be associated with increased prevalence of CAC.

Our hypothesis was based on two intervention studies showing improved vascular elasticity and reduced progression of coronary calcification after phylloquinone supplementation (10;11). Unexpectedly, we found higher prevalence of CAC in participants with detectable phylloquinone concentrations in the present study. However, in a previous study in the same study population, after full-adjustment a non-significant trend was observed towards and increased risk with a RR of 1.17 (95%CI: 0.96-1.42; $p_{trend}$=0.11) for the highest versus lowest quartile of phylloquinone intake. This is in concordance with the results we found in the present study. Therefore, our assumption that our results on phylloquinone intake were driven by the low relative validity of phylloquinone intake as estimated by the FFQ might be incorrect as we now observed the same direction of effect in plasma measurements.

These results are inconsistent with previous intervention studies, but the phylloquinone dosages that were used (10;11) were at least 2-4 times higher than the mean habitual dietary phylloquinone intake in observational studies. It is possible that the protective effect of phylloquinone on CAC can only be reached with supplementary dosages and not with dietary intake. Alternatively, participants with CAC at baseline may have had characteristics or risk factors that influenced overall nutritional status, which are reflected in the plasma phylloquinone concentrations (19). A possible explanation for this unexpected observation is that plasma phylloquinone may be a marker of an unmeasured biochemical or genetic risk factor for calcification. For example, the minor allele of VKORC1 rs8050894 (G) and the major allele of VKORC1 rs7294 (G) are associated with higher phylloquinone concentrations (20). These alleles are linked and have been reported to be part of haplotype sequences that are associated with decreased warfarin dose requirements (21;22). Furthermore, VKORC1 haplotypes that reduce the activity of the VKORC1 enzyme have been shown to be associated with a significant higher risk of vascular calcification in rats (23) and aortic calcification in humans (24). Hence, it is possible that similar polymorphisms in genes involved in the phylloquinone metabolism may result in

higher phylloquinone status due to reduced phylloquinone recycling or metabolism. Further, it is also possible that higher phylloquinone concentrations are caused by disturbed phylloquinone transport or cellular phylloquinone uptake. However, these potential mechanisms have not been investigated yet.

Strengths of this study include the prospective study design, measurement of vascular calcification and plasma phylloquinone concentrations in healthy women. Nevertheless our study has certain limitations to consider. Circulating phylloquinone concentrations are thought to reflect overall status but are highly correlated with triglycerides, due to it being transported on triglyceride lipoproteins (25). However, data about triglyceride concentrations were only available in a subsample of 63 women. In this group we adjusted for triglyceride concentrations but this did not change the results. Since we had so little data on triglyceride concentrations, we used non-HDL cholesterol as a proxy, since this is correlated with triglyceride concentrations (26). Adjusting for non-HDL in an additional analysis did not materially change our results. Furthermore, phylloquinone concentrations reflect recent intakes (27;28), since half-life time of phylloquinone is 1-2 hours. Although adjusting for time since last meal did not affect our results, no information was available about the content of the last meal before blood sampling. Therefore it could be that the use of a single plasma phylloquinone measure as a marker for long-term vitamin K status is imperfect. Unfortunately, this study did not measure baseline measures of vascular calcification which precludes evaluation of the predictive value of phylloquinone concentrations on progression of vascular calcification.

Taken together, this study shows that a normal phylloquinone status is not associated with reduced vascular calcification, but instead seemed to be associated with higher CAC prevalence. Further research is required to confirm our findings and to investigate possible underlying mechanisms for these associations.

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# Chapter 4

# Dairy intake and coronary heart disease or stroke

Based on:

Dairy intake and coronary heart disease or stroke- a population-based cohort study

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GW Dalmeijer

EA Struijk

YT van der Schouw

SS Soedamah-Muthu

WMM Verschuren

JMA Boer

JM Geleijnse

JWJ Beulens

**Abstract**

**Aim**: This study aimed to investigate the relationship between total dairy intake and dairy subtypes (high-fat dairy, low-fat dairy, milk and milk products, cheese and fermented dairy) with incident coronary heart disease (CHD) and stroke.

**Methods**: EPIC-NL is a prospective cohort study among 33,625 Dutch men and women. At baseline (1993-1997), dairy intake was measured with a validated food frequency questionnaire (FFQ). The incidence of both fatal and non-fatal CHD and stroke was obtained by linkage to the national registers.

**Results:** During 13 years follow-up, 1648 cases of CHD and 531 cases of stroke were documented. Total dairy intake was not significantly associated with risk of CHD (hazard ratio (HR) per standard deviation (SD) increase=0.99; 95%CI: 0.94–1.05) or stroke (0.95; 95%CI: 0.85–1.05) adjusted for lifestyle and dietary factors. None of the dairy subtypes were to CHD, while only fermented dairy tended to be associated (p=0.07) with a lower risk of stroke (0.92; 95% CI: 0.83-1.01). Hypertension appeared to modify the association of total and low-fat dairy with CHD (p *interaction*<0.02). Among participants without hypertension, but not among hypertensive participants, total (0.92; 95% CI: 0.85- 1.02) and low-fat (0.94; 95% CI 0.87- 1.02) dairy tended to be associated with a lower risk of CHD.

**Conclusion**: Our results provide no evidence that dairy products are associated with risk of CHD or stroke. High intakes of total and low-fat dairy may be associated with a lower risk of CHD among participants without hypertension, while fermented dairy could be associated with a reduced risk of stroke.

## Introduction

The intake of dairy products was thought to be associated with an increased risk of cardiovascular diseases (CVD) due to its relatively high content of saturated fat. Dietary recommendations target a diet limited in saturated fat, trans fat and cholesterol and therefore advise consuming fat-free or low-fat dairy products (1). Such recommendations have been based on the positive linear relationship among dietary saturated fat, LDL cholesterol, and CVD risk (2).

However, a recent dose-response meta-analysis of 17 prospective studies investigated the relation of total dairy, milk, and low-fat and high-fat dairy with risk of CVD or all-cause mortality. The study indicated that milk intake was modestly inversely associated with CVD risk (3). For coronary heart disease (CHD), stroke and all-cause mortality, no significant association with milk intake was observed. The authors also concluded that a limited number of studies are available to draw firm conclusions for specific subtypes of dairy like low- or high-fat dairy and cheese, in particular for stroke.

Meanwhile, different kinds of dairy products with varying nutrient compositions may have different effects on CHD or stroke risk. Minerals, protein and vitamins from dairy products can exert blood pressure- or cholesterol lowering effects, while saturated fat is associated with an increased risk of CHD (4). Indeed, one study showed that in particular the ratio of high-fat dairy to low-fat dairy products is associated with an increased risk of CHD (5).

In Sweden and The Netherlands large quantities and varieties of dairy products are consumed, compared with other European countries (6). Therefore the Dutch population provides an excellent opportunity to investigate the relation of dairy consumption and its subtypes in relation to CHD and stroke. The aim of this study is therefore to investigate the relationship of dairy intake, including the specific dairy subtypes high-fat dairy, low-fat dairy, milk and milk products, cheese and fermented dairy, with CHD and stroke in a large prospective cohort in the Netherlands.

**Materials and Methods**

*Study population and design*

The EPIC-NL cohort is the Dutch contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC) and consists of the Prospect-EPIC and MORGEN-EPIC cohorts (7). The Prospect-EPIC study includes 17.357 women aged 49-70 years living in Utrecht and vicinity who participated in the nationwide Dutch breast cancer screening program between 1993 and 1997. The MORGEN-EPIC cohort consists of 22,654 men (n=10,260) and women (n=12,349) aged 21-64 years selected from random samples of the Dutch population in three different towns. Participants were recruited in both studies from 1993 to 1997. Both cohorts used standardized questionnaires and followed identical protocols in the collection of biological samples (7). At baseline, a general and a food-frequency questionnaire (FFQ) were administered, and a medical examination was performed for blood pressure measurements, anthropometry, and blood sampling. All participants provided informed consent before study inclusion. The study complies with the Declaration of Helsinki and was approved by the institutional board of the University Medical Center Utrecht (Prospect) and the Medical Ethical Committee of TNO Nutrition and Food Research (MORGEN).

From the total cohort (n=40,011) subjects who did not give permission for linkage with vital status registries were excluded (n=2717). Additionally, participants without information on dietary intake (n=172) or with implausibly high or low scores for total food intake (outside the range of 800-4,200 kcal/d for men and 500-3,500 kcal/d for women) (n=448) were excluded. Furthermore, subjects with known cardiovascular disease (n=1099), cancer (n=1485) or diabetes (n=465) at baseline were excluded, leaving 33,625 participants for the present analysis.

*Dietary assessment*

Daily nutritional intakes were obtained from a self administered FFQ containing questions on the usual frequency of consumption of 79 main food items during the year preceding enrollment. This questionnaire allows estimation of the average daily consumption of 178 foods. A registered dietician checked the FFQ for inconsistencies, which were resolved by contacting the participant. The validity of the FFQ was assessed against 12 monthly 24-h recalls over a one-year period among 212 men and women. Spearman correlations were good for milk and milk products

(r=0.69 for men, r=0.77 for women) and moderate for cheese (r=0.56 and r=0.32, respectively)(8;9).

Total dairy included all dairy food products except for butter and ice cream. Milk and milk products included all kinds of milk, yogurt, coffee creamers, curd, pudding, porridge, custard, and whipping cream. Cheese included all types of cheese except for curd. Low-fat dairy is defined as milk and milk products with a fat content <2 g/100 g (skimmed or semi-skimmed milk products) or cheese with a fat content <20 g/100 g. High-fat dairy is defined as milk and milk products with a fat content ≥2 g/100 g (whole milk products) or cheese products with a fat content ≥20 g/100 g. Fermented dairy included buttermilk, yogurts and cheese.

*Outcome assessment*

Morbidity follow-up data on coronary heart disease (CHD) events and stroke events were obtained from the Dutch Centre for Health Care Information which holds a standardized computerized register of hospital discharge diagnoses. All diagnoses were coded according to the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9). Follow-up was complete until the first of January 2008. The database was linked to the cohort on the basis of birth date, gender, postal code and general practitioner with a validated probabilistic method (10). Information on vital status was obtained through linkage with the municipal registries. Causes of death were collected from Statistics Netherlands. Endpoints for the present analysis were CHD (ICD-9; 410-414, 427.5, 798.1, 798.2, 798.9) and stroke (ICD-9; 430-434, 436), whichever came first. These endpoints included both fatal and non-fatal cases of CHD and stroke. In a secondary analysis, we differentiated to non-fatal CHD obtained from hospital discharge diagnoses and CHD mortality based on causes of death. Finally, to compare our results with the meta-analysis (3), we used an endpoint of cardiovascular disease defined as a broad range of fatal and non-fatal vascular diseases including CHD, stroke, peripheral artery disease, congestive heart failure and pulmonary embolism (ICD-9: 410-414, 427.5, 428, 415.1, 443.9, 430-438, 440-442, 444, 798.1, 798.2, 798.9). In a subsample of our cohort, cases of CHD and acute myocardial infarction obtained from hospital discharge diagnoses were verified against hospital records. This showed that 91% of CHD events and 97% of acute myocardial infarction could be confirmed (11).

*Other measurements*

The general questionnaire contained questions on demographic characteristics, the presence of chronic diseases, and risk factors for chronic diseases. Smoking was categorized into current, past, and never smoker. Level of education was categorized as low (primary education up to those completing advanced elementary education), average (intermediate vocational education and higher general secondary education) or high (higher vocational education and university). Physical activity was assessed using a questionnaire validated in an elderly population and categorized according to the Cambridge Physical Activity Index (12). Because it was not possible to calculate a physical activity score for 14% of all participants missing scores were imputed using single linear regression modeling (SPSS MVA procedure). During the baseline physical examination screening, systolic and diastolic blood pressure measurements were performed twice in the supine position on the right arm using a Boso Oscillomat (Bosch & Son, Jungingen, Germany) (Prospect-EPIC) or on the left arm using a random zero sphygmomanometer (MORGEN-EPIC), from which the mean was taken. Hypertension was considered present when one or more of the following criteria were met: systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, self-reported antihypertensive medication use, or self-report of physician-diagnosed hypertension. Height, and weight were measured, and BMI was calculated. All measurements were performed according to standard operating procedures. In a 6.5% random sample of the baseline cohort HDL-cholesterol and LDL-cholesterol were measured using a homogeneous assay with enzymatic endpoint. These assays, including the haemolytic, icteric and lipemic indices (absorbance), were all performed on an autoanalyser (LX20, Beckman Coulter, Mijndrecht, the Netherlands).

*Data analysis*

Participant characteristics are presented as medians, means with standard deviations or percentages. Intakes of total dairy and dairy subtypes were adjusted for total energy intake according to the residual method (13). Total dairy and its subtypes were evaluated as continuous variables per standard deviation of the mean intake which is 265 g/d for total dairy, 34 g/d for high-fat dairy, 216 g/d for low-fat dairy, 265 g/d for milk and milk products, 13 g/d for cheese and 123 g/d for fermented dairy. Cox regression was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) of the relation between total dairy intake and both fatal

and non-fatal events of CHD or stroke. The analyses were repeated for different types of dairy, i.e. high-fat dairy, low-fat dairy, milk and milk products, cheese, fermented dairy and the ratio of high-fat to low-fat dairy. The presence of nonlinear associations of total dairy and its subtypes were explored by including the quadratic term of these intakes (per SD increase) in the model with the linear term. No evidence for nonlinear associations was found (with P values for quadratic terms ranging from 0.10 to 0.96).

All analyses were stratified for cohort. The duration of follow-up was calculated as the interval between date of study entry and the occurrence of a cardiovascular event, death, loss to follow-up, or January 1, 2008, whichever came first. The estimates were adjusted for age, sex and total energy intake (model 1). The second model additionally included smoking, BMI, education and physical activity. Further adjustment was made for the intake of coffee, ethanol, fruit, vegetables, fish, meat and bread (model 3).

The interaction between total dairy and possible effect modifiers, sex, age, BMI and hypertension was tested by including interaction terms into the model. In sensitivity analyses, we excluded cases occurring during the first 2 years and those participants censored in the first 2 years. To explore whether observed relations were explained by blood pressure, calcium or potassium, we adjusted for these variables in model 3. The proportionality assumption was checked visually by means of log minus log plots with no deviations detected. In the random sample (n=2,050) the Pearson correlation between total cholesterol or HDL-cholesterol and total dairy or its subtypes were tested. Two-sided P-values below 0.05 were considered to be statistically significant. All statistical analyses were conducted using SAS 9.2 (SAS Institute, Cary, US).

**Results**

During a mean follow-up of 13 years 1,648 incident cases of CHD, including 211 CHD deaths, and 531 stroke cases, including 128 stroke deaths, were documented. Table 1 shows the baseline characteristics of the study population.

**Table 1:** Mean (± SD) baseline characteristics and dietary intakes of 33,625 men and women from the EPIC-NL cohort

| *Variable* | |
|---|---|
| Follow up (years) | 13.1 |
| Gender (% male) | 25.5 |
| Age at recruitment (years) | 49.0 ± 11.9 |
| BMI ( kg/m$^2$) | 25.6 ± 4.0 |
| Physically active (CPAI)(%) | 34.6 |
| Systolic BP (mmHg) | 126.1 ± 18.7 |
| Diastolic BP (mmHg) | 77.8 ± 10.6 |
| Hypertension (%) | 36.3 |
| High education (%) | 20.6 |
| Current smoker (%) | 30.1 |
| Total energy (kcal/d) | 2041.0 ± 583.6 |
| Dietary intakes (g/d) [1,2] | |
| Total dairy | 392 (234 – 574) |
| High-fat dairy | 46 (30 – 67) |
| Low-fat dairy | 230 (106 – 385) |
| Milk and milk products | 356 (198 – 538) |
| Cheese | 16.1 (9.7 – 25.8) |
| Fermented dairy | 85 (45 – 176) |
| Fruit | 240 (136 – 354) |
| Vegetables | 130 (101-167) |
| Fish | 7.6 (3.3 – 14.9) |
| Meat | 105 (65 – 139) |
| Bread | 134 (99-176) |
| Coffee | 500 (375 – 750) |
| Tea | 250 (54 – 500) |
| Alcohol | 5.1 (0.7 - 15.8) |

CPAI; Cambridge Physical Acitivity Index

[1] All dietary variables were adjusted for total energy

[2] Intake of food groups and beverages is presented as median (interquartile range)

The median energy-adjusted total dairy intake was 392 g/d (quartile 1= 221 g/d; quartile 3=575 g/d). Low-fat dairy (median: 230 g/d) accounted for the largest part of dairy intake, compared with high-fat dairy (median: 46 g/d). The median daily intakes of milk and milk products, fermented dairy and cheese were 356 g/d, 85 g/d and 16 g/d, respectively. Participants with a high dairy intake were more active,

smoked less often, drank less alcohol and consumed more fruit and vegetables (data not shown).

Adjusted for age, gender and energy intake, dairy intake was not associated with risk of CHD (HR per SD: 0.97; 95%-CI: 0.92-1.02). Adjustment for lifestyle and dietary factors further attenuated the association (HR: 0.99; 95%CI: 0.94 - 1.05). None of the dairy subtypes were related to CHD risk, although for high-fat dairy we observed a slightly, but non-significantly decreased risk of CHD (HR: 0.97; 95%-CI: 0.92- 1.02) (table 2). Specifying these analyses to non-fatal CHD events and CHD mortality, we observed a borderline significant (p=0.10) inverse relation with a HR of 0.96 (0.90 – 1.01) per SD increase for non-fatal events, while a non-significant increased risk was observed for CHD mortality ($HR_{SD}$= 1.05; 0.92 – 1.20).

In additional analyses total dairy intake was categorized in quartiles. The association remains similar. After full-adjustment there was no association with CHD risk (HR 0.91; 0.91-1.06 for the highest versus lowest quartile).

Associations of dairy intake and risk of stroke were generally stronger than for CHD (table 3). Total dairy consumption was associated with a lower risk of stroke adjusted for age, gender and total energy with a HR of 0.90 (0.82-0.99) per SD increase. Fermented dairy (0.86; 0.78-0.95) and low-fat dairy (0.89; 0.81- 0.98) were also associated with a lower risk of stroke in model 1. However, after full adjustment, these associations attenuated to non-significant with a HR of 0.95 (0.85-1.05) for total dairy. Only fermented dairy tended to be associated (p=0.07) with a lower risk of stroke with a HR of 0.92 (0.83-1.01) per SD increase. Finally, when investigating the relation of milk and milk products with CVD, we observed a similar non-significant association as for CHD and stroke separately (HR=1.01; 95%-CI: 0.96-1.05).

We did not observe interactions between total dairy intake and sex, age and BMI. Only for presence of hypertension, we observed an interaction (p<0.02) with total dairy. Among participants without hypertension, total dairy (0.92; 0.85- 1.02) tended to be associated (p=0.07) with a lower risk of CHD, while this was not apparent for those with hypertension. Similar results were observed for milk and milk products (0.92; 0.85- 1.02) and low-fat dairy (0.94; 0.87- 1.02) among normotensive participants.

Adjusting all associations for blood pressure or excluding cases occurring in the first 2 years did not change our results (data not shown). The associations of total dairy (HR:

0.94; 0.78-1.13) and milk and milk products (HR: 0.95; 0.81- 1.11) with CHD among normotensive participants attenuated to non-significant when we adjusted for calcium and potassium. Including calcium and potassium almost completely explained the association of low-fat dairy (1.00; 0.87-1.15) with CHD among normotensive participants. In the random sample we found an association between high-fat dairy and HDL-cholesterol (Pearson *r* = 0.043, p=0.049). There were no others associations found between dairy intake and total cholesterol, or HDL cholesterol levels.

**Table 2:** Univariable and adjusted hazard ratios (95%CI) for the association of total dairy intake and dairy subtypes* with incident of (fatal or nonfatal) coronary heart disease among 33,625 subjects of the EPIC-NL study

| | *CHD (n=1,648)* | | |
|---|---|---|---|
| | Model 1[1] | Model 2[2] | Model 3[3] |
| Total dairy intake | 0.97 (0.92 – 1.02) | 1.00 (0.95 – 1.05) | 0.99 (0.94 – 1.05) |
| Milk and milk products | 0.97 (0.92 – 1.02) | 1.00 (0.95 – 1.05) | 0.99 (0.94 – 1.05) |
| Fermented dairy | 0.95 (0.91 – 1.00) | 0.99 (0.95 – 1.05) | 1.00 (0.95 – 1.05) |
| Cheese | 0.96 (0.91 – 1.01) | 0.98 (0.93 – 1.03) | 0.99 (0.94 – 1.04) |
| High-fat dairy | 0.96 (0.92 – 1.01) | 0.98 (0.93 – 1.03) | 0.97 (0.92 – 1.02) |
| Low-fat dairy | 0.98 (0.93 – 1.03) | 1.01 (0.96 – 1.06) | 1.01 (0.96 – 1.06) |
| Ratio high-fat to low-fat dairy | 1.01 (0.97 – 1.05) | 1.02 (0.98 – 1.06) | 1.02 (0.98 – 1.06) |

*All HRs are expressed per SD of the mean in g/d.
[1]Adjusted for gender, age and total energy intake.
[2]Adjusted for model 1 and physical activity, smoking, education and BMI.
[3]Adjusted for model 2 and intake of ethanol, coffee, fruit, vegetables, fish, meat and bread.

**Table 3:** Univariable and adjusted hazard ratios (95%CI) for the association of total dairy intake and dairy subtypes* with incident (fatal or nonfatal) stroke among 33,625 subjects of the EPIC-NL study

| | *Stroke (n=531)* | | |
|---|---|---|---|
| | Model 1[1] | Model 2[2] | Model 3[3] |
| Total dairy intake | 0.90 (0.82 – 0.99) | 0.93 (0.84 – 1.02) | 0.95 (0.85 – 1.05) |
| Milk and milk products | 0.91 (0.83 – 0.99) | 0.93 (0.85 – 1.02) | 0.95 (0.86 – 1.05) |
| Fermented dairy | 0.86 (0.78 – 0.95) | 0.90 (0.81 – 0.98) | 0.92 (0.83 – 1.01) |
| Cheese | 0.94 (0.86 – 1.02) | 0.96 (0.88 – 1.05) | 0.96 (0.88 – 1.06) |
| High-fat dairy | 0.98 (0.90 – 1.07) | 0.99 (0.91 – 1.07) | 0.99 (0.91 – 1.09) |
| Low-fat dairy | 0.89 (0.81 – 0.98) | 0.92 (0.83 – 1.01) | 0.94 (0.85 – 1.03) |
| Ratio high-fat to low-fat | 0.98 (0.93 – 1.03) | 0.98 (0.93 – 1.04) | 0.98 (0.93 – 1.04) |

*All HRs are expressed per SD of the mean in g/d.
[1]Adjusted for gender, age and total energy intake.
[2]Adjusted for model 1 and physical activity, smoking, education and BMI.
[3]Adjusted for model 2 and intake of ethanol, coffee, fruit, vegetables, fish, meat and bread.

**Discussion**

In this large cohort of 33,625 Dutch men and women, total dairy consumption was not associated with risk of CHD or stroke. However, among participants without hypertension, total dairy, milk and milk products and low-fat dairy consumption tended to be associated with a reduced risk of CHD. Fermented dairy tended to be associated with a reduced risk of stroke.

Strengths of this study include its prospective design with over 10 years of follow-up and its large sample size. However, certain limitations need to be addressed. Firstly, we relied on self-reported intakes of dairy using an FFQ. This FFQ was validated against 12 monthly 24-h recalls in a population of 121 subjects(9). This study showed good reproducibility (r= 067-0.85) and good relative validity for milk and milk products (r=0.69 for men, r=0.77 for women), but lower relative validity for cheese intake (r=0.56 and r=0.32 respectively). This may have diluted the relation of cheese intake with CHD or stroke risk. Since dairy intake was assessed at baseline only, the effects of participants subsequently changing their pattern of dairy consumption is uncertain. However, we excluded participants with prevalent diseases and, in a sensitivity analysis, cases obtained in the first 2 years who are most likely to alter their dietary habits with similar results. Such reverse causation is therefore unlikely to influence our results. Finally, as in any observational study, observed associations could be due to differences in other factors than dairy consumption. Despite adjustment for a large range of possible confounders, we cannot exclude unknown or unmeasured confounding.

The results from this study provided no evidence that total dairy or milk and milk products are associated with risk of either CHD or stroke. These findings are in line with a recent meta-analysis, that showed no significant association between total dairy or milk and milk products with risk of CHD (1.00; 0.96- 1.04) or stroke (0.87; 0.72-1.07) (3). Recently, the Netherlands Cohort Study could not detect an association between dairy products and all-cause or CVD mortality as well (14). Only milk and milk products were inversely associated with risk of CVD in the meta-analysis with a HR of 0.94 (95%-CI: 0.89- 0.99), an endpoint not primarily included in this study. However, when investigating the association of milk and milk products with a broad range of CVD in an additional analysis, we observed a similar non-significant association as we did for CHD and stroke. The previously reported reduced CVD risk

for milk and milk products (3) seems to be mainly driven by effects of dairy consumption on stroke, since the association of milk intake with stroke was stronger than with CHD. Albeit not significant, the association of total dairy and milk and milk products with stroke in our study was also stronger than for CHD.

Distinguishing between high-fat and low-fat dairy, we could not detect significant associations with CHD or stroke as well, consistent with most previous studies (3). Only the study by Hu et al. observed a lower risk of CHD with high intakes of low-fat dairy (5). Surprisingly, in our study, only high-fat dairy tended to be associated with a lower risk of CHD events, while it was associated with a non-significantly higher risk of CHD mortality. This suggests a specific relation of high-fat dairy with fatal CHD that could perhaps be explained by the high saturated fat content of these dairy products. A recent meta-analysis indeed showed similar results for high saturated fat intakes with an increased risk of fatal CHD (RR=1.32), while an RR of 0.99 was shown for total CHD (15;16).

Presence of hypertension appeared to modify the relation of total dairy, milk and milk products and low-fat dairy with risk of CHD. Only among participants without hypertension, total dairy, milk and milk products and low-fat dairy were associated with an approximately 8% reduced risk of CHD per 200-250 g/day, while no association was observed among participants with hypertension. These associations could be explained by effects of dairy consumption on blood pressure, since low-fat dairy intake is associated with reduced blood pressure (relative risk 0.87; 95%-CI 0.74-0.95) (17). However, adjusting the associations between dairy and CHD for systolic and diastolic blood pressure did not alter our findings. Moreover, blood pressure is more strongly related to stroke than CHD (18). Therefore, risk factors more strongly related to CHD like blood lipids could better explain our results. However, adjusting for baseline total- and HDL-cholesterol in part of cohort did not affect our results (data not shown). Minerals from dairy products could also be involved, since adjusting for potassium and calcium explained part of the association between dairy and CHD. However, this would then have to be driven by effects on other risk factors than blood pressure or blood lipids like inflammatory factors (19). Finally, residual confounding could be involved. The DASH-diet was reported to be more effective for participants with high sodium intakes (20). Possibly, hypertensive participants in our cohort already slightly reduced their sodium intake, while normotensive

participants did not. Unfortunately, a FFQ is not a valid tool to estimate sodium intake and we can therefore not address this effect.

We observed a borderline significant inverse association between intake of fermented dairy and risk of stroke. Larsson et al. investigated associations between fermented dairy products and risk of stroke and observed an inverse relation between cheese consumption and cerebral infarction, but not for other fermented dairy foods like yogurt or buttermilk (21). Consistently, Goldbohm et al. observed an inverse relation of fermented full-fat milk with all-cause mortality and non-significantly with stroke mortality (14). Altogether, these studies suggest an inverse relation between fermented dairy products and risk of stroke. Blood pressure- (22) and cholesterol- (23) lowering effects of fermented dairy could explain these relations. Specific tripeptides originating from milk fermentation, for example, have been suggested to reduce blood pressure (24), but a recent large randomized trial failed to confirm these results (25). Another explanation could be the effect of vitamin $K_2$ that is mainly present in fermented dairy products. Vitamin $K_2$ has previously shown to reduce vascular calcification and subsequent risk of CHD in two prospective cohorts (26;27), but the relation between vitamin $K_2$ and risk of stroke has not been investigated to date. Further research on the association between fermented dairy products and risk of stroke is therefore warranted.

In conclusion, our results provide no evidence that dairy products are associated with risk of CHD or stroke in a generally healthy Dutch population. Among participants without hypertension, high intakes of total and low-fat dairy could be associated with a lower risk of CHD, while fermented dairy may be associated with a reduced risk of stroke.

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# Chapter 5

# Vitamin K supplementation and MGP species

Based on:

The effect of menaquinone-7 supplementation on circulating species of matrix Gla protein

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GW Dalmeijer

YT van der Schouw

EJ Magdeleyns

N Ahmed

C Vermeer

JWJ Beulens

**Abstract**

**Objective:** To investigate whether menaquinone-7 (MK-7) supplementation increases carboxylation of MGP.

**Design:** A randomized, double-blind, placebo-controlled trial was performed. Sixty participants (40-65 y) were randomly allocated to supplementation of 180 µg/d, 360 µg/d of MK-7 or placebo during 12 weeks. At baseline, after 4 and 12 weeks, desphospho-uncarboxylated MGP (dp-ucMGP), desphospho-carboxylated MGP (dp-cMGP) and total uncarboxylated MGP (t-ucMGP) were measured by ELISA techniques. Furthermore, the ratio of uncarboxylated osteocalcin (ucOC) to carboxylated osteocalcin (cOC) was used as proxy of vitamin K status and various cardiovascular risk factors were measured.

**Results:** Dp-ucMGP decreased significantly and dose-dependently in the 180 µg and 360 µg MK-7 supplementation groups (*P* time*treatment <0.001) after 12 weeks, by 31% and 46% respectively, while dp-ucMGP levels remained unchanged after placebo treatment. The osteocalcin ratio also decreased significantly after 12-week supplementation with 180 µg (60%) and 360 µg (74%) MK-7 (*P* time*treatment <0.001), while levels remained unchanged after placebo treatment. These results indicate improved vitamin K status and good compliance to the study treatment. Changes over time of dp-cMGP (p=0.42) and t-ucMGP (p=0.23) levels did not differ between treatment arms. Other cardiovascular risk factors did not differ between treatments arms

**Conclusions:** Menaquinone supplementation dose-dependently decreases dp-ucMGP concentrations, but does not affect other MGP species. Dp-ucMGP may serve as a non-invasive marker of vitamin K status.

## Introduction

Vitamin K is a fat-soluble vitamin that occurs in two biologically active forms; phylloquinone (vitamin $K_1$) and menaquinones (vitamin $K_2$). The major dietary source of vitamin K is phylloquinone, found in leafy green vegetables and vegetable oils (1;2). The richest dietary sources of menaquinones are meat, eggs and fermented dairy like cheese and curd (3).

Vitamin K functions as a cofactor for the enzyme gamma-glutamyl carboxylase, catalyzing the gamma-glutamyl carboxylation of certain glutamic acid residues (Gla) in proteins (4). These vitamin K-dependent proteins include hepatic-coagulation factors like prothrombin (4), but also some extra-hepatic Gla-proteins in bone (osteocalcin (OC)) and the vessel wall (matrix Gla-protein (MGP)). MGP is a powerful inhibitor of vascular calcification (5). Coronary calcification is a strong, independent predictor of coronary events (6). The importance of MGP for vascular health was demonstrated in MGP-deficient mice, who all died of massive arterial calcification within 6-8 weeks after birth (7).

Observational studies have shown that high dietary menaquinone intake is associated with reduced risk of coronary vascular disease (CVD) and coronary artery calcification (8-11). This association may be explained by carboxylation of MGP by menaquinones. Human studies investigating the association between circulating MGP levels and CVD, however, showed inconsistent results (5-7). Inability of the MGP assays to discriminate between different MGP species might explain these inconsistencies. MGP exists as various species, which differ in their state of phosphorylation and/or carboxylation: phosphorylated (pMGP), non-phosphorylated (desphospho-MGP, dpMGP), carboxylated (cMGP) or uncarboxylated (ucMGP). Development of assays to measure circulating MGP species enabled the investigation thereof in the circulation (12). Cross-sectional studies suggested that low dephospho-uncarboxylated MGP (dp-ucMGP) levels were associated with high vitamin K status among hypertensive patients and older people (60-80 years)(13;14). Consistently, a randomized controlled trial showed a significant reduction in dp-ucMGP levels after supplementation with 500 µg/d of phylloquinone after three years (14). A 6-week randomized non—placebo controlled trial (15) and a exploratory pilot study among healthy adults (16) showed similar results. It is, however, unknown whether changes of dp-ucMGP already occur after a shorter period and what the

effect is of specific dosages of menaquinone. Finally, the effect of vitamin K supplementation on other MGP species has not been investigated to date.

The aim of this study is to investigate the short-term (after 4 and 12 weeks) effect of menaquinone-7 (MK-7) supplementation on different MGP species, including dp-ucMGP, at different dosages.

## Materials and Methods

*Design & Participants*

The study was performed according to a randomized, double-blind, placebo-controlled trial. Sixty participants were randomly assigned through a web-based application to one of the three treatment arms (placebo, 180 µg/d MK-7, 360 µg/d MK-7), stratified by gender. The participants were recruited through the Julius Center database of subjects who have indicated their interest in participating in studies, and complied with the following criteria: apparently healthy men and healthy postmenopausal women, aged 40-65 years, with a body mass index (BMI; in kg/m$^2$) between 18.5 and 30. The exclusion criteria were: using vitamin K antagonists, using chronic medication for cardiovascular diseases, using menopausal hormone therapy, having a known history with coagulation problems, smoking, habitual vitamin $K_2$ intake > 90 µg/day, vegans and known soy allergy. The participants gave written informed consent and the Institutional Review Board of the University Medical Center Utrecht approved the research protocol. The study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization of Good Clinical Practice guidelines, and appropriate regulatory requirements.

*Intervention*

In this intervention study participants received 180 µg/d, 360 µg/d MK-7 or placebo during 12 weeks. These two dosages are at the higher end of the range that can be achieved through for example a Dutch or Japanese diet (9;17). By using these dosages we wanted to ensure that if the supplementation is not effective, we can conclude that menaquinone intake feasible with diet or nutritional supplements has no effect on MGP levels.

Because studies investigating the effect of MK-7 supplementation on carboxylation of MGP were not available, the 12-week intervention period is based on a study investigating the effect on MK-7 supplementation on OC carboxylation (18). We assumed that similar to OC the carboxylation of MGP is occurring rapidly.

Two different capsules were used; MK-7 capsules containing 180 µg MK-7 and linseed oil and placebo capsules containing linseed oil (210 mg) only. Placebo and MK-7 containing capsules were similar in taste and appearance, and were

indistinguishable for participants and investigators. Both capsules were provided by Natthopharma (Oslo, Norway). The source of MK-7 was MenaQ7, a natural form of MK-7 produced by fermentation. Participants in the 360 µg MK-7 treatment arm received two MK-7 capsules daily. Those in the 180 µg MK-7 treatment arm received one MK-7 capsule and one placebo capsule daily, while the placebo arm received two placebo capsules daily. The participants were instructed to take the capsules with the evening meal and were asked to maintain their habitual food consumption, body weight and physical activity pattern.

*Study outline*

The participants visited our research units three times during the study; at baseline (visit 1), after 4 (visit 2) and 12 weeks (visit 3). At visit one, information on medication use (including nutritional supplements), medical history and lifestyle factors (smoking habits, alcohol consumption, physical activity and consumption of vitamin K rich products) were assessed with a questionnaire. Height, waist- and hip circumference were measured twice and average was taken. Participants gave written informed consent and were randomized to one of the three treatment arms. At each visit, fasting blood samples were drawn for biochemical analysis, and blood pressure, heart rate and body weight were measured. Blood pressure and heart rate were measured twice, after 5 minutes rest, in a sitting position and two minutes between each measurement with an automated and calibrated oscillomat (Omron HEM-907). Body weight was measured wearing indoor clothing, without shoes, wallet and keys with a calibrated floor scale.

Dietary intake

Dietary intake was assessed using a 3-day food record consisting of 3 non-consecutive days, including 2 week days and 1 weekend day. The food record had pre-specified sections for breakfast, lunch, dinner and snacks. All participants received verbal and written instructions. Food records were analyzed using Evry (Ensemble BV, Zoetermeer, the Netherlands), based on the Dutch national food compositions (NEVO) table. Energy and nutrient intake were calculated using the 2006 version of the NEVO table (19). Vitamin $K_1$ and $K_2$ intake were estimating using a database of 260 foods added to the NEVO (2006), as described previously (10).

*Biochemical analyses*

Plasma concentrations of dp-ucMGP, dp-cMGP, t-ucMGP, total-, HDL cholesterol, triglycerides, glucose, insulin, prothrombin time and serum concentrations of uncarboxylated OC (ucOC), carboxylated OC (cOC) were measured in fasting blood samples. cOC and ucOC were quantified in serum with the Gla-OC and Glu-OC test kits from Takara (Shinha, Japan). The measurements of dp-ucMGP and dp-cMGP were conducted with sandwich dual antibody ELISA. For both measurements the capture antibody was directed against the dpMGP sequence 3-15 (mAb-dpMGP; VitaK BV, Maastricht, The Netherlands). For dp-ucMGP the detecting antibody directed against the ucMGP sequence 35-49 (mAb-ucMGP, VitaK BV) while for dp-cMGP this was directed against the cMGP sequence 35-54 (mAb-cMGP, VitaK BV). The measurement of t-ucMGP was performed with a competitive mono-antibody ELISA as described previously (12). Plasma total cholesterol and triglycerides were measured using standard enzymatic procedures. HDL cholesterol was measured with the use of a homogeneous colorimetric technique. LDL cholesterol concentrations were estimated with the use of the Friedewald formula (20). Glucose concentrations were assed capillary with the use of a glucose oxidase method. Insulin was measured with an immunometric technique on an IMMULITE 1000 analyzer (Siemens Medical Solutions Diagnostics, Los Angeles, USA). We calculated the homeostasis model assessment of insulin resistance (HOMA-IR) to assess insulin resistance, with the equation HOMA-IR = (fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5 (21).

*Compliance & Safety*

Compliance was checked by return of leftover trial capsules and by measurements of OC ratio. Compliance was calculated with the following formula: compliance (%) = (number of capsules actually taken / number of capsules that should have been taken) × 100. Safety was assessed at each visit by monitoring adverse events and measurements of prothrombin time.

*Power calculation*

When the study was designed, no studies were available that investigated the effect of MK-7 supplementation on carboxylation of MGP. Therefore, we based our sample size calculation on a study of Schurgers et al. (18). They reported a 150% increase in cOC/ucOC ratio after supplementation with MK-7. Assuming a 2-sided a of 0.05, 10

participants per treatment arm were required to detect a 50% differences in dp-ucMGP levels with a power of 80%. To account for a dropout rate of 15% and using conservative approach, we recruited 20 participants per treatment arm.

*Data analysis*

Characteristics of the study population were described by means ± SD or median (interquartile range) for continuous variables and frequencies for categorical variables. A linear mixed model was used to estimate the effect of menaquinone supplementation on both primary and secondary outcomes with treatment arm as between-subjects factor and time as within-subjects factor. The primary outcome of interest was the interaction effect between treatment arm and time. We tested if gender was an effect modifier by stratifying the analyses by gender. Differences were considered significant at a P-value less than 0.05. Statistical analyses were done using PASW statistics 17.0.2 for Windows (SPSS Inc, Chicago, IL).

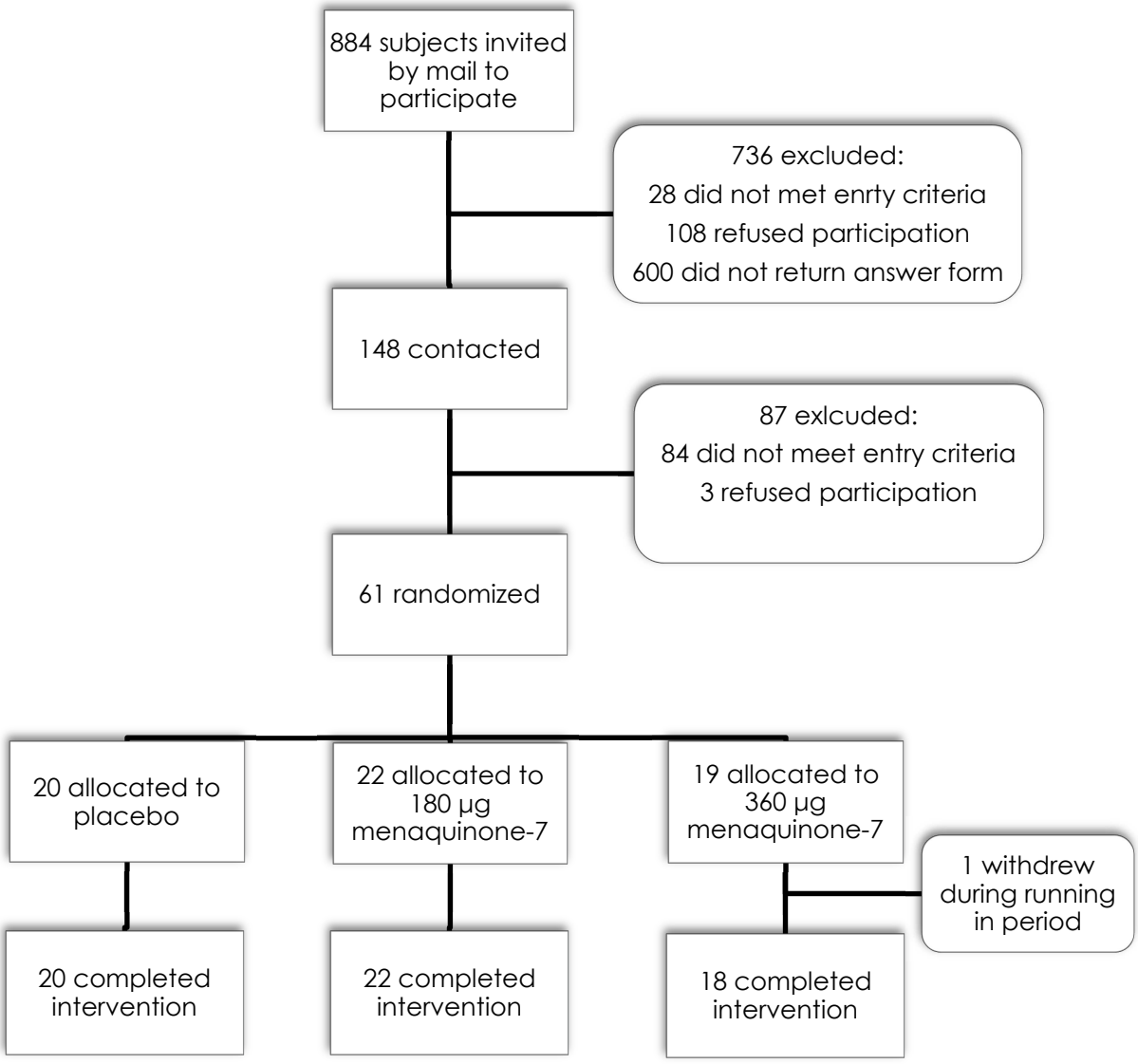



**Figure 1:** Flow chart of a double-blind, randomized, placebo-controlled trail of menaquinone-7 supplements and circulating species of matrix Gla protein in 60 healthy Dutch subjects

**Results**

In total, 884 potential participants were invited to take part in the study, of which 281 replied and 148 were interested to participate. During screening, 87 participants were excluded (84 did not meet entry criteria, 3 refused participation). Finally, 61 participants entered the study, of which one participant in the 360 µg/d treatment arm withdrew during the run-in period (figure 1). This participant was excluded from the analysis.

Table 1 shows the baseline characteristics across the 3 treatment arms. Demographic characteristics, vitamin K intake and cardiovascular risk factors were similar over the treatments arms (table 1). Compliance of the study was excellent as indicated by pill counts (rate 96%) and changes of OC ratio.

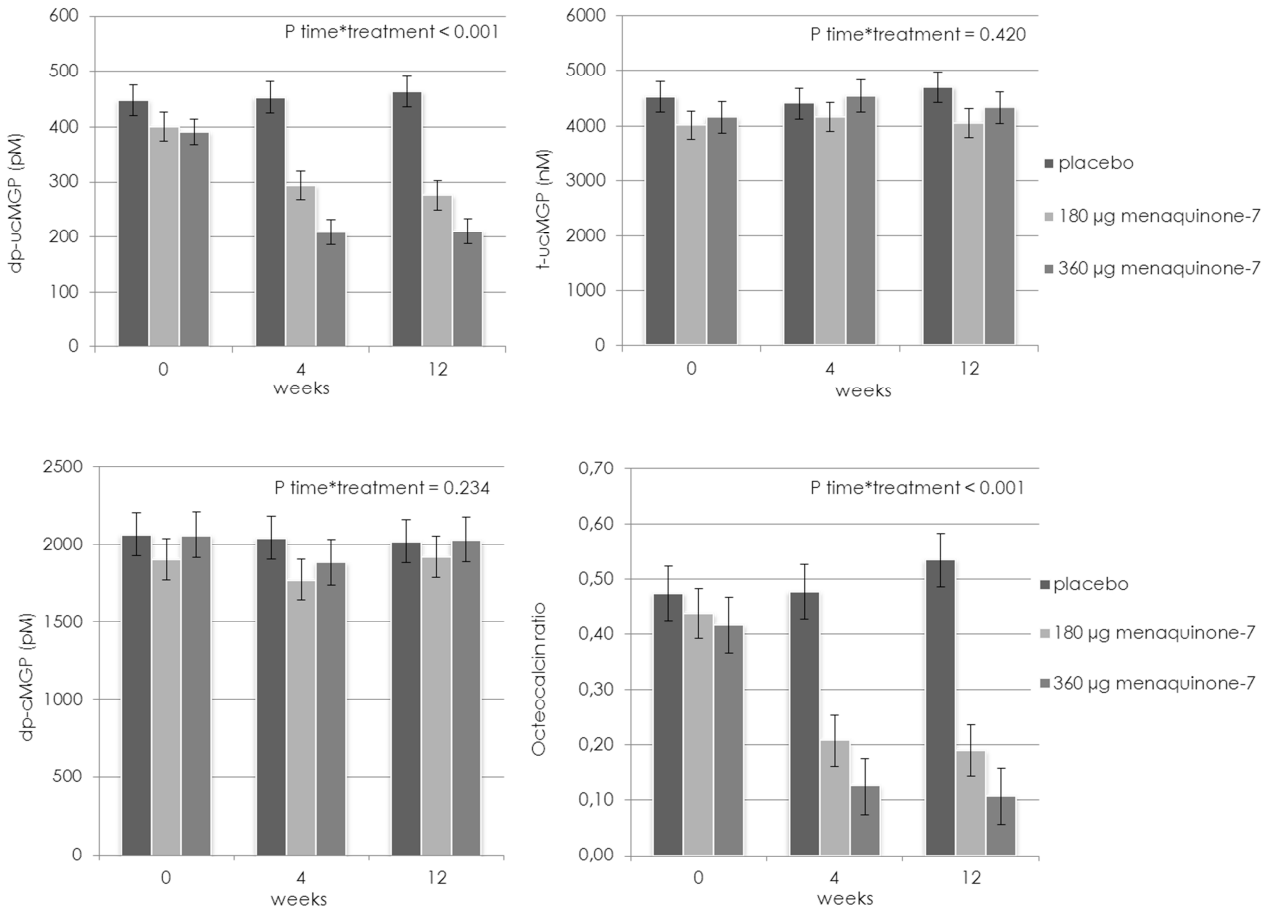
The effect of MK-7 supplementation on MGP species and OC ratio is shown in figure 2. Changes over time of OC ratio were significantly different between treatments arms (P time*treatment <0.001). After four weeks of supplementation with 180 µg/d MK-7, OC ratio decreased with 56.8% from 0.47 to 0.21 (SE 0.05), while after 12 weeks the mean OC ratio decreased further to 0.19 (59.6%). After 360 µg/d MK-7 supplementation, OC ratio decreased with 70.2 % from 0.42 to 0.12 after 4 weeks and after 12 weeks the mean OC ratio decreased further to 0.11 (74.5%). The OC ratio remained unchanged after placebo treatment. Similar results were observed for dp-ucMGP levels. Changes over time of dp-ucMGP levels were significantly different between treatment arms (P time*treatment <0.001). After 180 µg/d MK-7, dp-ucMGP levels decreased with 31.2% from 401 pmol/L (SE 27) to 294 pmol/L and 276 pmol/L after 4 and 12 weeks. After 360 µg/d MK-7 supplementation, dp-ucMGP levels decreased with 46.2% from 391 to 209 after four weeks and remained similar after 12 weeks (210 pmol/L). The dp-ucMGP levels did not change on placebo treatment.

Changes over time of the other MGP species, dp-cMGP (P=0.42) and t-ucMGP (P=0.23), did not differ between treatment arms. Changes over time of prothrombin time (P=0.92) and other risk factors of cardiovascular disease like blood lipid profile or blood pressure did not differ between treatments as well (table 2). Results were similar when we stratified for gender (data not shown).

**Table 1**: Baseline characteristics of the study population

| | *Placebo (n=20)* | *180 µg menaquinone (n=22)* | *360 µg menaquinone (n=18)* |
|---|---|---|---|
| Age (y) | 59.2 ± 2.7[1] | 59.5 ± 3.0 | 59.8 ± 3.3 |
| Men [n (%)] | 8 (40%) | 10 (46%) | 6 (33%) |
| BMI (kg/m$^2$) | 24.4 ± 2.5 | 24.9 ± 3.0 | 23.7 ± 1.9 |
| Mean systolic pressure (mm Hg) | 117 ± 13 | 126 ± 15 | 119± 18 |
| Mean diastolic pressure (mm Hg) | 68 ± 8.4 | 74 ± 10.9 | 72 ± 12.4 |
| Waist circumference (cm) | 88.7 ± 9.2 | 89.8 ± 8.3 | 87.7 ± 7.9 |
| Hip circumference (cm) | 100.1 ± 6.1 | 103.8 ± 17.3 | 99.6 ± 5.2 |
| Plasma total cholesterol (mmol/L) | 6.1 ± 0.8 | 5.7 ± 0.6 | 6.0 ± 1.1 |
| Plasma LDL cholesterol (mmol/L) | 4.0 ± 0.9 | 3.8 ± 0.7 | 3.9 ± 0.9 |
| Plasma HDL cholesterol (mmol/L) | 1.7 ± 0.5 | 1.5 ± 0.4 | 1.7 ± 0.5 |
| Triglyceride (mmol/L) | 0.9 ± 0.5 | 0.9 ± 0.3 | 0.8 ± 0.3 |
| Glucose (mmol/L) | 5.4 ± 0.4 | 5.5 ± 0.6 | 5.2 ± 0.4 |
| Protrombin time (sec) | 13.0 ± 0.6 | 13.0 ± 0.5 | 13.1 ± 0.5 |
| dp-ucMGP (pmol/L) | 448 ± 167 | 401 ± 131 | 391 ± 118 |
| dp-cMGP (pmol/L) | 2066 ± 673 | 1902 ± 586 | 2061 ± 661 |
| t-ucMGP (nmol/L) | 4528 ± 1464 | 4014 ± 1090 | 4153 ± 804 |
| cOC (ng/mL) | 5.80 ± 1.79 | 5.60 ± 1.76 | 6.55 ± 1.96 |
| ucOC (ng/mL) | 2.67 ± 1.40 | 2.40 ± 1.83 | 2.63 ± 1.64 |
| OC ratio | 0.47 ± 0.21 | 0.44 ± 0.33 | 0.42 ± 0.25 |
| Vitamin $K_1$ (µg/d) | 203 ± 159 | 179 ± 136 | 191 ± 167 |
| Vitamin $K_2$ (µg/d) | 26.0 ± 18.7 | 24.7 ± 16.5 | 23.5 ± 22.7 |

BMI, Body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; MGP, matrix Gla protein; dp-ucMGP, desphospho- uncarboxylated MGP; dp-cMGP, desphospho-carboxylated MGP; t-ucMGP, total uncarboxylated MGP; cOC, carboxylated osteocalcin; ucOC, uncarboxylated osteocalcin; OC ratio, osteocalcin ratio, defined as uncarboxylated osteocalcin divided by carboxylated osteocalcin;
[1]Mean ± SD (all such values)




MGP, matrix Gla protein; dp-ucMGP, desphospho- uncarboxylated MGP; dp-cMGP, desphospho- carboxylated MGP; t-ucMGP, total-uncarboxylated MGP; OC ratio, osteocalcin ratio (uncarboxylated osteocalcin/ carboxylated osteocalin)

**Figure 2:** mean values (SE) for dp-ucMGP, dp-cMGP, t-ucMGP and OC ratio during run-in and intervention in 60 randomized subjects in two treatment groups and the placebo group

**Table 2:** Changes over time of risk factors of cardiovascular by treatment

| | *Placebo* | *180 µg menaquinone* | *360 µg menaquinone* | *P time * treatment* |
|---|---|---|---|---|
| **Prothrombin time** | | | | |
| Baseline | 13.02 | 13.01 | 13.02 | 0.92 |
| Week 12[1] | -0.01 (-0.1%) | -0.10 (-0.8%) | -0.07 (-0.6%) | |
| P-value[2] | | 0.63 | 0.89 | |
| **Glucose** | | | | |
| Baseline | 5.39 | 5.48 | 5.24 | 0.99 |
| Week 12 | 0.04 (0.7%) | 0.05 (0.9%) | 0.05 (1.0%) | |
| P-value | | 0.51 | 0.37 | |
| **Triglyceride** | | | | |
| Baseline | 0.87 | 0.90 | 0.79 | 0.91 |
| Week 12 | -0.07 (-7.5%) | 0.03 (3.0%) | -0.01(-0.8%) | |
| P-value | | 0.48 | 0.72 | |
| **HDL cholesterol** | | | | |
| Baseline | 1.68 | 1.48 | 1.70 | 0.87 |
| Week 12 | 0.00 (0.2%) | 0.06 (4.0%) | 0.01 (0.8%) | |
| P-value | | 0.16 | 0.96 | |
| **LDL cholesterol** | | | | |
| Baseline | 4.04 | 3.79 | 3.82 | 0.71 |
| Week 12 | -0.17 (-4.2%) | -0.11 (-2.8%) | 0.03 (0.7%) | |
| P-value | | 0.44 | 0.65 | |
| **Cholesterol** | | | | |
| Baseline | 6.11 | 5.66 | 5.89 | 0.82 |
| Week 12 | -0.16 (-2.6%) | 0.00 (0.1%) | -0.26 (-4.4%) | |
| P-value | | 0.18 | 0.41 | |
| **BMI** | | | | |
| Baseline | 24.4 | 24.9 | 23.7 | 0.19 |
| Week 12 | -0.06 (-0.2%) | 0.14 (0.5%) | 0.18 (0.8%) | |
| P-value | | 0.40 | 0.50 | |
| **Systolic BP** | | | | |
| Baseline | 116.7 | 126.2 | 118.9 | 0.15 |
| Week 12 | 5.88 (5.0%) | -0.86 (-0.7%) | 7.6 (6.4%) | |
| P-value | | 0.17 | 0.46 | |
| **Diastolic BP** | | | | |
| Baseline | 68.4 | 73.8 | 71.7 | 0.77 |
| Week 12 | 0.83 (1.2%) | -0.82 (1.1%) | 3.14 (4.4%) | |
| P-value | | 0.05 | 0.11 | |
| **HOMA-ID** | | | | |
| Baseline | 0.70 | 1.08 | 0.91 | 0.78 |
| Week 12 | 0.21 (30.2%) | -0.04 (-3.3%) | 0.07 (7.3%) | |
| P-value | | 0.49 | 0.75 | |

BP, Blood pressure; HOMA-ID, homeostasis model assessment of insulin resistance

[1]change from baseline

[2]2-sided P-values for the differences from placebo

**Discussion**

In this 12-week, double blind, randomized, placebo-controlled trial, we showed that supplementation with MK-7 dose-dependently decreased dp-ucMGP by 31% to 46% after 12 weeks. These results were comparable to those of the OC ratio, although that showed larger effect sizes of 60% and 75%. The changes already occurred in the first 4 weeks of intervention. This relatively short period of MK-7 supplementation did not affect other MGP species, nor did it affect any of the cardiovascular risk markers.

The strengths of our study include its randomized, placebo-controlled design, excellent compliance and low drop-out rate. The present study was designed as a proof of principle study. For that reason we used relatively high dosages of MK-7. On average, the dietary menaquinone intake was 26 ± 19 µg/d. However, we provided dosages of 180 µg/d and 360 µg/d. Therefore, our study shows that high MK-7 supplementation improves MGP carboxylation in healthy adults, but cannot answer the question regarding the amount of vitamin K that is needed for optimal carboxylation of MGP in healthy adults. Additional dose-finding studies are needed to answer this research question.

However, there are two limitations to our study. First, the assays we used to analyse dp-ucMGP and dp-cMGP only detected the non-phosphorylated fraction of MGP. Biochemical tests for other circulating MGP species are not available at the moment. Second, circulating MGP levels do not necessarily reflect MGP tissue levels of the vasculature, we therefore can only speculate how circulating MGP levels as biomarker are related to tissue MGP.

Our study shows that MK-7 supplementation dose-dependently decreases dp-ucMGP concentrations, similar to its effect on osteocalcin in healthy adults. Thus far, only two intervention studies have been performed with MK-7; one exploratory pilot study and the other one in vitamin K deficient haemodialysis patients. Theuwissen et al. (16) showed that after 3-months of MK-7 supplementation with doses above 75 µg/d dp-ucMGP levels decreased while doses below 75 µg/d did not affect dp-ucMGP levels. Westerfeld et al. (15) assessed a six week dose-dependent MK-7 intervention trial in vitamin K deficient haemodialysis patients and showed a decrease of dp-ucMGP levels of 17.9%, 36.7% and 61.1% in the 45, 135, and 360 µg/d groups, respectively. These reductions were higher than the reductions

we have found. This is probably due to the 4.5 fold higher baseline dp-ucMGP levels in haemodialysis patients compared to our population.

Shea et al. (14) showed a ≈80% reduction in dp-ucMGP levels after three years of supplementation with 500 µg/d phylloquinone. This larger reduction compared to our study can have several reasons. Firstly, they used a higher dosage and different form of vitamin K, namely 500 µg/d phylloquinone. However, compared on a molar basis, 360 µg MK-7 provides about half the dosage of 500 µg phylloquinone, which corresponds to the differences found. Nevertheless, the bioavailability of MK-7 differs from that of phylloquinone; a study showed that with MK-7 supplementation higher and more stable serum levels of menaquinone are reached and it has a higher efficiency than phylloquinone in protein carboxylation (18), suggesting that MK-7 is utilized better than phylloquinone. However, this does not correspond with our results, which could be due to the shorter intervention time in our study. Secondly, the population differences could influence the response to vitamin K supplementation, since our participants were younger and dp-ucMGP levels were a little lower. Finally, their intervention was much longer. Although in our study reduction mostly occurred in the first 4 weeks, we cannot exclude that a longer intervention could further reduce dp-ucMGP levels. Altogether, our study showed that MK-7 supplementation dose-dependently reduces dp-ucMGP levels already within four weeks in healthy adults. Because the results for dp-ucMGP were similar to the OC ratio, this provides evidence that dp-ucMGP levels may serve as a non-invasive marker of vitamin K status.

None of these previous studies, however, investigated the effect on other MGP species. We did not find an effect of MK-7 supplementation on dp-cMGP and t-ucMGP. This is consistent with a non-randomised comparison of MK-7 users with placebo by Cranenburg et al (12). Based on a reduction of dp-ucMGP, one would expect that dp-cMGP levels would increase after MK-7 supplementation. However, our intervention did not affect dp-cMGP levels. It is possible that dp-cMGP species are not only better carboxylated but that the carboxylated precursor proteins are also better substrates to be phosphorylated (12). This would result in formation of p-cMGP species, which cannot yet be detected with an ELISA assay as previously described. Both the carboxylated and phosporylated fractions of MGP have high affinity to bind to the hydroxyappatite in the tissue and are therefore not readily

released into the circulation (22), which could explain that both dp-cMGP and t-ucMGP remained unchanged. Perhaps longer intervention periods are required to induce such changes in the circulation. More research into MGP phosphorylation is needed to investigate why dp-cMGP does not respond to vitamin K supplementation.

Several studies have consistently shown that dp-ucMGP may serve as a marker of vitamin K status (13;14). These lower dp-ucMGP levels could reduce the risk on CAC. So far, the association between dp-ucMGP levels and CAC is only investigated in 2 cross-sectional studies. One observed a borderline significant association between high dp-ucMGP and high CAC (23), while the other found no association (14). Based on previous studies, t-ucMGP appears to be a marker for presence of coronary calcification (13;24), rather than a marker for vitamin K status. This is confirmed by our study since t-ucMGP did not respond to vitamin K supplementation. Finally, for dp-cMGP several studies have investigated associations with CAC or mortality risk with inconsistent results, but no relation with vitamin K status has been found (12;23;25). Taken together, our study shows that dp-ucMGP is a marker for vitamin K status, whereas t-ucMGP and dp-cMGP do not respond to vitamin K supplementation. However, the biological meaning of these MGP species need to be further explored. Due to the relative short follow-up of our study it was not feasible to measure the effect of menaquinone supplementation on arterial calcification markers. Larger interventions with a longer follow up are needed to answer the question if menaquinone supplementation can reduce or prevent calcification.

Limited evidence from human studies suggest that high menaquinone intake improved the blood lipid profile (26;27). Our intervention of 12 weeks MK-7 supplementation, however, did not affect blood lipid profile. Although our study was designed to detect changes in MGP species, the study was adequately powered to detect changes as small as 2.5% in blood lipid profile. Hence, we conclude that supplementation with 360 µg/d MK-7 does not affect blood lipid profile in healthy adults. Like blood lipid profile, previous studies have suggested that vitamin K intake is inversely associated with insulin resistance (28-30). These results could not be confirmed by our intervention study. This is probably due to our study size, which was not large enough to detect an effect on insulin resistance.

In this study we choose for the dosages of 180 µg/d and 360 µg/d because these two dosages are at the higher range than can be achieved through diet. However, the results of this study are not generalizable to dietary menaquinones intake as it has been demonstrated that the bioavailability of vitamin K is dependent upon the nature of the food matrix (31). Observational studies or intervention studies with dietary menaquinones intake are necessary to give insight into the effect of dietary phylloquinone and menaquinone intake on dp-ucMGP circulating levels.

In conclusion, this study shows that supplementation with MK-7 during 12 weeks, dose-dependently decreases dp-ucMGP concentrations, but does not affect other MGP species. These changes already occur within 4 weeks of supplementation. Dp-ucMGP levels may serve as a non-invasive marker of vitamin K status in a healthy population.

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# Part II

# Chapter 6

# The association between MGP and coronary artery calcification

Based on:

Circulating matrix Gla protein is associated with coronary artery calcification and vitamin K status in healthy women

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GW Dalmeijer

YT van der Schouw

C Vermeer

EJ Magdeleyns

LJ Schurgers

JWJ Beulens

**Abstract**

Matrix Gla protein (MGP) is a vitamin K-dependent protein and an inhibitor of vascular calcification. Vitamin K is required for the carboxylation of MGP and can thereby reduce calcification. Circulating MGP species with different conformations have been investigated as markers for coronary artery calcification (CAC). In high-risk populations, high total uncarboxylated MGP (t-ucMGP) was associated with decreased CAC, while high non-phosphorylated uncarboxylated MGP (dp-ucMGP) was associated with a poor vitamin K status. This cross-sectional study investigated the association of MGP species with CAC, vitamin K status among 200 healthy women. Circulating dp-ucMGP, t-ucMGP and, non-phosphorylated carboxylated MGP (dp-cMGP) levels were measured by ELISA techniques and Agatston score by multi-detector computed tomography. The ratio of uncarboxylated to carboxylated osteocalcin was used as proxy of vitamin K status. A borderline significant (p=0.06) association between higher circulating dp-ucMGP levels and high CAC was observed (β=0.091, 95%-CI -0.01;0.19). In the entire study population, high t-ucMGP levels tended to be associated (p=0.09) with lower CAC (β=-0.36, 95%-CI:-0.78; 0.06). This association strengthened amongst women with CAC to a significant relation between high t-ucMGP levels and lower CAC (β=-0.55, 95%-CI -1.01;-0.10). Dp-cMGP was not associated with CAC. Low vitamin K-status was associated with high dp-ucMGP concentrations (β=0.138, 95%-CI 0.09; 0.19) but not with other MGP species. These results show that dp-ucMGP may serve as a biomarker of vitamin K status. Circulating dp-ucMGP and t-ucMGP may serve as markers for the extent of CAC, but these findings need to be confirmed.

## Introduction

Coronary artery calcification (CAC) is an important predictor for cardiovascular disease (CVD) (1). Matrix-Gla protein (MGP) is a vitamin K-dependent protein and a potent inhibitor of vascular calcification (2). The importance of MGP for vascular health was demonstrated in MGP-deficient animals, who all died of massive arterial calcification within 6-8 weeks after birth (3). Vitamin K is required for the function of MGP through its role as a cofactor for the enzyme gamma-glutamyl carboxylase, catalyzing the carboxylation of certain glutamic acid residues (Gla) in proteins (4). Previous studies have shown that high vitamin K intake is associated with reduced CAC and risk of CVD (5;6). These effects are thought to be mediated by increased activation of MGP (7).

MGP exists as various species, which differ in their state of phosphorylation and/or carboxylation: phosphorylated (pMGP), non-phosphorylated (desphospho-MGP, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP) (Figure 1). Studies showed that low dp-ucMGP levels were associated with a high vitamin K status (8). Dp-ucMGP has very low affinity for vascular calcification and, therefore, high vitamin K status or intake is leading to lower dp-ucMGP circulating levels. Such low dp-ucMGP concentrations are thought to be associated with reduced CAC, but studies, mainly in high-risk populations, have thus far shown inconsistent results ranging from no to a positive association (9-12).

Low circulating total uncarboxylated MGP (t-ucMGP) has been associated with high vascular burden (13), increased CAC in hemodialysis patients (14) and increased risk of mortality and cardiovascular disease among outpatients with stable coronary artery diseases(15). The phosphorylated fraction of t-ucMGP is thought to bind to arterial calcification thereby causing a decrease in the plasma circulation levels of t-ucMGP, leading to lower t-ucMGP at higher CAC (14). This relation is, however, not investigated in a healthy population. Finally, the association of dp-cMGP with vitamin K status or CAC has not been investigated in healthy populations.

This study will therefore investigate the determinants of different MGP species and the association of different MGP species with CAC, vitamin K status and intake among 200 healthy post-menopausal women.

| *Carboxylation:* | *Phosphorylation:* | | | | |
|---|---|---|---|---|---|
| | Desphospho MGP (dpMGP) | | Phosphorylated MGP (pMGP) | | |
| Uncarboxylated MGP (ucMGP) | Dp-ucMGP | + | p-ucMGP | → | Total uncarboxylated MGP (t-ucMGP) |
| | + | | + | | |
| Carboxylated MGP (cMGP) | Dp-cMGP | + | p-cMGP | → | Total carboxylated MGP (t-cMGP) |
| | ↓ | | ↓ | | |
| | Total desphospho-MGP (t-dpMGP) | | Total phosphorylated MGP (t-pMGP) | | |

**Figure 1:** Schematic overview of MGP phosphorylation and carboxylation

## Materials and Methods

*Study population*

The present study was designed as cross-sectional study among 200 postmenopausal healthy women. These 200 women were selected from a study among 1000 postmenopausal healthy women as previously described, conducted in 2002-2004 (16). Of these 1000 women, a random selection of 573 underwent a multislice CT examination at a second visit between January and December 2004. Of these women we randomly selected 100 women without coronary calcification (CAC score less than 1), 25 women with a CAC score between 1-10, 25 women with a CAC score between 10-100, and 50 women with a CAC score above 100 for this study. None of the women were using vitamin K antagonists. The Medical Ethical Committee of the University Medical Center Utrecht approved the study and written informed consent was obtained from all participants before enrolment. The study complied with the Declaration of Helsinki.

*Baseline Measurements*

At the visit in 2002-2004, smoking behavior and family history of CVD were assessed by a questionnaire. Height and weight were measured and body mass index (BMI) was calculated as weight divided by height squared ($kg/m^2$). Systolic and diastolic blood pressures (SBP and DBP) were measured twice at both arms with an automated and calibrated blood pressure device (DINAMAP™ XL, Critikon, Johnson & Johnson, Tampa, Florida, USA) with the subject in supine position. The mean of the duplicate measurements was calculated and the highest value between both arms was used. A venous blood sample was drawn after an overnight fast of at least 8h. Plasma total cholesterol, plasma triglycerides, and plasma glucose were measured using standard enzymatic procedures. High-density lipoprotein (HDL) cholesterol was measured by the direct method (inhibition, enzymatic). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. Smokers were categorized as current (<10, 11-20, >20), past or never smokers. Hypertension was defined as either using antihypertensive therapy or a SBP > 140 mmHg or a DBP >90 mmHg. Diabetes mellitus was defined as fasting blood glucose greater than 6.9 mmol/L and/or the use of antidiabetic medication.

*Coronary calcium measurement*

In 2004, the participants underwent a multi-detector computed tomography (MDCT) examination for the assessment of CAC. The amount of calcium in the coronary arteries was assessed with a MDCT scanner (Mx 8000 IDT 16, Philips Medical systems, Best, The Netherlands). Subjects were positioned within the gantry of the MDCT scanner in supine position. During a single breath hold, image of the heart, from the level of the tracheal bifurcation to below the base of the heart, were acquired using prospective ECH triggering at 50-80% of the RR-interval, depending on the heart rate. Scan parameters were 16 mm × 1.5 mm collimation, 205 mm field of view (FOV), 0.42s rotation time, 0.28s scan time per table position, 120 kVp and 40-70mAs (patients weight <70kg: 40mAs; 70-90 kg: 55mAs; >90kg:70mAs). Scan duration was approximately 10 s, depending on heart rate and patient size. Quantification of coronary calcium was performed on a separate workstation with software for calcium scoring (Heartbeat-CS, EBW, Philips Medical System, Best, The Netherlands). All regions with a density over 130 Hounsfield units were identified as potential calcifications. After completing a training-program, a trained scan reader, blinded for other results of the women manually selected only the calcifications within the coronary arteries (left main, left anterior descending, left circumflex, right coronary artery, or posterior descending artery). To reduce the influence of noise, the minimum size of a calcified lesion was set at 0.5 $mm^2$. The peak density in Hounsfield units and the area in $mm^2$ of each selected region were calculated. The Agatston (17) calcium score was obtained by multiplying the area by weighting factor that is dependent on the peak signal anywhere in the lesion. The score of individual lesions were added to obtain the Agatston calcium score for the entire coronary tree. The total calcium volume was calculated by multiplying the area of the calcified lesion (measured in square millimeters) by section thickness (1.5 mm and 3.0 mm). The calcium volume for each coronary vessel was computed by summing the volumes of the lesions in that vessel for all sections. Finally, the total volume from all the vessels became the calcium volume for a subject. The mass method uses volumetric, density information and a calibrationphantom of hydroxyapatite to calculate the actual mass of the calcified plaques (18). Calcium presence was defined as score >0. Reproducibility was assessed by having 199 scans read by two independent observers and by having 58 women undergo a second scan within 3 months. The

inter-reader reproducibility and the inter-scan reproducibility were excellent with intra-class correlation coefficients greater than 0.95 (19).

*Vitamin K status*

The osteocalcin ratio (OCR), defined as the ratio between the concentrations of circulating uncarboxylated osteocalcin and carboxylated osteocalcin, was used as a proxy for vitamin K status (20). A lower OCR reflects higher vitamin K status. Two commercially available test kits (Takara Bio Inc., Otsu, Shiga, Japan) were used to determine OCR.

*MGP species*

The measurements for dp-ucMGP and dp-cMGP were performed with a sandwich dual-antibody ELISA and t-ucMGP was performed with a competitive mono-antibody ELISA as described previously (11).

*Vitamin K intake data*

Energy and nutrient intake were estimated from a validated food frequency questionnaire (FFQ) (21). The FFQ contained questions on the usual frequency of consumption of 79 main food items during the year preceding enrolment. Overall, the questionnaire allows the estimation of the average daily consumption of 178 foods, by asking sub-items for several food items. The FFQ has been validated in pilot studies prior to the start of the study (21). Nutrient intake was calculated using the 1996 version of the NEVO (Dutch national food composition table) (22).

Because the Dutch national food composition table does not contain information on vitamin K contents of foods, concentrations of phylloquinone (vitamin $K_1$) and menaquinone (vitamin $K_2$) in a series of Dutch foods were assessed (23) or published data were used to update the dietary database for vitamin K (23-27). Reliability of the FFQ to estimate vitamin K intake was estimated against 12-24 h recalls in 58 women (21), showing a low relative validity of phylloquinone (r=0.24) and menaquinone-10 intake (r=0.23) but reasonable relative validity for intake of menaquinone (MK) and MK4-MK9 (correlations ranging from 0.51 for MK-7 to 0.72 for MK-5).

*Data analyses*

Differences between women with and without CAC were examined using an independent samples t-test for continuous variables or chi-square test for categorical

variables. To check differences in more extreme values of CAC, we also investigated differences between those with none or minor CAC compared to those with moderate to severe CAC (cutoff for Agatston score of 10).

We explored correlates of MGP species (logarithmically transformed because of non-normal distribution) by including the following exposures into a stepwise multivariable linear regression: age, BMI, triglyceride, OC ratio, vitamin K intake, Agatston score, hypertension, diabetes and smoking. P<0.20 was pre-specified for inclusion in the model.

To study the association between circulating MGP species and CAC, multivariate linear regression analysis was performed with circulating dp-ucMGP, dp-cMGP, and, t-ucMGP as the independent variables of interest and Agatston score (logarithmically transformed because of non-normal distribution) as the dependent variable. Age, smoking (never, former, current), diabetes (present or not), hypertension (present or not), were added to this model as confounders. In a separate model we also checked if adjusting for cholesterol and adjusting for systolic or diastolic blood pressure as continuous variables did influence the results.

To study the association between vitamin K status or vitamin K intake and circulating MGP species, multivariate regression analysis were performed with either vitamin K status (OCR), or vitamin K intake, phylloquinone or menaquinone, as the independent variables of interest and dp-ucMGP, dp-cMGP, t-ucMGP (logarithmically transformed) as the dependent variable. For, these analyses, energy, age, smoking, diabetes, hypertension, education attainment (low, middle and high), physical activity, alcohol consumption, and energy adjusted intake of protein, calcium, saturated fat, vitamin A, vitamin C and fiber were added to the model as confounders. The analyses described above were done in the whole study population but also separately in the group of women with CAC. A two-tailed p-value of <0.05 was considered to be statistically significant. Statistical analyses were conducted using PASW version 17.0.2 for Windows (SPSS CORP, Chicago, IL, USA).

**Results**

Table 1 shows the baseline characteristics of our study population. For five and 18 women, no blood samples were available for MGP and OCR measurement and they were therefore excluded from the analyses. The baseline characteristics of these women did not differ from the total study population. The mean age of the study population was 70 years. The median of the Agatston score was 0.2 with a range of 0-2264. The median of the circulating MGP levels of the different species were 579 pmol/l for dp-ucMGP (range 102-2852), 1994 pmol/l for dp-cMGP (range 1003-5185), and 3112 nmol/l for t-ucMGP (range 1520-6899). The median of the OCR was 0.33 with a range of 0.04-4.2. The mean intakes of phylloquinone and menaquinone were 217 µg/d and 31.5 µg/d, respectively.

Except for age and Agatston score, no population characteristics were significantly different between women with and without CAC. Comparing those with none or minor CAC (Agatston<10; n=125) and those with moderate to severe CAC (Agatson≥10; n=75) more distinct differences were observed. Apart from age and Agatston score, dp-cMGP was significantly higher (p=0.009) among those with (2308 ± 559) than without CAC (2035 ± 877), while t-ucMGP was lower (p=0.078) among those with (3108 ± 831) than without CAC (3310 ± 740). Also HDL cholesterol tended to be lower (p=0.052) among those with (1.33 ± 0.40) than without CAC (1.44 ± 0.37). In addition, vitamin $K_2$ intake tended to be lower (p<0.10) among those with (30.8 ±12.0) than without CAC (34.3 ±15.4). Similar results were observed when comparing those with Agatston<10 and those with Agatston>100. For dp-cMGP and t-ucMGP, the association strengthened while for HDL-cholesterol and vitamin $K_2$ the association was slightly attenuated.

*Independent correlates of circulating MGP species*

In the stepwise linear regression (table 2), the determinants OCR (p<0.001), BMI (p=0.003) Agatston score (p=0.129), hypertension (p=0.145) and smoking (p=0.180) explained 24.6% of the variability in plasma dp-ucMGP. Age (p=0.019), BMI (p= 0.006) and Agatston score (p=0.163) entered the model and explained 10% of the variability in dp-cMGP levels. For circulating t-ucMGP 8.5% of the variability was explained by diabetes (p=0.015), triglyceride levels (p=0.063), Agatston score (p=0.075) and OCR (p=0.137).

**Table 1:** mean (±SD) baseline characteristics of the study population

| *Variable* | *All women (n=200)* | *Women without CAC (n=100)* | *Women with CAC (n=100)* |
|---|---|---|---|
| Age[1] | 66.9 ±5.5 | 64.9 ±5.0 | 68.9 ±5.3 |
| BMI | 26.7 ±4.3 | 26.7 ±4.5 | 26.7 ±4.2 |
| Agatston score[1,2] | 0.2 (0-2264) | 0 (0-0) | 99 (1-2264) |
| | | | |
| Diabetes (%) | 5% | 5% | 4% |
| Hypertension (%) | 33% | 27% | 38% |
| SBP (mmHg) | 139 ±21 | 137 ±20 | 141 ±22 |
| DBP (mmHg) | 74 ±10 | 73 ±10.2 | 74 ±9 |
| HDL cholesterol (mmol/l) | 1.40 ±0.38 | 1.42 ±0.37 | 1.38 ±0.39 |
| LDL cholesterol (mmol/l) | 4.16 ±0.88 | 4.07 ±0.85 | 4.23 ±0.92 |
| High education (%) | 15% | 16% | 14% |
| Current smoking (%) | 43% | 49% | 37% |
| OCR | 0.36 (0.02-7.34) | 0.39 (0.02-7.34) | 0.32 (0.04-4.2) |
| Dp-ucMGP (pmol/l) | 579 (102-2852) | 575 (102-2852) | 579 (155-2630) |
| Dp-cMGP (pmol/l) | 1994 (1003-5185) | 1936 (1003-3852) | 2028 (1019-5185) |
| T-ucMGP (nmol/l) | 3112 (1520-6899) | 3144 (1757-5109) | 3094 (1520-6899) |
| Dietary intake | | | |
| Energy (kcal) | 1824 ±397 | 1827 ±403 | 1822 ±393 |
| Phylloquinone (µg/d)[2, 3] | 217 ±89 | 214 ±93 | 219 ±85 |
| Menaquinone (µg/d)[2, 3] | 31.5 ±12.0 | 32.7 ±13.2 | 30 ±10.7 |
| Vitamin A (µg/d)[2, 3] | 583 ±363 | 590 ±404 | 576 ±318 |
| Vitamin C (mg/d)[2, 3,] | 130 ±47 | 130 ±48 | 130 ±45 |
| Alcohol (g/d) | 4.8 (0.0-50.3) | 4.6 (0.0-47.2) | 4.8 (0-50.3) |
| Protein (g/d)[2, 3] | 69.6 ±10.2 | 70.2 ±9.3 | 68.9 ±11.1 |
| Saturated fat (g/d)[2, 3] | 29.4 ±5.1 | 29.8 ±5.4 | 29.0 ±4.8 |
| Fibre (g/d)[2, 3] | 22.7 ±4.1 | 22.6 ±4.2 | 22.7 ±3.9 |
| Calcium (mg/d)[2, 3] | 1079 ±290 | 1088 ±308 | 1070 ±273 |

BMI, Body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; OCR, osteocalcin ratio, defined as uncarboxylated osteocalcin divided by carboxylated osteocalcin; MGP, matrix Gla protein; dp-ucMGP, desphospho- uncarboxylated MGP; dp-cMGP, desphospho- carboxylated MGP; t-ucMGP, total uncarboxylated MGP

[1]p<0.05 between with and without CAC.

[2] Median (min-max)

[3]Energy-adjusted intake

**Table 2:** Determinants of MGP species in women (n=176) based on linear regression[1]

| | *dp-ucMGP* | | *dp-cMGP* | | *t-ucMGP* | |
|---|---|---|---|---|---|---|
| | *β-coefficient* | *P-value* | *β-coefficient* | *P-value* | *β-coefficient* | *P-value* |
| Age | - | - | 0.004 | 0.019 | - | - |
| BMI | 0.01 | 0.003 | 0.006 | 0.006 | - | - |
| Triglyceride | - | - | - | - | 0.022 | 0.063 |
| OCR | 0.14 | <0.001 | - | - | 0.018 | 0.137 |
| Agatston score | 8.1e-5 | 0.129 | 4.73e-5 | 0.163 | -4.551e-5 | 0.075 |
| Hypertension | 0.05 | 0.145 | - | - | - | - |
| Diabetes | - | - | - | - | 0.092 | 0.015 |
| Smoking | -0.033 | 0.180 | - | - | - | - |
| Model $r^2$ | 0.246 | | 0.10 | | 0.085 | |

CI, Confidence interval; MGP, matrix Gla protein; dp-ucMGP, desphospho- uncarboxylated MGP; dp-cMGP, desphospho- carboxylated MGP; t-ucMGP, total uncarboxylated MGP; OCR, osteocalcin ratio, defined as uncarboxylated osteocalcin divided by carboxylated osteocalcin;

[1] The outcome of MGP species was natural log-transformed to improve normality; the following exposures were entered: age, BMI triglyceride, OCR, vitamin K intake, Agatston score, hypertension, diabetes and smoking. P<0.20 was specified for inclusion

*Circulating MGP species and CAC*

Table 3 shows the associations between MGP species and CAC. Higher circulating dp-ucMGP levels were significantly associated with high CAC with a β of 0.140 (0.04-0.24) in the crude analysis. After adjustment for age and smoking, the association attenuated to a β of 0.107 (0.01;0.21), and after full adjustment the association attenuated to a borderline significant β of 0.091 (-0.01;0.19).

Higher dp-cMGP levels were significantly associated with high CAC with a β of 0.70 (0.20-1.20) in the crude analysis. However, after full adjustment, the association attenuated to a non-significant β of 0.37 (-0.09; 0.83). High t-ucMGP levels tended to be associated (p=0.09) with lower CAC (β -0.36, -0.78; 0.06) after full adjustment. However, among women with CAC high t-ucMGP levels were significantly associated with lower CAC with a β of -0.55 (-1.01; -0.10) after full adjustment. Dp-ucMGP was not associated with CAC in women with CAC (table 3). Adjusting for cholesterol and adjusting for systolic or diastolic blood pressure as a continuous variable did not affect these results (data not shown).

*Vitamin K status and intake and circulating MGP species.*

High OCR, was associated with high circulating dp-ucMGP circulating levels with a β of 0.146 (95%-CI 0.097; 0.196). After adjusting for classical cardiovascular risk factors and nutritional factors, the β was 0.138 (0.090; 0.189) and 0.138 (0.089; 0.187),

respectively. No associations between OCR and dp-cMGP or t-ucMGP were found (data not shown). Neither phylloquinone nor menaquinone intake were associated with any of the circulating MGP species in this population (data not shown).

**Table 3:** Association between MGP-species and (log-) agatston score

| | *All women(n=195)* | | | *Women with CAC (n=99)* | | |
|---|---|---|---|---|---|---|
| | *β-coefficient* | *95% CI* | *P-value* | *β-coefficient* | *95% CI* | *P-value* |
| dp-ucMGP | 0.140 | 0.04; 0.24 | 0.007 | 0.52 | -0.41; 1.45 | 0.27 |
| adjusted-1[1] | 0.107 | 0.01; 0.21 | 0.035 | 0.50 | -0.40; 1.40 | 0.28 |
| adjusted-2[2] | 0.091 | -0.01; 0.19 | 0.065 | 0.40 | -0.49;1.28 | 0.38 |
| dp-cMGP | 0.70 | 0.20; 1.20 | 0.01 | 0.45 | -0.04; 0.94 | 0.07 |
| adjusted-1[1] | 0.39 | -0.07; 0.85 | 0.10 | 0.39 | -0.08; 0.86 | 0.11 |
| adjusted-2[2] | 0.37 | -0.09; 0.83 | 0.12 | 0.36 | -0.10; 0.82 | 0.12 |
| t-ucMGP | -0.40 | -0.86; -0.06 | 0.09 | -0.58 | -1.04; -0.12 | 0.01 |
| adjusted-1[1] | -0.24 | -0.66; 0.17 | 0.25 | -0.47 | -0.94;-0.01 | 0.05 |
| adjusted-2[2] | -0.36 | -0.78; 0.06 | 0.09 | -0.55 | -1.01; -0.10 | 0.02 |

CI, confidence interval; MGP, matrix Gla protein; dp-ucMGP, desphospho- uncarboxylated MGP; dp-cMGP, desphospho- carboxylated MGP; t-ucMGP, total uncarboxylated MGP

[1]Adjusted for age, smoking

[2]Adjusted for age, smoking, diabetes and hypertension

**Discussion**

To our knowledge, this is the first study investigating the association of concentrations of different MGP species with CAC and vitamin K status in healthy women. We observed that high circulating dp-ucMGP levels were associated with low vitamin K status. High dp-ucMGP and low t-ucMGP concentrations tended to be associated with higher CAC. Among women with CAC, circulating t-ucMGP levels were significantly, inversely associated with the extent of CAC.

We hypothesized that dp-cMGP and t-ucMGP may serve as non-invasive biomarkers for arterial calcification. We could not confirm these relations, because we could not distinguish between women with and without CAC with t-ucMGP. However, a borderline significant inverse relation between t-ucMGP and CAC was observed in the entire population. This relation strengthened to a significant inverse association between circulating t-ucMGP levels and Agatston scores among women with CAC. These results are consistent with studies showing inverse associations between t-ucMGP concentrations and coronary calcification (14) or risk of mortality (15) in high risk populations. We now extend these associations to healthy women with coronary calcium. Similarly, other studies showed lower t-ucMGP concentrations in patient groups with high risk of calcifications compared to a healthy reference group (11;13;14;28). We could not find differences between those with and without CAC. This may be due to the different study population. We compared women with and without calcification within a healthy population, whereas other studies compared high-risk patient groups with healthy reference groups. The contrast between the two groups is therefore much smaller in our study, while our sample size was relatively small. Indeed, when comparing those with none or minor CAC to those with moderate to severe CAC, both t-ucMGP and dp-cMGP tended to differ between both groups. This suggests that these species of MGP may only be able to differentiate between non versus moderate to severe CAC.

In this study we also found a significant positive association between circulating dp-cMGP levels and CAC in crude analyses, but this attenuated to non-significant after adjustment for confounders. In addition, higher levels of dp-cMGP were observed among those with moderate to severe CAC than those with minor or no CAC. These results are in line with two previous studies showing that circulating dp-cMGP levels in aortic valve disease (11) and renal disease patients (11;29) were

higher than those of healthy controls. However, a prospective analysis among renal disease patients showed that low levels of dp-cMGP were associated with an increased risk of total and cardiovascular mortality (30). This seems to contradict the positive association between dp-cMGP and CAC in our study, but this is probably due to the prospective study design and the population of patients with high CAC compared to healthy women in our study.

Interestingly, the direction of the relations was different for t-ucMGP and dp-cMGP. It has been suggested that for t-ucMGP this can be explained by binding of the phosphorylated form to CAC leading to lower circulating levels in plasma (7). MGP indeed exerts its function by binding to either crystal nuclei in hydroxyapaptite or by binding to and thereby inhibiting bone morphogenetic protein-2, an osteogenic growth factor (11). However, for both mechanisms of action MGP has to be carboxylated. Therefore, one would expect dp-cMGP also to be inversely associated with CAC, while the opposite was found in our study. For dp-cMGP, it is currently unclear why the circulating levels in plasma raise with CAC. Further research is necessary to investigate if circulating dp-cMGP levels may serve as biomarker for arterial calcification. Phosphorylation plays an important role in the secretion of MGP. Wajih et al. (31) propose that phosphorylated MGP exits VSMCs via the secretory pathway, while non-phosphorylated MGP is released in vesicles. It is however unclear whether phosphorylation also plays a role in the activation of the calcification inhibitory properties.

In this study we also observed a borderline significant positive association between circulating dp-ucMGP levels and CAC. In a previous cross-sectional study, no association between dp-ucMGP and CAC in healthy older adults was observed (9). However, our findings are in line with results of case-control studies reporting higher circulating dp-ucMGP levels among patients with diseases characterized by vascular calcification, including those with aortic valve disease, aortic valve disease aortic stenosis and kidney disease (10-12).

We further hypothesized that dp-ucMGP was specifically associated with vitamin K status and intake. Because this form of MGP has very low affinity to bind to CAC, the influence by vitamin K intake is directly noticed in the circulation and therefore expected to be a sensitive marker of vitamin K status (7). We indeed found higher circulating levels of dp-ucMGP to go together with higher levels of OCR. In other

words, a poor vitamin K status was associated with high dp-ucMGP circulating levels. These results are consistent with cross-sectional of intervention studies showing low dp-ucMGP concentrations with high vitamin K status or after vitamin K supplementation (8;9;11). Our study shows that dp-ucMGP may also serve as a marker of vascular vitamin K status among healthy women. Since high dp-ucMGP concentrations also tended to be associated with high CAC, dp-ucMGP could perhaps explain the relation between vitamin K intake and CAC.

Unfortunately, we were not able to detect any significant associations of MGP species with vitamin K intake. This could be due to our relatively small sample size, since dietary assessments have relatively large variation leading to less power to detect associations. In addition, the relatively good vitamin K status of our study population could be involved. Vitamin K intake may be associated directly with measures of vitamin K status only when a low vitamin K status is present. McKeown et al. (30) indeed showed a linear relation between intake of phylloquinone and plasma concentrations of phylloquinone up to 150-200 µg/day that levelled of at higher levels. In our study, more than 75% of the women had a phylloquinone intake above 150 µg/day and only 4 women had a low vitamin K status as indicated by an OCR above 1.0. This could explain why we were unable detect any significant association in this range of intake.

This study has certain limitations that should be addressed. It was designed as a pilot study to verify the direction and the association between the different circulating MGP levels and CAC or vitamin K status. Therefore this study had a relatively small sample-size to detect small differences or associations with vitamin K intake with relatively large variation. We indeed observed several borderline significant associations that need to be confirmed with larger studies. As in any observational study, our result could be influenced by other factors than MGP circulating levels and vitamin K status. Although, we adjusted for several lifestyle and nutritional habits in our analyses, residual confounding may be present. In addition, the cross-sectional design of this study does not allow causal interpretation. Another limitation is the relative validity of our FFQ to estimate intake of vitamin K. Relative validity of our FFQ for vitamin $K_1$ intake was low, but for vitamin $K_2$ intake it was reasonable with correlation coefficients well in line with many other nutrients estimated using FFQ's (21).

In conclusion, this study shows that circulating dp-ucMGP may serve as a non-invasive marker of vitamin K status in the healthy population. Similar to high-risk populations, high circulating dp-ucMGP and low t-ucMGP levels may be associated with the extent of CAC among healthy women. Further confirmation of these findings in large prospective studies is warranted.

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# Chapter 7

# MGP and vascular calcification risk

Based on:

Circulating species of matrix Gla protein and the risk of vascular calcification in healthy women

*Submitted*

GW Dalmeijer
YT van der Schouw
EJ Magdeleyns
C Vermeer
SG Elias
BK Velthuis
PA de Jong
JWJ Beulens

**Abstract**

**Background:** Observational studies showed that high vitamin K intake is associated with reduced coronary artery calcification (CAC) and risk of cardiovascular disease (CVD). These effects are thought to be mediated by increased activation of the vitamin K-dependent protein matrix Gla protein (MGP). The aim of this study is to investigates the association between circulating MGP species and vascular calcification among healthy women.

**Methods and results:** In a prospective cohort of 571 women, MGP levels were measured by ELISA techniques at baseline. Calcification was measured in the coronary arteries, aortic valve, mitral valve, and aortic arch by multi-detector computed tomography after 8.5 years follow-up. CAC was present if Agatston score was ≥10 and the calcification score was calculated as the sum of the calcified areas. Multivariate adjusted relative risks (RR) and odds ratios (OR) were estimated using modified Poisson regression and multinomial logistic regression per standard deviation. Of the women, 24% had no calcification while 5% had calcification in all areas. High desphospho-uncarboxylated MGP (dp-ucMGP) levels, reflecting poor vitamin K status, were borderline significantly (p=0.08) associated with more CAC ($RR_{SD}$ 1.07;95%CI 0.99-1.15) and calcification areas ($OR_{(4\ areas\ vs\ no\ calcification)}$ 1.49; 95%CI 0.95-2.35). High total uncarboxylated MGP (t-ucMGP) levels were borderline significantly associated with less calcification ($OR_{(4\ areas\ vs\ no\ calcification)}$ 0.63; 95%CI 0.36-1.10). Desphospho-carboxylated MGP was not associated with calcification.

**Conclusion:** High dp-ucMGP levels may be associated with more calcification; while high t-ucMGP levels may be associated will less calcification areas.

## Introduction

Observational studies showed that high vitamin K intake is associated with reduced coronary artery calcification (CAC) and risk of cardiovascular disease (CVD) (1-4). These effects are thought to be mediated by increased activation of the vitamin K-dependent protein matrix Gla protein (MGP) (5). The importance of MGP for vascular health was demonstrated in MGP-deficient animals, who all died of massive arterial calcification within 6-8 weeks after birth (6). Vitamin K is required for the function of MGP through its role as a cofactor for the enzyme gamma-glutamyl carboxylase, catalyzing the carboxylation of glutamic acid residues into gammacarboxyglutamate (Gla) at five well-defined places in the protein (7). Mature MGP also carries three phosphoserine (PSer) residues and both Gla and Pser act as strong calcium binding groups rendering MGP its high affinity for calcium ions and crystals.

Since neither carboxylation nor phosphorylation occurs to completeness in the normal population, MGP exists as various species, which differ in their state of phosphorylation and/or carboxylation: phosphorylated (pMGP), non-phosphorylated (desphospho-MGP, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP). Development of assays to measure circulating MGP species enabled the investigation of these species in the circulation.(8) Total uncarboxylated MGP (t-ucMGP) is the sum of non-phosphorylated and phosphorylated uncarboxylated MGP (dp-ucMGP and p-ucMGP); and mainly consists of p-ucMGP. Due to its phosphorylated group p-ucMGP has a strong calcium-binding capacity, irrespective of the Gla content. Among patients at risk of vascular calcification, high levels of t-ucMGP were associated with decreased calcification (9-11). In a cross-sectional study we also found an inverse association between circulating t-ucMGP levels and the amount of calcification in women with clear CAC (12).

High dp-ucMGP levels (13-16) are regarded as a marker of low vitamin K status, resulting in a decreased capacity to inhibit artery calcification. Case-control studies have reported higher dp-ucMGP plasma levels among patients at risk of vascular calcification (8;17-20). However, these studies did not measure calcification. So far, high circulating dp-ucMGP levels were associated with more calcifications in chronic kidney disease patients and in patients using oral anticoagulants (17;21). We also found a borderline significant association between dp-ucMGP levels and vascular

calcification in healthy post-menopausal women in a cross-sectional study (12), while in another study no association between dp-ucMGP and vascular calcification was observed among healthy adults (14).

Theoretically, desphospho-carboxylated MGP (dp-cMGP) forms the mirror image of dp-ucMGP and two cross-sectional studies have investigated the association with calcification. In kidney disease patients low dp-cMGP levels were associated with more calcification (19). However, this association could not be confirmed among healthy women (12).

Taken together, the studies that investigated the association between MGP species and vascular calcification were cross-sectional and mostly among patients at risk of vascular calcification. We therefore, performed a prospective study to investigate the association between circulating MGP species and vascular calcification among 571 healthy postmenopausal women. We have previously shown that high menaquinone (vitamin $K_2$) intake was associated with lower presence of CAC in this study (1).

## Materials and Methods

*Study population*

We used data from a sample of 571 postmenopausal healthy women as detailed previously (22). In brief, these women were selected from participants of the PROSPECT cohort study, one of the two Dutch cohorts participating in the European Prospective Investigation into Cancer and Nutrition (EPIC). In PROSPECT, a total of 17,357 healthy breast cancer screening participants, aged 49-70 years, living in Utrecht and surrounding areas, were enrolled between 1993 and 1997. Between October 2002 and April 2004, 1,996 women were randomly selected from 5,844 participants of the PROSPECT study who were post-menopausal and did not use contraceptives of hormone therapy, and 1,000 agreed to participate. Of these 1,000 women, a random selection of 573 underwent a multislice CT examination at a second visit between January and December 2004. Two participants were excluded because they used vitamin K antagonists, leaving 571 participants for analysis. The study was approved by the Institutional Review Board of the University Medical Center Utrecht, and written informed consent was obtained from all participants.

*Baseline measurements*

At baseline in 1993-1997, all participants filled in a general questionnaire containing questions on demographic characteristics, presence of chronic diseases, and risk factors for chronic diseases, such as hypertension, reproductive history, family history, smoking habits, drinking of alcohol and physical activity.

Systolic and diastolic blood pressures were measured twice using the left arm with the subjects in sitting position after 10 min of rest with an automated and calibrated oscillomat (Bosch & Son, Jungingen, Germany) and the average value was used. Body height was measured to the nearest 0.5 cm with a wall mounted stadiometer (Lameris, Utrecht, TheNetherlands). Body weight was measured in light indoor clothing without shoes to the nearest 0.5 kg with a floor scale (Seca, Atlanta, GA, USA). Body mass index (BMI) was calculated as weight divided by height squared ($kg/m^2$). Blood samples were taken to prepare citrated plasma and serum, which were subsampled and stored under liquid nitrogen at -196ºC.

*Vascular calcification measurements*

In 2004, the participants underwent a multi-detector computed tomography (MDCT, Mx 8000 IDT 16, Philips Medical systems, Best, The Netherlands) as previous described (23). The amount of calcium in the coronary arteries was quantified on a separate workstation with software for calcium scoring (Heartbeat-CS, EBW, Philips Medical System, Best, The Netherlands). The Agatston (24) calcium score was obtained by multiplying the area by weighting factor that is dependent on the peak signal anywhere in the lesion. The score of individual lesions were added to obtain the Agatston calcium score for the entire coronary tree. Reproducibility was assessed by having 199 scans read by two independent observers and by having 58 women undergo a second scan within 3 months. The inter-reader reproducibility and the inter-scan reproducibility were excellent with intra-class correlation coefficients greater than 0.95 (23).

The tomography MDCT-scans were visually scored with regard to aortic, aortic valve and mitral valve calcification by a certificated radiologist with 10 years of experience in chest CT. The radiologist was blinded for subject characteristics and outcome status. Calcification of the heart valves was anatomically subdivided into calcification of the aortic valve leaflets (AVL) and the mitral valve leaflets (MVL). AVL and MLV calcification was graded as absent, mild (one leaflet affected), severe (2 or three leaflets affected). Aortic arch calcification was graded as absent, mild (≤3 foci), moderate (4–5 foci or 1 calcification extending over ≥3 slices) and severe (>5 foci or 1 calcification extending over ≥3 slices).

*MGP species*

The measurement of t-ucMGP was performed with a competitive mono-antibody ELISA as described previously (8). This ELISA identifying all MGP species carrying the uncarboxylated Gla-domain (i.e.: whether phosphorylated or not). Its characteristics are best understood by hypothesizing that the vast majority of circulating t-ucMGP consist of p-ucMGP which, despite the absence of Gla residues has a high affinity for calcium via its phosphoserine residues. In contrast to t-ucMGP, dp-ucMGP and dp-cMGP were conducted with a dual antibody ("sandwich") ELISA. For both measurements the capture antibody was directed against the dpMGP sequence 3-15 (mAb-dpMGP; VitaK BV, Maastricht, The Netherlands). For dp-ucMGP the detecting antibody directed against the ucMGP sequence 35-49 (mAb-ucMGP,

VitaK BV) while for dp-cMGP this was directed against the cMGP sequence 35-54 (mAb-cMGP, VitaK BV). All measurements were performed in duplicate and average values are given throughout this paper.

Because 200 of the 571 women of our prospective study were also included in our previous cross-sectional study (12), we were able to estimate reliability of the MGP levels by comparing the levels in both studies. The samples of the cross-sectional study were collected in 2002-2004, while the samples of our prospective study were collected in 1993-1997. The correlations between these samples were reasonable with Spearman correlation coefficients of 0.67 for dp-ucMGP and 0.51 for t-ucMGP.

*Vitamin K intake*

Energy and nutrient intake were estimated from a validated food frequency questionnaire (FFQ) (25). The FFQ contained questions on the usual frequency of consumption of 79 main food items during the year preceding enrolment. Overall, the questionnaire allows the estimation of the average daily consumption of 178 foods, by asking sub-items for several food items. The FFQ has been validated in pilot-studies prior to the start of the study (25). Nutrient intake was calculated using the 1996 version of the NEVO (Dutch national food composition table)(26).

Because the Dutch national food composition table does not contain information on vitamin K contents of foods, concentrations of phylloquinone (vitamin $K_1$) and menaquinones (MK-n, vitamin $K_2$) in a series of Dutch foods were assessed (27) or published data were used to update the dietary database for vitamin K (27-31). Reliability of the FFQ to estimate vitamin K intake was estimated against 12-24 h recalls in 58 women (25), showing a low relative validity of phylloquinone (r=0.24) and MK-10 intake (r=0.23) but reasonable relative validity for intake of total menaquinones and MK4-MK9 (correlations ranging from 0.51 for MK-7 to 0.72 for MK-5).

*Data analyses*

Characteristics of the study population are presented as the mean (SD) for continuous variables and frequencies (percentages) for categorical variables.

Calcification was measured in the coronary arteries (continuous), aortic valve (none, mild and severe), mitral valve (none, mild and severe) and aortic arch (none, mild, moderate and severe) by multi-detector computed tomography. To combine these

calcification scores we dichotomized each of the four areas into present or absent. Because of the continuous measurement of CAC we categorized this as calcification absent if Agatston score was <10 and present if Agatston score was ≥ 10. The total calcification score was calculated as the sum of all calcified areas (grades 0-4).

Intakes of phylloquinone and menaquinone were adjusted for energy intake using the regression residual method (32).

We first analyzed the association between MGP species and presence of CAC. Because of the high prevalence of CAC, an odds ratio will overestimate the effect size (33), we therefore used a modified Poisson regression model (33) to estimate relative risk (RR) and 95% confidence interval (CI). We did not analyze the association between vitamin K intake and presence of CAC because this had been done in a previous study (1).

Next, we analyzed the total calcification score. Due to the categorical outcome (0-4), we used multinomial logistic regression per standard deviation of vitamin K intake or MGP species to estimate odds ratios (OR). The groups with one to four calcification areas were compared with the group without calcification in any area.

In model 1 the RRs or ORs were adjusted for age and follow-up time and in model 2 we additionally included smoking (never, former or current), BMI and blood pressure. In model 3 we additionally adjusted for HDL cholesterol.

To study the association between vitamin K intake and circulating MGP species, multivariable linear regression analyses were performed with phylloquinone and menaquinone intake as the independent variable of interest and dp-ucMGP, dp-cMGP and t-ucMGP (logarithmically transformed) as the dependent variable. For these analyses, age, BMI and smoking were added to the model as confounders.

The presence of a nonlinear association of vitamin K intake and MGP species were explored by including the quadratic term of vitamin K or MGP species in the model with the linear term. In case this quadratic term was significant, the possibility of a non-linear relation was further examined nonparametrically with restricted cubic splines.(34) This likelihood ratios test was used for nonlinearity, comparing the model with only the linear term to the model with the linear and cubic spline terms.

To handle missing data we used multiple imputations. We assumed that the missing data were at random. We generated 10 imputed datasets and used Rubin's rules to combine the estimates of the parameters (35). A two-tailed p-value of <0.05 was considered to be statistically significant. Statistical analyses were conducted using IBM SPSS (version 20 for Windows and SAS (version 9.2 for windows).

**Results**

Table 1 shows the baseline characteristics of the study population. The mean (± SD) follow-up time was 8.5 (1.3) years. In these women we observed that 43% had CAC, 22% aortic valve calcification, 11% mitral valve calcification and 62% had aortic calcification. The mean phylloquinone (vitamin $K_1$) and menaquinones (vitamin $K_2$) intake was 226 ± 96 µg/d and 35 ± 14 µg/d, respectively.

**Table 1:** Mean (±S.D.) baseline characteristics of the study population

| | *N* | *Mean (±S.D.)* |
|---|---|---|
| Follow up time | 571 | 8.5 (1.3) |
| Age (years) | 571 | 57.3 ± 5.2 |
| BMI (kg/m$^2$) | 571 | 25.7 ± 4.0 |
| Waist hip ratio (cm) | 569 | 0.78 ± 0.05 |
| Current smoker (%) | 571 | 18.2 |
| | | |
| Systolic blood pressure (mmHg) | 571 | 131 ± 18.8 |
| Diastolic blood pressure (mmHg) | 571 | 78.3 ± 9.7 |
| Total cholesterol (mmol/L) | 527 | 6.2 ± 1.1 |
| HDL cholesterol (mmol/L) | 526 | 1.53 ± 0.42 |
| | | |
| Energy (kcal) | 570 | 1687 ± 378 |
| Vitamin C (mg/d)[1] | 570 | 123 ± 46 |
| Vitamin K (µg/day)[1] | 570 | 261 ± 98 |
| Phylloquinone (µg/day)[1] | 570 | 226 ± 96 |
| Menaquinone (µg/day)[1] | 570 | 35 ± 13.5 |
| | | |
| Dp-ucMGP (pmol/L) | 548 | 150 ± 109 |
| Dp-cMGP (pmol/L) | 547 | 884 ± 374 |
| T-ucMGP (nmol/L) | 548 | 3807 ± 1041 |

BMI, Body mass index; MGP, matrix Gla protein; dp-ucMGP, desphospho-uncarboxylated MGP; dp-cMGP, desphospho carboxylated MGP; t-ucMGP, total uncarboxylated MGP
[1] energy-adjusted intake

Table 2 shows the associations between MGP species and calcification. Circulating dp-ucMGP was associated with increased CAC with a RR per SD of 1.12 (95%CI: 1.05-1.19, p=0.001) in the crude analysis. This association attenuated to borderline significant (p=0.08) after adjustment for age and follow-up time ($RR_{SD}$: 1.06; 95%CI: 0.99-1.13). Similar results were observed after adjusting for smoking, BMI and blood pressure with a $RR_{SD}$ 1.07 (95%CI: 1.00-1.15, p=0.06) and after adjustment for HDL-cholesterol ($RR_{SD}$: 1.07; 95%CI: 0.99-1.15, p=0.08).

The associations between dp-ucMGP and the number of calcified areas showed the same pattern. High dp-ucMGP circulating levels were associated with more

calcification areas with an $OR_{(4\ areas\ vs\ no\ calcification)}$ per SD of 1.80 (95%CI: 1.24-2.60, p=0.002) in the crude analysis. This association attenuated after full adjustment to an $OR_{(4\ areas\ vs\ no\ calcification)}$ per SD of 1.49 (95%CI: 0.95-2.35, p=0.09).

Circulating dp-cMGP was not associated ($RR_{SD}$: 1.04 95%CI: 0.95-1.13, p=0.77) with coronary calcification in the crude analysis. Similar results were observed after full adjustment with a $RR_{SD}$ of 1.01 (95%CI: 0.92-1.11, p=0.85). There was no association between dp-cMGP circulating levels and the amount of calcified areas with an $OR_{(4\ ares\ vs\ no\ calcification)}$ per SD of 1.07 (95%CI: 0.61-1.86, p=0.82) after full adjustment.

High t-ucMGP circulating levels were borderline significantly associated with less calcification areas with an $OR_{(4\ areas\ vs\ no\ calcification)}$ of 0.63 (95%CI: 0.36-1.10, p=0.10) after full-adjustment. However, circulating t-ucMGP was not associated with presence of CAC with a $RR_{SD}$ 1.06 (95%CI: 0.98-1.16, p=0.17) after full adjustment. This result was due to the presence of a nonlinear relation as indicated by a significant quadratic term (p=0.03). Spline regression showed evidence of a U-shaped relation (p=0.13) between t-ucMGP and coronary calcification with lower presence of CAC up to 3.2 nM, but increasing RRs at higher levels (figure 1).

Table 3 shows associations between vitamin K intake and calcification. After full adjustment, high phylloquinone intake was not associated with the number of calcified areas $OR_{(4\ areas\ vs\ no\ calcification)}$ 1.14 (95%CI: 0.72-1.81, p=0.57). In contrast, higher menaquinones intake was significantly associated with decreased odds for four areas with calcification. Per 1 SD increase in menaquinone, the odds of all four areas with calcification was significantly decreased by 61% (OR 0.39; 95%CI: 0.21-0.74, p=0.004). On the other hand, no significant relationship was observed between menaquinone intake and calcification in one till three areas.

Both a high phylloquinone intake (β: -0.043; 95%CI: -0.070;-0.016) and menaquinone intake (β:-0.032; 95%CI: -0.059;-0.009) were associated with lower dp-ucMGP circulation levels after adjustment for age, BMI and smoking. Neither phylloquinone nor menaquinones intakes were associated with dp-cMGP nor t-ucMGP levels (data not shown).

The association between menaquinone intake and calcification was not explained by dp-cMGP and t-ucMGP levels and only partially by dp-ucMGP circulating levels.

**Table 2:** Relative risks (95%-CI) of circulating levels of MGP species with coronary calcification and Odds Ratios (95%-CI) of MGP species with calcification among 571 post-menopausal women

| | CAC | | calcified areas | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *0* | *1* | | *2* | | *3* | | *4* | |
| | *N=249* | | *n=138* | *n=181* | | *n=168* | | *n=57* | | *n=27* | |
| | RR* | 95%CI | | OR* | 95%CI | OR* | 95%CI | OR* | 95%CI | OR* | 95%CI |
| **Dp-ucMGP** | | | | | | | | | | | |
| Crude | 1.12 | 1.05-1.19 | 1 | 1.40 | 1.06-1.85 | 1.48 | 1.12-1.95 | 1.66 | 1.20-2.30 | 1.80 | 1.24-2.60 |
| Model 1[1] | 1.06 | 0.99-1.13 | 1 | 1.33 | 1.00-1.76 | 1.33 | 0.99-1.79 | 1.50 | 1.06-2.13 | 1.49 | 0.98-2.26 |
| Model 2[2] | 1.07 | 1.00-1.15 | 1 | 1.28 | 0.95-1.72 | 1.25 | 0.91-1.71 | 1.47 | 1.02-2.12 | 1.51 | 0.97-2.35 |
| Model 3[2] | 1.07 | 0.99-1.15 | 1 | 1.28 | 0.95-1.72 | 1.25 | 0.91-1.71 | 1.47 | 1.02-2.12 | 1.49 | 0.95-2.35 |
| **Dp-cMGP** | | | | | | | | | | | |
| Crude | 1.04 | 0.95-1.13 | 1 | 1.27 | 0.99-1.63 | 1.43 | 1.12-1.83 | 1.12 | 0.79-1.59 | 1.18 | 0.75-1.85 |
| Model 1[1] | 1.01 | 0.93-1.10 | 1 | 1.27 | 0.99-1.64 | 1.43 | 1.09-1.86 | 1.13 | 0.78-1.63 | 1.09 | 0.65-1.83 |
| Model 2[2] | 1.01 | 0.93-1.11 | 1 | 1.22 | 0.94-1.58 | 1.34 | 1.01-1.77 | 1.05 | 0.71-1.56 | 1.10 | 0.63-1.91 |
| Model 3[3] | 1.01 | 0.92-1.11 | 1 | 1.22 | 0.94-1.59 | 1.33 | 1.00-1.76 | 1.05 | 0.71-1.56 | 1.07 | 0.61-1.86 |
| **T-ucMGP** | | | | | | | | | | | |
| Crude | 1.04 | 0.95-1.14 | 1 | 0.83 | 0.65-1.06 | 1.06 | 0.85-1.33 | 0.99 | 0.73-1.36 | 0.61 | 0.38-0.98 |
| Model 1[1] | 1.08 | 0.99-1.18 | 1 | 0.89 | 0.70-1.13 | 1.20 | 0.94-1.55 | 1.14 | 0.81-1.59 | 0.75 | 0.44-1.27 |
| Model 2[2] | 1.06 | 0.98-1.16 | 1 | 0.84 | 0.65-1.09 | 1.09 | 0.83-1.41 | 1.02 | 0.72-1.46 | 0.62 | 0.35-1.09 |
| Model 3[3] | 1.06 | 0.98-1.16 | 1 | 0.84 | 0.65-1.09 | 1.08 | 0.83-1.41 | 1.02 | 0.72-1.46 | 0.63 | 0.36-1.10 |

MGP, matrix Gla protein; dp-ucMGP, desphospho-uncarboxylated MGP; dp-cMGP, desphospho carboxylated MGP; t-ucMGP, total uncarboxylated MGP

*All RRs and ORs are expressed per SD of the mean.

[1]Adjusted for age, follow-up time

[2] Adjusted for age, follow-up time, smoke habits, BMI and blood pressure

[3] Adjusted for age, follow-up time, smoke habits, BMI, blood pressure and HDL-cholesterol

After adding dp-ucMGP circulating levels to the models the RR and OR changed with less than 10%.

**Table 3:** Odds Ratios (95%-CI) of vitamin K intake with calcification among 571 post-menopausal women

| Calcified areas | 0 | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|---|
| | N=138 | N=174 | | N=165 | | N=55 | | N=26 | |
| | | OR* | 95%CI | OR* | 95%CI | OR* | 95%CI | OR* | 95%CI |
| **phylloquinone** | | | | | | | | | |
| Crude | 1 | 0.86 | 0.69-1.08 | 1.01 | 0.81-1.26 | 0.90 | 0.65-1.23 | 1.18 | 0.83-1.69 |
| Model 1[1] | 1 | 0.83 | 0.66-1.04 | 0.95 | 0.75-1.21 | 0.84 | 0.60-1.19 | 1.17 | 0.75-1.82 |
| Model 2[2] | 1 | 0.84 | 0.66-1.06 | 0.94 | 0.73-1.21 | 0.84 | 0.59-1.19 | 1.14 | 0.72-1.80 |
| Model 3[3] | 1 | 0.83 | 0.66-1.06 | 0.94 | 0.73-1.21 | 0.83 | 0.59-1.18 | 1.14 | 0.72-1.81 |
| **menaquinone** | | | | | | | | | |
| Crude | 1 | 0.86 | 0.69-1.07 | 0.88 | 0.71-1.10 | 0.87 | 0.64-1.19 | 0.37 | 0.22-0.65 |
| Model 1[1] | 1 | 0.86 | 0.69-1.08 | 0.90 | 0.71-1.14 | 0.93 | 0.67-1.29 | 0.35 | 0.20-0.64 |
| Model 2[2] | 1 | 0.88 | 0.69-1.11 | 0.91 | 0.70-1.17 | 0.93 | 0.66-1.30 | 0.37 | 0.19-0.72 |
| Model 3[2] | 1 | 0.88 | 0.69-1.11 | 0.93 | 0.72-1.20 | 0.94 | 0.67-1.32 | 0.39 | 0.21-0.74 |

MGP, matrix Gla protein; dp-ucMGP, desphospho-uncarboxylated MGP; dp-cMGP, desphospho carboxylated MGP; t-ucMGP, total uncarboxylated MGP

*All ORs are expressed per SD of the mean.

[1] Adjusted for age, follow-up time

[2]Adjusted for age, follow-up time, smoke habits, BMI and blood pressure

[3] Adjusted for age, follow-up time, smoke habits, BMI, blood pressure and HDL-cholesterol

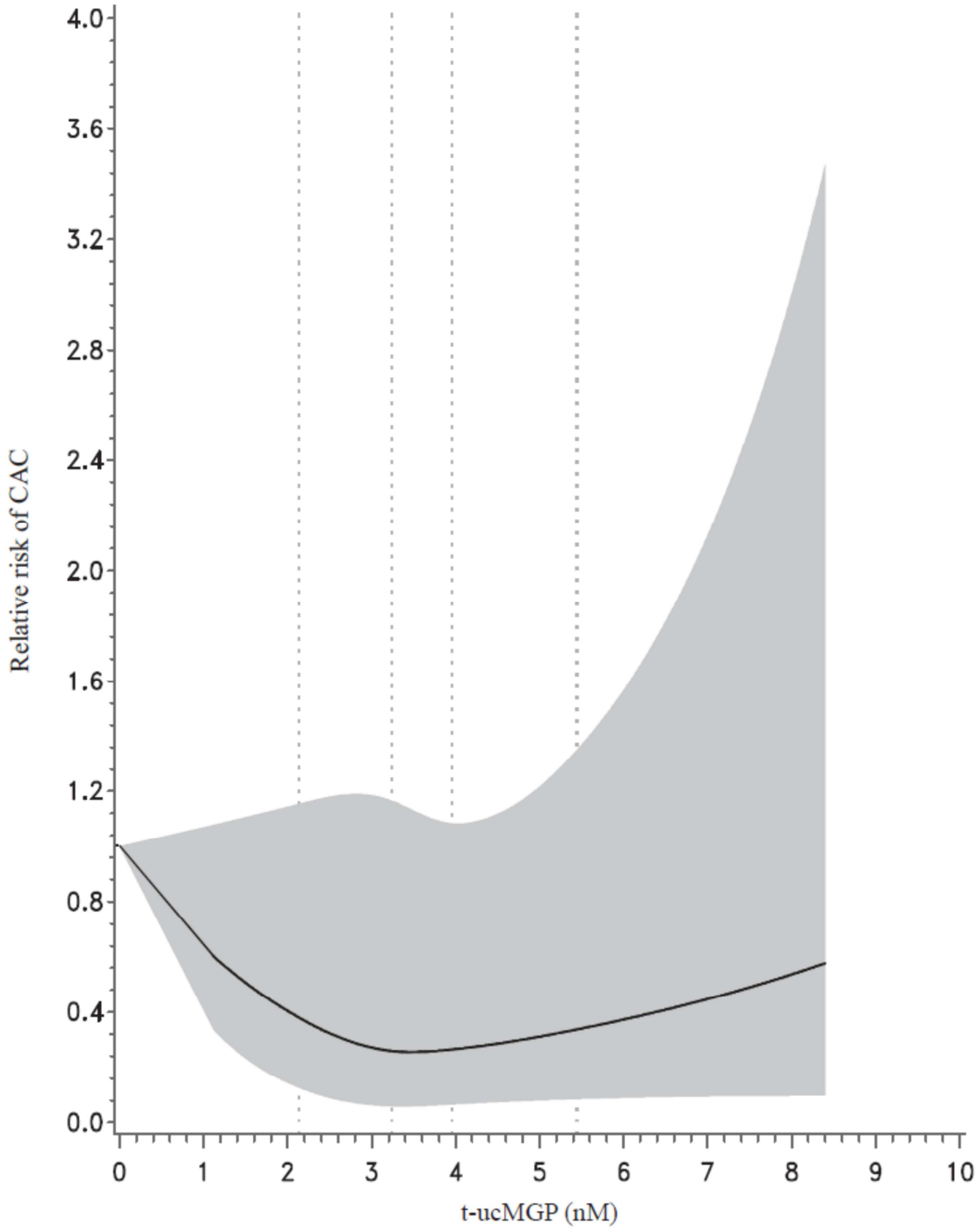


**Figure 1** Association between t-ucMGP circulating levels and relative risk of CAC modelled continuously using splines; RR(—) with 95%CI in grey

**Discussion**

This prospective study shows that high dp-ucMGP levels may be associated with higher risk of CAC and with calcification in more areas. In addition, high t-ucMGP levels seem to be associated with less calcification areas. We did not observe an association between dp-cMGP and CAC or number of calcification areas. Furthermore, high menaquinones intake was associated with less calcification areas. Besides, high menaquinone intake was associated with lower dp-ucMGP levels. However, plasma dp-ucMGP levels only partially explain the association between menaquinones intake and vascular calcification.

A previous study in the same study population showed that high menaquinones intake, but not phylloquinone, was associated with reduced coronary calcification (1). We now extend these results by showing that high intake of menaquinone is associated with fewer calcification areas. Intake of phylloquinone, however, was not associated with calcification. These results are in line with previous observational studies on phylloquinone and menaquinones intake in relation to CAC or coronary heart disease (1-4).

We observed borderline significant associations between high circulating dp-ucMGP levels and CAC but also with more calcification areas in post-menopausal women free of clinical cardiovascular disease. This is in line with the results of our cross-sectional study, among 200 of these women, showing a borderline significant association between high dp-ucMGP and high CAC (12). Similarly, previous studies reported higher circulating dp-ucMGP levels among patients with diseases characterized by vascular calcification (17;21). However, in another cross-sectional study among healthy older adults, no association between dp-ucMGP and CAC was found (14). Because this form of MGP has very low affinity to bind to calcification, the influence by vitamin K intake is directly noticed in the circulation and is therefore expected to be a sensitive marker of vitamin K intake and status (5). Previous cross-sectional and intervention studies have already shown that high dp-ucMGP is associated with a low vitamin K status and can be used as a marker of vitamin K status (13;14). We also found that low circulating levels of dp-ucMGP go together with high phylloquinone and menaquinone intake. Altogether, these results suggest that a decrease of dp-ucMGP could explain the reduced risk of CAC with high vitamin K intake. However, we observed that dp-ucMGP circulating levels explained

only 2.3% of the association between menaquinone intake and calcification, while dp-cMGP and t-ucMGP levels had no influence. This suggests that other mechanisms could also be involved.

The dp-cMGP assay only became available recently and has only been used on a limited scale. Consistent with our pilot study (12) we did not find an association between dp-cMGP levels and more calcification. Schlieper et al. (19) observed that circulating dp-cMGP levels were 12% lower in kidney disease patient with more extensive calcification compared with patients with fewer calcifications. This difference is probably due to the population of patients with high CAC compared to the healthy women in our study. The biological meaning of dp-cMGP is therefore still unclear and requires further investigation.

Similar to high-risk populations, we found that low circulating t-ucMGP levels were associated with more calcification areas (9-11). Due to the presence of a U-shaped relation between t-ucMGP and CAC we did not find an association when performing a linear regression analysis. However, similar to the association with number of calcification areas, spline regression also showed slightly lower RRs for CAC up to 3.2 nM t-ucMGP, which increased at higher levels. We assume that t-ucMGP mainly consists of phosphorylated ucMGP species. The phosphoserines equip the molecule with strong calcium-binding groups irrespective of its Gla content and this fraction has a strong affinity for vascular calcification. Thereby causing a decrease in the plasma circulating levels of t-ucMGP, leading to lower t-ucMGP levels at higher CAC (9). Therefore, t-ucMGP may act differently in persons with or without calcification.

Strengths of this study include the prospective study design, measurements of three different species of MGP, vascular calcification and vitamin K intake in healthy women. Nevertheless, our study has certain limitations to consider. The assays we used to analyse dp-ucMGP and dp-cMGP only detected the non phosphorylated fraction of MGP. Biochemical tests for other circulating MGP species are not available at this time. Further, circulating MGP levels do not necessarily reflect MGP tissue levels of the vasculature, we therefore can only speculate how circulating MGP levels as biomarker are related to tissue MGP. Unfortunately, vascular calcification was not measured at baseline, and therefore we could not investigate the progression of vascular calcification during follow-up.

Our study population was limited to post-menopausal women, which limits generalizability of our results to men. However, this study population is particularly relevant to explore effects on vascular calcification, because these women are particularly prone to have reduced bone mineral density (36), which is strongly and independently associated with arterial calcification (36). Further this study had a relatively small sample size to detect small differences or associations with MGP levels with relatively large variation. We indeed observed several borderline significant associations which are in line with previous studies.

Finally, the dp-ucMGP and dp-cMGP levels in our study were low compared to a healthy reference population (8). This could be due to the possible degradation of MGP in our samples, because the samples have been stored for a long time. However, we compared the levels with a previous study (12) and found reasonable Spearman correlation coefficients of 0.67 for dp-ucMGP and 0.51 for t-ucMGP. We therefore assume that degradation of MGP did not substantially affect the ranking of individuals and did not systematically bias the associations we found.

Taken together, this study shows that high dp-ucMGP levels, reflecting a poor vitamin K status, may be associated with more calcification, also in a healthy population. High menaquinones intake was associated with less calcification, but dp-ucMGP could not fully explain this association. Consistent with high-risk populations, high t-ucMGP levels seem to associate will less calcification areas.

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# Chapter 8

# MGP and cardiovascular disease risk in diabetes patients

Based on:

Matrix Gla protein species and risk of cardiovascular events in type 2 diabetes patients

*Under revision Diabetes care*

GW Dalmeijer
YT van der Schouw
EJ Magdeleyns
C Vermeer
WMM Verschuren
JMA Boer
JWJ Beulens

**Abstract**

**Objective**: To investigate the relationship of circulating matrix Gla protein (MGP) species; with incident cardiovascular disease (CVD) or coronary heart disease (CHD) in type 2 diabetes patients.

**Research Design and Methods**: EPIC-NL is a prospective cohort study among 40,011 Dutch men and women. At baseline (1993-1997) 518 participants were known with type 2 diabetes. MGP levels were measured by ELISA techniques in baseline plasma samples. The incidence of fatal and non-fatal CVD and CHD was obtained by linkage to national registers. Cox proportional hazard models were used to calculate hazard ratios (HRs), adjusted for sex, waist hip ratio, physical activity and history of CVD or CHD.

**Results:** During 10 years of follow-up, 160 cases of CVD and 99 cases of CHD were documented. Higher circulating desphospho-uncarboxylated MGP (dp-ucMGP) levels were significantly associated with higher risk of CVD with a HR per SD of 1.21 (95%CI: 1.06-1.38) after adjustment. Higher circulating dp-ucMGP levels were not related to risk of CHD ($HR_{SD}$ 1.12 (95%CI: 0.94-1.34).

Circulating desphospho-carboxylated MGP (dp-cMGP) levels were not associated with CVD risk ($HR_{SD}$: 0.96; 95%CI: 0.81-1.14) or with CHD risk ($HR_{SD}$: 0.78; 95%CI: 0.58-1.03). Circulating total-uncarboxylated MGP (t-ucMGP) levels were not associated with CVD risk with a $HR_{SD}$ 0.99 (95%CI: 0.85-1.16) or CHD risk ($HR_{SD}$: 0.94; 95%-CI:0.76-1.15).

**Conclusion**: High dp-ucMGP levels were associated with increased CVD risk among type 2 diabetes patients, while other MGP species were not related to CVD risk. These results suggest that a poor vitamin K status is associated with increased CVD risk.

## Introduction

Coronary artery calcification (CAC) is an independent predictor of cardiovascular disease (CVD) (1). Matrix Gla protein (MGP) is a vitamin K dependent protein and a potent inhibitor of vascular calcification (2). The importance of MGP for vascular health was demonstrated in MGP-deficient animals, who all died of massive arterial calcification within 6-8 weeks after birth (3). Vitamin K is required for the function of MGP through its role as a cofactor for the enzyme gamma-glutamyl carboxylase, catalyzing the carboxylation of glutamic acid residues (Glu) into gammacarboxyglutamate (Gla) at five well-defined places in the protein (4). Human studies showed that high vitamin K intake is associated with reduced CAC and reduced risk of CVD (5-8). These effects are thought to be mediated by increased activation of MGP (9).

MGP exists as various species, which differ in their state of phosphorylation and/or carboxylation: phosphorylated (pMGP), non-phosphorylated (desphospho-MGP, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP). Total uncarboxylated MGP (t-ucMGP) is thought to be the sum of desphospho-uncarboxylated MGP (dp-ucMGP) and phosphorylated-uncarboxylated MGP (p-ucMGP) and mainly consists of p-ucMGP.

Development of assays to measure circulating MGP species enabled the investigation of these species in the circulation (10). These studies have shown that dp-ucMGP is a marker for vitamin K status with high dp-ucMGP level reflecting a low vitamin K status (11-16). In line with these results, several studies indeed showed that high dp-ucMGP levels were associated with more calcification, but not consistently (12;15;17;18). Theoretically, desphospho-carboxylated MGP (dp-cMGP) forms the mirror image of dp-ucMGP and is hypothesized to be associated with lower calcification, but results from human, observational studies are inconsistent (15;19). Finally, in cross-sectional studies high t-ucMGP has been associated with decreased calcification (15;20-22).

Vascular calcification has emerged as a strong and independent risk factor for CVD (1), but the association of MGP species with CVD events has not been investigated to date. Diabetes mellitus is associated with severe cardiovascular complications, including vascular calcification and accelerated atherosclerosis, leading to

increased morbidity and mortality in diabetic patients (23-25). Therefore, we performed a prospective study to investigate the association between circulating MGP species and CVD or coronary heart disease (CHD) risk among a high-risk population, i.e. type 2 diabetes patients.

## Research Design and Methods

*Study population and design*

The EPIC-NL cohort is the Dutch contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC) and consists of the Prospect-EPIC and MORGEN-EPIC cohorts (26). The Prospect-EPIC study includes 17,357 women aged 49-70 years living in Utrecht and vicinity who participated in the nationwide Dutch breast cancer screening program between 1993 and 1997. The MORGEN-EPIC cohort consists of 22,654 men and women aged 21-64 years selected from random samples of the Dutch population in three different towns. Participants were recruited in both studies from 1993 to 1997. At baseline, a general and a food-frequency questionnaire (FFQ) were administered, and a physical examination was performed for blood pressure measurements, anthropometry, and non-fasting blood sampling. All participants provided informed consent before study inclusion. The study complies with the Declaration of Helsinki and was approved by the institutional board of the University Medical Center Utrecht (Prospect) and the Medical Ethical Committee of TNO Nutrition and Food Research (MORGEN).

Three sources of ascertainment of diabetes were used: self-report, hospital discharge diagnoses and urinary strip test (in the Prospect part of the cohort only). Potential cases of diabetes ascertained by these sources were verified against medical and pharmacy records. Details of the ascertainment sources and verification procedures are described else-were (27).

At baseline, 615 participants were verified to have type 2 diabetes. Those who did not give permission for linkage with vital status registries were excluded (n=10). After exclusion of participants with missing data on CVD (n=25) and blood samples (n=62) 518 participants were left for analysis.

*MGP species*

The measurement of plasma t-ucMGP was performed with a competitive mono-antibody ELISA as described previously (10). This ELISA identifies all MGP species carrying the uncarboxylated Gla-domain, irrespective of whether the MGP species are phosphorylated or fragmented. In contrast to t-ucMGP, analyses of dp-ucMGP and dp-cMGP were conducted with a dual antibody ("sandwich") ELISA. Both assays use a monoclonal antibody against the dpMGP sequence 3-15 as capture antibody (mAb-dpMGP; VitaK BV, Maastricht, The Netherlands). The dp-ucMGP

assay is based on the use of detection monoclonal antibody directed against the ucMGP sequence 35-49 in human MGP (mAb-ucMGP, VitaK BV) while for dp-cMGP this was directed against the cMGP sequence 35-54 in human MGP (mAb-cMGP, VitaK BV). All measurements were performed in duplicate and average values are given throughout this paper.

*Outcome assessment*

Morbidity follow-up data on cardiovascular disease (CVD) events were obtained from the Dutch Centre for Health Care Information, which holds a standardized computerized register of hospital discharge diagnoses. All diagnoses were coded according to the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9). Follow-up was complete until the first of January 2008. The database was linked to the cohort on the basis of birth date, gender, postal code and general practitioner with a validated probabilistic method (28). Information on vital status was obtained through linkage with the municipal registries. Causes of death were collected from Statistics Netherlands. Endpoints for the present analysis were CVD (ICD-9; 410-414, 427.5, 428, 415.1, 443.9, 430-438, 440-442, 444, 798.1, 798.2, 798.9) and coronary heart disease (CHD) (ICD-9; 410-414, 427.5, 798.1, 798.2, 798.9). These endpoints included both fatal and non-fatal cases of CVD and CHD.

*Other measurements*

The general questionnaire contained questions on demographic characteristics, the presence of chronic diseases, and risk factors for chronic diseases. Smoking was categorized into current, past, and never smoker. Level of education was categorized as low (primary education up to those completing advanced elementary education), average (intermediate vocational education and higher general secondary education) or high (higher vocational education and university). Physical activity was assessed using a questionnaire validated in an elderly population and categorized according to the Cambridge Physical Activity Index (29). During the baseline physical examination screening, systolic and diastolic blood pressure measurements were performed twice in the supine position on the right arm using a Boso Oscillomat (Bosch & Son, Jungingen, Germany) (Prospect-EPIC) or on the left arm using a random zero sphygmomanometer (MORGEN-EPIC), from which the mean was taken. Height, and weight were measured, and BMI was calculated. Time since diabetes diagnosis was calculated by subtracting the age of diagnosis

from the age at baseline examination. HbA1c concentrations were measured in erythrocytes using an immunoturbidimetric latex test. All measurements were performed according to standard operating procedures.

*Data analysis*

Participant characteristics are presented as means with standard deviations or percentages. The duration of follow-up was calculated as the interval between date of study entry and the occurrence of a cardiovascular event, death, loss to follow-up, or January 1, 2008, whichever came first.

Cox proportional hazard models were used to calculate crude and adjusted hazard ratios (HRs) and their 95% CIs for the associations between MGP species (continuous) and CVD or CHD. Potential confounding factors (age, gender, BMI, waist to hip ratio, smoking habits, physical activity, education, systolic and diastolic blood pressure and total cholesterol) were selected based on univariable associations of potential confounders with both CVD or CHD and MGP species, and whether adjustment for the confounder changed the HR by > 10%. Variables selected this way were incorporated into a multivariate model by means of a stepwise selection approach. The confounders that were entered into the model were age, sex, waist to hip ratio and CPAI (model 1). The second model additionally included history of CVD or CHD (model 2). In a separate model, we also checked if adjusting for duration of diabetes and HbA1c levels influenced the results. We repeated the analyses after excluding not verified type 2 diabetes patients (n=67).

The possibility of a non-linear relation was examined nonparametrically with restricted cubic splines (30), and no evidence for nonlinear associations was found.

For handling missing data for confounders we used multiple imputations. We assumed that the missing data were at random. We generalized 10 imputed datasets and used Rubin's rules to combine the estimates of the parameters (31). Two-sided P-values below 0.05 were considered to be statistically significant. All statistical analyses were conducted using IBM SPSS (version 20 for Windows).

**Results**

Table 1 shows the baseline characteristics of the study population. The mean age of the study population was 58.1 years and 17.8% were men. The diabetes duration was on average 6.3 years and mean HbA1c was 8.0%. The medians of the circulating MGP levels of the different species were 156 pmol/L with an interquartile range (IQR) of 91-258 for dp-ucMGP, 1062 pmol/L for dp-cMGP (IQR 716-1240), and 4308 nmol/L for t-ucMGP (IQR 3514-5079). During a mean follow-up of 9.9 years 160 incident cases of CVD were documented, of which 99 were CHD.

**Table 1:** Baseline characteristics of 518 diabetes patients

| | | *N* |
|---|---|---|
| Age (years) | 58.1 ± 7.1 | 518 |
| Male sex (%) | 17.8 | 518 |
| BMI (kg/m$^2$) | 29.6 ± 4.9 | 516 |
| Waist/hip ratio | 0.89 ± 0.08 | 515 |
| Currently smoking (%) | 23.4 | 484 |
| Physically inactive[1] (%) | 18.9 | 510 |
| High education (%) | 7.9 | 515 |
| Systolic bloodpressure (mmHg) | 144 ± 22 | 515 |
| Diastolic bloodpressure (mmHg) | 83 ± 10 | 498 |
| Total cholesterol (mmol/L) | 6.0 ± 1.2 | 479 |
| HDL-cholesterol (mmol/L) | 1.15 ± 0.33 | 466 |
| Duration of diabetes (years) | 6.3 ± 7.0 | 496 |
| Using OBGL drugs | 43% | 396 |
| HbA1c (%) | 8.03 ± 1.78 | 496 |
| Dp-ucMGP (pmol/L) | 217 ± 210 | 518 |
| Dp-cMGP (pmol/L) | 1062 ± 623 | 518 |
| T-ucMGP (nmol/L) | 4354 ± 1284 | 518 |

BMI, Body mass index; MGP, matrix Gla protein; dp-ucMGP, desphospho-uncarboxylated MGP; dp-cMGP, desphospho carboxylated MGP; t-ucMGP, total uncarboxylated MGP, OBGL; oral blood glucose lowering

[1]Inactive according to the Cambridge physical activity index

Table 2 shows the association between MGP species and CVD or CHD risk. After adjustment for age, gender, waist to hip ratio, Cambridge physical activity index and history of CVD, higher circulating dp-ucMGP levels were significantly associated with higher risk of CVD with a $HR_{SD}$ of 1.21 (95%-CI 1.06-1.38, p=0.01). Higher circulating dp-ucMGP levels were significantly associated with higher risk of CHD in crude analyses ($HR_{SD}$ 1.24; 95%-CI 1.06-1.45, p=0.01). After full adjustment, the association attenuated to non-significant ($HR_{SD}$ 1.12; 95%-CI: 0.94-1.34, p=0.21).

Circulating dp-cMGP levels were not associated with CVD risk both in crude analyses and after full adjustment ($HR_{SD}$ 0.96; 95%-CI 0.81-1.14, p=0.64). They were borderline significantly associated with a lower CHD risk ($HR_{SD}$ 0.78; 95%-CI 0.58-1.03, p=0.08) after full adjustment.

No association between circulating t-ucMGP levels and CVD or CHD risk was observed.

Adjusting for diabetes duration and HbA1c levels did not affect these results (data not shown). Excluding not verified type 2 diabetes patients yielded comparable results (data not shown).

**Table 2:** Crude and adjusted hazard ratios (95%CI)* for the association of MGP species with incident (fatal or nonfatal) cardiovascular and coronary heart disease among 518 diabetes subjects

| | CVD (n=160) | CHD (n=99) |
|---|---|---|
| **Dp-ucMGP** | | |
| Crude | 1.37 (1.21-1.54) | 1.24 (1.06-1.45) |
| Model[1] | 1.29 (1.13-1.48) | 1.17 (0.99-1.40) |
| Model[2] | 1.21 (1.06-1.38) | 1.12 (0.94-1.34) |
| **Dp-cMGP** | | |
| Crude | 1.02 (0.88-1.18) | 0.86 (0.67-1.11) |
| Model[1] | 0.95 (0.80-1.12) | 0.78 (0.59-1.03) |
| Model[2] | 0.96 (0.81-1.14) | 0.78 (0.58-1.03) |
| **T-ucMGP** | | |
| Crude | 0.99 (0.85-1.16) | 0.92 (0.75-1.12) |
| Model[1] | 0.98 (0.84-1.15) | 0.91 (0.74-1.11) |
| Model[2] | 0.99 (0.85-1.16) | 0.94 (0.76-1.15) |

* HRs are expressed per SD

[1]Adjusted for age, gender, waist to hip ratio, CPAI

[2]Adjusted for model 1 + history of CVD or history of CHD

**Conclusions**

To our knowledge, this is the first study investigating the association of circulating MGP species with the risk of cardiovascular events. In this prospective study we observed that high circulating dp-ucMGP levels, reflecting a poor vitamin K status, were associated with increased CVD risk in type 2 diabetes patients. We did not observe a significant association between dp-cMGP or t-ucMGP and CVD risk.

No previous studies investigated the association between dp-ucMGP levels and CVD events, so far only the association between dp-ucMGP and mortality has been investigated. These studies have been performed in high risk populations such as patients with aortic stenosis or heart failure and generally showed strong positive associations between dp-ucMGP and risk of total or cardiovascular mortality (17;32;33). Only a study by Schlieper et al. (19) found that haemodialysis patients with low dp-ucMGP levels had a non-significant HR of 1.71 (95%-CI 0.92-3.17) for all-cause mortality and a non-significant HR of 1.83 (95%-CI 0.90-3.70) for cardiovascular mortality but these HRs were not adjusted for other risk factors. In line with most studies on mortality, several studies, but not all, have shown that high dp-ucMGP levels are associated with increased calcification (12;15;17;18). We now extended these findings by showing that high circulating dp-ucMGP levels are also associated with increased CVD risk among diabetes patients. Since studies have consistently shown that high dp-ucMGP levels are associated with a poor vitamin K status (11-16), these results suggest that a poor vitamin K status is associated with increased CVD risk.

Theoretically dp-cMGP forms the mirror image of dp-ucMGP and therefore we expected that high levels of dp-cMGP would be associated with lower CVD risk. No previous studies investigated the association between dp-cMGP levels and CVD events, but the association with mortality has been investigated. No association between dp-cMGP levels and mortality risk in patients with symptomatic aortic stenosis or chronic heart failure was observed (32;33). This is consistent with a previous cross-sectional study from our group, where we did not find an association between dp-cMGP and calcification (15). However, in haemodialysis patients low levels of dp-cMGP were associated with increased CVD mortality risk (HR 2.7; 95%-CI 1.2-6.2) (19). In our study, we could not detect an association between dp-cMGP and CVD risk. However we observed a borderline significant association of high dp-cMGP

levels with a reduced risk of CHD, similar to the results in haemodialysis patients (19). Because of the relatively small sample size of our study, we may have had limited power to detect a somewhat weaker association. Therefore, our results and those of Schlieper et al.(19) suggests that there might be an association between high dp-cMGP and reduced CHD risk. To further clarify the role of dp-cMGP in CVD and CHD risk, more and larger population studies, as well as physiological studies are warranted.

One previous study investigated the association between t-ucMGP and CVD or mortality risk (34). In coronary artery disease (CAD) patients each 1000 nM higher t-ucMGP level was associated with a 16% lower CVD risk and a 22% lower risk of all-cause mortality. However, the association of t-ucMGP levels with mortality was limited to participants without diabetes, whereas there was no significant association in diabetes patients (HR 1.10; 95%-CI 0.85-1.41). We also found no association between t-ucMGP levels and CVD risk in diabetes patients. This could be due to population differences: the study mentioned above resulted from a cohort selected for CAD, which are people with coronary artery calcification whilst our participants were selected for the presence of diabetes. We assume that t-ucMGP may act differently in persons with or without calcification, because t-ucMGP mainly consists of phosphorylated ucMGP species. The phosphoserines equip the molecule with strong affinity for vascular calcification, thereby causing a decrease in the plasma circulating levels of t-ucMGP, leading to lower t-ucMGP levels at higher levels of calcification (20). Our study has certain limitations to consider. The assays for dp-ucMGP and dp-cMGP only detected the non-phosphorylated fraction of MGP. Biochemical tests for other circulating MGP species are currently not available. Further, circulating MGP levels do not necessarily reflect MGP tissue levels of the vasculature. We therefore can only speculate how circulating MGP levels as biomarker are related to tissue MGP. Futhermore, the dp-ucMGP levels and dp-cMGP levels in our study were low compared to a healthy reference group (10). This could be due to the possible degradation of MGP in our samples, because of samples have been stored for a long time in straws. Finally, our study was limited to type 2 diabetes patients, which limits generalizability of our results to the general population. However, this study population is particularly relevant to explore associations with CVD risk, because approximately one half of patients with type 2 diabetes will die of cardiovascular causes (35;36).

In conclusion, the findings in this prospective study among type 2 diabetes patients' shows that high circulating dp-ucMGP levels are associated with increased CVD risk. Circulating dp-cMGP and t-ucMGP levels were not related to CVD risk. These results suggest that a poor vitamin K status is associated with increased CVD risk.

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# Chapter 9

## Dp-ucMGP and cardiovascular disease risk

Based on: Circulating desphospho-uncarboxylated matrix Gla protein and the risk of coronary heart disease or stroke
*submitted*

GW Dalmeijer
YT van der Schouw
EJ Magdeleyns
C Vermeer
WMM Verschuren
JMA Boer
JWJ Beulens

**Abstract**

**Objective:** Matrix Gla protein (MGP) is a vitamin K dependent protein and a potent inhibitor of vascular calcification. Desphospho-uncarboxylated MGP (dp-ucMGP) is a marker for vitamin K status with high dp-ucMGP level reflecting a low vitamin K status. This study investigated the association of dp-ucMGP with incident coronary heart disease (CHD) or stroke.

**Design and Methods:** A prospective case-cohort study with a representative baseline sample of 1406 participants and 1154 and 380 incident cases of CHD and stroke, respectively, was nested within the EPIC-NL study. Dp-ucMGP levels were measured by ELISA technique in baseline plasma samples. The incidence of fatal and non-fatal CHD and stroke was obtained by linkage to national registers. Cox proportional hazard models were used to calculate hazard ratios (HRs) per standard deviation (SD) and per quartile of circulating dp-ucMGP levels, adjusted for cardiovascular risk factors.

**Results:** This case-cohort study had an average follow-up of 11.5 years. Circulating dp-ucMGP levels were not associated with CHD risk with a HR per SD of 1.00 (95% CI: 0.93-1.07) and a $HR_{Q4\ vs\ Q1}$ of 0.94 (95% CI: 0.79-1.13) after adjustment. Dp-ucMGP was not associated with stroke risk with a HR per SD of 0.98 (95% CI: 0.90-1.08) and a $HR_{Q4\ vs\ Q1}$ of 1.09 (95% CI: 0.78-1.51).

**Conclusion:** Circulating dp-ucMGP levels were not associated with CHD or stroke risk. This study could not confirm that high dp-ucMGP levels, reflecting a poor vitamin K status, are associated with increased CHD or stroke risk.

## Introduction

Coronary artery calcification (CAC) is an independent predictor of cardiovascular disease (CVD) (1). Matrix Gla protein (MGP) is a vitamin K dependent protein and a potent inhibitor of vascular calcification (2). The importance of MGP for vascular health was demonstrated in MGP-deficient animals, who all died of massive arterial calcification within 6-8 weeks after birth (3). Vitamin K is required for the function of MGP through its role as a cofactor for the enzyme gamma-glutamyl carboxylase, catalyzing the carboxylation of glutamic acid residues (Glu) into gammacarboxyglutamate (Gla) at five well-defined places in the protein (4). Human studies showed that high vitamin K intake is associated with reduced CAC and reduced risk of CVD (5-8). These effects are thought to be mediated by increased carboxylation of MGP (9).

MGP exists as various species, which differ in their state of phosphorylation and/or carboxylation: phosphorylated (pMGP), non-phosphorylated (desphospho-MGP, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP).

Development of assays to measure circulating MGP species enabled the investigation of these species in the circulation (10). These studies have shown that desphospho-uncarboxylated MGP (dp-ucMGP) is a marker for vitamin K status with high dp-ucMGP level reflecting a low vitamin K status (11-16). In line with these results, several studies showed that high dp-ucMGP levels were associated with more calcification (15;17;18) but not consistently (12). Studies linking MGP species to manifest cardiovascular disease are still rare; we found that high dp-ucMGP levels were associated with increased CVD risk among type 2 diabetes patient (Chapter 8 of this thesis). Studies performed in high risk populations investigating the association between dp-ucMGP levels and total mortality risk were inconsistent (18-20).

To date, the association of circulating dp-ucMGP levels with coronary heart disease (CHD) or stroke events in the general population has not been investigated.

Therefore, we performed a case-cohort study to investigate the association between circulating dp-ucMGP levels and CHD or stroke risk.

## Methods

*Study population and design*

The EPIC-NL cohort is the Dutch contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC) and consists of the Prospect-EPIC and MORGEN-EPIC cohorts (21). The Prospect-EPIC study includes 17,357 women aged 49-70 years living in Utrecht and vicinity who participated in the nationwide Dutch breast cancer screening program. The MORGEN-EPIC cohorts consists of 22,654 men and women aged 21-64 years selected from random samples of the Dutch population in three different towns.

Participants were recruited in both studies from 1993-1997. At baseline, a general and a food-frequency questionnaire (FFQ) were administrated, and a physical examination was performed for blood pressure measurements, anthropometry, and non-fasting blood sampling. All participants provided informed consent before study inclusion. The study complies with the Declaration of Helsinki and was approved by the institutional board of the University Medical Centre Utrecht (Prospect) and the Medical Ethical Committee of TNO nutrition and Food Research (MORGEN).

For the present study, we used a case-cohort design, including all incident CHD (n=1167) and stroke cases (n=385) until January 1, 2008, and a representative subcohort (n=1,413 including 98 of incident CHD cases and 27 stroke cases) randomly selected from the baseline EPIC-NL cohort. After exclusion of participants using vitamin K antagonists (n=21; 15 cases and 6 subcohort participants) and with missing dp-ucMGP data (n=3; 2 cases, and 1 subcohort participant) 2,941 participants (1,535 cases and 1,406 subcohort participants) were left for the analyses.

*Baseline measurements*

The general questionnaire contained questions of demographic characteristics, the presence of chronic diseases, and risk factors of chronic diseases. Smoking was categorized into current, former, and never smoker. Physical activity was assessed using a questionnaire validated in an elderly population and categorized to the Cambridge Physical Activity index (22). During the baseline physical examination, systolic and diastolic blood pressure were measured twice in the supine position on the right arm using a Boso Oscillomat (Bosch & Son, Jungingen, Germany)(Prospect-EPIC) or on the left arm using a random zero sphygmomanometer (MORGEN-EPIC),

from which the mean was taken. Height, and weight were measured and BMI was calculated. All measurements were performed according to standard operating procedures. Blood samples were taken to prepare citrated plasma and serum, which were subsampled in straws and stored under liquid nitrogen at -196°C.

*Dp-ucMGP*

The measurement of dp-ucMGP was performed with a dual antibody ("sandwich") ELISA. This ELISA use a monoclonal antibody against the dpMGP sequence 3-15 as capture antibody (mAb-dpMGP; VitaK BV, Maastricht, The Netherlands) and use a monoclonal antibody directed against the ucMGP sequence 35-49 in human MGP (mAb-ucMGP, VitaK BV).

*Outcome assessment*

Morbidity follow-up data on cardiovascular disease events were obtained from the Dutch centre of Heath Care Information, which holds a standardized computerized register of hospital discharge diagnoses. All diagnoses were coded according to the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9). Follow-up was complete until the first of January 2008. The database was linked to the cohort on the basis of birth date, gender postal code and general practitioner with a validated probabilistic method (23). Information on vital status was obtained through linkage with the municipal registries. Causes of death were collected from Statistics Netherlands. Endpoints of the present analysis were coronary heart diseases (CHD) (ICD-9; 410-414, 427.5, 798.1, 798.2, 798.9 and ICD-10; I20-I26, I46, R96) and stroke (ICD-9; 430-434, 436 and ICD-10; I60-I66). These analyses included both fatal and non-fatal cases of CHD and stroke.

*Data analyses*

Participant characteristic are presented as means with standard deviations or percentages. The duration of follow-up was calculated as the interval between data of study entry and the occurrence of CHD or stroke event, death or censoring, whichever came first. Modified Cox regression models that account for the case-cohort design according to the unweighted method (24) were used to estimate associations. Age was used as an underlying time variable in the Cox models, with age at recruitment as entry time and the age at diagnosis of CHD or stroke, death, loss to follow-up, or censoring of the end of the follow-up as exit time. All analyses were stratified for cohort. Covariates (potential confounders) were entered into the

model as follows: model 1 included age and BMI; model 2 additionally included gender, smoking status, blood pressure and physical activity index. In the final model cholesterol ratio, triglycerides, history of CHD or stroke was added. These models were also used for the analyses of circulating dp-ucMGP levels with ischemic stroke. To test for linear trends across categories, we modeled dp-ucMGP levels by including the median value of each category as continuous variables. The square of this term was included in the model with the linear term to test whether nonlinear association was present but this was not detected. The interaction between dp-ucMGP and possible effect modifiers age and sex was tested by including interaction terms in to the model. Two-sided P-values below 0.05 were considered to be statistically significant. Data analysis were conducted using SAS 9.2 for windows (SAS Institute, Cary,NC, USA).

**Results**

The median of the follow up was with an average follow-up of 12.0 years. Table 1 shows the baseline characteristics of the study population. The median of circulating dp-ucMGP levels was 114 pmol/L with an interquartile range of 67-197. The mean age of the study population was 49.5 years and 24.4% were men. Participants with high circulating dp-ucMGP levels had a higher BMI, blood pressure, cholesterol ratio, glucose levels and had a lower menaquinone intake.

**Table 1:** Mean (±S.D.)* baseline characteristics of the study population

| | *Total population* | *Dp-ucMGP Q1* | *Dp-ucMGP Q2* | *Dp-ucMGP Q3* | *Dp-ucMGP Q4* |
|---|---|---|---|---|---|
| Dp-ucMGP (pmol/l) | 167 ± 98 | 47 ± 90 | 103 ± 86 | 168 ± 86 | 348 ± 87 |
| Follow up time | 11.4 ± 3.1 | 11.4 ± 2.9 | 11.5 ± 2.8 | 11.4 ± 2.8 | 11.3 ± 2.8 |
| Male (%) | 24.4 | 29.5 | 25.3 | 22.9 | 17.5 |
| Age (years) | 49.5 ± 11.8 | 43.9 ± 11.9 | 48.9 ± 11.4 | 52.4 ± 10.7 | 55.5 ± 9.5 |
| BMI (kg/m$^2$) | 26.2 ± 4.6 | 24.9 ± 4.2 | 25.9 ± 4.1 | 26.6 ± 4.1 | 27.6 ± 4.1 |
| Waist hip ratio (cm) | 0.86 ± 0.07 | 0.85 ± 0.06 | 0.86 ± 0.06 | 0.86 ± 0.07 | 0.87 ± 0.07 |
| Smoking (pack years) | 9.9 ± 0.4 | 11.0 ± 0.6 | 10.1 ± 0.6 | 9.8 ± 0.7 | 8.7 ± 0.7 |
| | | | | | |
| Systolic BP (mmHg) | 128 ± 19 | 125 ± 18 | 126 ± 17 | 128 ± 17.6 | 133 ± 17 |
| Diastolic BP (mmHg) | 79 ± 12 | 78 ± 11 | 79 ± 11 | 80 ± 11 | 81 ± 10 |
| Total cholesterol (mmol/L) | 5.6 ± 1.1 | 5.4 ± 1.0 | 5.5 ± 1.0 | 5.7 ± 1.0 | 5.6 ± 1.0 |
| HDL cholesterol (mmol/L) | 1.36 ± 0.45 | 1.41 ± 0.41 | 1.41 ± 0.40 | 1.32 ± 0.38 | 1.30 ± 0.39 |
| Total cholesterol/HDL ratio | 4.4 ± 1.5 | 4.2 ± 1.4 | 4.3 ± 1.3 | 4.5 ± 1.3 | 4.6 ± 1.3 |
| Triglyceride (mmol/L) | 1.8 ± 1.2 | 1.7 ± 1.1 | 1.6 ± 1.1 | 1.9 ± 1.1 | 1.9 ± 1.1 |
| Glucose (mmol/L) | 5.1 ± 1.8 | 5.0 ± 1.7 | 5.1 ± 1.6 | 5.2 ± 1.6 | 5.3 ± 1.6 |
| | | | | | |
| Energy (kcal) | 2209 ± 605 | 2191 ± 556 | 2212 ± 535 | 2198 ± 532 | 2234 ± 542 |
| Vitamin K (µg/day)[1] | 221 ± 118 | 238 ± 109 | 223 ± 105 | 219 ± 104 | 202 ± 106 |
| Phylloquinone (µg/day)[1] | 190 ± 116 | 206 ± 107 | 190 ± 103 | 189 ± 102 | 174 ± 104 |
| Menaquinone (µg/day)[1] | 30 ± 16 | 30 ± 15 | 32 ± 14 | 29 ± 14 | 27 ± 14 |

BMI, Body mass index; BP, blood pressure, MGP, Matrix gla protein; dp-ucMGP, desphospho-uncarboxylated MGP;
*means were adjusted for gender and age except for the variable age and gender
[1]energy-adjusted intake

Table 2 shows the association between circulating dp-ucMGP and CHD or stroke risk. Circulating dp-ucMGP levels were not associated with risk of CHD with a HR per SD of 1.03 (95%-CI 0.98-1.10). After adjustment for age, BMI, gender, smoking status, blood pressure, physical activity, cholesterol ratio, triglycerides, and history of CVD, circulating dp-ucMGP levels were not associated with CHD risk with a HR per SD of 1.00 (95%-CI 0.93-1.07). In additional analyses circulating dp-ucMGP plasma levels were categorized in quartiles. After full adjustment circulating dp-ucMGP was not

associated with risk of CHD when comparing the highest quartile with the lowest quartile with a $HR_{(Q4 \text{ versus } Q1)}$ of 0.94 (95%-CI 0.79-1.13, P for trend 0.51). Similar results were observed for the associations between dp-ucMGP and stroke risk. Circulating dp-ucMGP levels were not associated with risk of stroke with a $HR_{SD}$ of 0.98 (95%-CI 0.90-1.08) and ($HR_{(Q1 \text{ versus } Q4)}$ 1.09, 95%-CI 0.78-1.51 P for trend 0.52). Restricting to ischemic stroke did not affect these results (data not shown). Interaction with age and gender was not detected.

**Table 2:** Hazard ratios (95%CI) for the association of baseline dp-ucMGP levels with risk of CVD and CHD

| | *dp-ucMGP per SD* | *dp-ucMGP Q1* | *dp-ucMGP Q2* | *dp-ucMGP Q3* | *dp-ucMGP Q4* |
|---|---|---|---|---|---|
| **CHD** | | | | | |
| Cases | | 262 | 287 | 354 | 349 |
| Crude | 1.03 (0.98-1.10) | 1 | 0.94 (0.79-1.12) | 1.11 (0.94-1.31) | 1.06 (0.90-1.26) |
| Model 1[1] | 1.02 (0.96-1.08) | 1 | 0.92 (0.77-1.09) | 1.06 (0.89-1.25) | 0.99 (0.83-1.18) |
| Model 2[2] | 0.99 (0.93-1.06) | 1 | 0.95 (0.80-1.14) | 1.06 (0.89-1.24) | 0.96 (0.80-1.14) |
| Model 3[3] | 1.00 (0.93-1.07) | 1 | 0.97 (0.81-1.16) | 1.03 (0.87-1.22) | 0.94 (0.79-1.13) |
| **Stroke** | | | | | |
| Cases | | 75 | 90 | 114 | 126 |
| Crude | 1.02 (0.93-1.10) | 1 | 0.97 (0.71-1.32) | 1.11 (0.82-1.51) | 1.15 (0.85-1.57) |
| Model 1[1] | 1.01 (0.94-1.10) | 1 | 0.98 (0.72-1.34) | 1.13 (0.83-1.53) | 1.18 (0.86-1.62) |
| Model 2[2] | 0.98 (0.89-1.07) | 1 | 1.02 (0.74-1.39) | 1.13 (0.83-1.54) | 1.09 (0.78-1.51) |
| Model 3[3] | 0.98 (0.90-1.08) | 1 | 1.01 (0.74-1.39) | 1.12 (0.81-1.53) | 1.09 (0.78-1.51) |

Matrix Gla protein; dp-ucMGP, desphospho-uncarboxylated MGP
All analyses are adjusted for cohort
[1]adjusted for age and BMI
[2]adjusted for age, BMI, gender, smoking status , blood pressure and physical activity index
[3]adjusted for age, BMI, gender, smoking status, blood pressure, physical activity index, cholesterol ratio, triglyceride, history of CHD or stroke

**Discussion**

To our knowledge this is the first study investigating the association of dp-ucMGP with the risk of CHD and stroke events in the general population. In this case-cohort study we did not observe that high dp-ucMGP levels, reflecting poor vitamin K status, were associated with increased CHD or stroke risk.

We previously reported that higher circulating dp-ucMGP levels were significantly associated with increased risk of CVD with a HR per SD of 1.21 (95%-CI 1.06-1.38) among type 2 diabetes patients, while higher dp-ucMGP levels were not related to risk of CHD ($HR_{SD}$ 1.12; 95%-CI 0.94-1.34) (chapter 8 of this thesis). As far as we know there are no other studies that investigated the association between dp-ucMGP and cardiovascular events. However, several studies were performed in populations with high cardiovascular risk using mortality risk as outcome. Although this is not the same as CVD risk, CVD is an important cause of mortality in populations with a high cardiovascular risk. The results in these high risk populations were inconsistent; in calcific valvular aortic stenosis patients (19) and chronic heart failure patients (20), high dp-ucMGP levels were associated with increased all-cause mortality risk. However, in chronic kidney disease patients high dp-ucMGP levels were not associated with mortality risk (18;25). Furthermore, participants of high risk populations had markedly elevated circulating dp-ucMGP levels compared to matched healthy controls (18-20). In the present study, which was population-based, dp-ucMGP levels were much lower and the variation in concentrations was relatively small, which may have limited the contrast between dp-ucMGP levels to detect an association between circulating dp-ucMGP levels and CHD or stroke. Furthermore, it is unclear whether high risk populations suffer from generalized vitamin K deficiency, leading to high levels of dp-ucMGP, or whether the disease state by itself causes higher dp-ucMGP levels. Previous studies showed that high circulating dp-ucMGP levels were associated with vascular calcification risk (15;17;18). Hence, it is possible that the association found in the high risk populations is explained by the higher prevalence of vascular calcification in high risk populations, compared to our general population. Studies have suggested that vitamin K and MGP may be more effective when vascular calcification is already present. An intervention study showed that phylloquinone supplementation slows the progression of CAC especially in adults with preexisting CAC (6). This is in line with the proposed mechanism of action of

MGP, which is thought to inhibit calcification by binding to the hydroxyapetite in the vessel wall or by binding to and thereby inhibiting bone morphogenetic protein-2, an osteogenic growth factor (26). However, dp-ucMGP does not contain the phosphorylated or carboxylated fractions that allow MGP to bind calcium. Perhaps dp-ucMGP reflects other forms of MGP that do so.

Another explanation could be the type of cardiovascular endpoint used in this study. In our study among type 2 diabetics (chapter 8 of this thesis) we found an association between higher circulating dp-ucMGP levels and increased CVD risk. However, this association was stronger than the associations with CHD and stroke risk. Further analyses showed that the association with CVD was mainly driven by diseases like peripheral vascular diseases (HR 1.42 CI95%: 1.16-1.75). So, perhaps dp-ucMGP is more strongly related to other forms of CVD than CHD or stroke. This could be explained by two types of vascular calcification; intimal calcification and medial calcification. Intimal calcification is seen in sites of atherosclerotic plaques, in which cellular necrosis, inflammation, and cholesterol deposition occur. The presence of this type of calcification is associated with atherosclerotic burden (27). The other type of cardiovascular calcification is medial calcification which occurs in the elastic lamina of large- and medium- to small-size arteries and is particularly present in peripheral arteries (27). Medial calcification is a metabolite-induced calcification in the absence of lipid deposits, leading to upregulation of osteogenic genes and proteins with subsequent matrix mineralization, bone and cartilage formation (28). This process requires a complex regulatory network involving both stimulating and inhibitors of calcification such as matrix Gla protein, osteoprotegerin and osteopontin(28). It is thus possible that the association between dp-ucMGP and calcification differ between the two types of calcification.

The strengths of this study include its prospective design, long follow up, adjustment for a large range of potential confounders and generalizability to the general population. However, certain limitations need to be addressed. Circulating dp-ucMGP levels were measured at a single time point; at baseline, and the time course or release of dp-ucMGP and its persistence in the circulation is unknown. Further, the circulating dp-ucMGP levels in our study were low compared to a healthy reference group (10). This could be due to the possible degradation of dp-ucMGP in our samples, because our samples have been stored for a long time. However, in our study with type 2 diabetes patients where we had similar low

dp-ucMGP levels, we did detect an association between dp-ucMGP levels and vascular calcification risk (Chapter 8 of this thesis). These plasma samples were stored under the same conditions as the present study. Finally, in another study (chapter 7) we were able to estimate reliability of the MGP levels by comparing the samples of that study (collected in 1993-1997) with samples with a shorter storage time (collected in 2002-2004) and found a Spearman correlation coefficient of 0.67, which is reasonable. We therefore assume that degradation of MGP did not substantially affect the ranking of individuals among the dp-ucMGP distribution, and did not systematically bias the associations we found. Nevertheless, the degradation of dp-ucMGP in our samples may have led to a very small dispersion of circulating dp-ucMGP levels, which could be a reason that we did not find an association between circulating dp-ucMGP levels and CHD risk in this population. However, excluding people with circulating dp-ucMGP levels lower than 100 pmol/L or 50 pmol/L did not affect our results. Furthermore circulating dp-ucMGP levels were associated with age as expected. We do therefore not think that degradation brought about the null result of this study.

Taken together, this study showed no association between high dp-ucMGP levels, reflecting a poor vitamin K status, and higher CHD or stroke risk in a healthy population. Because this is the first study that investigated the association between dp-ucMGP levels and CHD or stroke risk in the general population, further research is required to confirm our findings and to investigate the association with specific endpoints like peripheral vascular calcification.

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# Part III

# Chapter 10

General discussion

## General discussion

This thesis aimed to study the role of matrix Gla protein (MGP) carboxylation in the association of high vitamin K intake with reduced vascular calcification and cardiovascular disease (CVD) risk. In this chapter, the findings presented in this thesis are placed in a broader context, and the implications for future research are discussed.

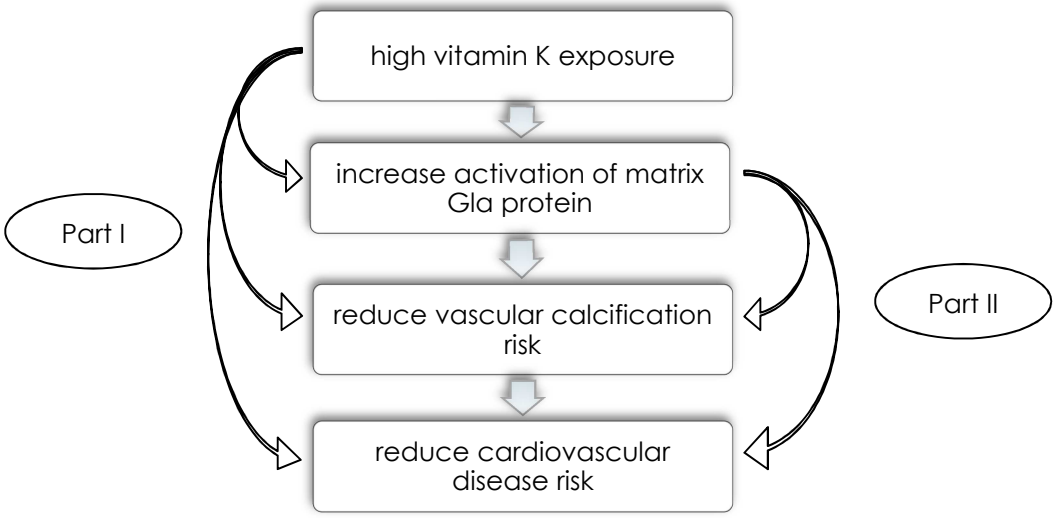


**Figure 1:** Schematic overview of the hypotheses considered in part I and II

In the first part of this thesis we focused on the association between vitamin K exposure and circulating MGP species, vascular calcification or CVD risk (see figure 1).

*Measurement of vitamin K exposure*

Accurate assessment of vitamin K exposure is of great importance to obtain valid estimates for the associations between phylloquinone or menaquinone exposure and vascular calcification or CVD risk. The majority of population-based studies reporting on the association between vitamin K exposure and vascular calcification or coronary heart disease (CHD) have relied on vitamin K intake as the measure of exposure. Observational studies did not find an association between the intake of phylloquinone and vascular calcification or CHD risk (1-5). However, for intake of menaquinones observational studies consistently showed that high intake is associated with reduced vascular calcification or CHD risk (2-5). As in most cohort studies, we used a food frequency questionnaire (FFQ) to assess vitamin K intakes of

the participants. Despite its feasibility to apply for large cohorts and measuring long-term dietary intake the FFQ has certain limitations since it depends on the long-term memory of study participants, which can lead to over- or under-reporting of food items. Phylloquinone is found in healthy foods like green vegetables, therefore self-reported intakes may be subject to over-reporting. Dietary sources of menaquinones are generally not associated with an overall healthy diet, so intake of menaquinone data may be less prone to over-reporting and residual confounding to the same extent as phylloquinone. In line with this, the relative validity of our FFQ for phylloquinone intake was low (r=0.24), but reasonable for the intake of menaquinones (correlations ranging from 0.51 for MK-7 to 0.72 for MK-5), except for MK-10 (r=0.23)(5) with correlation coefficients well in line with those of many other nutrients estimated using FFQs (6).

Another limitation of food record based estimate of vitamin K intake is the limited information on vitamin K content of foods. The Dutch food consumption table does not contain information on phylloquinone and menaquinone content of foods. Therefore, we used concentrations of phylloquinone and menaquinones in a series of Dutch foods assessed at the Biochemistry Laboratory, Maastricht University (7) and published data by others were used to update the dietary database for vitamin K (8-11). Despite these efforts, the database is not complete. Although the most important sources of vitamin K were included in the database, variation within a food product e.g. cheese is not always taken into account and more research is required to compile more elaborate food composition data of vitamin K in which concentrations of phylloquinone and menaquinone from representative food items from different food supplies and seasons are taken into account.

Because of these limitations, results found for vitamin K intake need to be confirmed by results of relations of other markers of vitamin K exposure or markers of the putative underlying mechanism with CVD. People do not eat single nutrients, but rather consume food products that consist of combination of nutrients. Studies into single nutrients may not be able to adequately take into account interactions between nutrients that are in the same food product. Furthermore, it is possible that the absorption of a single nutrient differs depending on de food matrix it is consumed with. Therefore, an alternative approach that we used in this thesis was to focus on food product intake instead of single nutrient intake. The association of a food

product, which is a main source of a specific nutrient, and disease risk could confirm the relation of a nutrient with disease risk. We therefore investigated the association between dairy intake, which is an important source of menaquinones, and dairy subtypes with CHD and stroke risk in chapter 4. These analyses did not provide evidence that dairy intake is associated with risk of CHD or stroke. This does not necessarily mean that that menaquinone intake is not associated with CHD or stroke risk. Although we did not account for other sources of menaquinones, in our study population dairy intake contributed 72.0%. It thus seems more likely that other nutrients like saturated fat and calcium of which dairy is also an important source can dilute the association of menaquinone with CHD or stroke (12). However, in our study we observed that only high fermented dairy intake tended to be associated with a lower stroke risk. This could be an indication that menaquinone is the underlying mechanism because of all dairy subtypes fermented dairy contributes 66.1% of menaquinone intake. Further research is required to confirm our finding and to investigate if this association is indeed brought about by menaquinone.

In addition to studying food groups, we also used nutritional biomarkers in biological samples, i.e. plasma phylloquinone, to further investigate the relation with coronary calcification. Nutritional biomarkers are unlikely to be influenced by dietary intake alone. Nutritional biomarkers also take into account other aspects that may influence nutrient concentrations like preparation methods, but also metabolism and efficiency of uptake from the digestive tract (13). Furthermore, genetic, environmental, and lifestyle factors may also influence nutrient concentrations. A concern about nutritional biomarkers is that they do not always reflect long-term intake of diet. Some nutritional biomarkers respond to dietary intake within hours while others responds over weeks or months. Another concern is that the relation between nutrient intake and levels in plasma or serum is rarely linear and that relation could flatten with high intake (13). In particular, for phylloquinone, a previous study indeed showed that the relation of plasma phylloquinone with intake phylloquinone was linear up to ca. 100-150 microgram and leveled off at higher intakes (14).

In chapter 3 we investigated the association of phylloquinone concentrations with CAC and vascular calcification. This study showed that higher phylloquinone concentrations were not associated with reduced vascular calcification, but

seemed to be associated with increased CAC risk. This was in contrast with our hypothesis. A possible explanation for this unexpected observation is that single plasma phylloquinone measure as a marker for long-term vitamin K intake is imperfect. This is because circulating phylloquinone concentrations have a very short half-life time of 1-2 hours (15), and therefore they reflect only very recent phylloquinone intakes (16;17). Furthermore, in our study the relation between phylloquinone intake and circulation phylloquinone concentrations was low with a Pearson correlation of 0.14. This correlation is in line with other studies with correlations of 0.13 and 0.19 (18;19), although one found a moderate correlation of 0.51 (20). The current understanding of phylloquinone physiology and metabolism only partially explains the variation in vitamin K exposure between individuals (21). It is plausible that dietary intakes of phylloquinone contribute more than can be currently quantified, but methodological limitations may attenuated diet-plasma correlations. Taking this into account it is possible that plasma phylloquinone may be a better marker for an unmeasured biochemical or genetic risk factor for calcification than for phylloquinone intake.

The uncarboxylated fractions of vitamin K-dependent proteins, which can also be measured in the circulation, are proposed as functional indicators of vitamin K exposure of certain tissues (21). Thus far, conformation-specific tests have been developed for the vitamin K-dependent blood coagulation II, prothrombin, osteocalcin (OC) and MGP. An advantage of measuring uncarboxylated forms of vitamin K dependent proteins is that insufficiencies measured in circulating forms theoretically reflect what occurs at the level of the different target tissues. Uncarboxylated prothrombin (PIVKA-II) reflects hepatic vitamin K status and changes according to vitamin K intake. However, is not useful as a nutritional biomarker because it only detects overt vitamin K deficiency and does not have the sensitivity to detect differences in usual intake of K vitamins in the generally healthy population (22). Of the uncarboxylated Gla proteins, the effect of vitamin K intake on ucOC is most frequently studied. These studies have shown that ucOC is very responsive to supplementation with either phylloquinone and menaquinone (15;23-29). UcOC is therefore suggested as functional marker of vitamin K exposure. However, ucOC may not necessarily reflect vitamin K status in vascular tissue.

Assays to measure the vascular vitamin K-dependent protein desphospho-uncarboxylated MGP (dp-ucMGP) only recently became available (30). Cross-sectional comparisons between users of vitamin K supplements and controls suggested that dp-ucMGP could also reflect vitamin K exposure (31;32). This was confirmed by an intervention study with phylloquinone supplementation (33), a non-placebo controlled trial and an exploratory pilot study with menaquinone supplementation (29;34). In chapter 5 of this thesis, we showed that supplementation with menaquinone during 12 weeks, dose-dependently decreased circulating dp-ucMGP concentrations. In addition, we found that these changes already occurred within 4 weeks of supplementation with a size of effect similar as size of effect on ucOC. Moreover, in one of our observational studies (chapter 6) we observed a Pearson correlation of 0.6 between ucOC and dp-ucMGP. Therefore, we conclude that circulating dp-ucMGP clearly reflects the vitamin K supplementation status, and may serve as biomarker of vitamin K exposure. This is confirmed by result from chapter 7 and an cross-sectional study of Shea et al (32), in which an association of high dietary vitamin K intake with lower circulating dp-ucMGP concentrations was shown. However, more observational studies are required to give more insight into the association between dietary vitamin K intake and circulating dp-ucMGP concentrations.

Although both OC and MGP are associated with vitamin K exposure, one limitation should be kept in mind for these markers. Both proteins also have biological functions in bone and vascular tissues, respectively. It is probable that this biological function also influences the ucOC and dp-ucMGP concentrations in the circulation. This possibility must be taken into account when interpreting our results.

Taken together, as for most nutrients, there is not a gold standard to measure vitamin K exposure in epidemiological studies. However, dp-ucMGP concentrations may be promising to serve as a biomarker of vitamin K exposure.

In the second part of this thesis we therefore focused on investigating the association of circulating dp-ucMGP in particular and other MGP species with vascular calcification or CVD risk (see figure 1). We first investigated the association between circulating MGP species and vascular calcification and second we studied the association between circulating MGP species and CVD risk.

*MGP*

Theoretically, low uncarboxylated and high carboxylated MGP (cMGP) concentrations would be associated with reduced calcification risk. However, in practice it is more complicated due to the fact that MGP undergoes two types of post-translational modification; i.e. carboxylation and phosphorylation. Although the function of phosphoserine residues is not precisely known, it has been suggested that phosphorylated MGP residues may also contribute to MGP's calcification-inhibitory activity (35). As a result of both post-translational modifications MGP exist in the circulation as various distinct species according to its state of phosphorylation and/or carboxylation: phosphorylated carboxylated (p-cMGP), phosphorylated uncarboxylated (p-ucMGP), desphospho-carboxylated MGP (dp-cMGP) and desphospho-uncarboxylated dp-ucMGP (dp-ucMGP). The use of circulating biomarkers to predict vascular calcification is an attractive possibility because it may quickly, noninvasively, and cost-effectively predict disease and monitor efficacy of therapy. Unfortunately, there are currently no assays available to measure each individual circulating MGP species or even the total circulating MGP amount. To date, it is possible to measure circulating dp-ucMGP, dp-cMGP and total-ucMGP (t-ucMGP), which is the sum of p-ucMGP and dp-ucMGP. Within this context, we investigated whether these measurable circulating species are associated with vascular calcification or CVD risk.

*Dp-ucMGP*

Based on the effects of vitamin K supplementation on dp-ucMGP and its association with vitamin K intake, we expected that higher circulating dp-ucMGP concentrations, reflecting low vitamin K exposure, would result in a decreased capacity to inhibit calcification. In this thesis we indeed observed borderline significant associations between high circulating dp-ucMGP concentrations and CAC, both in a cross-sectional and a prospective study described in chapter 6 and 7, respectively. Furthermore, in the prospective study we also found borderline significant association between high circulating dp-ucMGP concentrations and higher number of calcified areas. Moreover, in type 2 diabetes patients, a high-risk population, we observed that high circulating dp-ucMGP concentrations were associated with increased CVD risk (chapter 8). Unfortunately, circulating dp-ucMGP concentrations were not associated with CHD or stroke risk in the general population. The different results of chapter 8 compared to the other studies may be due to

different study populations. The prevalence of vascular calcification in type 2 diabetes patients is much higher than in the general population. Studies have suggested that vitamin K and MGP may be more effective when vascular calcification is already present. An intervention study showed that phylloquinone supplementation slows the progression of CAC especially in adults with preexisting CAC (33). This is in line with proposed mechanism of action of MGP, which is thought to inhibit calcification by binding to the hydroxyapatite already present in the vessel wall or by binding to and thereby inhibiting bone morphogenetic protein-2, an osteogenic growth factor (36). However, dp-ucMGP does not contain the phosphorylated or carboxylated fractions that allow MGP to bind calcium, but perhaps reflects other forms of MGP that do. However, in chapter 6 and 7 we did observe an association between dp-ucMGP and vascular calcification in a general population sample, namely postmenopausal women. This is probably due to the fact that vascular calcification is more directly affected by MGP than CVD.

Another explanation for the non-significant finding in chapter 8 could be the different types of cardiovascular endpoint used in these studies. In the general population we used CHD and stroke as outcome while in the type 2 diabetes population we studied CVD and CHD risk. When looking at specific types of CVD outcomes in the type 2 diabetes population, we found a stronger association between dp-ucMGP concentrations and 'other CVD' than for CHD or stroke (HRs 1.42 (95%CI 1.16-1.75) for other CVD, and 1.12 (95%CI 0.94-1.34) and 1.05 (95CI 0.73-1.49) for CHD and stroke, respectively). Although the number of 'other CVD' cases was small (n= 44), our findings may suggest that dp-ucMGP is especially associated with other forms of CVD than CHD or stroke like peripheral vascular diseases. This could suggest that dp-ucMGP differentially affects different types of vascular calcification.

There are two types of vascular calcification; intimal calcification and medial calcification. Intimal calcification is seen in sites of atherosclerotic plaques, in which cellular necrosis, inflammation, and cholesterol deposition occur. The presence of this type of calcification is associated with arterial obstruction (37). The other type of cardiovascular calcification is medial calcification, which occurs in the elastic lamina of large- and medium- to small-size arteries, is particularly present in peripheral arteries, and is associated with vascular stiffness (37). Medial calcification

is a metabolite-induced calcification in the absence of lipid deposits, leading to upregulation of osteogenic genes and proteins with subsequent matrix mineralization, bone and cartilage formation (38). This process requires a complex regulatory network involving both stimulating and inhibitors of calcification such as MGP, osteoprotegerin and osteopotin (38). It is thus possible that the associations between dp-ucMGP and the two types of calcification differ.

More observational studies are required to investigate whether circulating dp-ucMGP concentrations indeed is associated differently with the different types of calcification or with specific CVD endpoints, like peripheral vascular diseases. Furthermore, long-term clinical trials are needed to confirm the hypothesis that the reduction in dp-ucMGP concentrations due to vitamin K supplementation leads to less calcification. In conclusion, circulating dp-ucMGP has the potential to become a biomarker for vitamin K exposure and may contribute to vascular calcification or CVD risk marker

*Dp-cMGP*

Since carboxylation of MGP is vitamin K-dependent, our expectation was that the dp-cMGP species would respond to changes in vitamin K supplementation in an opposite direction as the dp-ucMGP species. However, in chapter 5 of this thesis circulating dp-cMGP concentrations remained unchanged after menaquinone supplementation. One possible explanation for these results could be that the dp-cMGP species that are synthesized upon high vitamin K intake remain in the tissue and are not readily secreted into the circulation. Another explanation could be found in the second post-translational phosphorylation. It could be speculated that due to high vitamin K intake, dp-cMGP species are not only better carboxylated but also better phosphorylated (35). This would result in formation of p-cMGP species, which remain undetected with the dp-cMGP assay. It is clear that more fundamental research into MGP phosphorylation is necessary to investigate why dp-cMGP species do not respond to vitamin K intake.

We did not find an association between dp-cMGP and vascular calcification or CVD risk. However, one observational study found an association between low dp-cMGP and more extensive calcification in a high risk population (39). The biological meaning of dp-ucMGP is therefore still unclear and more observational studies, as well as physiological studies are warranted. Taken together, circulating dp-cMGP is

not suited as biomarker for vitamin K exposure or as risk marker for vascular calcification or CVD.

*T-ucMGP*

We did not find an association between menaquinone intake and circulating t-ucMGP concentrations and therefore not suited as biomarker for vitamin K exposure. With the t-ucMGP assay, the total pool of circulating uncarboxylated MGP can be measured, regardless of the phosphorylation status. Previous studies in populations at high risk of vascular diseases found that low circulating t-ucMGP concentrations were associated with more vascular calcification (40-42). In our pilot study (chapter 6) to verify the direction and the association between the different circulating MGP species and CAC we also observed that high t-ucMGP concentrations tended to be associated with lower CAC in the entire population. This association strengthened to a significant association between high t-ucMGP concentrations and lower CAC amongst women with CAC. Similarly, in chapter 7 we observed that low circulating t-ucMGP concentrations were associated with more calcified areas. However, in this study we did not find a linear association between circulating t-ucMGP levels and CAC. Spline regression showed evidence of a U-shaped relation between t-ucMGP and coronary calcification with lower presence of CAC up to up to 3.2 nM t-ucMGP, but increasing presence of CAC at higher levels. Therefore, the association of t-ucMGP with CAC appears to differ depending on the amount of CAC already present. This can be explained by binding of MGP to crystal nuclei in hydroxyapatite (30). Because t-ucMGP concentrations are >1000-fold higher than dp-ucMGP, we assume that t-ucMGP mainly consists of phosphorylated ucMGP species (p-ucMGP). The t-ucMGP levels therefore mainly reflect p-ucMGP. The phosphorserines equip the molecule with strong calcium binding groups irrespective of its Gla content and this fraction has a strong affinity for vascular calcification. Due to this affinity the p-ucMGP species will adhere to the sites of calcification thereby causing a decrease in circulating concentrations of p-ucMGP. This might also lead to lower t-ucMGP concentrations if vascular calcification is present. Therefore, presence of CAC can distort the associations observed. Similar results have been found for other markers of CAC such as osteoprotegerin, another inhibitor of coronary calcifications. The associations of osteoprotegerin with CAC in human studies was opposite to what was expected

from experimental work and a U-shaped association of osteoprotegerin with CAC was also observed (43).

Taken together, it seems that the association between high circulating t-ucMGP concentrations and CAC is mainly present in people that have vascular calcification. If this hypothesis it true then circulating t-ucMGP concentrations can only be used as a biomarker for presence and amount of calcification in people with vascular calcification and not as a risk marker for vascular calcification and CVD. Future research in subjects with and without vascular calcification is necessary to give more insight in the association between circulating t-ucMGP and vascular calcification. In addition, research to determine the relation between t-ucMGP and vascular calcification progression is needed.

Overall, the aim of this thesis was to investigate the role of MGP carboxylation in the association of high vitamin K intake with reduced vascular calcification and CVD risk. In the first part of this thesis we showed that high vitamin K intake decreases circulating dp-ucMGP concentrations and therefore circulating dp-ucMGP may serve as biomarker for vitamin K exposure. In the second part we observed that increased dp-ucMGP levels were associated with more vascular calcification and increased CVD among type 2 diabetes patients. This suggest that dp-ucMGP may form a link between vitamin K and vascular calcification or CVD, although we could not confirm this in the general populations. Furthermore, this thesis suggests that the type of vascular calcification has an effect on the association between circulating dp-ucMGP concentrations and vascular calcification. Neither dp-cMGP nor t-ucMGP were associated with vitamin K intake, but circulating t-ucMGP might be a biomarker for presence of vascular calcification.

*Implications and recommendations for future research*

We would recommend to (further) investigate the following topics:

- Compile more elaborate food consumption data of vitamin K and more accurate data on intake of vitamin K
- The association between dietary vitamin K intake and circulating dp-ucMGP concentrations
- The association between circulating dp-ucMGP and different types of calcification (intimal and medial), the progression of vascular calcification or specific CVD endpoints

- The association between circulating t-ucMGP and different types of calcification (intimal and medial) and the progression of vascular calcification
- If long-term vitamin K supplementation can lead to less vascular calcification due to reduction in dp-ucMGP concentrations
- Fundamental research to investigate the processes of underlying MGP synthesis, transport, carboxylation and phosphorylation

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# Chapter 11

Summary

Summary in Dutch

Acknowledgements

About the author

Publication list

The overall aim of this thesis was to investigate the role of matrix Gla protein (MGP) carboxylation in the association between high vitamin K intake and reduced vascular calcification and CVD risk. We hypothesized that high vitamin K exposure will increase the carboxylation of MGP, which will reduce vascular calcification and lowers cardiovascular disease risk.

**Chapter 2** provides an overview of the literature on vitamin K intake in relation to vascular calcification and cardiovascular disease risk. In observational studies, vitamin K intake was estimated with food frequency questionnaires (FFQ). For the intake of menaquinones, observational studies consistently showed that high intakes are associated with reduced coronary artery calcification (CAC) and cardiovascular disease (CVD) risk. However, for phylloquinone intake, observational studies did not show associations with CAC and CVD risk. Nevertheless, intervention studies showed improved vascular elasticity and reduced progression of coronary calcification after phylloquinone supplementation.

In **chapter 3** of this thesis, we investigated the association between circulating phylloquinone concentrations with CAC and vascular calcification among 508 postmenopausal women. This association is of interest because circulating phylloquinone concentrations could be a better measure for phylloquinone exposure than phylloquinone intake estimated by FFQ, because of its low relative validity. We showed that detectable phylloquinone concentrations (>0 nmol/L) were not associated with reduced vascular calcification compared to non-detectable phylloquinone concentrations (0 nmol/L), but on the contrary seemed to be associated with an increased prevalence of CAC with a prevalence ratio of 1.34 (95%CI: 1.01-1.77).

**Chapter 4** presents the relation between dairy intake, which is an important dietary source of menaquinones, and the risk of coronary heart disease (CHD) or stroke, using data from a prospective cohort among 40.011 Dutch men and women. Our results provide no evidence that total dairy intake is associated with risk of CHD or stroke. None of the dairy subtypes were associated with CHD risk, while only fermented dairy tended to be associated with a lower risk of stroke with a hazard ratio (HR) of 0.92 (95%CI: 0.83-1.01). Among participants without hypertension total and low-fat dairy tended to be associated with a lower risk of CHD.

We investigated the effect of menaquinone-7 supplementation on circulating MGP species in a double-blind, randomized, placebo-controlled trial among 60 healthy participants in **chapter 5**. Participants received 180 µg/d, 360 µg/d menaquinone-7 or placebo supplements. After 12 weeks of supplementation dp-ucMGP decreased dose dependently in the 180 µg/d, 360 µg/d supplementation groups by 31% and 46% respectively, but did not affect other MGP species. Changes in dp-ucMGP levels already occurred after 4 weeks of supplementation. Therefore, dp-ucMGP may serve as a biomarker for vitamin K exposure.

The second part of this thesis focused on the association between in particular dp-ucMGP and other circulating matrix Gla protein species and vascular calcification or CVD risk.In chapter 6 and 7 of this thesis, circulating MGP species in relation to vascular calcification were studied. **Chapter 6** describes the association between circulating MGP species and CAC in a cross-sectional study, which consisted of 200 healthy postmenopausal women. A borderline significant association between higher circulating dp-ucMGP concentrations and high CAC was observed. High total uncarboxylated MGP (t-ucMGP) concentrations tended to be associated with lower CAC. This association strengthened amongst women with CAC to a significant association between high t-ucMGP concentrations and lower CAC. Desphospho-carboxylated MGP (dp-cMGP) was not associated with CAC. In continuation of chapter 6, we also performed a prospective study in **chapter 7** to investigate the association between circulating MGP species and vascular calcification among 571 healthy postmenopausal women. After 8.5 years follow-up 24% of the women had no calcification, while 5% had calcification in all measured areas, namely in the coronary arteries, aortic valve, mitral valve and aortic arch. High circulating dp-ucMGP concentrations were borderline significantly associated with more CAC ($RR_{SD}$ 1.07; 95%CI: 0.99-1.15) and the number of calcification areas ($OR_{(4\ areas\ vs\ no\ calcification)}$ 1.49; 95%CI: 0.95-2.35). High t-ucMGP levels tended to be associated with less calcified areas ($OR_{(4\ areas\ vs\ no\ calcification)}$ 0.63; 95%CI: 0.36-1.10). Dp-cMGP was not associated with vascular calcification.

Vascular calcification has emerged as a strong and independent risk factor for CVD, but the association of circulating MGP species with CVD events has not been investigated. Therefore, in **Chapter 8** of this thesis, we investigated the relation between circulating MGP species and risk of CVD among a high-risk population, i.e.

type 2 diabetes patients. Higher circulating dp-ucMGP concentrations, reflecting poor vitamin K exposure, were associated with higher CVD risk with a hazard ratio (HR) per standard deviation (SD) of 1.21 (95%CI: 1.06-1.38), while circulating dp-cMGP and t-ucMGP concentrations were not associated with CVD risk. **Chapter 9** describes a case-cohort study that investigated the association between circulating dp-ucMGP concentrations and CHD and stroke risk. After an average follow up of 11.5 years, circulating dp-ucMGP concentrations were not associated with CHD or stroke risk. Therefore, this study could not confirm that high dp-ucMGP concentrations, reflecting poor vitamin K exposure, are associated with increased CHD or stroke risk in the general population.

In **chapter 10**, the main findings presented in this thesis are discussed and implications for further research are given. Overall, in the first part of this thesis we showed that high vitamin K intake decreases circulating dp-ucMGP concentrations and therefore circulating dp-ucMGP may serve as biomarker for vitamin K exposure. In the second part we observed that increased dp-ucMGP levels were associated with higher vascular calcification. Furthermore, higher circulating dp-ucMGP concentrations, reflecting poor vitamin K exposure, were associated with higher CVD risk however, only in a high risk population.

# Chapter 11

Summary

Summary in Dutch

Acknowledgements

About the author

Publication list

In dit promotie onderzoek is onderzocht of carboxylatie (activiteit) van het matrix Gla eiwit (MGP) een rol speelt in de associatie tussen een hoge vitamine K inname enerzijds en minder aderverkalking en een lager risico op hart- en vaatziekten anderzijds. Onze hypothese was dat een hoge vitamine K inname leidt tot hogere carboxylatie van MGP en dat gecarboxyleerd MGP aderverkalking vermindert waardoor een lager risico op hart- en vaatziekten ontstaat.

In **hoofdstuk 2** wordt een overzicht gegeven van de literatuur over vitamine K inname in relatie tot vasculaire verkalking en het risico op hart- en vaatziekten. In observationele studies is met behulp van voedselfrequentie vragenlijsten de vitamine K inname gemeten. In deze observationele studies wordt consequent een associatie gevonden tussen een hoge menaquinone (vitamine $K_2$) inname en een lager risico op coronaire aderverkalking (CAC) en een lager risico op hart- en vaatziekten. Er werd geen associatie gevonden tussen phylloquinone (vitamine $K_1$) inname en het risico op CAC of hart- en vaatziekten. Echter, interventie studies laten zien dat phylloquinone suppletie leidt tot verbeterde vasculaire elasticiteit en tot lagere progressie van coronaire verkalking.

In bovenstaande observationele studies is phylloquinone blootstelling gebaseerd op phylloquinone inname, gemeten met behulp van een voedselfrequentie vragenlijst waarvan de relatieve validiteit voor phylloquinone vrij laag is. Wellicht is circulerende phylloquinone concentratie een betere maat voor phylloquinone blootstelling. In **hoofdstuk 3** van dit proefschrift is daarom in 508 postmenopauzale vrouwen de associatie tussen circulerende phylloquinone concentraties en CAC onderzocht. We vonden het tegenovergestelde dan verwacht, namelijk dat in de groep vrouwen met detecteerbare phylloquinone concentraties (>0nmol/l) de prevalentie van CAC hoger was dan in de groep vrouwen met niet-detecteerbare phylloquinone concentraties ( 0 nmol/l) met een prevalentie ratio van 1.34 (95%CI: 1.01-1.77).

Zuivel producten, een belangrijke bron van menaquinonen, zijn mogelijk geassocieerd met het risico op coronaire hartziekten en beroerte. Daarom hebben we in **hoofdstuk 4** van dit proefschrift de relatie tussen inname van totaal zuivel en verschillende type zuivelproducten en het risico op coronaire hartziekten en beroerte onderzocht in een prospectief cohort van 40.011 Nederlandse mannen en vrouwen. Totaal zuivel was niet statistisch significant gerelateerd aan het risico op coronaire hartziekten ($HR_{SD}$ 0.99, 95%CI: 0.94-1.05) en beroerte ($HR_{SD}$ 0.95, 95%CI: 0.85-1.05). Een

hogere inname van gefermenteerde zuivel leek gerelateerd aan een lager risico op beroerte ($HR_{SD}$ 0.92, 95%CI: 0.83-1.01). Andere typen zuivelproducten waren niet geassocieerd met risico op coronaire hartziekten of beroerten.

In **Hoofdstuk 5** van dit proefschrift hebben we het effect van menaquinone-7 suppletie op verschillende vormen van MGP bestudeerd in een dubbelblinde, gerandomiseerde placebogecontroleerde interventie studie bij 60 volwassenen. De deelnemers ontvingen per dag een dosis van 180 µg menaquinone-7 of 360 µg menaquinone-7 of een placebo. Na 12 weken was de concentratie desfosfo-descarboxy MGP (dp-ucMGP) dosisafhankelijk gedaald met 31% in de 180 µg/d groep en 41% in de 360 µg/d groep. Deze daling was al zichtbaar na 4 weken suppletie. Menaquinone-7 suppletie had geen effect op de concentratie van andere vormen van MGP. Deze studie suggereert dat circulerende dp-ucMGP concentratie gebruikt kan worden als bio-marker voor vitamine K blootstelling.

In het tweede deel van dit proefschrift hebben we ons gericht op het verband tussen verschillende vormen van MGP, in het bijzonder dp-ucMGP, en het risico op aderverkalking en hart- vaatziekten. In hoofdstuk 6 en 7 van dit proefschrift hebben we de associatie tussen circulerende MGP soorten en aderverkalking onderzocht. In **hoofdstuk 6** rapporteren we over de relatie tussen circulerende MGP soorten en het risico op CAC. We hebben gebruik gemaakt van een cross-sectionele studie die bestond uit 200 gezonde postmenopauzale vrouwen. Hogere dp-ucMGP concentraties waren bijna significant geassocieerd met meer CAC (β=0.091, 95%CI: -0.78; 0.06). Hoge totaal-descarboxy MGP (t-ucMGP) concentraties leken geassocieerd met minder CAC. Desfosfo-carboxy (dp-cMGP) was niet geassocieerd met CAC. In voortzetting op hoofdstuk 6, hebben we ook een prospectieve studie gedaan in **hoofdstuk 7** om de relatie tussen circulerende MGP soorten en vasculaire verkalking in 571 gezonde postmenopauzale vrouwen te bestuderen. Na 8,5 jaar volgen had 24% van deze vrouwen geen verkalking, terwijl 5% verkalking had in alle gebieden die gemeten waren, namelijk in de coronaire slagaderen, aortaklep, mitralisklep en de aortaboog. Hoge dp-ucMGP concentraties waren bijna significant gerelateerd aan meer CAC ($RR_{SD}$ 1.07; 95%CI: 0.99-1.15) en aantal verkalkte gebieden ($OR_{(4\ gebieden\ vs\ geen\ verkalking)}$ 1.49; 95%CI: 0.95-2.35). Hoge t-ucMGP leek gerelateerd te zijn met minder verkalkte gebieden ($OR_{(4\ gebieden\ vs\ geen\ verkalking)}$ 0.63; 95%CI: 0.36-1.10). Dp-cMGP was niet gerelateerd aan vasculaire verkalking.

Vasculaire verkalking is een sterke onafhankelijke risicofactor voor hart- en vaatziekten, maar de associatie tussen verschillende vormen van MGP en het risico op hart- en vaatziekten is nog niet onderzocht. Daarom hebben we in **hoofdstuk 8** van dit proefschrift in een hoog risico groep, namelijk in 518 diabetes type 2 patiënten, de relatie tussen concentraties van verschillende vormen van MGP en het risico op hart- en vaatziekten onderzocht. Hoge dp-ucMGP concentraties waren gerelateerd aan een hoger risico op hart- en vaatziekten ($HR_{SD}$ 1.21; 95%CI: 1.06-1.38), terwijl dp-cMGP and t-ucMGP niet gerelateerd waren aan het risico op hart- en vaatziekten. **Hoofdstuk 9** beschrijft een case-cohort studie met een representatieve steekproef van 1406 deelnemers en 1534 hart- vaatziekten patiënten waarin de relatie tussen dp-ucMGP concentraties en het risico op coronaire hartzieken en beroerte wordt onderzocht in de algemene populatie. Na een gemiddelde follow-up van 11,5 jaar is er geen relatie gevonden tussen circulerende dp-ucMGP concentraties en het risico op coronaire hartziekten en beroerte.

In **hoofdstuk 10** worden de belangrijkste bevindingen uit dit proefschrift bediscussieerd en worden implicaties voor vervolgonderzoek beschreven. Samenvattend, in het eerste deel van dit proefschrift rapporteren we dat een hoge vitamine K inname de dp-ucMGP concentraties verlaagt en hierdoor kan de dp-ucMGP concentraties mogelijk gebruikt worden als biomarker voor vitamine K blootstelling. In het tweede deel van het proefschrift beschrijven we dat hoge dp-ucMGP concentraties mogelijk geassocieerd zijn met meer vasculaire verkalking. Bovendien, is een hogere dp-ucMGP concentratie geassocieerd met een hoger risico op hart- en vaatziekten, maar alleen in een hoog risico groep.

# Chapter 11

Summary

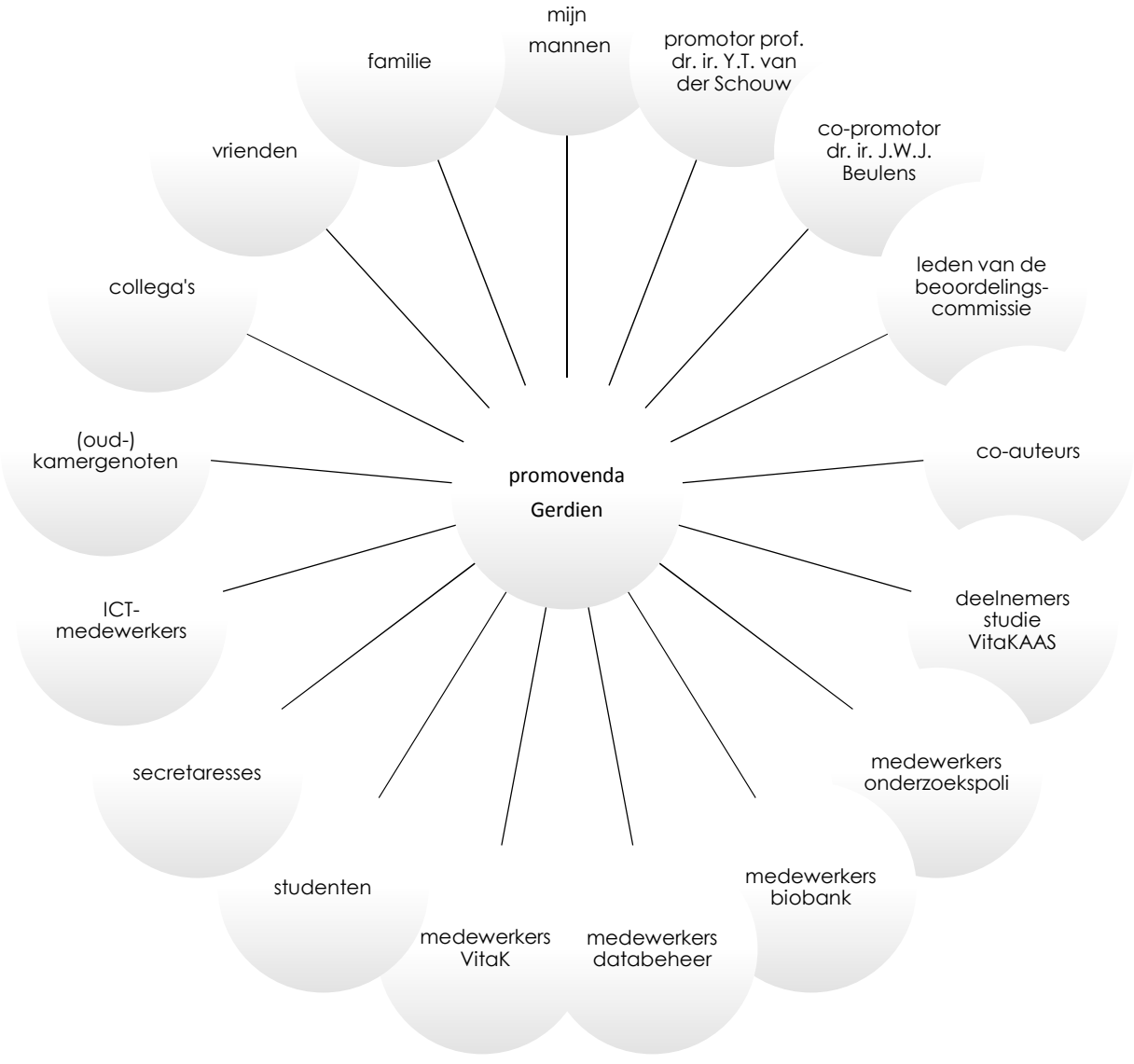
Summary in Dutch

Acknowledgements

About the author

Publication list

Dit proefschrift is af! Al staat mijn naam op de voorkant, er zijn veel andere mensen belangrijk geweest bij het tot stand komen van dit proefschrift. Iedereen die mij op wat voor manier dan ook heeft geholpen: ontzettend bedankt! Aangezien het zo wel een heel kort dankwoord is en ik op deze manier veel mensen tekort doe, wil ik een aantal mensen in het bijzonder noemen. Ik ben zelf erg schematisch ingesteld en heb daarom in figuur 1 weergegeven welke partijen aan dit proefschrift hebben bijgedragen.




**Figuur 1:** Partijen die hebben bijgedragen aan het tot stand komen van dit proefschrift.

Mijn bijzondere dank gaat uit naar mijn promotor Yvonne van der Schouw en mijn co-promotor Joline Beulens, want jullie hebben ervoor gezorgd dat ik mij kon ontwikkelen tot de wetenschapper die ik nu ben. Beste Yvonne, jouw goede en fijne

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# Chapter 11

Summary

Summary in Dutch

Acknowledgements

About the author

Publication list

Gerdien Dalmeijer was born on May 22, 1980 in De Bilt, The Netherlands. She started the bachelor program Nutrition and Health at Wageningen University and obtained her degree in 2003. She continued with the master program Nutrition and Health with the specialization of Epidemiology and Public Health at Wageningen University. As part of the master program she conducted three research projects. The first project focused on indicators of caesarian sections, at The National Institute for Public Health and the Environment (RIVM). The second project focused on dietary betaine and choline intake in relation to risk of cardiovascular disease, at the Julius Center for Health Sciences and Primary Care of the University Medical Center Utrecht. The third project focused on the quality of life in celiac disease patients, at Umeå University, Sweden. After obtaining her master degree in Nutrition and Health she worked from 2006-2009 as research nutritionist at Unilever Vlaardingen.

In June 2009, she started her PhD study at the Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht under the supervision of prof. dr. ir. Y.T. van der Schouw and dr. ir. J.W.J. Beulens. The results of this work are described in this thesis. In 2012, she obtained a Master of Science degree in epidemiology. Notably, she had an oral presentation at EPI-NPAM 2011 entitled "The relationship between dairy intake and incident cardiovascular disease", within the MSc program Clinical Epidemiology her thesis research was awarded for best poster presentation 2012. During the October 2012 meeting of the Dutch organization for scientific research, she was nominated for the young investigators award, Foppe ten Hoor (finished second place). In 2012 she received a conference travel award of the Circulatory Heath Program commission of University Medical Center Utrecht. Furthermore, in 2013 she received the professor Frits de Waard award for the best and most original epidemiogal research by a student in medicine. Currently, Gerdien Dalmeijer works as post-doctoral researcher at the Julius Center, UMC Utrecht.

# Chapter 11

Summary

Summary in Dutch

Acknowledgements

About the author

Publication list

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