

The role of vitamin K in vascular calcification, cardiovascular disease risk and type 2 diabetes

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The role of vitamin K in vascular calcification, cardiovascular disease risk and type 2 diabetes

De rol van vitamine K in slagaderverkalking en het ontstaan van
cardiovasculaire ziekten en type 2 diabetes

(met een samenvatting in het Nederlands)

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1

General introduction

Vitamin K

Vitamin K is a fat soluble vitamin occurring in two different forms in the human diet, phyloquinone (vitamin K₁) and menaquinones (vitamin K₂). Phyloquinone is mainly derived from green leafy vegetables, while menaquinones mainly occur in fermented animal products like cheese and meat¹. Phyloquinone accounts for up to 75% of the total vitamin K intake. Phyloquinone and menaquinones have the same chemical structure, but differ in the length and saturation degree of the side chain². Menaquinones are denoted as menaquinone-n, where n stands for the number of isoprenoid residues and are often divided in short chain menaquinones (menaquinone-4 to menaquinone-6) and long-chain menaquinones (menaquinone-7 to menaquinone-10). Menaquinones have a longer half-life time and higher bioavailability² and are therefore suggested to be more effective in activating vitamin K dependent proteins like matrix Gla protein and osteocalcin.

Vitamin K is known for its importance in the conversion of Gla residues in prothrombin's and other factors involved in blood coagulation. From 1970 onwards, multiple other vitamin K dependent proteins have been identified as well^{3,4} (*Figure 1*). Matrix Gla Protein (MGP) is dependent on vitamin K for its carboxylation and phosphorylation⁵. Inactive MGP, dephosphorylated-uncarboxylated MGP (dp-ucMGP), serves as a marker for vitamin K status, where low dp-ucMGP levels represents long term high vitamin K intake⁶ (*Figure 2*). Osteocalcin is another vitamin K dependent protein, due to its dependency on vitamin K for carboxylation⁷. Osteocalcin is a marker of bone formation and seems to be involved in the process of bone mineralization and may play a role in the glucose metabolism⁷.

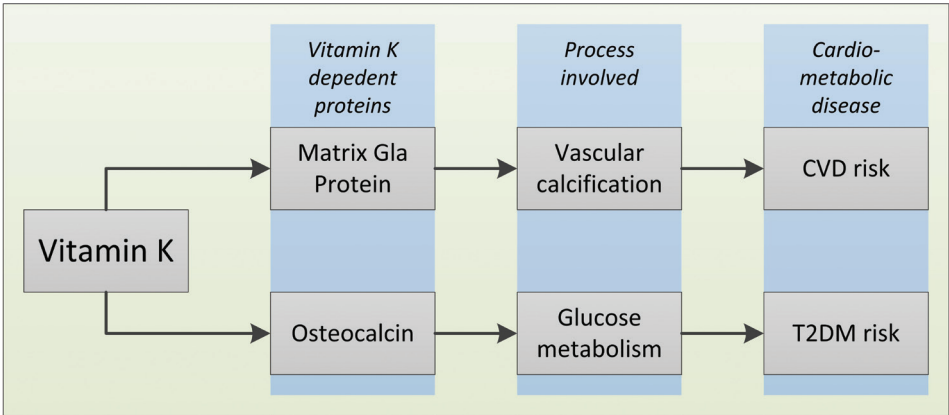


Figure 1. Vitamin K dependent proteins and the cardio-metabolic diseases involved.

Assessment of dietary vitamin K intake in epidemiological studies commonly relies on self-report based on a food frequency questionnaire (FFQ). The FFQ is suitable to rank individuals in macro- and micro nutrients, but are often imprecise and limited by recall bias and incomplete nutritional databases. An objective alternative to measure nutritional status are biomarkers. Various nutritional biomarkers exist to estimate vitamin K status in blood, each with their respective limitations. Circulating phylloquinone represents dietary phylloquinone intake, but only of the previous days because of the short half-life time⁸ and circulating menaquinones are generally not detected in circulation⁷. Therefore, (un)carboxylated forms of vitamin K dependent proteins (MGP and osteocalcin) are often used as marker for vitamin K status. These proteins reflect both phylloquinone and menaquinone intake, but are subject to metabolism⁸; therefore, these proteins are functional markers for vitamin K status but cannot be directly translated to vitamin K intake.

The observational studies on vitamin K and chronic diseases may be hampered by reverse causation or confounding⁹ and the intervention studies are limited by having a short duration and focus on intermediate risk factors. Mendelian Randomization studies, in which genetic variants for vitamin K status are used as instrumental variables, may provide support for the causal association between vitamin K and cardiovascular disease (CVD) risk. This thesis aims to investigate the association between vitamin K and CVD using cohort studies and assess the causality of this association using a Mendelian Randomization approach, and we finally aim to investigate the effect of vitamin K on vascular calcification.

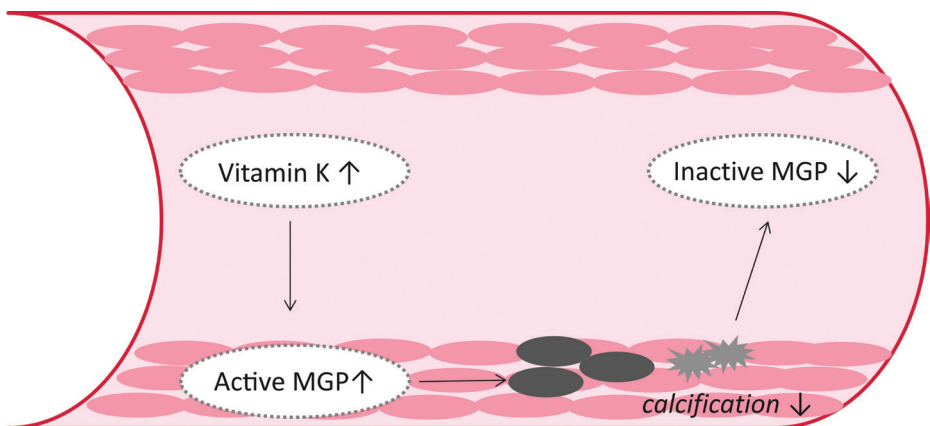


Figure 2. Vitamin K, MGP and vascular calcification

Vascular calcification

Vascular calcification is associated with a 3- to 4 fold increase risk of CVD¹⁰. Vascular calcification is highly prevalent in patients with diabetes and chronic kidney disease, about 70% of the patients with type 2 diabetes have vascular calcification¹¹. In the past, vascular calcification was seen as a passive process but in the last decade, this view has changed and vascular calcification is seen as an actively regulated and reversible process¹². Vascular calcification is a process sharing many features with bone formation, like the mineralization of vascular smooth muscle cells (VSMC)¹³. VSMC differentiate to osteoblast-like cells and causes a deposition of bone matrix cells in blood vessels.

MGP is a strong inhibitor of vascular calcification¹⁴. This function of MGP was first shown in MGP knock-out mice, who all died of massive vascular calcification within 6-8 weeks after birth¹⁵. In line with this, other animal studies showed that treatment with vitamin K antagonists resulted in vascular calcification, which was reversed by vitamin K supplementation¹⁶. This was confirmed in humans showing that warfarin treatment causes vascular calcification¹⁷. Indeed, multiple observational studies showed that low levels of inactive MGP, reflecting a high vitamin K intake, are associated with less vascular calcification and reduced CVD risk⁸. Similarly, a high vitamin K intake has been associated with a decreased CVD risk in population based studies¹⁸⁻²⁰. However, the associations for vitamin K intake was mainly found for menaquinone intake, while often no association was found for phylloquinone intake¹⁸⁻²⁰. Conversely, four out of five randomized placebo controlled trials showed that both phylloquinone and menaquinone supplementation improved surrogate markers of cardiovascular health like vascular stiffness²¹⁻²⁵.

Bone formation markers may also inhibit mineralization of VSMC by blocking hydroxyapatite formation. Osteocalcin, osteopontin, osteonectin, osteoprotegerin, alkaline phosphatase and sclerostin are proposed as possible inhibitors of vascular calcification and are studied in this thesis.

Vascular calcification was long considered as one entity, but calcification can occur in different layers of the arterial wall. Intimal arterial calcification is an end-stage of atherosclerotic plaque formation and may stabilize the plaque. Calcification in the medial layer of the artery, also called Mönckeberg media sclerosis, is a deposit of hydroxyapatite with a high degree of crystallization²⁶. Medial calcification decreases vessel compliance and is highly prevalent in aging, diabetes mellitus and chronic kidney disease²⁷. Intimal

calcification is thought to have a spotty/patchy look on radiographs, whereas medial calcification outlines the artery with a smooth layer of calcium, resulting in linear or annular calcifications^{28,29}. Several mechanisms have been proposed how vascular calcification can lead to cardiovascular disease. Intimal calcification may cause lumen narrowing and plaque instability and so appears to be related with acute vascular diseases³⁰, while medial calcification may increase arterial stiffness, which may result in hypertension, chronic vascular disease such as peripheral arterial disease and heart failure³⁰. The distinction between intimal and medial calcification might be valuable in order to develop prevention and treatment strategies for both types of calcification. Although several studies assessed the risk factors for intimal and medial calcification³¹⁻³⁷, intimal and medial calcification are rarely compared within the same population^{31,34}. Therefore, we aim to assess risk factors for intimal and medial calcification and identify possible biomarkers for intimal versus medial calcification.

Vitamin K and risk of type 2 diabetes

In 2007, osteocalcin was suggested as a hormone playing a role in the glucose metabolism by increasing beta cell proliferation³⁸. This was supported by a study showing that injection of uncarboxylated osteocalcin reduced blood glucose and improved insulin sensitivity in mice³⁹. However, it is debated which form of osteocalcin is the active form herein. Based on mice studies, increased vitamin K intake and thus decreased uncarboxylated osteocalcin would result in abnormal glucose metabolism³⁹. In contrast, the majority of human studies showed that higher vitamin K intake in observational studies is associated with lower risk of type 2 diabetes and vitamin K supplementation reduced insulin resistance⁴⁰⁻⁴². Since the literature regarding vitamin K intake, the different subtypes of osteocalcin and type 2 diabetes is still conflicting we will assess the association between osteocalcin and the risk of type 2 diabetes and assess the causal association between vitamin K status and type 2 diabetes using a Mendelian Randomization approach.

Objectives of this thesis

The objectives of this thesis are to investigate the role of vitamin K in the progression of vascular calcification, the role of vitamin K in intimal and medial calcification, and whether vitamin K (causally) reduces cardiovascular disease (CVD) and diabetes risk.

Outline of this thesis

In **chapter 2**, we describe the validity and reproducibility of a FFQ to estimate vitamin K intake. **Chapter 3** describes to what extent vitamin K intake is associated with all-cause and cause specific (CVD, cancer and other causes) mortality in the Dutch EPIC-NL cohort. The assessment of a causal association between vitamin K status, measured with circulating phyloquinone and inactive MGP, and coronary heart disease risk using a Mendelian Randomization approach in three large European studies (EPIC-InterAct, DIAGRAM and UK Biobank) is described in **chapter 4**. **Chapter 5** describes the association between MGP and plaque stability and cardiovascular events in patients with severe atherosclerotic disease. **Chapter 6** shows the results of a randomized double blind placebo controlled trial which assessed the effect of menaquinone-7 supplementation on vascular calcification, measured with NaF PET/CT scans in people with diabetes and vascular disease.

The risk factors for intimal and medial calcification in patients with or at high risk of CVD are described in **chapter 7**. In **chapter 8**, the association between bone markers and CVD risk in patients with type 2 diabetes from the EPIC-NL cohort is described.

The last part of this thesis focuses on the association between vitamin K and diabetes incidence, starting with the association between different forms of osteocalcin and the risk of type 2 diabetes in the Dutch EPIC-NL cohort (**chapter 9**). **Chapter 10** describes a Mendelian Randomization study which assessed the causal association between genetically predicted circulating phyloquinone and the risk of type 2 diabetes.

In **chapter 11** we discuss the conclusions from all data presented in this thesis and challenges for future research.

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Reproducibility and relative validity of a food frequency questionnaire to estimate intake of dietary phylloquinone and menaquinones

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Abstract

Objective: This study aims to investigate the reproducibility and relative validity of the Dutch food frequency questionnaire (FFQ), to estimate intake of dietary phyloquinone and menaquinones compared with 24-hour dietary recalls (24HDR) and plasma markers of vitamin K status.

Methods: In a cross-sectional study among 63 men and 58 women, the FFQ was completed 3 times over a 1-year period and the reproducibility was calculated over these measurements. Twelve monthly 24HDR were collected to estimate relative validity. Additionally, the relative validity of the FFQ compared with plasma phyloquinone and desphospho-uncarboxylated Matrix Gla protein (dp-ucMGP), was assessed cross-sectionally among 507 postmenopausal women.

Results: Intra-class correlations showed a good reproducibility, with correlations ranging from 0.65 to 0.83. The relative validity for phyloquinone intake compared with 24HDR was lower for women ($r_s=0.28$) than men ($r_s=0.40$). The relative validity, compared with 24HDR, for intake of short-chain menaquinones were ranging between 0.30 and 0.34. Long-chain menaquinones showed good relative validity ($r_s=0.60$ -0.69). Plasma phyloquinone concentrations were weakly correlated with phyloquinone intake ($r_s=0.16(0.07$ -0.24)). Plasma dp-ucMGP was negatively but weakly correlated with phyloquinone intake ($r_s=-0.09(-0.18$; -0.01)) and long-chain menaquinones ($r_s=-0.13(-0.21$; -0.04)), but not with short-chain menaquinones ($r_s=-0.04(-0.13$; 0.05)).

Conclusions: The FFQ is reproducible to rank subjects for phyloquinone and menaquinone intake. The relative validity of our FFQ, compared to 24HDR, to estimate intake of phyloquinone and short-chain menaquinones was low, but the relative validity for long-chain menaquinones was good. The relative validity of our FFQ, compared to plasma phyloquinone and dp-ucMGP was relatively low for both phyloquinone and menaquinone intake.

Background

Vitamin K is a fat-soluble vitamin occurring in two biological active forms; phyloquinone and menaquinones. Phyloquinone is mainly present in green, leafy vegetables, while menaquinones are mostly found in animal products, like milk, meat, and fermented products as cheese¹. Menaquinones are divided in short- and long-chain menaquinones based on the length of the side chain. In addition, short- and long-chain menaquinones are also derived from different food sources². Phyloquinone and menaquinones differ in the length and saturation degree of the side chain, leading to higher bioavailability of the longer chained menaquinones.

Several observational studies, conducted in Europe, suggested that a high phyloquinone and/or menaquinone intake was associated with a reduced risk of chronic diseases such as coronary heart disease³⁻⁵, type 2 diabetes⁶ and cancer⁷. Some of these studies indicated that particularly long-chain menaquinones may be more beneficial for cardiovascular health than phyloquinone^{3-5,8}, due to their higher bioavailability⁹. Most of these studies relied on food frequency questionnaires (FFQ) to estimate intake of dietary phyloquinone and menaquinones. To date, five studies assessed the relative validity of an FFQ to estimate total vitamin K intake, with correlations ranging from 0.31 to 0.83⁹⁻¹³. However, none of these validation studies distinguished between phyloquinone and menaquinones intake.

Several studies investigated dietary phyloquinone intake in relation to plasma phyloquinone concentrations with correlations ranging from 0.18 to 0.51¹⁴⁻¹⁸. The validity of an FFQ to estimate dietary menaquinones using biomarkers as reference has not been investigated. Matrix Gla Protein is a vitamin K dependent protein and its inactive form, desphospho-uncarboxylated MGP (dp-ucMGP), is suggested as a valid marker for vitamin K status, with high values indicating a low vitamin K intake^{19,20}. However, no observational studies used dp-ucMGP as marker to validate the FFQ.

Previous vitamin K studies within the EPIC-NL cohort, reported on validity of the FFQ, but these studies used a vitamin K database developed in 1996^{4,6,21}. Recently, the vitamin K content of foods in this database was updated and completed. Therefore, this study investigates the reproducibility and relative validity of the FFQ to estimate intake of dietary phyloquinone and menaquinones compared to both 12 24-hour dietary recalls (24HDR) and plasma markers of vitamin K status, with the updated vitamin K database.

Methods

FFQ

The self-administered, semi-quantitative Dutch-EPIC FFQ contains questions on the average consumption frequency during the past year for 79 main food items. Participants can indicate their answers in times per day, per week, per month or per year, or as never. For several food items, additional questions were asked about the consumption frequency for different sub items, preparation methods or additions. The questionnaire contains colour photographs of two to four differently-sized portions of 21 foods. In total, the habitual consumption of 178 food items can be calculated from the information obtained. The validity of the Dutch EPIC-FFQ to estimate vitamin K intake was studied with two different reference methods and two different study populations: (1) The reproducibility and relative validity of the FFQ for vitamin K intake estimates against 12 24HDR in 121 participants; and (2) this FFQ has been used to estimate vitamin K intakes in a study among 507 healthy postmenopausal women²². Biomarkers of vitamin K status were measured for further validation of the FFQ²².

Study population – validity in comparison with 24HDR

A detailed description of the design of the FFQ and validation of this FFQ has been published previously^{23,24}. In brief, 960 healthy participants were recruited from the EPIC-NL cohort by mail. Out of the 240 who responded positively, we randomly selected 134 participants, equally distributed across the four towns, in 20-year age groups and of both sexes. In total, 121 participants, 63 men and 58 women completed the study. During 1991–1992, the FFQ was self-administered three times in intervals of 6 months, assessing the past year. A 6 month interval between the FFQs was used to assess the effect of seasons. The 24HDR were administered monthly throughout a year and were evenly divided over Mondays to Saturdays. The weighted average of 24HDR was calculated with a weight of one for weekdays and two for weekend days to correct for the underrepresentation of Saturdays and Sundays. All participants gave informed consent prior to inclusion. The study complied with the Declaration of Helsinki and was approved by the local ethics committees.

Study population – validity in comparison with biomarkers

To compare the FFQ with biomarkers, we used data from healthy postmenopausal women as described previously²². Women were selected from participants of the Prospect study, one of the two Dutch cohorts participating in the EPIC-NL cohort²⁵. Between October

2002 and April 2004, 1,996 postmenopausal women were randomly selected from 5,844 participants, of which 1,000 agreed to participate. Of these 1,000 women, 573 women were randomly selected for this study. The FFQ was administered at baseline of the EPIC-NL cohort; non-fasting blood samples were collected at the same time point. Plasma phylloquinone levels were missing for 64 women, two participants were excluded because they used vitamin K antagonists, leaving 507 participants for the validation using the biomarkers as reference method.

Vitamin K database

Nutrient intake was calculated according to the 1993 version of the Dutch food composition table²⁶. This table does not contain information on vitamin K contents of all foods. Therefore, Wageningen University and Research centre has recently developed a food composition database for vitamin K. The concentrations of phylloquinone, short-chain menaquinones and long-chain menaquinones in a series of Dutch foods were additionally assessed (9.5% of the database) at the R&D Group VitaK, Maastricht University according to previously published procedures²⁷. These values were supplemented with earlier analyses by Schurgers and Vermeer¹, the Dutch food composition table 2013²⁶ and the USDA National Nutrient Database for Standard Reference (1.5% of the database)^{28,29}. Finally, the database was completed by estimating phylloquinone and menaquinone content of mixed dishes through recipes and ingredient information. Short-chain menaquinones were defined as menaquinone-4 to menaquinone-6 and long-chain menaquinones as menaquinone-7 till menaquinone-10.

Biomarkers for vitamin K status

Plasma phylloquinone levels were measured by high-pressure liquid chromatography³⁰. Matrix Gla Protein is a vitamin K dependent protein and its inactive form, dp-ucMGP, is suggested as a valid marker for vitamin K status^{31,32}. The measurement of dp-ucMGP was conducted with a dual antibody ("sandwich") ELISA (mAb-dpMGP; R&D Group VitaK, Maastricht, the Netherlands). All measurements were performed in duplicate and average values were used in the statistical analyses. Detailed information regarding these biomarkers is described in the supplementary information.

Statistical analyses

We assessed whether relative validity of the FFQ against 24HDR differed for men and women by including an interaction term for sex in a linear regression of the FFQ against 24HDR. Because this interaction was significant for menaquinones ($p=0.047$), we present

the results stratified by sex. Stratification was only performed in the validation with the 24DHR, since the biomarker study was performed only among women. To assess the reproducibility, Spearman correlation coefficients (r_s) were calculated and intra-class correlation coefficients were calculated between the three FFQs. To assess relative validity, Spearman correlation coefficients and weighted kappa's, assessed in tertiles of dietary vitamin K intake, were calculated for the first FFQ compared with the weighted average of the 24HDR. Vitamin K intake was adjusted for energy intake using the residual method³³. Absolute agreement between two measurements was determined using Bland–Altman plots, in which the difference was plotted against the mean of the two dietary assessment methods. Linear regression analysis was performed to investigate whether the bias was proportional to the level of phyloquinone and menaquinones.

For validation with biomarkers as reference method, Spearman correlation coefficients were calculated for dietary vitamin K intake, as estimated from the first FFQ, compared with dp-ucMGP and plasma phyloquinone levels. The association between the different forms of vitamin K intake and phyloquinone concentrations was adjusted for 'time till last meal' because of the non-fasting plasma levels. Time of last meal was included as one question in the baseline questionnaire, and minutes between time of blood sampling and time of last meal was calculated. The association between menaquinones intake and plasma phyloquinone was assessed as a negative control. Finally, plasma phyloquinone concentrations were plotted per decile (median intake) of phyloquinone to determine if plasma phyloquinone reaches a plateau at a higher phyloquinone intake, as suggested in a previous study³⁴. A two tailed p-value <0.05 was considered statistically significant. Statistical analyses were performed using Statistical Analysis Systems version 9.2 (SAS Institute, Cary, NC, USA).

Results

The reproducibility and validity of the FFQ, compared to 24HDR, was assessed in men and women, with a mean age of 42.6 and 49.0 years respectively, and a body mass index (BMI) of 25.5 kg/m² for men and 24.9 kg/m² in women. The validity compared with biomarkers was assessed in postmenopausal women, aged 57.8 years old, with a BMI of 25.9 kg/m².

Reproducibility

Reproducibility of the FFQ was generally good, with correlation coefficients ranging from 0.63 (0.43;0.76) to 0.83 (0.73;0.90) at 6 months (*Table 1*). These correlations coefficients

were slightly lower for the FFQ at 12 months, but still showed good reproducibility in both men and women. The intra-class correlations were well in line with these correlation coefficients and ranged between 0.65 (0.52;0.76) and 0.83 (0.75;0.88).

Validity in comparison with 24HDR

The mean phyloquinone and menaquinones measured by the FFQ and 24HDR are shown in *Supplementary Table 1*. In both men and women, the FFQ slightly overestimated the phyloquinone and menaquinone intake compared to the 24HDR.

The relative validity for phyloquinone was 0.40 (0.16;0.59) among men and 0.28 (0.02;0.50) among women (*Table 2*). Relative validity of the FFQ to estimate menaquinone intake was 0.54 (0.34;0.70) among men and 0.57 (0.37;0.72) among women. For short-chain menaquinones, the relative validity was 0.30 (0.06;0.51) and 0.34 (0.09;0.55) for men and women respectively. Long-chain menaquinones were correlated with the 24HDR in both men ($r_s=0.65(0.48;0.77)$) and women ($r_s=0.66(0.49;0.79)$). Relative validity of the FFQ to estimate total vitamin K intake was comparable to the relative validity observed in phyloquinone intake. Similar results were observed for weighted kappa's with a low to moderate relative validity for total vitamin K, phyloquinone and short-chain menaquinones and reasonable relative validity for long-chain menaquinones.

Bland-Altman plots showed that negative associations were found between the difference and mean intake of total vitamin K intake, phyloquinone, total menaquinone and short-chain menaquinones intake (*Supplementary Figure 1*). This suggests presence of proportional bias in the FFQ; under-reporting at higher vitamin K levels. These plots showed no evidence for proportional bias for long-chain menaquinones. The crude values were comparable to the energy adjusted values for all forms of vitamin K (*Table 2*).

Validity in comparison with biomarkers

Among 507 women in the biomarker study, the mean daily intake of phyloquinone and menaquinones was 178.5µg and 48.2µg, respectively (*Supplementary Table 2*). Plasma phyloquinone concentrations were weakly, but significantly, correlated with phyloquinone intake ($r_s=0.16(0.07;0.24)$) (*Table 3*). At the higher deciles of phyloquinone intake ($\approx 150/200$ µg/day), the plasma phyloquinone concentrations levelled off, but did not reach a plateau (*Supplementary Figure 2*). Plasma phyloquinone concentrations were not correlated with menaquinone intake ($r_s=0.00(-0.08;0.09)$). The results were comparable

for both crude and energy adjusted intakes. Adjustment for time till last meal showed similar results (*Table 2*).

Plasma dp-ucMGP concentrations were negatively correlated with both phyloquinone ($r_s = -0.09(-0.18; -0.01)$) and menaquinones ($r_s = -0.12(-0.21; -0.03)$). Short-chain menaquinones were not associated with dp-ucMGP ($r_s = -0.04(-0.13; 0.05)$), while long-chain menaquinones were correlated with dp-ucMGP ($r_s = -0.13(-0.21; -0.04)$).

Table 1. Reproducibility at 6 and 12 months for intakes of vitamin K estimated by means of the EPIC-NL FFQ (n=121)

	Vitamin K (total)	Phylloquinone	Menaquinones	Short-chain menaquinones	Long-chain menaquinones
Men (n=63)					
<i>Crude</i>					
Spearman (r_s)					
6 month	0.80 (0.68; 0.87)	0.77 (0.65; 0.86)	0.73 (0.58; 0.82)	0.76 (0.63; 0.85)	0.75 (0.62; 0.84)
12 month	0.71 (0.55; 0.81)	0.71 (0.56; 0.81)	0.59 (0.39; 0.73)	0.67 (0.50; 0.78)	0.64 (0.46; 0.77)
ICC *	0.71 (0.60; 0.80)	0.70 (0.59; 0.80)	0.73 (0.62; 0.81)	0.75 (0.65; 0.83)	0.71 (0.61; 0.80)
<i>Energy-adjusted</i>					
Spearman (r_s)					
6 month	0.78 (0.66; 0.86)	0.75 (0.62; 0.84)	0.74 (0.60; 0.83)	0.76 (0.63; 0.84)	0.75 (0.61; 0.84)
12 month	0.71 (0.56; 0.81)	0.68 (0.51; 0.79)	0.63 (0.45; 0.77)	0.64 (0.47; 0.77)	0.64 (0.46; 0.76)
ICC *	0.70 (0.59; 0.73)	0.69 (0.58; 0.79)	0.72 (0.61; 0.81)	0.68 (0.56; 0.77)	0.71 (0.60; 0.80)
Women (n=58)					
<i>Crude</i>					
Spearman (r_s)					
6 month	0.74 (0.59; 0.84)	0.65 (0.46; 0.77)	0.75 (0.61; 0.84)	0.75 (0.60; 0.84)	0.80 (0.68; 0.88)
12 month	0.72 (0.56; 0.82)	0.70 (0.54; 0.81)	0.68 (0.51; 0.80)	0.66 (0.47; 0.78)	0.77 (0.64; 0.86)
ICC	0.67 (0.55; 0.78)	0.65 (0.52; 0.76)	0.79 (0.69; 0.86)	0.75 (0.65; 0.83)	0.82 (0.74; 0.88)
<i>Energy-adjusted</i>					
Spearman (r_s)					
6 month	0.67 (0.50; 0.79)	0.63 (0.43; 0.76)	0.80 (0.68; 0.88)	0.72 (0.56; 0.82)	0.83 (0.73; 0.90)
12 month	0.75 (0.60; 0.84)	0.72 (0.57; 0.82)	0.76 (0.62; 0.85)	0.67 (0.49; 0.79)	0.79 (0.66; 0.87)
ICC	0.67 (0.55; 0.78)	0.65 (0.52; 0.76)	0.80 (0.71; 0.87)	0.72 (0.61; 0.81)	0.83 (0.75; 0.88)

ICC = intraclass correlation

Table 2. Relative validity for vitamin K intakes estimated by means of the EPIC-NL FFQ versus 12 24-hour dietary recalls in 121 Dutch men and women aged 20-70 years.

	Vitamin K (total)	Phylloquinone	Menaquinones	Short-chain menaquinones	Long-chain menaquinones
Men (n=63)					
<i>Crude</i>					
Spearman (r_s)	0.46 (0.24; 0.64)	0.40 (0.17; 0.59)	0.52 (0.31; 0.68)	0.30 (0.05; 0.51)	0.69 (0.53; 0.80)
Weighted kappa	0.26 (0.08; 0.43)	0.23 (0.06; 0.41)	0.31 (0.15; 0.47)	0.26 (0.08; 0.44)	0.41 (0.25; 0.57)
<i>Energy-adjusted</i>					
Spearman (r_s)	0.43 (0.21; 0.61)	0.40 (0.16; 0.59)	0.54 (0.34; 0.70)	0.30 (0.06; 0.51)	0.65 (0.48; 0.77)
Weighted kappa	0.23 (0.07; 0.40)	0.23 (0.05; 0.41)	0.46 (0.30; 0.62)	0.18 (0.02; 0.35)	0.44 (0.27; 0.61)
Women (n=58)					
<i>Crude</i>					
Spearman (r_s)	0.24 (-0.02; 0.47)	0.28 (0.02; 0.50)	0.39 (0.15; 0.59)	0.29 (0.04; 0.51)	0.60 (0.40; 0.74)
Weighted kappa	0.10 (-0.07; 0.28)	0.16 (-0.02; 0.34)	0.33 (0.15; 0.51)	0.24 (0.06; 0.43)	0.44 (0.27; 0.61)
<i>Energy-adjusted</i>					
Spearman (r_s)	0.22 (-0.05; 0.45)	0.28 (0.02; 0.50)	0.57 (0.37; 0.72)	0.34 (0.09; 0.55)	0.66 (0.49; 0.79)
Weighted kappa	0.13 (-0.05; 0.32)	0.16 (-0.02; 0.34)	0.38 (0.23; 0.54)	0.24 (0.06; 0.43)	0.41 (0.26; 0.57)

Table 3. Relative validity (Spearman (r_s)) of vitamin K intakes estimated by means of the EPIC-NL FFQ versus plasma phylloquinone and dp-ucMGP in 507 postmenopausal Dutch women.

	Vitamin K (total)	Phylloquinone	Menaquinones	Short-chain menaquinones	Long-chain menaquinones
Plasma phylloquinone (n=507)					
<i>Crude</i>	0.16 (0.07; 0.24)	0.16 (0.07; 0.24)	0.01 (-0.07; 0.09)	0.06 (-0.02; 0.15)	0.00 (-0.08; 0.09)
<i>Energy-adjusted</i>	0.16 (0.08; 0.25)	0.16 (0.07; 0.24)	0.00 (-0.08; 0.09)	0.04 (-0.05; 0.12)	0.02 (-0.08; 0.09)
<i>Energy-adjusted *</i>	0.16 (0.08; 0.25)	0.16 (0.07; 0.24)	0.00 (-0.08; 0.09)	0.03 (-0.05; 0.12)	0.00 (-0.09; 0.08)
Dp-ucMGP (n=505)					
<i>Crude</i>	-0.12 (-0.20; -0.03)	-0.10 (-0.19; -0.01)	-0.12 (-0.20; -0.03)	-0.06 (-0.14; 0.03)	-0.13 (-0.21; -0.04)
<i>Energy-adjusted</i>	-0.11 (-0.20; -0.02)	-0.09 (-0.18; -0.01)	-0.12 (-0.21; -0.03)	-0.04 (-0.13; 0.05)	-0.13 (-0.21; -0.04)
<i>Energy-adjusted *</i>	-0.11 (-0.20; -0.02)	-0.09 (-0.18; -0.01)	-0.12 (-0.20; -0.03)	-0.04 (-0.13; 0.05)	-0.13 (-0.21; -0.04)

* Adjusted for time till last meal

Discussion

This study shows that the FFQ is to reproducibly rank participants according to their dietary intakes of phyloquinone and menaquinones. The relative validity of the FFQ compared to 24HDR varied by vitamin K form and sex, but was relatively good for long-chain menaquinones. The FFQ showed a weak validity, when using biomarkers as reference method.

To date, five validation studies, using a dietary assessment method as a reference, were performed⁹⁻¹³. These studies validated the FFQ against 3 to 12 day food records, and showed correlations ranging between 0.31 and 0.83^{10-13,16}. The highest correlations were found in elderly men and women ($r=0.83$)⁹ and Korean adults ($r=0.63$)¹³. The relative validity for energy adjusted total vitamin K intake in our study was 0.43 and 0.22, for men and women respectively. This was comparable to other validation studies performed in pregnant women¹², Quebec men and women¹⁰, and a study in elderly Finnish women¹¹. Since vitamin K was not divided into its sub forms¹¹, we cannot further compare our results to these previous validation studies⁹⁻¹³. Previous observational studies in the EPIC-NL cohort relied on the vitamin K content database from 1996⁴⁻⁶. Compared to the studies using the previous database, the relative validity for total vitamin K and phyloquinone intake increased, while it remained comparable for menaquinone intake. Similar results were found for both sexes and crude and energy adjusted values.

The low relative validity of phyloquinone probably results from a low relative validity of the FFQ to estimate vegetable intake, 0.38 and 0.31 for men and women, since green vegetables are the main source of phyloquinone²⁴. The relative validity for vegetable intake of our FFQ is comparable to other FFQs, with correlations ranging between 0.19 and 0.53³⁵⁻³⁷. Furthermore, measurement error may be present for vegetable intake because it comes from raw and boiled vegetables or as an ingredient in mixed meals, which are often forgotten by the participant in recall³⁸.

The relative validity for menaquinone was moderate, but there were differences between the short-chain and long-chain menaquinones. We observed a low relative validity for short-chain menaquinones ($r_s=0.30$). The most important source of short-chain menaquinone intake was meat (55%), but the validity of meat was 0.47 in men and 0.70 in women. So this does not completely explain the relative low validity of short-chain menaquinones. However, this could be explained by the precision of the FFQ, since

menaquinone content is highly correlated with fat content of animal products, and the FFQ did not differentiate the specific fat content of the meat. Long-chain menaquinones were mainly derived from cheese (68.5%) in the Dutch population. The relative validity for cheese differed for men ($r_s=0.64$) and women ($r_s=0.38$)²³. However, the relative validity of milk was good, 0.71 and 0.79 for men and women respectively. Despite the low validity for cheese in women, the relative validity of long-chain menaquinones was good.

Several previous studies estimated validity of phyloquinone intake with dietary biomarkers of vitamin K status, mainly plasma phyloquinone concentrations^{14–18}. The correlation coefficient observed in our study is similar to the correlation of 0.19 in elderly men and women¹⁵. Since this study also compared plasma phyloquinone measurements with phyloquinone intake estimated by a FFQ, this study is comparable to ours¹⁵. Studies using weighted food records or 24HDR, in healthy adults^{16,18} and elderly¹⁷, showed coefficient ranging between 0.18 to 0.30^{16–18}. Only Booth *et al.* showed a higher correlation of 0.51 between plasma phyloquinone concentrations and phyloquinone intake in healthy adults¹⁴. However, the dietary assessment was done by three sets of 4-day weighed food records, providing a much more accurate assessment than a FFQ. Since plasma phyloquinone concentrations are sensitive to fluctuations in intake, a precise assessment of phyloquinone intake shortly before blood sampling may result in better agreement between measures of intake and blood concentrations¹⁴.

Using dp-ucMGP as biomarker for status of both phyloquinone and menaquinones, we observed similar results for both forms of vitamin K. Both showed relatively low, but significant correlations with dp-ucMGP. Until now, only intervention studies showed the link between vitamin K supplementation and dp-ucMGP levels^{31,39,40}. The relative validity was lower for short-chain menaquinones compared to long-chain menaquinones. This could be explained by the lower half-life time for short-chain than long-chain menaquinones, which may affect dp-ucMGP levels to a lesser extent⁴¹. Since the correlation of phyloquinone, with an equally short half-life time, with dp-ucMGP was higher than for short-chain menaquinones, this cannot explain these results. The low validity could also be the result of the low relative validity of the FFQ to estimate short-chain menaquinones.

For both biomarkers, a relatively good vitamin K status of our study population may also explain the relatively low correlations. McKeown *et al.*, showed a linear relation between phyloquinone intake and plasma phyloquinone concentrations up to 150-200 µg/day

that levelled off at higher levels³⁴. Although the figure showed a trend, we did not observe a clear plateau as in previous research³⁴.

Strengths of this study include the 12 24HDR as reference method for assessing relative validity, which is sufficient to eliminate within-person variability for most nutrients. However, this reference method may have correlated errors with the FFQ, such as the reliance on memory, and use of the same food composition database for calculations of nutrient intakes. This may lead to an overestimated relative validity. To overcome this limitation, we also included biomarkers in the study. Of note, the relative validity against biomarkers is probably underestimated because factors like bio-availability and absorption play a role. For menaquinones, we used menaquinones food composition data measured by the VitaK laboratory of Maastricht University¹. Furthermore, the vitamin K database is partly based on the USDA database which may be not applicable to the Dutch foods⁴². However, only 1.5% food composition database was derived from the USDA database. For food products that were not covered by these sources, we imputed the vitamin K content based on similar food products and recipes of certain foods. Finally, the samples for serum phyloquinone were non-fasting samples. Phyloquinone concentrations are correlated with triglycerides because of being transported on triglyceride lipoproteins⁴³. Unfortunately, data on triglycerides were not available. Although adjustment for time till last meal did not change the correlation, the non-fasting plasma phyloquinone concentrations may have affected our results.

Conclusions

The FFQ is reproducible to rank subjects for phyloquinone and menaquinone intake. The relative validity of our FFQ, compared to 24HDR, to estimate intake of phyloquinone and short-chain menaquinones was low, but the relative validity for long-chain menaquinones was good. The relative validity of our FFQ, compared to plasma phyloquinone and dp-ucMGP was relatively low for both phyloquinone and menaquinone intake.

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Disclosures

None.

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Supplementary information

Plasma phylloquinone

Plasma phylloquinone levels were measured by high-pressure liquid chromatography. Low and high control specimens had average values of 1.2 and 4.8 nmol/L, with intra- and inter-assay coefficients of variation of 4.1 and 4.7%, respectively. For this analysis, the lower limit of detection for this assay was 0.1 nmol/L. Although this assay also has capacity to detect menaquinone-4 to -6, no detectable menaquinones were measured so only phylloquinone is reported.

Dp-ucMGP

The measurement of dp-ucMGP was conducted with a dual antibody (“sandwich”) ELISA. The capture antibody was directed against the dpMGP sequence 3-15 (mAb-dpMGP; R&D Group VitaK, Maastricht, the Netherlands). The detecting antibody was directed against the ucMGP sequence 35-49 (mAb-ucMGP, VitaK BV). All measurements were performed in duplicate and average values were used in the statistical analyses. The intra-assay and inter-assay coefficients of variation have been found to be 5.6% and 9.9%, respectively.

Supplementary Table 1. Mean values and standard deviations of energy and vitamin K intakes estimated by means of the EPIC-NL FFQ and 12 24-hour dietary recalls in 121 Dutch men and women.

Nutrient	Males (n=63)				Females (n=58)			
	FFQ		Recalls		FFQ		Recalls	
	Mean	SD	Mean	SD	mean	SD	Mean	SD
Energy	11075	2703	11302	2228	7965	1616	7525	1531
Vitamin K (total)	218	65	215	90	188	54	193	98
Phylloquinone	171	61	164	88	150	48	145	92
Menaquinones	47	17	51	23	38	13	47	31
SMK	30	8	35	21	21	6	32	29
LMK	16	12	15	10	16	10	15	9
<i>Energy-adjusted</i>								
Vitamin K (total)	196	54	181	75	204	53	224	106
Phylloquinone	155	53	138	72	161	49	167	98
Menaquinones	41	13	43	20	42	13	57	39
SMK	26	5	29	17	24	5	39	37
LMK	15	10	13	9	17	11	18	10

Energy is presented in kilojoule and vitamin levels in μg . SMK: short-chain menaquinones (MK4-MK6). LMK: long-chain menaquinones (MK7- MK10)

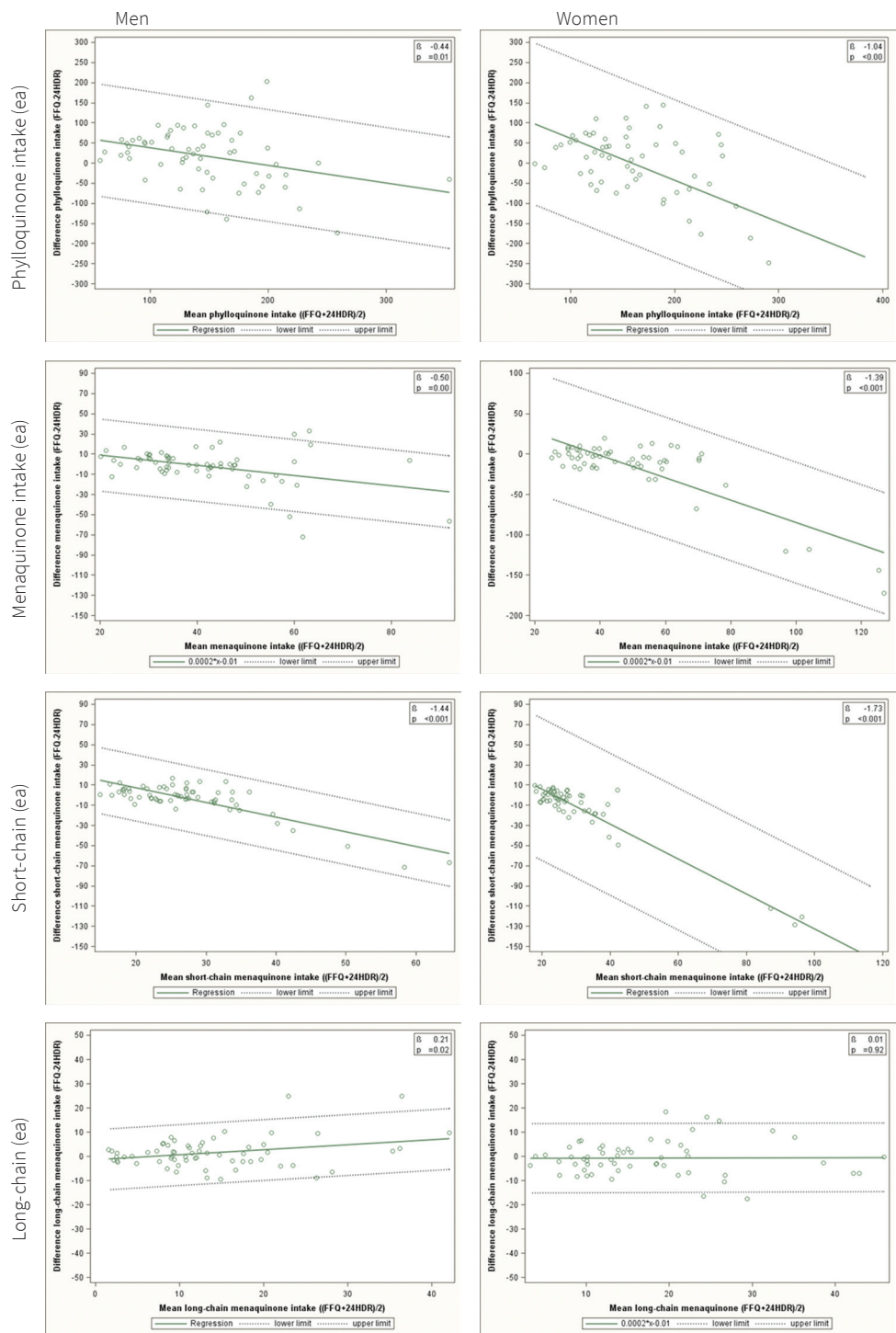
Supplementary Table 2. Mean values and standard deviation of nutrient intakes estimated by means of the EPIC-NL FFQ in 507 postmenopausal women.

Nutrient	Crude		Energy adjusted	
	Mean	SD	Mean	SD
Vitamin K (total)	216.0	72	226.7	71
Phylloquinone	170.9	67	178.5	68
Menaquinones	45.1	17	48.2	16
SMK	24.5	9	26.6	8
LMK	20.0	12	21.2	12

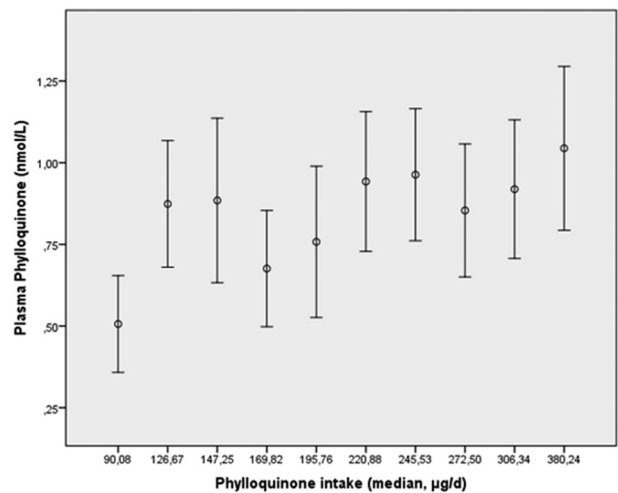
Energy is presented in kilojoule and vitamin levels in μg

SMK: short-chain menaquinones (MK4-MK6)

LMK: long-chain menaquinones (MK7- MK10)



Supplementary Figure 1. Bland-Altman plots for the difference between FFQ and 24 hour recall for energy-adjusted phyloquinone and menaquinones.



Supplementary Figure 2. Plasma phylloquinone concentrations according to decile category of phylloquinone intake, in 507 postmenopausal women.



Vitamin K intake and all-cause and cause specific mortality

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Abstract

Background & Aims: Vitamin K has been associated with various health outcomes, including non-fatal cardiovascular diseases (CVD) and cancer. However, little is known about the association between vitamin K intake and all-cause and cause specific mortality. This study aims to investigate the association between vitamin K intake and all-cause and cause-specific mortality.

Methods: This prospective cohort study included 33,289 participants from the EPIC-NL cohort, aged 20-70 years at baseline and recruited between 1993 and 1997. Dietary intake was assessed at baseline with a validated food frequency questionnaire and intakes of phylloquinone, and total, short chain and long chain menaquinones were calculated. Information on vital status and causes of death was obtained through linkage to several registries. The association between the different forms of vitamin K intake and mortality was assessed with Cox proportional hazards, adjusted for risk factors for chronic diseases and nutrient intake.

Results: During a mean follow-up of 16.8 years, 2,863 deaths occurred, including 625 from CVD (256 from coronary heart disease (CHD)), 1,346 from cancer and 892 from other causes. After multivariable adjustment, phylloquinone and menaquinones were not associated with all-cause mortality with hazard ratios for the upper vs. the lowest quartile of intake of 1.04 (0.92;1.17) and 0.94 (0.82;1.07) respectively. Neither phylloquinone intake nor menaquinone intake was associated with risk of CVD mortality. Higher intake of long chain menaquinones was borderline significantly associated ($p_{\text{trend}} = 0.06$) with lower CHD mortality with a $HR_{10\mu g}$ of 0.86 (0.74;1.00). None of the forms of vitamin K intake were associated with cancer mortality or mortality from other causes.

Conclusions: Vitamin K intake was not associated with all-cause mortality, cancer mortality and mortality from other causes.

Background

Vitamin K is a fat-soluble vitamin, which occurs as phyloquinone and menaquinones. Phyloquinone mostly occurs in green, leafy vegetables while menaquinones mostly occur in animal products¹. Menaquinones (MK) can be further subdivided, based on the length of the side chain, into short chain menaquinones (MK-4 till MK-6) and long chain menaquinones (MK-7 till MK-10).

Vitamin K is a cofactor in the carboxylation of vitamin K dependent proteins, such as matrix Gla protein (MGP), osteocalcin and prothrombin². Vitamin K has been associated with reduced risks of various health outcomes, including cardiovascular diseases (CVD) and cancer³⁻⁷. Vitamin K is thought to be associated with a decreased risk of CVD by reducing vascular calcification, through its function as a cofactor in the carboxylation of Matrix-Gla Protein (MGP), an inhibitor of vascular calcification⁸⁻¹⁰. In animal studies, the carboxylation of MGP and the inhibition of vascular calcification by vitamin K, has been consistently shown¹¹⁻¹³. The association of MGP with vascular calcification and CVD risk in humans is confirmed by observational studies^{8,14,15}.

Vitamin K intake has also been hypothesized to reduce the risk of cancer by inhibiting cell growth¹⁶. Studies on the dietary forms of vitamin K show anti-carcinogenic effects in cell lines, when administering a dose of phyloquinone or menaquinones¹⁶. Various theories for the effect of vitamin K on cancer exist, that all come down to vitamin K leading to cell growth arrest, apoptosis or halting the initiation of the cell cycle¹⁶. However, the few observational studies in humans that have been performed showed conflicting results for cancer mortality^{17,18}.

Thus far, four studies investigated the association between vitamin K intake and mortality^{3,17-19}, of which three studies investigated all-cause mortality^{3,18,19}. One study suggested that a high vitamin K intake was associated with a reduced risk of all-cause and cause-specific mortality¹⁸, while two other studies were not able to detect an association^{3,19}. The present study aims to investigate the association of phyloquinone and menaquinone intake with all-cause and cause-specific mortality in the EPIC-NL cohort.

Methods

Study population and design

The Dutch contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC-NL) consists of the Prospect (n=17,357) and the MORGEN (n=22,654) cohort. The cohorts were established simultaneously in 1993-1997. Prospect included women aged 49-70, who participated in the Dutch breast cancer screening, residing in Utrecht and vicinity. MORGEN included a general population sample of men and women, aged 20-59 from Amsterdam, Doetinchem and Maastricht. The rationale and design of EPIC-NL are described elsewhere²⁰.

For this study, participants who did not consent to linkage with disease- and vital status registries were excluded (n=2,369). Participants with missing questionnaires (n=176) or implausible energy intake (the upper and lower 0.5% of the ratio of energy intake over estimated basal metabolic rate) were excluded (n=346). Furthermore, participants with prevalent CVD, cancer or diabetes at baseline were excluded (n=3,756), and those using vitamin K antagonists (Warfarin and Coumarin) (n=75), leaving 33,289 participants for analyses.

Baseline measurement

At baseline a general questionnaire including a food frequency questionnaire (FFQ) were administered. The baseline questionnaire contained information on demographic characteristics, presence of chronic diseases, chronic disease risk factors and lifestyle. Additionally, a physical examination was performed including weight, height, waist circumference and blood pressure²⁰. Weight and height were used to calculate Body Mass Index (BMI) by dividing the weight by height squared (kg/m^2). Hypertension is defined as a measured systolic pressure of >140 mmHg or a diastolic pressure of >90 mmHg at baseline and/or the use of antihypertensive medication or self-reported hypertension²⁰.

Prevalent CVD and diabetes were determined at baseline by linkage to the national medical registry (1990-1997) and were self-reported in the baseline questionnaire. Cancer was determined through linkage with the cancer registry (1989-1997) and was also self-reported at baseline.

Dietary assessment

Dietary intake was assessed using a self-administered validated FFQ, allowing the estimation of the average daily intake of 178 foods over 77 main food categories in the year preceding enrolment. Information on preparation methods, additions, dietary supplements, special diets, brand names, frequency of selected sub-items and portion sizes was collected.

The 1993 Dutch food consumption table (NEVO) was used to calculate energy and nutrient intakes²¹. This table does not include information on vitamin K content of all food products²¹. Therefore, the phyloquinone, short chain menaquinones and long chain menaquinones concentrations in a series of Dutch foods were assessed at the R&D Group VitaK, Maastricht University. These values were supplemented with earlier analyses by Schurgers and Vermeer and the U.S. food composition database^{1,22,23}. Finally the vitamin K database was completed by estimating phyloquinone and menaquinone content through recipes and ingredient information. The relative validity of the FFQ for vitamin K, with this database, has been assessed against 12 monthly 24-hour recalls among 121 men and women^{24,25}. The relative validity for energy adjusted phyloquinone, menaquinones, short- and long- chain menaquinone intake were 0.34, 0.56, 0.32 and 0.66, respectively²⁶.

Outcome assessment

Information on morbidity and mortality was collected through linkage to several registries. The vital status of participants and cause of death were obtained through linkage with the municipal population registries and 'Statistics Netherlands', respectively. Causes of death were coded according to the International Classification of Diseases (ICD-9 and ICD-10).

The outcome is categorized into all-cause, cancer specific (ICD-9 140–239; ICD-10 C00–D48), stroke (ICD-10 I60–I66), CHD (ICD-10 I20–I25, I46, R96) and overall CVD mortality (including ICD codes for stroke, CHD and ICD-10 I26, G45, I60–I67, I69, I70–I74, I50). Lung cancer mortality was analyzed separately, because a previous study suggested a specific association of vitamin K intake with lung cancer¹⁷. Participants who were lost to follow-up were considered censoring events. Follow-up is complete up to December 31st 2012.

Statistical analyses

Baseline characteristics were summarized as mean $\pm$ SD or median (IQR) as appropriate for continuous variables. Data were missing at random, therefore missing values were

multiple imputed 5 imputations and combined according to Rubin's rules²⁷. Confounding variables, vital status and vitamin K levels were used as predictors of missing values.

Person time for each individual was calculated as the month of entry until the month of death, loss to follow-up or December 31st 2012, whichever came first. Consequently, Cox proportional hazards models were used to estimate Hazard Ratio's (HR) for mortality (all-cause and cause-specific) with 95% Confidence Intervals (CI). HR's were calculated for phylloquinone and total-, short chain- and long chain menaquinone intake as quartiles and as continuous variables. Phylloquinone and menaquinones were analyzed per 50 µg and 10 µg intake, respectively, based on previous studies in this cohort⁶. Intake of nutrients was adjusted for energy intake using the residual method²⁸.

Confounders were selected based on a more than 10% change of the association between the different sub forms of vitamin K and mortality when including the confounder in the model. We adjusted for risk factors of chronic diseases and dietary factors in three separate models. The first model was adjusted for age and sex. The second model was additionally adjusted for risk factors for chronic diseases; BMI, physical activity (inactive/moderately inactive/moderately active/active), smoking status (never/former/current), education (low/middle/high), total energy intake and alcohol consumption. The third, and final model was further adjusted for energy-adjusted intakes of mono unsaturated fatty acids (MUFA), poly unsaturated fatty acids (PUFA), saturated fatty acids (SFA), fibre, vitamin C, and plant protein for phylloquinone. For menaquinones the third model was additionally adjusted for energy-adjusted intakes of animal protein and calcium instead of plant protein. Because the participants were derived from two different cohorts, all analyses were stratified for cohort and the means of the strata were presented.

We checked whether interaction of age and sex for all forms of vitamin K was present, by including interaction terms in the model ($p < 0.05$). P-values for linear trends were calculated by including the median of each quartile as a continuous variable in the model. The presence of non-linear associations was explored with a quadratic term (the square of the median in the model) with the linear term, but no evidence of a non-linear association was observed.

In sensitivity analyses, we additionally adjusted for hypertension and hyperlipidemia, because a low vitamin K intake is associated with increased arterial stiffness and vitamin K is transported by apolipoproteins⁵. Other sensitivity analyses excluded cases in the first

2 years of follow-up and excluded cases with unrelated causes of death, such as poisoning or traffic accidents. Statistical analyses were performed using Statistical Analysis Systems version 9.2 (SAS Institute, Cary, NC, USA).

Results

Baseline characteristics are summarized in *Table 1*. The study population had a mean age of 48.6 ± 12.0 years and 26.3% was male, 45.9 % had a normal BMI and 42.5% was physically active. Participants with a high intake of phylloquinone and menaquinones were more often female, older, more often overweight or obese and less often smokers. Participants with a high phylloquinone intake were less often high educated and had a high intake of vitamin C, fibre, MUFA and PUFA. Participants with a high menaquinone intake more often had hypertension, less often hyperlipidemia and had a higher intake of saturated fat and calcium.

During a mean follow-up of 16.8 ± 2.9 years 2,863 participants died, of whom 625 from CVD, 1,346 from cancer and 892 from non-CVD and non-cancer causes. *Table 2* shows the HR's for phylloquinone- and menaquinone intake with all-cause mortality. Phylloquinone intake was not associated with all-cause mortality with a $HR_{Q4vs.Q1}$ of 1.04 (0.92;1.17) after adjustment for risk factors for chronic disease and nutrient intake. Menaquinone intake was not associated with all-cause mortality ($HR_{Q4vs.Q1}$ 0.95 (0.83;1.10)). A higher intake of long chain menaquinone was associated with a reduced risk of all-cause mortality in the age and sex adjusted model ($HR_{10\mu g}$: 0.97 (0.94;1.00)). However, this association was no longer present after adjustment for risk factors for chronic diseases.

Phylloquinone intake was also not associated with CVD mortality in any of the models (*Table 3*). A higher intake of long chain menaquinone intake was associated with a reduced CVD mortality in the age and sex adjusted model (model 1, $HR_{Q4vs.Q1}$: 0.76 (0.60;0.95)), but no association was found in the second and third model. Total menaquinones and short chain menaquinones did not consistently show associations in the final models.

CVD mortality was further split into death due to coronary heart disease (CHD) and death due to stroke (*Table 4*). A higher intake of menaquinones, especially long chain menaquinones, tended to be associated with reduced CHD mortality (model 3, $HR_{10\mu g}$ 0.86 (0.74;1.00)). However, no significant association was found in the quartiles of long chain

menaquinones and CHD mortality (model 3, $HR_{Q4vs.Q1}$ 0.70 (0.45;1.11). No association was observed between vitamin K intake and stroke mortality.

Table 5 presents the HR's for cancer mortality. No significant associations were found between phylloquinone or menaquinone intake and cancer mortality. Furthermore, no associations were found between vitamin K intake and lung cancer mortality (n=296). The association between long chain menaquinones and lung cancer mortality was $HR_{Q4vs.Q1}$ 0.99 (0.86;1.23) in the third model.

During follow up, 892 participants died of other causes than CVD or cancer. The most common causes of death in this group were diseases of the respiratory system (20.6%), diseases of the nervous system (11.8%), diseases of the digestive system (11.5%) and external causes such as accidents or drugs (11.3%). Intake of menaquinones and phylloquinone was not associated with mortality from other causes.

No interaction was present between sex and age for the intake of vitamin K sub forms and mortality ($p>0.12$). Hyperlipidemia and hypertension were included in the model as possible mediators, but this did not affect the results (data not shown), nor did excluding the cases of the first 2 years (n=79) or the unrelated causes of death, such as poisoning and traffic accidents (n=278) (data not shown).

Table 1. Baseline characteristics of 33,289 Dutch males and females, presented by quartiles of phyloquinone and menaquinone intake

	Phylloquinone intake				Menaquinone intake			
	Q1 (n=8,285)	Q2 (n=8,437)	Q3 (n=8,387)	Q4 (n=8,180)	Q1 (n=8,353)	Q2 (n=8,426)	Q3 (n=8,344)	Q4 (n=8,169)
Men (%)	33.7	29.8	24.9	16.5	32.5	31.0	24.3	16.9
Age (years)	46.3 ± 12.3	47.4 ± 11.8	48.9 ± 11.8	52.1 ± 11.1	45.2 ± 12.9	47.5 ± 12.1	49.8 ± 11.4	52.2 ± 10.2
BMI (kg/m ²)	25.2 ± 3.9	25.3 ± 3.8	25.6 ± 3.9	26.1 ± 4.1	24.9 ± 3.8	25.4 ± 3.8	25.8 ± 4.0	26.2 ± 4.1
Waist circumference (cm)	85.5 ± 11.6	84.7 ± 11.3	85.1 ± 11.3	85.6 ± 11.2	83.5 ± 11.2	85.0 ± 11.3	85.5 ± 11.3	85.8 ± 11.4
Physically inactive (%)	8.4	6.3	6.1	6.8	8.3	6.5	6.4	6.5
Current smoker (%)	32.8	29.6	29.6	29.0	33.7	32.7	28.5	25.8
High education (%)	25.6	22.3	19.8	16.1	20.1	20.5	21.9	21.4
Systolic BP (mmHg)	124.2 ± 18.1	124.8 ± 18.0	126.2 ± 18.8	128.2 ± 19.5	123.4 ± 18.1	125.0 ± 18.3	126.6 ± 18.7	128.3 ± 19.3
Diastolic BP (mmHg)	77.4 ± 10.7	77.6 ± 10.5	77.9 ± 10.5	78.2 ± 10.7	76.8 ± 10.4	77.6 ± 10.5	78.1 ± 10.8	78.5 ± 10.6
Hypertension (%)	39.7	41.4	36.7	40.1	31.4	34.7	37.3	40.0
Hyperlipidemia (%)	3.5	3.7	7.4	7.6	8.1	7.8	7.6	6.5
Diet								
Energy (kcal/day)	2044.1 ± 619	2133 ± 616	2105 ± 614	1979 ± 581	2075.2 ± 644	2121.9 ± 617	2067.3 ± 594	1997.5 ± 579
Vitamin K (µg/day) *	129.3 ± 23.6	171.9 ± 17.9	212.6 ± 20.1	296.5 ± 58.5	176.2 ± 64.0	194.2 ± 66.0	208.3 ± 67.2	230.6 ± 72.0
Phylloquinone (µg/day) *	87.7 ± 17.5	129.9 ± 10.5	169.3 ± 13.3	251.4 ± 55.7	150.0 ± 63.5	157.5 ± 65.9	162.5 ± 67.1	166.9 ± 71.1
Menaquinones (µg/day) *	41.6 ± 15	42.0 ± 14.5	43.3 ± 14.6	45.1 ± 15.5	26.2 ± 4.9	36.7 ± 2.4	45.8 ± 3.0	63.7 ± 11.3
Short chain *	24.9 ± 8.2	25.1 ± 7.2	25.8 ± 7.3	27.0 ± 8.2	19.0 ± 4.9	24.5 ± 5.0	27.6 ± 6.1	31.8 ± 8.3
Long chain *	16.3 ± 12.2	16.4 ± 11.4	17.0 ± 11.5	17.6 ± 11.8	6.8 ± 4.1	11.7 ± 5.0	17.7 ± 6.2	31.4 ± 11.8
Fat (g/day) *								
SFA	31.7 ± 6.0	32.4 ± 5.6	32.8 ± 5.6	33.3 ± 6.0	29.6 ± 5.4	32.2 ± 5.0	33.5 ± 5.3	35.1 ± 6.1
MUFA	28.9 ± 5.1	29.5 ± 4.9	29.8 ± 5.0	29.8 ± 5.3	28.3 ± 5.4	29.6 ± 4.8	30.0 ± 4.9	30.0 ± 5.1
PUFA	14.3 ± 3.8	14.9 ± 3.7	15.2 ± 3.7	15.3 ± 4.1	15.4 ± 4.2	15.1 ± 3.7	14.9 ± 3.7	14.3 ± 3.7
Vitamin C (mg/day) *	94.5 ± 39.2	106.8 ± 41.7	113.0 ± 44.9	122.5 ± 49.5	104.1 ± 49.2	105.6 ± 42.7	110.5 ± 42.5	116.6 ± 44.7
Calcium (mg/day) *	1029.4 ± 376	1039.7 ± 344	1065.5 ± 338	1117.0 ± 341	859.4 ± 309	975.8 ± 297	1092 ± 286	1329.4 ± 332
Fiber (g/day) *	21.1 ± 4.6	22.9 ± 4.4	23.8 ± 4.3	25.5 ± 4.7	23.4 ± 5.3	23.2 ± 4.7	23.4 ± 4.5	23.3 ± 4.6
Protein (g/day) *	74.0 ± 11.3	74.9 ± 10.3	76.0 ± 10.3	77.9 ± 11.1	66.7 ± 9.2	73.5 ± 8.0	77.8 ± 8.1	84.9 ± 9.2
Alcohol intake (g/day)	6.4 (17.9)	5.9 (15.4)	5.2 (14.2)	3.7 (12.4)	4.3 (15.4)	5.7 (15.4)	5.8 (14.9)	5.3 (14.6)

*Energy adjusted, mean ± SD, median (IQR) or %. Abbreviations: BMI = Body Mass Index, BP = blood pressure, short chain menaquinones = menaquinone-4 till menaquinone-6, long chain menaquinones = menaquinone-7 till menaquinone-10, SFA = saturated fatty acids, MUFA = mono unsaturated fatty acids, PUFA = poly unsaturated fatty acids.

Table 2. Hazard Ratio's (95%-confidence interval) for the association between intake of phyloquinone and menaquinones and all-cause mortality.

	Q1 (ref)	Q2	Q3	Q4	P _{fortrend}	Continuous*	P _{fortrend} *
Phylloquinone							
No. of cases	666	600	742	855		2,863	
Model 1	1.0	0.83 (0.74;0.92)	0.95 (0.86;1.06)	0.94 (0.84;1.04)	0.02	1.00 (0.97;1.03)	1.00
Model 2	1.0	0.85 (0.76;0.95)	0.97 (0.88;1.08)	0.94 (0.85;1.04)	0.07	1.00 (0.97;1.02)	0.59
Model 3	1.0	0.89 (0.79;0.99)	1.04 (0.93;1.16)	1.04 (0.93;1.17)	0.01	1.02 (0.99;1.05)	0.19
Menaquinones							
No. of cases	658	658	749	798			
Model 1	1.0	0.89 (0.80;0.98)	0.93 (0.84;1.02)	0.91 (0.82;1.00)	0.71	0.98 (0.96;1.01)	0.18
Model 2	1.0	0.90 (0.80;1.00)	0.94 (0.85;1.05)	0.94 (0.84;1.04)	0.53	0.99 (0.97;1.02)	0.31
Model 3	1.0	0.91 (0.81;1.02)	0.96 (0.85;1.09)	0.95 (0.83;1.10)	0.50	1.00 (0.96;1.03)	0.43
Short chain							
No. of cases	680	680	694	809			
Model 1	1.0	0.98 (0.88;1.08)	0.98 (0.87;1.08)	1.07 (0.97;1.19)	0.11	1.03 (0.99;1.08)	0.19
Model 2	1.0	0.97 (0.87;1.08)	0.93 (0.83;1.03)	0.97 (0.87;1.08)	0.78	0.98 (0.93;1.03)	0.28
Model 3	1.0	0.98 (0.87;1.11)	0.95 (0.83;1.09)	1.02 (0.86;1.21)	0.91	1.00 (0.91;1.09)	0.99
Long chain							
No. of cases	673	683	722	785			
Model 1	1.0	0.86 (0.77;0.96)	0.86 (0.77;0.96)	0.85 (0.77;0.94)	0.79	0.97 (0.94;1.00)	0.04
Model 2	1.0	0.92 (0.82;1.02)	0.94 (0.85;1.05)	0.96 (0.97;1.03)	0.53	1.00 (0.97;1.03)	0.58
Model 3	1.0	0.92 (0.82;1.02)	0.95 (0.85;1.07)	0.96 (0.84;1.09)	0.86	1.00 (0.96;1.04)	0.41

*phyloquinone per 50 µg/day, menaquinones per 10 µg/day.
Model 1: Adjusted for sex and age.
Model 2: Additionally adjusted for BMI, physical activity, smoking category, education, energy intake, alcohol intake.
Model 3: Additionally adjusted for MUFA, PUFA, SFA, fibre, vitamin C, plant protein. Model 3 for menaquinones is adjusted for animal protein and calcium instead of plant protein.

Table 3. Hazard Ratio's (95%-confidence interval) for the association between intake of phyloquinone and menaquinones and CVD-specific mortality.

	Q1	Q2	Q3	Q4	P fortrend	Continuous *	P fortrend *
Phylloquinone							
No. of cases	137	142	149	197		625	
Model 1	1.0	0.95 (0.75;1.20)	0.93 (0.74;1.18)	1.04 (0.84;1.30)	0.41	1.01 (0.96;1.07)	0.75
Model 2	1.0	0.96 (0.76;1.21)	0.92 (0.73;1.17)	1.00 (0.80;1.26)	0.69	0.99 (0.94;1.05)	0.80
Model 3	1.0	1.00 (0.80;1.26)	0.99 (0.77;1.26)	1.10 (0.86;1.41)	0.52	1.01 (0.95;1.07)	0.68
Menaquinones							
No. of cases	132	150	174	169			
Model 1	1.0	1.01 (0.80;1.28)	1.08 (0.86;1.36)	0.99 (0.78;1.24)	0.82	0.99 (0.94;1.04)	0.62
Model 2	1.0	1.00 (0.79;1.26)	1.05 (0.84;1.32)	0.96 (0.76;1.21)	0.75	0.98 (0.93;1.03)	0.44
Model 3	1.0	1.01 (0.79;1.30)	1.08 (0.83;1.40)	0.99 (0.73;1.34)	0.90	0.97 (0.90;1.05)	0.44
Short chain							
No. of cases	146	138	145	196			
Model 1	1.0	0.93 (0.73;1.17)	0.94 (0.75;1.18)	1.21 (0.98;1.50)	0.02	1.09 (0.99;1.20)	0.09
Model 2	1.0	0.89 (0.71;1.13)	0.84 (0.67;1.06)	1.00 (0.80;1.25)	0.27	0.99 (0.89;1.09)	0.82
Model 3	1.0	0.90 (0.69;1.16)	0.86 (0.64;1.16)	1.04 (0.73;1.49)	0.44	0.98 (0.81;1.18)	0.83
Long chain							
No. of cases	158	147	161	159			
Model 1	1.0	0.80 (0.63;1.00)	0.83 (0.67;1.04)	0.76 (0.60;0.95)	0.55	0.94 (0.88;1.01)	0.10
Model 2	1.0	0.84 (0.67;1.06)	0.90 (0.72;1.13)	0.85 (0.67;1.06)	0.99	0.97 (0.91;1.04)	0.41
Model 3	1.0	0.84 (0.66;1.06)	0.89 (0.70;1.14)	0.83 (0.62;1.09)	0.95	0.97 (0.88;1.06)	0.45

* phyloquinone per 50 µg, menaquinones per 10 µg.

Model 1: Adjusted for sex and age.

Model 2: Additionally adjusted for BMI, physical activity, smoking category, education, energy intake, alcohol intake.

Model 3: Additionally adjusted for mufa, pufa, saturated fat, fibre, vitamin C, plant protein. Model 3 for menaquinones is adjusted for animal protein and calcium instead of plant protein.

Table 4. Hazard Ratio's (95%-confidence interval) for the association between intake of phyloquinone and menaquinones and CHD- and stroke mortality.

	Q1	Q2	Q3	Q4	P _{fortrend}	Continuous *	P _{fortrend} *
CHD							
Phylloquinone [#]	67	63	54	72		256	
	1.0	0.87 (0.61;1.24)	0.71 (0.49;1.04)	0.85 (0.59;1.24)	0.79	0.97 (0.89;1.07)	0.57
Menaquinone *	60	63	77	56			
	1.0	0.91 (0.62;1.33)	1.06 (0.71;1.58)	0.77 (0.47;1.26)	0.31	0.89 (0.79;1.01)	0.08
Short chain *	59	53	64	80			
	1.0	0.87 (0.57;1.31)	0.97 (0.61;1.53)	1.18 (0.67;2.08)	0.45	0.99 (0.74;1.34)	0.97
Long chain *	70	63	71	52			
	1.0	0.85 (0.60;1.21)	0.95 (0.66;1.37)	0.70 (0.45;1.11)	0.44	0.86 (0.74;1.00)	0.06
Stroke							
Phylloquinone [#]	33	34	47	53		167	
	1.0	1.05 (0.64;1.71)	1.33 (0.84;2.13)	1.23 (0.75;1.99)	0.51	1.03 (0.92;1.16)	0.50
Menaquinone *	30	42	47	44			
	1.0	1.16 (0.72;1.88)	1.19 (0.72;1.98)	1.06 (0.58;1.92)	0.82	0.97 (0.84;1.12)	0.67
Short chain *	39	36	43	49			
	1.0	0.99 (0.60;1.64)	1.18 (0.67;2.07)	1.21 (0.60;2.42)	0.31	0.90 (0.63;1.28)	0.54
Long chain *	42	43	43	39			
	1.0	0.87 (0.56;1.36)	0.84 (0.53;1.34)	0.68 (0.40;1.18)	0.36	0.98 (0.82;1.17)	0.82

[#] Model adjusted for sex, age, BMI, physical activity, smoking category, education, energy intake, alcohol intake, saturated fat intake, plant protein, vitamin C, fibre, mufa and pufa. *Model adjusted for sex, age, BMI, physical activity, smoking category, education, energy intake, alcohol intake, mufa, pufa, saturated fat, fibre, vitamin C, animal protein and calcium. † phyloquinone per 50 µg, menaquinones per 10 µg.

Table 5. Hazard Ratio's (95%-confidence interval) for the association between intake of phyloquinone and menaquinones and cancer mortality.

	Q1 (ref)	Q2	Q3	Q4	P _{fortrend}	Continuous*	P _{fortrend} *
Phylloquinone							
No. of cases	323	285	350	388		1346	
Model 1	1.0	0.82 (0.70;0.96)	0.95 (0.82;1.10)	0.93 (0.80;1.08)	0.11	0.99 (0.96;1.03)	0.73
Model 2	1.0	0.85 (0.72;0.99)	0.98 (0.82;1.11)	0.95 (0.82;1.11)	0.16	0.99 (0.96;1.03)	0.75
Model 3	1.0	0.87 (0.74;1.02)	1.02 (0.87;1.20)	1.01 (0.85;1.19)	0.08	1.01 (0.97;1.05)	0.71
Menaquinones							
No. of cases	323	315	338	370			
Model 1	1.0	0.88 (0.75;1.02)	0.87 (0.75;1.02)	0.89 (0.77;1.04)	0.79	0.99 (0.96;1.03)	0.68
Model 2	1.0	0.89 (0.76;1.04)	0.90 (0.77;1.05)	0.93 (0.79;1.08)	0.65	0.99 (0.95;1.04)	0.95
Model 3	1.0	0.90 (0.77;1.07)	0.92 (0.77;1.10)	0.96 (0.78;1.18)	0.50	1.03 (0.98;1.08)	0.30
Short chain menaquinones							
No. of cases	311	331	335	369			
Model 1	1.0	1.04 (0.89;1.22)	1.03 (0.88;1.20)	1.08 (0.93;1.26)	0.63	1.02 (0.96;1.10)	0.49
Model 2	1.0	1.04 (0.89;1.21)	1.00 (0.86;1.17)	1.01 (0.87;1.18)	0.65	0.99 (0.92;1.06)	0.73
Model 3	1.0	1.10 (0.92;1.31)	1.11 (0.90;1.36)	1.20 (0.93;1.54)	0.42	1.06 (0.93;1.20)	0.40
Long chain menaquinones							
No. of cases	320	324	330	372			
Model 1	1.0	0.88 (0.75;1.02)	0.85 (0.73;0.99)	0.88 (0.76;1.03)	0.85	0.98 (0.94;1.03)	0.39
Model 2	1.0	0.93 (0.79;1.08)	0.93 (0.79;1.09)	0.98 (0.84;1.15)	0.45	1.01 (0.96;1.05)	0.75
Model 3	1.0	0.94 (0.80;1.10)	0.95 (0.80;1.12)	1.01 (0.84;1.23)	0.54	1.03 (0.97;1.09)	0.41

*phyloquinone per 50 µg, menaquinones per 10 µg.

Model 1: Adjusted for sex and age.

Model 2: Additionally adjusted for BMI, physical activity, smoking category, education, energy intake, alcohol intake.

Model 3: Additionally adjusted for MUFA, PUFA, SFA, fibre, vitamin C, plant protein. Model 3 for menaquinones is adjusted for animal protein and calcium instead of plant protein.

Discussion

This study found no significant associations between phylloquinone or menaquinone intake and all-cause or cause specific mortality. Only the intake of long chain menaquinones may be associated with a reduced risk of CHD.

To date, four studies investigated the association between vitamin K intake and mortality^{3,17-19}, of which three studies investigated all-cause mortality^{3,18,19}. Concordant with our findings, two studies did not observe an association between vitamin K intake or its sub forms, and the risk of all-cause mortality^{18,19}. Only Cheung *et al.* found a significant association between total vitamin K intake and all-cause mortality in patients with chronic kidney disease comparing adequate with inadequate intake¹⁹. In our study population 92.3% of the male and 97.4% of the female participants had an adequate vitamin K intake, according to the cut-off values used by Cheung *et al.* of 120 and 90 µg/day for men and women respectively. Therefore, the association observed by Cheung *et al.* is probably due to the high prevalence (77%) of an inadequate vitamin K intake in a population of renal patients. Another Dutch cohort study, the Rotterdam study, found an inverse association between menaquinone intake and all-cause mortality (RR_{T3vs.T1} 0.74(0.59-0.92)), but not for phylloquinone intake³. That study is comparable to our study regarding study population and design. However, in that study a higher percentage of participants died of CHD than in our study. Since CHD mortality contributed more to all-cause mortality in the Rotterdam study, the association for all-cause mortality in that cohort is likely driven by CHD mortality. Indeed, a higher menaquinone intake was strongly associated with a reduced CHD mortality in the Rotterdam study.

Two earlier studies investigated the association of total vitamin K, phylloquinone and menaquinone intake with CVD mortality^{18,19}. In line with our results, these studies did not observe an association of phylloquinone or menaquinone intake with CVD mortality^{18,19}, except for the study in patients with chronic kidney disease by comparing adequate with inadequate total vitamin K intake¹⁹. Our results on CHD mortality were in the same direction as the results from the Rotterdam study, where a higher intake of long chain menaquinones was associated with a reduced risk of CHD mortality³. However, our results were borderline significant, which may be due to the relatively small group deceased of CHD.

To date, most studies focused on CHD incidence, which is logical given the hypothesized underlying mechanisms of reducing coronary calcification through MGP carboxylation. Consistent with this hypothesis, previous studies found inverse associations of menaquinone intake, especially long chain menaquinones, with CHD incidence and coronary calcification³⁻⁵. Although we did not observe strong associations, our results on CHD mortality are in line with these findings. The mentioned studies investigated more specific outcomes, such as specific markers of CHD (e.g. coronary calcification) or disease incidence, whereas we investigated mortality risk. None of the studies found an association of phyloquinone with CHD, consistent with our study. This discrepancy between phyloquinone and menaquinones intake could be explained by the differences in their metabolism²⁹. Because phyloquinone is cleared rapidly by the liver and menaquinones are redistributed by the liver and transported by LDL, they have a longer half-life time and may be more available to extra hepatic tissues²⁹. We did not detect an association of vitamin K with stroke mortality, which is in line with our previous findings on total stroke incidence³⁰.

Finally, two cohort studies investigated the association between phyloquinone and menaquinone intake and cancer mortality^{17,18}. The PREDIMED study observed an inverse association between phyloquinone intake and cancer mortality¹⁸, while in the EPIC-Heidelberg cohort an inverse association between menaquinone intake and cancer mortality was found¹⁷. In our study we were not able to detect any association between phyloquinone or menaquinone intake and cancer mortality. The discrepancy for phyloquinone intake may be explained by the substantially higher dietary phyloquinone intake observed in the PREDIMED study due to the interventional study design (Q4, mean intake was 626.4 µg)¹⁸. The different finding for menaquinone intake observed in EPIC-Heidelberg could partly be explained by the different male/female ratio of the study population¹⁷. Nimptsch *et al.* showed that higher menaquinone intake was strongly associated with a decreased risk of lung- and prostate cancer and not with breast cancer¹⁷. Our study population consists of 26 % males only, leading to only a few cases of prostate cancer (n=16). However, if we restricted our analyses to lung cancer (n=296), we did not observe any associations between menaquinone intake and lung cancer mortality.

Strengths of the present study are its large sample size, allowing us to subdivide CVD mortality into CHD- and stroke mortality, the long follow-up period and the extensive adjustment for confounders. Due to the data from 'Statistics Netherlands', the information on mortality causes was as complete as possible. Since the FFQ was only assessed at

baseline, subjects may have altered their food consumption during follow up, which could result in misclassification. However, long-term reproducibility of the FFQ was assessed in the EPIC-Heidelberg cohort, after a follow up of 5.7 years and they found a reasonable reproducibility for the main sources of vitamin K (meat 0.57, dairy 0.62 and vegetables 0.41)³¹. Moreover, excluding those participants most likely to change their dietary habits, i.e. cases occurring in the first 2 years, did not affect our results. Finally, the validity of the FFQ to estimate vitamin K intake is a limitation of this study. The validity of the FFQ, against 12 24 hour recalls, to estimate long chain menaquinones intake is relatively good ($r=0.66$), while the relative validity for phyloquinone intake is weak ($r=0.33$). Therefore, we may not be able to detect a weak association due to the relatively low validity.

In conclusion, this study did not find an association between vitamin K intake and all-cause or cause specific mortality. Our study suggests that a higher intake of long chain menaquinones may be associated with a reduced risk of CHD mortality.

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Disclosures

None.

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**Circulating phylloquinone,
inactive Matrix Gla protein
and coronary heart
disease risk: a two-sample
Mendelian Randomization
study**

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Abstract

Background: Multiple observational studies and small-scale intervention studies suggest that high vitamin K intake is associated with improved markers for cardiovascular health. Circulating phylloquinone solely represents phylloquinone (vitamin K₁) intake, while dephosphorylated uncarboxylated Matrix Gla Protein (dp-ucMGP) represents both phylloquinone and menaquinone (vitamin K₂) intake.

Objective: This study aims to investigate the causal relationship between genetically predicted vitamin K concentrations and the risk of CHD via a two-sample Mendelian Randomization approach.

Methods: We used data from three studies: the European Prospective Investigation into Cancer and Nutrition (EPIC)-CVD case-cohort study, CARDIOGRAMplusC4D and the UK Biobank, resulting in 103,097 CHD cases. Genetically predicted vitamin K concentrations were measured using SNPs related to circulating phylloquinone and dp-ucMGP. We calculated a genetic risk score (GRS) including four SNPs (rs2108622, rs2192574, rs4645543 and rs6862071) related to circulating phylloquinone levels from a genome wide association study. Rs4236 was used as an instrumental variable for dp-ucMGP. Inverse-variance weighted (IVW) analysis was used to obtain Risk Ratios (RRs) for the causal relationship between phylloquinone and dp-ucMGP concentrations and CHD risk.

Results: Using the genetic score for circulating phylloquinone, we found that circulating phylloquinone was not causally related to CHD risk (RR 1.00 (95%-CI: 0.98;1.04)). Lower genetically predicted dp-ucMGP concentration was associated with a lower CHD risk with a RR of 0.96 (95%-CI: 0.93;0.99) for every 10 µg/L decrease in dp-ucMGP.

Conclusions: This study did not confirm a causal relationship between circulating phylloquinone and lower CHD risk. However, lower dp-ucMGP levels may be causally related with a decreased CHD risk. This inconsistent result may reflect the influence of menaquinones in the association with CHD.

Background

Vitamin K occurs in two biologically active forms, phylloquinone and menaquinones. Phylloquinone is mainly derived from green leafy vegetables, while menaquinones mostly occur in animal products. Phylloquinone and menaquinones have the same 2-methylated-1,4 naphthoquinones ring structure, but differ in the length and saturation degree of the side chain¹. Circulating phylloquinone is a marker for vitamin K status but solely reflects phylloquinone intake or supplementation²⁻⁴. Matrix Gla protein (MGP) is a vitamin K dependent protein since it requires vitamin K for activation⁵. Inactive MGP, dephosphorylated uncarboxylated MGP (dp-ucMGP), serves as a circulating marker for vitamin K, where low dp-ucMGP levels represents long term high vitamin K intake⁶. Both phylloquinone and menaquinone supplementation substantially reduce dp-ucMGP levels⁷.

Observational studies for circulating phylloquinone show contradicting results: lower circulating phylloquinone was a risk factor for incident cardiovascular disease (CVD) risk⁸, but studies focusing on coronary calcium showed contradicting results ranging from no to a positive association^{9,10}. Randomized placebo-controlled trials show that dietary phylloquinone supplementation reduces arterial elasticity¹¹, and progression of coronary calcium in adults treated for hypertension⁴.

MGP is a strong inhibitor of arterial calcification¹² and dp-ucMGP is therefore suggested to reduce coronary heart disease (CHD) risk¹³. A considerable number of observational studies showed that low dp-ucMGP levels, thus higher overall vitamin K intake, are associated with reduced coronary calcification levels and lower CVD risk¹³. In line with observational studies, dietary menaquinone supplementation may be effective in reducing coronary calcification⁶.

The current evidence points towards a beneficial role for vitamin K in prevention of cardiovascular disease risk. However, observational studies may be limited by reverse causation or confounding, while intervention studies are hampered by a short duration and focus on intermediate outcomes¹⁴. Mendelian randomization (MR), where genetic variants are used as an instrumental variable, is an alternative design to minimize bias and infer causality¹⁴. To date, two MR studies have been performed focusing on vitamin K concentrations and CHD risk^{15,16}. One MR study suggested that increased circulating phylloquinone is associated with an increased risk of CHD¹⁵. No association was found between dp-ucMGP levels and CHD risk in a small-scale MR study¹⁶. The MR study for

phylloquinone was performed in a large dataset, but recently more genetic information in multiple cohort studies became available. Furthermore, the MR-study on dp-ucMGP was limited by a small-sample size.

Therefore, this study aims to investigate the causal relation between both circulating phylloquinone and dp-ucMGP and CHD using a two sample Mendelian randomization approach in the European Prospective Investigation into Cancer and Nutrition (EPIC)-CVD case cohort study, CARDIOGRAMplusC4D and the UK Biobank.

Methods

Study population

For these analyses, we used data from three studies: EPIC-CVD, CARDIOGRAMplusC4D and the UK Biobank. Individual participant data was only available in EPIC-CVD. EPIC-CVD is a case-cohort study of cardiovascular disease nested within EPIC. EPIC consists of 366,521 women and 153,457 men, aged 35 to 70 years, recruited between 1991-1999, from 23 recruitment centres across 10 European countries. Participants were recruited in different ways in different countries and recruitment centres, as described previously¹⁷. This case-cohort design includes a randomly selected subcohort of EPIC including 13,985 participants. The subcohort is supplemented with 9,296 participants who developed CHD during follow up. Due to the random selection of the subcohort from the baseline cohort, the subcohort also contains 531 participants that developed CHD during follow up. The EPIC study was approved by the ethical review boards of the International Agency for Research on Cancer and all recruitment centres. All participants gave informed consent prior to inclusion.

Summarized data was included from CARDIOGRAMplusC4D and the UK Biobank. CARDIOGRAMplusC4D is a meta-analysis of 48 studies involving 60,801 CAD cases and 123,504 controls¹⁸. Data on coronary artery disease / myocardial infarction have been contributed by CARDIOGRAMplusC4D investigators and have been downloaded from www.CARDIOGRAMPLUSC4D.ORG. The UK Biobank (www.ukbiobank.ac.uk) recruited over 500,000 men and women (aged 37 to 73 years) between 2006 and 2010 (application number 29916)¹⁹. The UK Biobank study was approved by the North West Multi-Centre Research Ethics Committee and all participants provided written informed consent to participate. Including CARDIOGRAMplusC4D and the UK Biobank increased our dataset to 103,097 CHD cases.

Genotyping

For EPIC-CVD, DNA was extracted from buffy coat from citrated blood plasma. EPIC-CVD participants were genotyped with the Human Core Exome array, Illumina 660 Quad array and Omni Exome Express array. Single nucleotide polymorphisms (SNPs) were removed with minor allele count <2 or Hardy Weinberg p-value <1e-6. Missing genotypes were imputed using the 1000 Genomes reference panel. CARDIOGRAMplusC4D are genotyped using different assays, as described previously¹⁸. Genotyping in the UK Biobank was performed using the Affymetrix UK BiLEVE Axiom array and the Affymetrix UK Biobank Axiom Array²⁰.

Circulating phylloquinone

We included four SNPs that were related to circulating phylloquinone (both plasma and serum) in a small genome wide association study (GWAS) (rs2108622, rs2192574, rs4645543 and rs6862071)²¹. Rs2108622 (chromosome 19) is a *CYP4F2* variant, which functions as a phylloquinone oxidase²². Previous studies showed that rs2108622 was associated with altered phylloquinone metabolism and warfarin dosage²³. Rs2192574 (chromosome 2) is a variant on the *CTNNA2* locus, whereas a GWAS indicated an association between the *CTNNA2* locus and bone mineral density²⁴. Vitamin K is suggested to increase bone mineral concentration, via activation of osteocalcin, another vitamin K dependent protein²⁵, which may explain the correlation between vitamin K and the *CTNNA2* locus. Rs4645543 (chromosome 8; *KCNK9*) and rs6862071 (chromosome 5; *CDO1*) are SNPs on the potassium channel, and tumor suppressor gene respectively²⁶. The mechanism by which these genes influence phylloquinone concentration is unknown.

All SNPs passed the threshold of $p < 5 \times 10^{-6}$, but not $p < 5 \times 10^{-8}$ ²¹. No linkage disequilibrium (LD) at $R^2 > 0.9$ was present among these four SNPs. Pleiotropic effects were investigated by searching the GWAS catalogue and PhenoScanner for the SNPs or their proxies ($R^2 > 0.8$)^{27,28}, checking possible confounders with a threshold of $p < 5 \times 10^{-6}$.

We calculated a genetic risk score (GRS) to take into account the differences in the effect of the individual SNP on circulating phylloquinone levels. The genetic variants are uncorrelated and therefore the estimates from a weighted regression analysis using summarized data are equal to a genetic risk score using individual level data²⁹. We refer to the analysis as a genetic risk score as this is likely to be more familiar to applied readers. The weights of the individual SNPs are shown in *Table 1*. The F-statistic for the GRS for circulating phylloquinone was 280.4.

Dp-ucMGP

A candidate gene study identified three SNPs covering the MGP gene (chromosome 12) into the 3' and 5' flanking regions, that were associated with dp-ucMGP levels: rs2098435, rs2430692 and rs4236¹⁶. LD was present among these three SNPs ($R^2 > 0.75$). Rs4236 showed the strongest association with dp-ucMGP (beta/SE), and was therefore included as the instrumental variable. Furthermore, pleiotropic effects were investigated by searching in the GWAS catalog and Phenoscanner, and no pleiotropic effects were identified^{27,28}. SNP rs4236 was complete for all participants with available genetic data. The F-statistic for rs4236 was 117.8.

Table 1. Genetic variants associated with circulating phylloquinone and dp-ucMGP as reported in prior studies

	SNP	Chromosome	Nearest gene	β	p-value
Phylloquinone ²¹	Rs2108622	19	CYP4F2	0.16	8.78×10^{-7}
	Rs2192574	2	CTNAA2	0.28	1.82×10^{-6}
	Rs4645543	8	KCNK9	0.42	2.00×10^{-7}
	Rs6862071	5	CDO1	0.94	2.29×10^{-5}
Dp-ucMGP ¹⁶	Rs4236	12	MGP	-3.74	0.001 [#]

[#] identified from candidate gene study

CHD ascertainment

In EPIC-CVD, incident CHD cases were defined as first CHD event, both fatal and non-fatal as defined by ICD 10 codes: I20-I25. This includes myocardial infarction, acute ischaemic heart diseases, chronic ischaemic heart disease and angina pectoris. Several methods were used to ascertain CHD events including self-report, linkage to primary- and secondary-care registers and death registries³⁰. Case ascertainment in CARDIOGRAMplusC4D differed per study centre, as described previously³¹. In the UK Biobank, endpoints were derived from linkage with hospital admissions data in England, Scotland and Wales and national death register data to identify the date of the first known CHD after the date of baseline assessment, identified by ICD 10 codes: I20-I25.

Statistical analyses

In EPIC-CVD individual participant data was analysed. Prentice-weighted Cox proportional hazards regression, with robust standard errors were used to account for the case-cohort design, was performed to assess the association between individual SNPs, the GRS and CHD risk. Age was the underlying time scale, with entry time defined as the participant's age at recruitment and exit time as age at first fatal or non-fatal CHD event or censoring

(whichever came first). All models were analysed in strata of country and adjusted for sex, study centre, principal components and genetic array. Hazard ratios from EPIC-CVD and the odds ratios from CARDIOGRAMplusC4d and UK Biobank were pooled to obtain risk ratios (RR) using random-effect meta-analysis. Then, the inverse-variance weighted (IVW) method was applied to assess the association between genetically predicted circulating phylloquinone, dp-ucMGP and CHD risk, were calculated using the formula reported by Burgess *et al.*³².

To assess the robustness of the main result for the GRS for circulating phylloquinone, we performed a MR-Egger analysis using the 'MendelianRandomization' package in R³³. Circulating phylloquinone is carried predominantly in triglyceride rich lipoproteins and the GWAS also reported beta-coefficients adjusted for fasting triglycerides²¹. Therefore, we performed sensitivity analyses in EPIC-CVD only; using these adjusted beta-coefficients and additionally adjusted the association between the GRS and CHD risk for triglycerides and hours of fasting. The study identifying the dp-ucMGP SNPs also adjusted for BMI, alcohol intake and smoking status¹⁶. We performed sensitivity analyses including the same confounders. Missing values on triglycerides (6.6%), hours of fasting (32.4%) and alcohol intake (0.04%) were imputed using 10 imputations using the 'mice' package in R and combined according to Rubin's Rules³⁴.

To explain the differences between CARDIOGRAMplusC4D and EPIC-CVD and UK Biobank for rs2108622 and rs4645543, we requested study-level results from CARDIOGRAMplusC4D and excluded studies with participants of non-European descent and small studies ($n < 1,000$ cases) as sensitivity analyses. All analyses are performed using R Software version 3.1.1.

Results

Baseline characteristics of the EPIC-CVD participants are described in *Supplementary Table 1*. The IVW analyses showed no association between genetically-predicted concentrations of circulating phylloquinone risk and CHD risk in EPIC-CVD (HR_{GRS} of 0.99 (95%-CI: 0.90;1.07) per ln-nmol/L increase in circulating phylloquinone) (*Figure 1*). Similar results were observed in CARDIOGRAMplusC4D (OR 1.06(95%-CI: 0.96-1.18)) and the UK Biobank (OR 1.00 (95%-CI: 0.95-1.04)). There was no heterogeneity between the three studies ($I^2=0.0$) or genetic variants ($I^2=1.49$, $p=0.68$). However, a causal relation was found between rs2108622 (*CYP4F2*) and CHD risk in CARDIOGRAMplusC4D (OR 1.34 (95%-CI: 1.18;1.51)).

To assess the difference in studies for rs2108622, sensitivity analysis was performed excluding small scale studies ($n < 1,000$ cases) and participants of non-European descent from CARDIOGRAMplusC4D and this slightly decreased the RR (1.21 (95%-CI: 1.03;1.41)). The MR-Egger method indicated no significant pleiotropy (intercept=0.02, $p=0.09$), but the causal estimate slightly decreased to 0.95 (95%-CI: 0.88;1.02) per ln-nmol/L increase in circulating phylloquinone. EPIC-CVD data only, additional adjustment for triglycerides and hours of fasting did not change the results with a HR of 0.90 (95%-CI: 0.83;1.26) for the GRS (*Supplementary Table 2*).

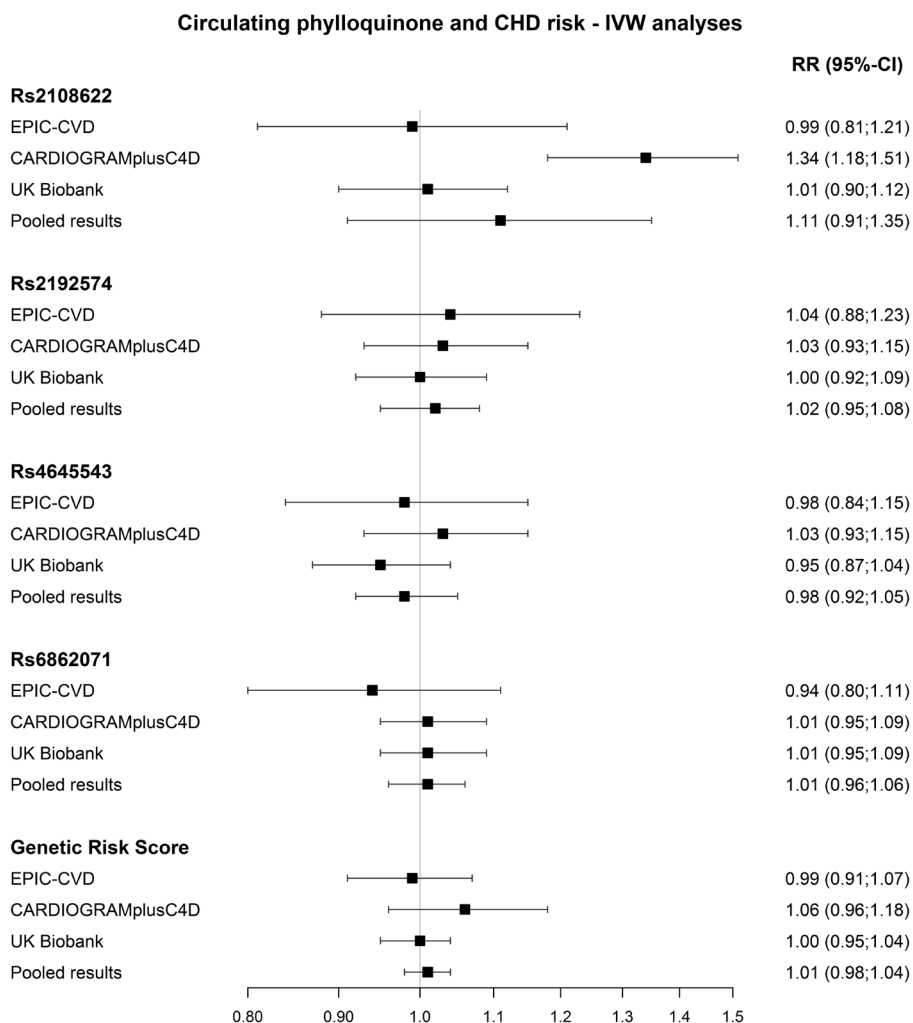


Figure 1. The results of the Mendelian Randomization analyses for circulating phyllloquinone in EPIC-CVD (n=9,296 cases), CARDIOGRAMplusC4D (n=60,801 cases) and UK Biobank (n=33,000 cases), and the pooled association (n=103,097 cases). The results are derived from the IVW analyses and presented as RR per ln-nmol/L increase in circulating phyllloquinone.

Dp-ucMGP

No causal relation was found between rs4236 and risk of CHD in EPIC-CVD with a HR of 0.99 (95%-CI: 0.91;1.07) (*Figure 2*). Adding CARDIOGRAMplusC4D and UK Biobank data resulted in a RR of 0.96 (95%-CI: 0.93;0.99) per 10 µg/L decrease in dp-ucMGP (p=0.02). Additional adjustment for smoking, drinking and alcohol intake, in EPIC-CVD only, did not change the results (HR 0.94 (95%-CI: 0.79;1.11)) (*Supplementary Table 2*).

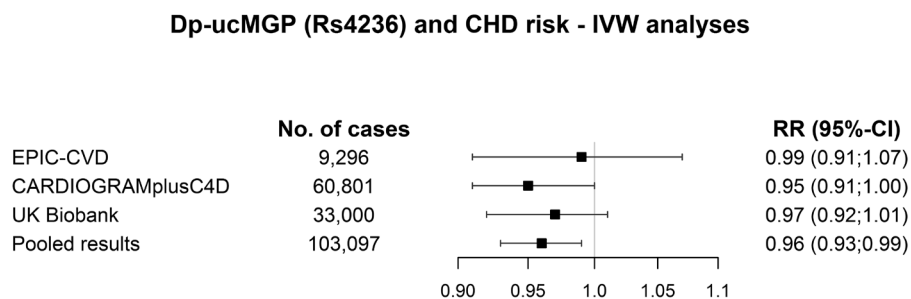


Figure 2. The results of the Mendelian Randomization analyses for circulating dp-ucMGP (rs4236) in EPIC-CVD, CARDIOGRAMplusC4D and UK Biobank, and the pooled association (EPIC-CVD, CARDIOGRAMplusC4D and UK Biobank). The results are derived from the IVW analyses and presented as RR per every 10 µg/L decrease in dp-ucMGP.

Discussion

This study did not provide evidence for an association between genetically predicted phyloquinone concentrations and CHD risk. However, lower genetically predicted dp-ucMGP concentrations, representing long-term increased vitamin K intake, may be causally related with a decreased CHD risk.

Our results are in contrast with a previous MR-study showing that higher genetically predicted phyloquinone concentrations were associated with an increased CAD/MI risk in the CARDIOGRAM data¹⁵. This well-conducted and reproducible study used CARDIOGRAM (2011) and CARDIOGRAMplusC4D (2013), while we used the CARDIOGRAMplusC4D 2015 release. The difference in results between the study from Schooling and our study were driven by two genetic variants: rs4645543 (*KCNK9* gene) and rs2108622 (*CYP4F2* gene). The results for rs4645543 differed in CARDIOGRAMplusC4D 2015 release and the CARDIOGRAM 2011 release. The major differences between the releases are the studies including participants from non-European descent; however, excluding studies from non-European

descent did not alter the results. Furthermore, restricting to the studies (n=12) in the CARDIOGRAMplusC4D 2015 release that were also in the CARDIOGRAM 2011 release, did not explain the discrepancies as well. Therefore, we cannot fully explain the discrepancy between the results of Schooling using earlier CARDIOGRAM releases and our study. Rs2108622 (*CYP4F2* gene) was positively associated with CHD risk in CARDIOGRAMplusC4D, like in all CARDIOGRAM releases, while no association was found in EPIC-CVD or in the UK Biobank. We excluded small-scale studies and studies from non-European descent in CARDIOGRAMplusC4D, and this slightly decreased the risk ratios, but we could not fully explain the discrepancy for rs2108622 in CARDIOGRAMplusC4D compared to EPIC-CVD or the UK Biobank. This genetic variant may be the most reliable marker for phylloquinone concentrations since the *CYP4F2* gene functions as a phylloquinone oxidase, and could alter the phylloquinone metabolism²². However, we did not detect a causal relation between this genetic variant and CHD risk.

In contrast with phylloquinone, decreased genetically predicted dp-ucMGP concentrations, reflecting increased vitamin K intake, were causally related with a decreased CHD risk. Liu *et al.* did not detect a causal relation between rs4236 and risk of CVD using an MR approach¹⁶. However, the direction of the association is comparable to our study (HR 0.87 (95%-CI: 0.71;1.08)). The small sample size of this study (CVD events, n=87) may explain the non-significant finding¹⁶. Indeed, our study only detected a causal relation in the pooled dataset of over 100,000 CHD cases.

The differences between phylloquinone and dp-ucMGP could be explained by the different characteristics of both biomarkers to assess vitamin K status. Circulating phylloquinone is the primary circulating form of vitamin K and solely represents phylloquinone intake of the previous days¹³. Inactive MGP, dp-ucMGP, is considered the best marker for vitamin K since dp-ucMGP reflects the bioavailability of both phylloquinone and menaquinone intake over multiple weeks to months. Menaquinones should be taken into account since menaquinones have a higher absorption and bioavailability than phylloquinone, and therefore may be more beneficial to human health¹. Moreover, dp-ucMGP is functionally associated with the mechanism explained the reduced CHD risk. Therefore, the association found for dp-ucMGP may be the most reliable marker to reflect vitamin K intake. However, candidate gene studies also suggested that rs4236 was associated with total MGP³⁵. Therefore, this SNP may also reflect the causal association between MGP itself and CHD rather than vitamin K.

To date, the literature regarding circulating phylloquinone and CHD risk is inconsistent. Observational studies focusing on circulating phylloquinone and differences in coronary artery calcium showed contradicting results ranging from no to a positive association^{9,10}, while intervention studies showed that phylloquinone supplementation reduces arterial elasticity and coronary calcification^{4,11}. It is possible that the protective effect can be reached with supplementary dosage and not with dietary intake or circulating levels of phylloquinone⁹. The majority of the observational studies showed that low dp-ucMGP levels are associated with reduced cardiovascular disease (CVD) risk and presence of coronary calcification levels¹³. Our MR-study is comparable to previous observational studies, since we were not able to detect an association between circulating phylloquinone and CHD risk. However, higher genetically predicted dp-ucMGP levels were associated with a reduced CHD risk, which could be explained by the influence of menaquinones.

A particular strength in two-sample MR study is the large sample size which can detect small effect sizes. Several issues may compromise the value of Mendelian randomization approach in assessing causality. First, genetic variants determining significant proportions of variance in the risk factor are needed. While the SNPs did not reach genome wide significance, the F-statistic did not show an indication of weak instrument bias. Additionally, the F-statistic for dp-ucMGP SNP showed no weak instrument bias as well. However, since this SNP is identified in a small scale candidate gene study, the effect size of the SNP may be overestimated³⁶. A GWA study on dp-ucMGP should be performed to verify the SNPs used in this MR-study. Circulating phylloquinone and dp-ucMGP are not available in EPIC-CVD, CARDIOGRAMplusC4D or UK Biobank, and therefore we were not able to assess the strength of our instrument in our study population. Furthermore, Mendelian Randomization studies assume that instruments are associated exclusively via the risk factor of interest, and the instrument is not associated with possible confounders. Therefore, we assessed possible pleiotropy using the MR-Egger, and we did not find an indication for pleiotropy in this study. However, our power to detect pleiotropy with a limited number of genetic instruments was low. Additionally, we cannot exclude the possibility of unmeasured pleiotropy for the genetic variants for phylloquinone, since the biological pathways by which the genetic variants influence phylloquinone concentrations is not clear yet. Furthermore, our instrument for dp-ucMGP has also been associated with levels of proteins encoded by other genes in the region (eg, *ART*)³⁷, which suggests potential alternative pathways via which any causal effect could be acting.

In conclusion, this study did not provide evidence for a causal association between circulating phyloquinone concentrations and CHD risk, but decreased dp-ucMGP concentrations, representing long-term increased vitamin K intake, may be causally associated with a decreased CHD risk. Whether this association reflects the influence of menaquinones in the association with CHD or MGP as a causal factor leading to CHD needs to be further investigated.

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Disclosures

None.

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Supplementary information

Supplementary table 1. Baseline characteristics for EPIC-CVD cases and subcohort members.

	Cases (N=9,296)	Subcohort (N=13,985)
Age at recruitment (yrs)	58.8±8.2	52.4±9.4
Male sex	60.9	61.6
Low education level*	45.3	41.5
BMI (kg/m ²)	27.1±4.1	26.2±4.3
Physical inactivity	29.4	23.6
Current smokers	37.4	26.5
Alcohol intake (grams/day)	5.9 (16.8)	6.0 (16.93)
SBP (mmHg)	143.5±20.8	132.4±19.6
DBP (mmHg)	85.6±11.1	81.6±10.8
Triglycerides (mmol/L)	1.6 (1.2)	1.1 (0.9)
Total cholesterol (mmol/L)	6.4±1.2	5.9±1.1
HDL (mmol/L)	1.3±0.4	1.5±0.4
LDL (mmol/L)	5.1±1.2	4.5±1.2
HbA1c (mmol/mol)	5.6 (0.65)	5.4 (0.44)

Variables are depicted as mean ± standard deviation or median (interquartile range) or as %. * None or primary school completed, BMI = body mass index; SBP = systolic blood pressure; Tchol = total cholesterol.

Supplementary table 2. Adjusted hazard ratios (95% CI) for the individual SNPs and GRS for risk of CHD in EPIC-CVD.

	HR (95% CI)
<i>Circulating phylloquinone</i>	
Rs2108622	0.79 (0.85;1.83)
Rs2192574	1.25 (0.93;1.69)
Rs4645543	0.89 (0.62;1.26)
Rs6862071	0.86 (0.66;1.11)
GRS	0.90 (0.83;1.26)
<i>Dp-ucMGP</i>	
Rs4236	0.94 (0.79;1.01)

Adjusted for sex, centre, principal components and genetic array. The SNPs for circulating phylloquinone are additionally adjusted for triglycerides and hours of fasting. The dp-ucMGP SNP is additionally adjusted for BMI, smoking and alcohol intake.



**Matrix Gla Protein, plaque
stability and cardiovascular
events in patients with
severe atherosclerotic
disease**

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Abstract

Objective: This study aims to investigate whether plasma Matrix-Gla Protein (MGP) species, desphospho-uncarboxylated (dp-uc) MGP and total-uncarboxylated (t-uc) MGP, are associated with plaque levels of uncarboxylated (uc) MGP, markers of plaque stability, and cardiovascular disease (CVD) risk.

Methods: From the Athero-Express biobank, we selected carotid plaque samples of 100 patients who underwent carotid endarterectomy. The level of agreement between plasma MGP species and plaque ucMGP levels was assessed using Weighted Kappa's. We analyzed histological characteristics of plaque composition (plaque haemorrhage, lipid and calcification content). Logistic regression analyses were used to assess the association between plasma MGP and plaque characteristics. Furthermore, CVD endpoints (n=20) were collected over a mean follow-up of 2.6 years.

Results: Weighted Kappa statistics of plasma dp-ucMGP and t-ucMGP and plaque ucMGP were 0.08 (-0.33;0.49) and 0.14 (-0.20;0.48). Higher dp-ucMGP levels tended to be associated with less plaque haemorrhage ($OR_{per\ 500nM} 0.96 (0.92;1.00)$). No association was found for lipid and calcification content. Cox proportional hazard models showed no association between dp-ucMGP ($HR_{per\ 200pM} 0.92 (0.75;1.12)$) and an inverse association between t-ucMGP ($HR_{per\ 500nM} 0.79 (0.62;0.99)$) and cardiovascular events.

Conclusions: Plasma dp-ucMGP and t-ucMGP concentrations do not reflect plaque ucMGP levels. Elevated dp-ucMGP levels may be associated with less plaque haemorrhage, suggestive of more stable plaques. T-ucMGP was not related with markers of plaque stability; however, elevated plasma t-ucMGP levels were associated with reduced CVD risk.

Background

Vascular calcification is associated with increased cardiovascular disease (CVD) risk, and Matrix-Gla protein (MGP) is an inhibitor of vascular calcification¹. Proposed mechanisms for inhibiting vascular calcification all require vitamin K dependent carboxylation of MGP². MGP exists in various species, differing in their phosphorylation and/or carboxylation state. High non-phosphorylated and uncarboxylated MGP (dp-ucMGP), representing poor vitamin K status, is associated with an increased CVD risk^{3,4}. Total uncarboxylated MGP (t-ucMGP) includes dp-ucMGP, but mainly consists of phosphorylated uncarboxylated MGP⁵. Circulating t-ucMGP has been proposed as a biomarker for prevalent vascular calcification^{3,6}; however, the association between t-ucMGP and CVD risk showed inconsistent results ranging from no to an inverse association with cardiovascular events^{5,7}. Vitamin K antagonists treatment resulted in high dp-ucMGP levels and accelerated plaque calcification⁴, and shifted atherosclerotic plaque to vulnerable plaques in mice⁸. No studies have been performed investigating plasma MGP levels and plaque stability in humans.

The aim of this study was to assess whether plasma dp-ucMGP and t-ucMGP levels are associated with ucMGP levels in plaque, markers of plaque stability, and cardiovascular events in a very high-risk population of patients with occlusive atherosclerotic disease in the carotid artery.

Methods

Study population

Athero-Express is an ongoing biobank study of patients undergoing carotid and femoral endarterectomy in two Dutch centers, from 2002 onwards. Details of the study design have been described previously⁹. Blood samples are taken before surgery and were stored at -80 degrees. Patients gave informed consent prior to study inclusion and the study was approved by the local medical ethical committee. All patients underwent clinical follow-up 1 year after surgical intervention and completed postal questionnaires 1, 2, and 3 years after the operation.

This study included 100 patients who underwent carotid endarterectomy for stroke, transient ischemic attack and amurosis fugax or asymptomatic patients between 2002 and 2006. A patient group was selected based on estimated glomerular filtration rate (eGFR) and calcification level (assessed with histological assessment); 25 patients with eGFR

between 30 and 60 ml/min/1.73m², 25 patients with eGFR levels above 60 ml/min/1.73m², 25 patients with no plaque calcification, and 25 patients with heavy calcification staining were selected for this study. Selection was based on eGFR and calcification levels since these factors highly affect plasma MGP levels and thus ensure sufficient variation in MGP levels. Patients with missing data on plasma MGP levels (n=2), plaque ucMGP levels (n=8) or cardiovascular events occurrence (n=3) were excluded, leaving 90 patients for the comparison between plasma MGP levels and plaque ucMGP levels, 98 patients for plasma MGP and plaque stability markers, and 95 patients for analyses with plasma MGP levels and cardiovascular events.

Plaque stability

Atherosclerotic plaques were harvested during carotid endarterectomy according to a standardized protocol. The segment with the greatest plaque burden was considered as the culprit lesion. Detailed description of the histological assessment has been published previously⁹. Plaque haemorrhage was defined as haemorrhage within the tissue of the plaque, and scored as absent or present. The lipid core size was expressed as the percentage of total plaque area and scored as <40% and ≥40%, and calcification was semi-quantitatively scored as no/minor, moderate/heavy staining.

Matrix Gla Protein

UcMGP in plaque was visualized by immunohistochemistry using a monoclonal ucMGP antibody, and the intensity of staining was scored by two independent coworkers as 'no/minor', 'moderate' or 'heavy' staining. Conflicting scores were resolved in consensus with a third person. Plasma dp-ucMGP measurements were performed with a sandwich dual-antibody ELISA and plasma t-ucMGP levels were examined with a competitive mono-antibody ELISA, as described previously¹⁰.

Statistical analyses

Baseline characteristics were summarized as percentages or as median (interquartile range), since the variables were not normally distributed. Dp-ucMGP and t-ucMGP levels were divided in tertiles. Three types of analyses were performed: First, Weighted Kappa statistics were calculated to assess agreement between tertiles of plasma dp-ucMGP and t-ucMGP levels and plaque ucMGP levels. Secondly, we assessed the association between dp-ucMGP and t-ucMGP and markers of plaque stability using logistic regression analyses adjusted for age and eGFR. Finally, associations of dp-ucMGP and t-ucMGP with cardiovascular events (fatal and non-fatal) were analysed with Cox proportional hazard

regression models. Based on literature, we investigated whether age, gender, current smoking (yes/no), eGFR (using the MDRD formula) and degree of contralateral stenosis (0-50%/ 50-70%/70-99%/100%) were confounders in this association. Only age and eGFR were associated with dp-ucMGP and t-ucMGP, and included as confounders. Gender, smoking status and degree of stenosis were included in a sensitivity analysis. All analyses were performed in R version 3.2.2. P-values <0.05 were considered statistically significant.

Results

The study population had a mean age of 70 years, and 56.4% was male with a mean BMI of 26 kg/m² (Table 1). Participants with higher dp-ucMGP levels were older, less often current smokers and had lower eGFR levels, while patients with higher t-ucMGP levels had higher eGFR levels.

There was no association between dp-ucMGP (weighted kappa=0.08; -0.33;0.49) or t-ucMGP (0.14; -0.20; 0.48) concentrations and plaque ucMGP levels. The levels of plasma dp-ucMGP and t-ucMGP by plaque ucMGP levels are shown in Figure 1.

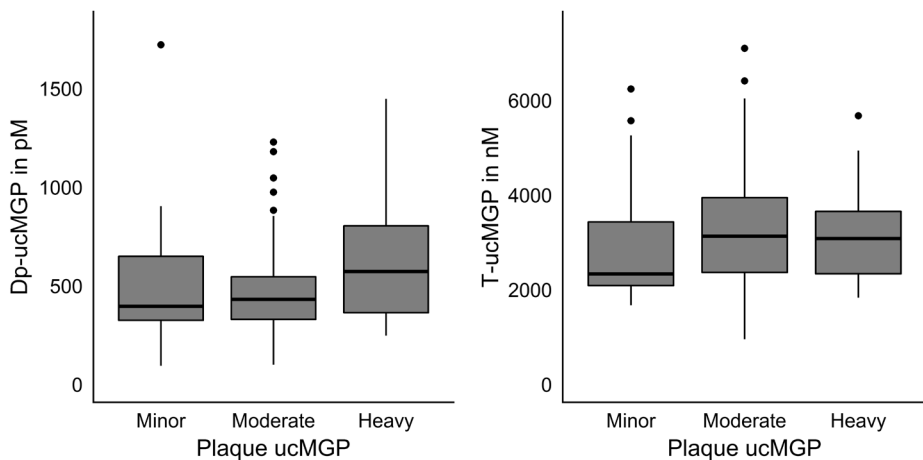


Figure 1. Dp-ucMGP and t-ucMGP in pM and nM respectively, presented by the different categories of plaque ucMGP

Table 1. Baseline characteristics by tertiles of dephosphorylated uncarboxylated MGP (dp-ucMGP) and total uncarboxylated MGP (t-ucMGP).

	Dp-ucMGP			T-ucMGP		
	Tertile 1 (n=33) 95–364 pM	Tertile 2 (n=32) 365–573 pM	Tertile 3 (n=33) 576–2,800 pM	Tertile 1 (n=33) 956–1,504 nM	Tertile 2 (n=32) 2,496–3,575 nM	Tertile 3 (n=33) 3,593–7,653 nM
Age, years	64.0 (60.0–72.0)	70.0 (65.8–76.0)	73.0 (68.0–79.0)	72.0 (64.0–77.0)	74.5 (62.8–79.0)	68.0 (61.0–71.0)
Males	19 (57.6)	23 (71.9)	15 (45.5)	19 (57.6)	17 (53.1)	21 (63.6)
BMI	24.6 (23.6–27.6)	26.6 (23.4–29.4)	26.0 (22.4–28.3)	26.6 (23.3–28.3)	25.2 (23.4–27.8)	25.7 (23.5–28.4)
Current smokers	14 (42.4)	10 (31.2)	10 (30.3)	11 (33.3)	9 (28.1)	14 (42.4)
Diabetes	8 (24.2)	10 (31.2)	11 (33.3)	8 (24.2)	6 (18.8)	15 (45.5)
Hypertension	23 (69.7)	23 (71.9)	30 (90.9)	25 (75.8)	28 (87.5)	23 (69.7)
Contralateral stenosis	9 (27.3)	8 (25.0)	8 (24.2)	10 (30.3)	9 (28.1)	7 (21.2)
<i>Laboratory results</i>						
eGFR, mL/min/1.73m ²	77 (65–90)	67 (55–78)	55 (50–65)	64 (53–82)	65 (55–73)	78 (52–85)
Total cholesterol, mg/dL	4.6±0.9	4.6±1.1	4.2±1.2	4.4±1.2	4.4±1.0	4.5±1.1
HDL, mg/dL	1.2 (1.0–1.4)	1.1 (1.0–1.3)	0.9 (0.7–1.3)	1.2 (0.9–1.3)	1.0 (0.9–1.4)	1.1 (1.0–1.3)
LDL, mg/dL	2.7 (2.1–3.4)	3.1 (2.2–3.4)	2.5 (1.6–3.2)	2.7 (2.0–3.6)	2.6 (2.1–3.3)	2.6 (2.0–3.3)
DpucMGP, pM	296 (199–341)	459 (426–531)	833 (661–1,180)	433 (330–646)	493 (350–818)	445 (353–576)
t-ucMGP, nM	3,021 (2,225–4,455)	3,315 (2,569–4,060)	2783 (2,144–3,612)	2,060 (1,710–2,225)	3,073 (2,780–3,279)	4,466 (4,117–5,366)
<i>Plaque characteristics</i>						
Calcified plaque	16 (48.5)	16 (50.0)	19 (57.6)	20 (60.6)	16 (50.0)	15 (45.5)
Fat content <40%	23 (69.7)	21 (65.6)	27 (81.8)	23 (69.7)	22 (68.8)	26 (78.8)
Plaque hemorrhage	29 (87.9)	23 (71.9)	14 (42.4)	22 (66.7)	24 (75.0)	20 (60.6)
<i>Plaque ucMGP</i>						
Mo/heavy staining	22 (66.7)	29 (90.6)	17 (51.5)	21 (63.6)	24 (75.0)	23 (69.7)

Values are presented as medians (IQR), means ± SD, or numbers (%). HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Higher plasma dp-ucMGP levels were suggested to be associated with less plaque haemorrhage (OR 0.96 (0.92;1.00)) (Table 2). No associations were observed between dp-ucMGP levels and fat content and plaque calcification. Plasma t-ucMGP was not associated with plaque characteristics.

During 2.6 years follow-up, 20 cardiovascular events occurred, of which 17 participants deceased. Dp-ucMGP (per 200 pM) was not associated with cardiovascular events (HR 0.92 (0.75;1.12)) adjusted for age and eGFR (Table 2). Each 500 nM higher t-ucMGP level was associated with a reduced CVD risk (HR 0.79 (0.62;0.99)) adjusted for age and eGFR. Including gender, smoking status and degree of stenosis did not substantially change the results (dp-ucMGP: 0.88 (0.72;1.08), t-ucMGP: 0.79 (0.62;0.99)).

Table 2. Results of the logistic regression analyses for plasma MGP and plaque characteristics and the results of the Cox proportional hazard models for plasma MGP and cardiovascular events (n=20).

	Calcified plaque	Fat content <40%	Plaque haemorrhage	Cardiovascular events
	OR (95%-CI)	OR (95%-CI)	OR (95%-CI)	HR (95%-CI)
Dp-ucMGP	0.99 (0.94;1.04)	0.98 (0.94;1.02)	0.96 (0.92;1.00)	0.92 (0.75;1.12)
T-ucMGP	0.98 (0.93;1.01)	0.99 (0.95;1.03)	0.98 (0.95;1.02)	0.79 (0.62;0.99)

Dp-ucMGP: dephosphorylated uncarboxylated MGP, t-ucMGP: total uncarboxylated MGP. The OR/HR represents every 200pM increment in dp-ucMGP and 500nM increment in t-ucMGP. All models are adjusted for age and eGFR.

Discussion

This is a first study comparing plasma MGP levels with plaque MGP levels and plaque stability, but the study is exploratory in nature because of the small sample size and semi quantitative measures. The sample size was also too small to investigate the association between plaque ucMGP and CVD events. We hypothesized that elevated dp-ucMGP was linked with unstable plaques and increased CVD risk^{3,4,8}. In our study, higher plasma dp-ucMGP levels tended to be associated with less plaque haemorrhage. This inverse association seems counterintuitive at first. However, active MGP (low dp-ucMGP levels) is an inhibitor of calcification¹. Calcification rich plaques may cause more plaque stability; therefore, low dp-ucMGP levels may represent stable plaques¹¹. Nevertheless, no association was found between dp-ucMGP and calcification levels. The

association between dp-ucMGP and calcification might be different in patients with severe atherosclerotic disease or this could be due to a lack of power.

Previous studies proposed t-ucMGP as a marker of prevalent vascular calcification^{3,6} instead of an active player in vascular calcification. However, our study did not find an association between t-ucMGP and different plaque characteristics. To date, the literature is still inconclusive regarding the association between t-ucMGP and CVD risk^{3,6}. It is suggested that t-ucMGP may act differently in persons with or without calcification⁵. Calcified vessels may hamper the MGP phosphorylation¹². Since t-ucMGP mainly consists of phosphorylated ucMGP levels, this could result in lower circulating t-ucMGP levels¹². Future research, with an enhanced sample size, is necessary to give more insight in the association between t-ucMGP and CVD risk.

In conclusion, plasma dp-ucMGP and t-ucMGP concentrations did not reflect plaque ucMGP levels. Elevated dp-ucMGP levels may be associated with less plaque haemorrhage, suggestive of more stable plaques. T-ucMGP was not linked with plaque stability, nevertheless high plasma t-ucMGP levels were associated with reduced CVD risk.

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Disclosures

None.

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**The effect of menaquinone-7
supplementation on
vascular calcification in
people with diabetes: a
randomized, double-blind,
placebo-controlled trial**

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Abstract

Aims: To assess if menaquinone supplementation, compared to placebo, decreases vascular calcification in people with type 2 diabetes and known CVD.

Methods and results: In this double-blind, randomized placebo-controlled trial, we randomly assigned men and women with type 2 diabetes and CVD to 360 µg/day menaquinone-7 or placebo during 6 months. Femoral arterial calcification at baseline and 6 months was measured with ¹⁸F-NaF PET scans as target-to-background ratios (TBR). Calcification mass on conventional computed tomography (CT) scan was measured as secondary outcome. Inactive matrix-Gla protein (MGP) concentrations, a vitamin K dependent protein, were measured to assess compliance. Linear regression analyses were performed with either TBR or CT-calcification at follow up as dependent variable, and treatment and baseline TBR or CT-calcification as independent variables. We randomized 35 patients to the menaquinone-7 group (33 completed follow up) and 33 to the placebo group (27 completed follow up). After 6 months intervention, TBR tended to increase in the menaquinone-7 group compared to placebo (0.25 (95%-CI: -0.02;0.51), p=0.06). Log-transformed CT-calcification mass, did not increase in the intervention group compared to placebo (0.50 (95%-CI: -0.23; 1.36), p=0.18). Menaquinone-7 supplementation reduced inactive MGP compared to placebo with -205.6 pmol/l (95%-CI: -255.8; -155.3) (p<0.01). No adverse events were reported.

Conclusion: Menaquinone-7 supplementation tended to increase vascular calcification measured with ¹⁸F-NaF PET activity compared to placebo. No effect was found on conventional CT, although the effect was directionally concordant. Therefore, this study does not support that menaquinone-7 supplementation inhibits vascular calcification.

Background

Vascular calcification is associated with a three- to four-fold increased risk of cardiovascular events^{1,2}. In the past, vascular calcification was seen as a passive process of calcium deposition, but recent evidence has shown that vascular calcification is an active process regulated by stimulators and inhibitors³. Matrix Gla Protein (MGP), a vitamin K dependent protein, functions as an inhibitor of vascular calcification⁴. This function of MGP was first shown in MGP knock-out mice, which resulted in initiation and progression of vascular calcification⁵. Multiple observational studies since showed that low levels of inactive MGP, dephosphorylated-uncarboxylated MGP (dp-ucMGP), are associated with less vascular calcification and reduced cardiovascular disease risk⁶. Vitamin K is suggested to reduce vascular calcification and risk of CVD by carboxylating MGP.

Vitamin K is fat soluble, and occurs in two different forms, phyloquinone (vitamin K₁) and menaquinones (vitamin K₂). Phyloquinone is mainly derived from green leafy vegetables, while menaquinones mainly occur in fermented animal products like cheese and meat⁷. Phyloquinone and menaquinones have the same chemical structure, but differ in the length and saturation degree of the side chain⁸. Menaquinones have a longer half-life time and a higher bioavailability, and may therefore be more beneficial for cardiovascular health than phyloquinone⁸.

Multiple observational studies showed a reduced CVD risk with high vitamin K intake⁶. Thus far, two studies investigated the effect of phyloquinone supplementation on vascular calcification and showed reduced progression in older men and women⁹ and chronic kidney disease patients¹⁰. The effect of menaquinones on vascular calcification has not been investigated to date, although interventions with menaquinones showed reduced vascular stiffness^{11,12}. Furthermore, none of the trials investigated the effect of vitamin K and vascular calcifications in people with type 2 diabetes, while vascular calcification is prevalent in about 70% of people with type 2 diabetes¹. Finally, vascular calcification is an active process, but none of the studies measured the active vascular calcification. ¹⁸Fluoride sodium positron emission tomography (¹⁸F-NaF PET) activity is a promising technique to detect early changes in active vascular calcification before calcifications become CT visible. Therefore, this double-blind randomized placebo controlled trial aims to investigate the effect of 6 months menaquinone-7 (MK-7) supplementation on active vascular calcification, measured with ¹⁸F-NaF PET activity, in people with type 2 diabetes and a history of CVD.

Methods

Study design and study population

This study is a double-blind, randomized placebo controlled trial. Participants were recruited through a pre-existing diabetes cohort study (Utrecht participants only)¹³, a Julius Center database of subjects who are interested in participating in studies, and via out-patients clinics of the University Medical Center Utrecht and Diaconessenhuis Utrecht. Participants complied with the following criteria: men and women above 40 years, with diagnosed type 2 diabetes and pre-existing CVD, since vascular calcification is highly prevalent in this patient group, and an estimated glomerular filtration rate (eGFR) above 30, because renal dysfunction could theoretically result in higher ¹⁸F-NaF uptake. Exclusion criteria were vitamin K antagonist use, use of (multi)vitamins with vitamin K and/or unwillingness to stop before randomization and known coagulation problems such as deep vein thrombosis. All participants gave written informed consent prior to participation. This trial was approved by the institutional review board of the University Medical Center Utrecht and registered in clinical trial registries (NCT02839044 / NTR5287).

Intervention

Participants were randomized to 360 µg MK-7 daily or a placebo supplement during 6 months. Participants received daily two tablets of MK-7 (Nattopharma, Oslo, Norway) or placebo (manufactured by Legosan, Kumla, Sweden), which were similar in taste and appearance. Participants were asked to take the tablets with their evening meal. At the start and at the end of the study, MK-7 and placebo tablets were measured for MK-7 content to assess the stability of the tablets (Maastricht University, The Netherlands). During the whole study, the MK-7 content was stable. Participants returned their leftover tablets and we calculated the compliance as number of tablets actually taken divided by the number of tablets that should have been taken. Dp-ucMGP serves as a marker for vitamin K status, where low dp-ucMGP levels represents long term high vitamin K intake¹⁴. Therefore, dp-ucMGP was measured at baseline, three months and six months, to assess whether participants were compliant to the treatment. Dp-ucMGP was measured with the sandwich ELISA method using the IDS Automated Analyser IDS-iSYS InaKtif MGP assay (Maastricht University, The Netherlands).

Participants were randomized in a 1:1 ratio, stratified for sex, performed by the data management department through a computerized method. A list of numbers was

created and the researcher only received a medication number. During the study, the randomization code was kept at the data management and pharmacy departments.

Study endpoints

Active vascular calcification is measured as femoral arterial wall ^{18}F -NaF PET activity. ^{18}F -NaF PET activity is promising to detect early changes in active deposition of vascular calcification before calcifications become CT visible, due to the binding of the tracer to hydroxyapatite, thereby enabling to detect micro-calcifications. The type of calcification which mostly resembles bone formation is medial calcification. Since medial calcification is prevalent in the lower extremities, we choose the femoral artery as our outcome measurement¹⁵. ^{18}F -NaF PET CT scans were conducted at baseline and after 6 months on a Siemens Biograph 40 scanner (Siemens Healthcare, Erlangen, Germany). Ninety minutes before imaging participants received an intravenous injection of 2.0 MBq/kg ^{18}F -NaF, with a maximum dosage of 200 MBq. The primary outcome was the target-to-background ratio (TBR) measured in the femoral artery.

The maximal standardized uptake value (SUVmax) was measured in the left and right femoral artery, from the bifurcation of the femoral artery to the femur condyles (IntelliSpace Portal v8.0, Philips Healthcare, Best, the Netherlands). Slice thickness was 5 mm with a slice interval of 4 mm. The mean of the SUVmax of the left and right femoral artery was calculated (mean number of 47 measurements per patient). Secondly, the blood-pool was determined in the superior vena cava on consecutive slices starting at the aortic arch. Three measurements of the mean standardized uptake value (SUVmean) were performed and an average was used. The femoral TBR was calculated by the SUVmax in the femoral artery divided by the SUV mean in the vena cava. Vascular calcification on conventional CT images was measured as a secondary outcome. Calcification in both left and right femoral artery was measured using in-house developed software (iX Viewer, Image Sciences Institute, Utrecht, The Netherlands), using a threshold of 130 Hounsfield units for calcium. Calcification mass score was computed as the product of the volume of the lesion (in ml) and the mean attenuation (in HU) of the lesion¹⁶. Similar to the PET/CT images, we measured calcification from the bifurcation of the femoral artery to the femur condyles. Both TBR and arterial calcification measurements were performed by one investigator (RvA), blinded for treatment and patient characteristics. Three investigators, blinded for all characteristics, assessed 10 randomly selected participants (5 baseline and 5 follow up measurements) to calculate the inter-observer reliability for TBR measurements and 20 randomly selected participants for calcification mass (10 baseline and 10 follow

up measurements). The intra class correlation for the inter-observer reliability was 0.98 (95%-CI: 0.94-0.99) for TBR measurements and 0.998 (95%-CI:0.995-0.999) for calcification mass using conventional CT imaging.

Other measurements

Participants visited the University Medical Center Utrecht at baseline, after three, and after six months. During these visits, lifestyle questionnaires were obtained, including disease history, medication use and smoking habits. During each visit, anthropometric measurements including height, weight, waist- and hip circumference, were performed twice and a mean value was calculated. Blood pressure was measured twice, in sitting position using an automated oscillomat (Omron HEM-907). Non-fasting blood samples were drawn and HbA1c, creatinine and lipid levels were measured. eGFR was calculated using the MDRD formula. Finally, participants were asked to fill out a three-day food record to obtain the dietary vitamin K intake. The food record included three non-consecutive days including two week days and one weekend day, and consisted of a pre-specified format for breakfast, lunch, dinner and snacks. The food records were analyzed using Evry (Ensemble BV, Zoetermeer, the Netherlands), based on the Dutch national food composition table (2013)¹⁷ and a previously described vitamin K food content database¹⁸. The energy adjusted intakes of total vitamin K, phylloquinone and menaquinones were calculated, using the residual method.

Statistical analyses

The sample size was calculated based on a previous study including ¹⁸F-NaF PET CT, showing a mean TBR of 1.96¹⁹. With a power of 80%, a 2-sided α of 5%, a standard deviation of the difference in TBR between baseline and follow up of 0.41 and 15% drop-out rate, 70 participants were required to detect a 15% difference in TBR. A 15% difference was also found in previous studies investigating the effect of vitamin K on coronary artery calcification^{9,10}.

Baseline characteristics were described as percentages, mean $\pm$ SD or median (interquartile range) as appropriate per treatment arm and clinically relevant baseline differences between treatment arms were visually assessed. Intention-to-treat analyses, including all participants with a complete follow up, were performed to compare the intervention and placebo group. The primary endpoint was TBR and log transformed calcification mass was included as secondary endpoint. Absolute differences between baseline and follow up were calculated. Linear regression analyses were performed with the follow

up calcification measurement as outcome, and treatment and baseline calcification measurement as independent variables (for example: TBR at follow up ~ intervention + TBR at baseline). We used linear regression models, since analyzing change scores does not appropriately control baseline imbalances²⁰. Since baseline CT calcification mass, phylloquinone intake and a low ABI differed between the intervention and placebo group at baseline, we additionally adjusted for these variables in a secondary analysis. Due to the baseline difference in calcification mass between treatment arms and the strong predictive value of baseline calcification mass on progression of calcification mass^{21,22}, we assessed the correlation between baseline and difference between baseline and follow up measurements for calcification mass and TBR using the Spearman's correlation coefficients. Since baseline calcification mass was correlated with change in calcification mass, we excluded participants with no calcification at baseline and with a calcification mass score > 1000 (arbitrary chosen) as post-hoc analyses.

All measurements and analyses were performed blinded to treatment. No missing data occurred for the imaging, the outcome measurement of interest. In the adjusted models, 2 participants had missing information on phylloquinone intake and median phylloquinone intake was imputed. Analyses were performed in R version 3.2.2 and a p-value below 0.05 was considered statistically significant.

Results

A total number of 108 patients were screened for participation in this study, 40 patients were ineligible, mainly because of the absence of CVD, or declined to participate (*Figure 1*). 68 out of the 70 participants were randomized, 35 to the MK-7 group and 33 in the placebo group, and 2 could not be randomized for logistic reasons. Six participants were lost to follow up (MK-7 group: 2, placebo group: 6). Reasons for loss to follow up were unrelated to the intervention and mainly due to discomfort of the PET/CT scan.

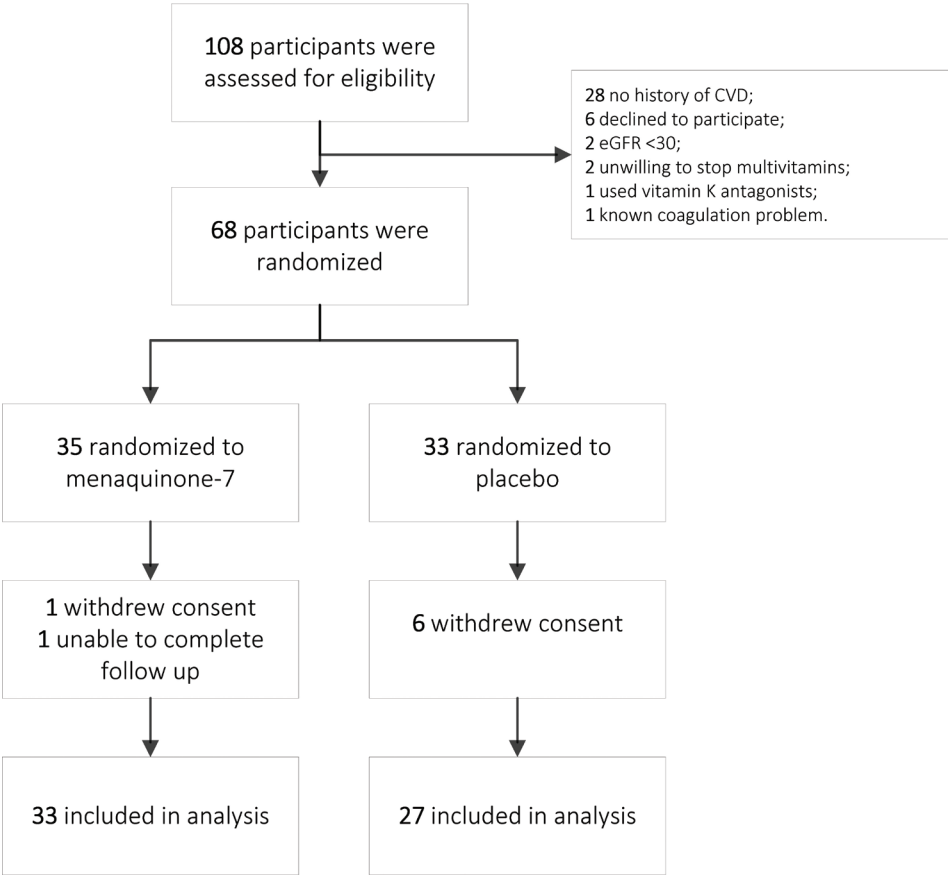


Figure 1. Flow chart of study participation

Baseline characteristics are described in *Table 1*. The study included 16 females (24%) and the mean age was 69.1 (SD: 8.4) years. Participants in the MK-7 group had higher baseline calcification levels, higher phylloquinone intake and more often a low ABI compared to the placebo group. Baseline characteristics of participants with a complete follow up were comparable to those randomized (*Supplementary Table 1*).

Table 1. Baseline characteristics of all participants who are randomized.

	Vitamin K (n=35)	Placebo (n=33)
Age _(years)	69.1 ± 8.4	69.1 ± 8.4
Females	9 (25.7)	7 (21.2)
BMI _(kg/m²)	31.1 ± 5.6	31.1 ± 5.0
Systolic BP _(mmHg)	136.0 ± 20.5	137.8 ± 14.3
Diastolic BP _(mmHg)	69.8 ± 11.4	73.5 ± 9.5
Current smoker	6 (17.1)	4 (12.1)
Higher educated	15 (42.9)	13 (39.4)
Vitamin D supplements	23 (65.7)	24 (72.7)
ABI ≤ 0.9	16 (45.7)	9 (27.3)
<i>Laboratory measurements</i>		
HbA1c _(mmol/mol)	57.1 ± 14.8	59.6 ± 17.1
eGFR _(ml/min/1.73m²)	79.2 ± 26.2	86.6 ± 26.1
Total cholesterol _(mmol/L)	4.5 ± 1.3	4.2 ± 1.2
HDL cholesterol _(mmol/L)	1.1 ± 0.3	1.1 ± 0.3
LDL cholesterol _(mmol/L)	2.1 ± 0.9	2.0 ± 0.9
Triglycerides _(mmol/L)	2.8 (1.8-3.4)	1.9 (1.5-2.7)
Dp-ucMGP _(pmol/L)	613 (512.9-684.2)	614.8 (489.4-743.0)
<i>Energy adjusted vitamin K intake</i>		
Total vitamin K _(mg)	166.7 (137.8-288.2)	141.1 (116.8-192.8)
Phylloquinone _(mg)	123.8 (99.8-225.4)	94.1 (73.1-149.1)
Menaquinones _(mg)	48.8 (34.6-57.0)	47.6 (33.0-52.2)
<i>Calcification measurements</i>		
TBR	2.2 ± 0.7	2.1 ± 0.6
CT calcification mass	196.0 (32.5-424.0)	44.9 (9.6-409.5)

Values are mean ± SD, median (IQR) or n (%).

BMI: Body Mass Index, HbA1c: Hemoglobin A1c, eGFR: estimated Glomerular Filtration Rate, HDL: high density lipoprotein, LDL: low density lipoprotein, dp-ucMGP: dephosphorylated-uncarboxylated Matrix Gla Protein, CT: Computed Tomography, TBR: Target to Background Ratio

The absolute differences between baseline and 6 months intervention are presented in Figure 2. After 6 months intervention, TBR tended to increase with 0.25 in the MK-7 group (95%-CI: -0.02;0.51; p=0.06) compared with placebo. Log-transformed calcification mass slightly increased in the MK-7 group compared to placebo (0.50; 95%-CI: -0.24;1.23; p=0.18), although this result was not statistically significant (Table 2). Adjustment for baseline characteristics (baseline calcification mass, phylloquinone intake and low ABI) did not alter these results (Table 2).

TBR and calcification mass were modestly correlated at baseline ($r=0.47$; 95%-CI: 0.27-0.64). Furthermore, baseline calcification mass was modestly correlated with change in calcification mass between baseline and 6 months ($r=0.53$; 95%-CI: 0.32-0.69), while TBR at baseline was not correlated with change in TBR levels during follow up ($r=-0.01$; 95%-CI: -0.35-0.15). Finally, change in TBR was not correlated with change in calcification mass ($r=0.14$; 95%-CI: -0.12-0.38). Based on the correlation between calcification at baseline and change in calcification mass, we excluded participants with no calcification at baseline ($n=3$), which resulted in a similar effect of MK-7 compared to placebo on TBR during 6 months intervention (*Table 2*), whereas calcification mass significantly increased (0.22; 95%-CI: 0.04-0.41; $p=0.02$). When excluding participants with a high calcification mass at baseline ($n=3$) the results were similar to those in the full study population for both TBR and calcification mass.

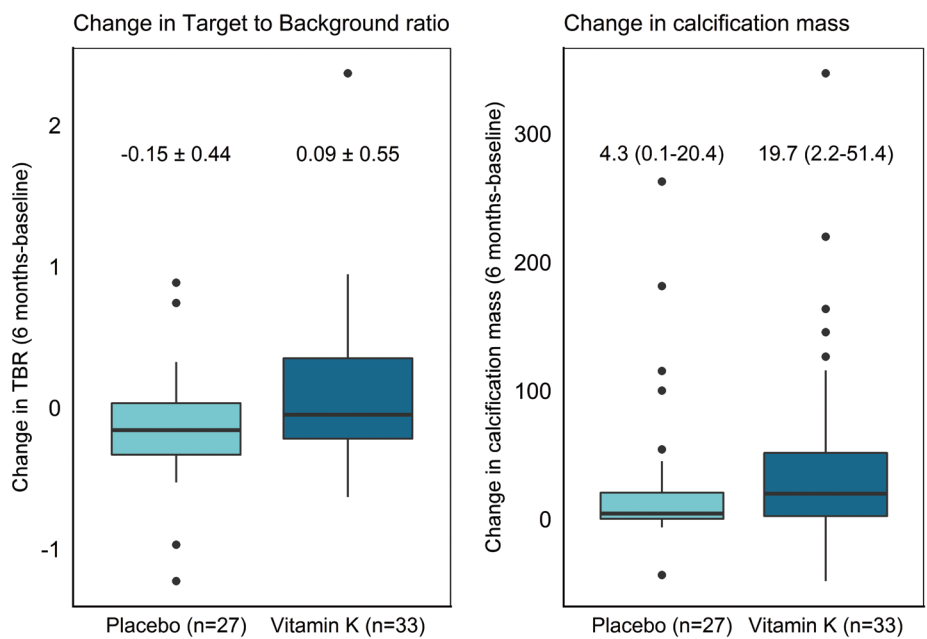


Figure 2. Absolute changes in TBR and calcification mass (CT) in the placebo and vitamin K group between baseline and 6 months intervention.

Table 2. Result of linear regression analyses and sensitivity analyses excluding the participants without baseline calcification and with high baseline calcification levels.

	Linear regression	P-value
TBR		
Model 1	0.25 (-0.02; 0.51)	0.06
Model 2	0.31 (0.02; 0.60)	0.03
<i>Sensitivity analyses 1</i>		
Model 1	0.24 (-0.03; 0.52)	0.08
Model 2	0.32 (0.02; 0.62)	0.04
<i>Sensitivity analyses 2</i>		
Model 1	0.24 (-0.04; 0.52)	0.09
Model 2	0.34 (0.04; 0.64)	0.03
Calcification mass*		
Model 1	0.50 (-0.24; 1.23)	0.18
Model 2	0.40 (-0.36; 1.16)	0.30
<i>Sensitivity analyses 1</i>		
Model 1	0.22 (0.04; 0.41)	0.02
Model 2	0.27 (0.08; 0.47)	<0.01
<i>Sensitivity analyses 2</i>		
Model 1	0.42 (-0.32; 1.16)	0.26
Model 2	0.41 (-0.37; 1.19)	0.29

Model 1: Linear regression models included outcome measurement as dependent variable and intervention and baseline measures were used as independent values.

Model 2: Additionally adjusted for calcification mass, phyloquinone intake and ankle brachial index.

Sensitivity analyses 1: exclusion of 3 participants with no baseline calcification mass (CT), n=57

Sensitivity analyses 2: exclusion of 6 participants with baseline calcification mass score above 1000 (CT), n=54

* Log transformed values. TBR: target to background ratio

MK-7 supplementation significantly reduced inactive MGP concentrations already after 3 months intervention compared to placebo (-205.6 pmol/L; 95%-CI: -255.8;-155.3; $p<0.01$) (Figure 3). This effect of MK-7 compared to placebo was sustained after 6 months (-202.7; 95%-CI: -272.5;-132.8; $p<0.01$) pmol/L, indicating high compliance. According to the pill-count, the compliance was also high; 97.4% (95%-CI: 92.3%-99.1%) in the intervention group and 97.8% (95%-CI: 94.2%-99.7%) in the placebo group. No serious adverse events were reported.

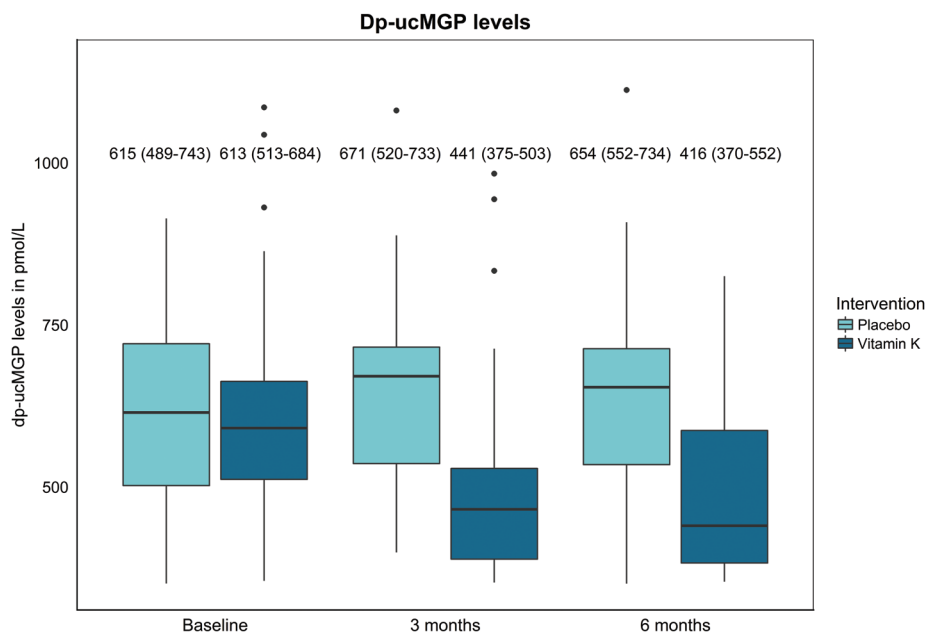


Figure 3. Dp-ucMGP levels in the placebo and vitamin K group at baseline, 3 months and 6 months intervention, including median and interquartile ranges.

Discussion

In contrast to our hypothesis and despite excellent compliance, vascular calcification on ^{18}F -NaF PET scan, albeit not statistically significant, tended to increase after MK-7 supplementation compared with placebo during 6 months intervention. No effect of MK-7 supplementation on CT calcification mass was found, but changes in calcification mass were directionally concordant with changes in ^{18}F -NaF PET activity.

Strengths of our study are its randomized, double-blind, placebo-controlled design with high compliance and inter-observer reliability for assessment of imaging. Secondly, this is the first study investigating the effect of MK-7 supplementation in patients with type 2 diabetes. Finally, an innovative measurement of vascular calcification was used as primary endpoint, allowing sensitive detection of changes in vascular calcification. However, some limitations need to be addressed. The drop-out rate was relatively high, especially in the placebo group, and although the reasons for drop-out were unrelated to the treatment, it reduced the power to detect effects of vitamin K supplementation. Since the baseline

characteristics of the subjects who completed follow up are comparable to all randomized participants, this selection bias did probably not influence our results to a large extent.

This is the first study assessing the effect of MK-7 on active vascular calcification. Our results are inconsistent with the majority of previous studies investigating the effect of phylloquinone supplementation on vascular calcification using CT imaging, showing a protective effect of vitamin K supplementation in older men and women and participants with chronic kidney disease^{9,10}. So far, the effect of MK-7 was only studied in association with arterial stiffness, which showed a beneficial effect of MK-7 supplementation^{11,12}. Only one study did not detect an effect of MK-7 supplementation on arterial stiffness and physical function during six months follow up, which was probably due to the low-dose MK-7 supplementation (100µg) and relative short follow-up period to detect changes in arterial stiffness²³. The MK-7 dosage in our study was much higher compared to this previous trial. The follow-up period in our study was similar to the previous study, but ¹⁸F-NaF PET scan is designed to detect early changes in calcification; therefore, it is not likely that the follow-up period explains our unexpected result.

Based on these previous studies, we hypothesized that MK-7 supplementation would decrease vascular calcification, resulting in lower ¹⁸F-NaF PET activity. Contrary to our hypothesis, we found increased ¹⁸F-NaF activity compared to the placebo group, although these results just lacked statistical significance. One reason for this unexpected result might be the difference in calcification mass at baseline, which was higher in the MK-7 than placebo group, despite randomization. Previous studies on determinants of coronary artery calcification (CAC) progression showed that a high baseline CAC burden was the strongest predictor of CAC progression^{21,22}. In line with this, in our study a high baseline calcification mass was correlated with an increase in calcification mass during follow up. Since calcification mass was higher in the MK-7 group at baseline, this may explain the higher progression of vascular calcification in this group compared to the placebo group. However, excluding participants with high baseline calcification mass only slightly attenuated the effect of MK-7 supplementation on change in calcification mass compared to placebo, and did not alter our results for TBR. Therefore, the difference in baseline calcification mass could only partly explain our results.

Another explanation for these results may concern the interpretation of ¹⁸F-NaF PET activity as marker of vascular calcification. ¹⁸F-NaF PET activity is promising to detect early changes in active deposition of vascular calcification before calcifications become

CT visible, due to the binding of the tracer to hydroxyapatite, thereby enabling to detect micro-calcifications²⁴. Our study is the second trial using this outcome. One other trial investigating the effect of etidronate on vascular calcification in patients with pseudoxanthoma elasticum, found no effect on ¹⁸F-NaF PET activity in the femoral artery, while the CT images showed a significant reduction of vascular calcification in the intervention group²⁵. This challenges our understanding of what ¹⁸F-NaF PET activity in femoral arteries actually represents. In addition, some areas of macro-calcification showed no ¹⁸F-NaF uptake, while other areas with micro-calcification showed high ¹⁸F-NaF uptake²⁶. Macro-calcifications have a larger volume but a small surface area, while micro-calcifications have a large surface area. Since ¹⁸F-NaF only binds to the outer surface, our results could indicate that MK-7 supplementation shifts macro- to micro calcification, in which the ¹⁸F-NaF uptake increases as seen in our results²⁶. In our study, changes in ¹⁸F-NaF PET activity were directionally concordant with changes in calcification mass; therefore, this does not fully explain our results. Currently, multiple studies are investigating whether ¹⁸F-NaF PET activity can identify atherosclerotic plaques (for example NCT02607748), which are essential for the interpretation of our study results. High-resolution imaging may shed more light on this in future studies with MK-7. Finally, our hypothesis could be incorrect in the role of vitamin K in vascular calcification in people with type 2 diabetes. Ongoing clinical trials of the effect of menaquinone-7 on vascular calcification are awaited.

In conclusion, in contrast to our hypothesis, vitamin K supplementation did not result in lower ¹⁸F-NaF activity compared to placebo. Although no effect of MK-7 supplementation on calcification mass was found using conventional CT imaging, changes of calcification mass were directionally concordant with changes in ¹⁸F-NaF PET activity. Therefore, this study does not support the use of vitamin K supplementation to reduce progression of vascular calcification. Results of currently ongoing trials with vitamin K supplementation and further research investigating the interpretation of ¹⁸F-NaF PET activity coupled with higher resolution imaging are needed to fully understand the effects of vitamin K on vascular calcification.

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Disclosures

None.

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Supplementary information

Supplementary Table 1. Baseline characteristics of all participants with complete follow up stratified by treatment.

	Vitamin K (n=33)	Placebo (n=27)
Age (years)	69.8 ± 8.1	69.2 ± 7.2
Females	7(21.2)	7 (25.9)
BMI (kg/m ²)	31.0 ± 5.7	31.4 ± 5.2
Systolic BP (mmHg)	136.4 ± 20.6	136.5 ± 14.7
Diastolic BP (mmHg)	69.2 ± 11.5	73.3 ± 9.6
Current smoker	4 (12.1)	3 (11.1)
Higher educated	14 (42.5)	11 (40.7)
Vitamin D supplements	21 (63.6)	20 (74.1)
ABI ≤ 0.9	15 (45.5)	6 (22.2)
<i>Laboratory measurements</i>		
HbA1c (mmol/mol)	57.1 ± 14.8	59.6 ± 17.1
eGFR (ml/min/1.73m ²)	80.7 ± 26.2	87.0 ± 26.4
Total cholesterol (mmol/L)	4.5 ± 1.3	4.2 ± 1.2
HDL cholesterol (mmol/L)	1.1 ± 0.3	1.1 ± 0.3
LDL cholesterol (mmol/L)	2.1 ± 0.9	2.0 ± 0.9
Triglycerides (mmol/L)	2.8 (1.8-3.4)	1.9 (1.5-3.2)
Dp-ucMGP (pmol/L)	607.6 (512.3-672.8)	632.7 (529.9-768.1)
<i>Energy adjusted vitamin K intake</i>		
Total vitamin K (mg)	171.0 (139.8-293.3)	133.7 (114.8-183.2)
Phylloquinone (mg)	126.2 (101.2-231.9)	81.7 (67.4-130.5)
Menaquinones (mg)	50.6 (36.5-57.2)	43.7 (32.9-54.9)
<i>Calcification measurements</i>		
TBR	2.2 ± 0.7	2.1 ± 0.6
CT calcification mass	215.9 (37.6-447.3)	44.9 (10.1-442.5)

Values are mean ± SD, median (IQR) or n (%).

BMI: Body Mass Index, HbA1c: Hemoglobin A1c, eGFR: estimated Glomerular Filtration Rate, HDL: high density lipoprotein, LDL: low density lipoprotein, dp-ucMGP: dephosphorylated-uncarboxylated Matrix Gla Protein, CT: Computed Tomography, TBR: Target to Background Ratio



Intimal and medial calcification in relation to cardiovascular risk factors

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Abstract

Background: Arterial calcification is associated with an increased cardiovascular risk. Intimal arterial calcification (IAC) and medial arterial calcification (MAC) may represent different pathophysiological mechanisms driven by separate risk factors.

Objective: We aimed to assess specific risk factors associated with IAC and MAC.

Methods: We conducted a cross-sectional study in patients with or at risk of vascular disease from the Second Manifestations of ARterial Disease study (n=520) and from the Hoorn Diabetes Care System cohort (n=198). Non-contrast CT scanning of the lower extremities was performed and calcification in the femoral and crural arteries was scored as absent, predominant IAC, predominant MAC or indistinguishable. Multinomial regression models were used to assess the associations between cardiovascular risk factors and calcification patterns. Biomarkers for inflammation, calcification and vitamin K status were measured in a subset of patients with IAC (n=151) and MAC (n=151).

Results: Femoral calcification was found in 77% of the participants, of whom 38% had predominant IAC, 28% had predominant MAC and 11% were scored as indistinguishable. The absolute agreement between the femoral and crural arteries was high (69%). Higher age, male sex, statin use and history of coronary artery disease were associated with higher prevalences of femoral IAC and MAC compared to absence of calcification. Smoking and low ankle-brachial index (ABI) were associated with higher prevalence of predominant IAC and high ABI was associated with less IAC. Compared to patients with IAC, patients with MAC more often have diabetes and high ABI and are less often smokers. Inactive Matrix Gla Protein may be associated with increased MAC prevalence, while osteonectin was associated with decreased risk of MAC compared to IAC.

Conclusions: When femoral calcification is present, the majority of the patients have a predominant arterial calcification type throughout the lower extremity. These calcification patterns, predominant IAC or predominant MAC, have different associated risk factor profiles.

Background

Arterial calcification is associated with increased cardiovascular risks¹. Intimal arterial calcification (IAC), considered a representation of underlying atherosclerotic plaque burden, has long been held responsible for this association, whereas other types of arterial calcification were often considered innocent². In recent years, increasing interest is directed towards medial arterial calcification (MAC) and other forms of non-atherosclerotic calcification, such as calcification of the internal elastic lamina².

Based on radiology-pathology correlation studies, IAC is thought to have a spotty/patchy look on radiographs, whereas MAC and internal elastic lamina calcification tend to outline the artery with a smooth layer of calcium, resulting in linear or annular calcifications on radiographs^{3,4}. These calcification patterns may represent different pathophysiological mechanisms driven by separate risk factors which may subsequently lead to different forms of CVD. While IAC is characterized by inflammation and lipid deposition in plaques, MAC is suggested to reflect a process of active calcification resembling bone formation⁵⁻⁸. Therefore, the distinction might be valuable in order to develop prevention and treatment strategies for both types of calcification.

Several studies have related MAC to risk factors and outcomes⁶⁻¹¹. These studies found that advanced age⁷⁻¹⁰, diabetes^{6,8-10}, and kidney disease⁶ are risk factors associated with MAC, and despite some heterogeneity, most studies report no relationships with hypertension or dyslipidaemia⁶⁻⁹. Studies on IAC, such as predominant in the coronary arteries, report positive associations with traditional cardiovascular risk factors, including smoking and dyslipidaemia^{6,8,12}. However, the available information is limited; as most of these studies were performed in selected groups and only two studies assessed both MAC and IAC^{6,8}.

In this study we aim to assess the prevalence of predominant IAC and predominant MAC, measured in the lower extremity arteries using computed tomography (CT), and the association with cardiovascular risk factor profiles. Secondly, we aim to identify whether markers for calcification, inflammation and vitamin K status are potential biomarkers for MAC compared to IAC.

Methods

Study population

This study included patients of two ongoing cohort studies, the Second Manifestations of ARterial disease (SMART) study and the Diabetes Care System (DCS) cohort. This sub-study was approved by the medical ethics review board at the University Medical Center Utrecht (14/444), and all participants gave their written informed consent. Only patients with bilateral lower limb amputations were excluded.

The SMART study is an ongoing prospective cohort study comprising patients with cardiovascular disease (CVD) and patients at high risk for CVD due to hypertension, diabetes or other cardiovascular risk factors. Details of the study have been described elsewhere¹³. Patients that were newly referred to the University Medical Center Utrecht with either manifest vascular disease or important cardiovascular risk factors were asked to participate. Newly referred patients, from March 2015 till December 2017, were invited for an additional unenhanced thin-slice CT scan of the legs, of which 548 participants agreed to participate and 520 participants signed the informed consent form and underwent the CT scan.

Participants with type 2 diabetes from all 103 general practitioners (GPs) in the West-Friesland region in the Netherlands are included in the Hoorn Diabetes Care System (DCS) cohort. All participants are monitored annually and the clinical measures, obtained during the standardized examinations, are used for clinical research. Participants were approached individually for specific research projects. Details of this study have been described previously¹⁴. Participants with an annual visit between June 2017 and February 2018 were invited for an additional CT scan of the legs. In total, 200 participants agreed to participate of whom 198 participants signed the informed consent and underwent the CT scan.

Risk factor measurement

In SMART, all patients underwent a comprehensive screening at baseline, including questionnaires on medical history and medication use. In DCS, this information was extracted from the medical records, which were obtained during the annual visits. Medical history was categorized as coronary artery disease (CAD), cerebrovascular disease (CVA), abdominal aortic aneurysm (AAA) and peripheral arterial disease (PAD) which is diagnosed as Fontaine stage II-IV. In both cohorts, smoking was self-reported and classified as never,

former and current smoker. Physical examinations were performed including weight, height and blood pressure measurement (in seated position). Fasting blood samples were drawn for measurement of blood lipids, HbA1c, C-reactive protein (CRP) and creatinine levels. Glomerular filtration rate (eGFR) was estimated using the CKD-EPI formula¹⁵. Hypertension was defined as a systolic blood pressure (SBP) of ≥ 140 mmHg, a diastolic blood pressure (DBP) ≥ 90 mmHg and/or use of antihypertensive medication. The SMART definition of hyperlipidemia was used and defined as total cholesterol ≥ 5 mmol/L, LDL-cholesterol ≥ 3.2 mmol/L and/or use of lipid-lowering medication.

SMART participants were diagnosed with diabetes mellitus when there was a referral diagnosis of diabetes mellitus, self-reported diabetes mellitus, the use of glucose-lowering agents or a baseline fasting plasma glucose ≥ 7 mmol/L and a definitive diagnosis of diabetes during the first year of follow-up. In the DCS cohort, type 2 diabetes mellitus was reported when a patient had at least one or more classic symptoms and fasting plasma glucose ≥ 7.0 mmol/L or random plasma glucose ≥ 11.1 mmol/L or in the absence of symptoms, two elevated fasting plasma glucose concentration on two different time points when no symptoms are present.

Ankle brachial index (ABI) measurements were conducted by experienced professionals using a Vasoguard dopplerprobe (8MHz). For each ankle, the highest SBP of the posterior tibial and dorsalis pedis (both measured twice) was used for the ABI calculations. An average SBP was calculated for each arm from at least 2 measurements of the brachial artery. The arm with the highest average was used for the ABI calculations. The leg-specific ABIs were thus calculated by dividing the highest average arm SBP by the highest of the ankle pressures of that leg. Finally, ABI was classified as low (≤ 0.9) or high (≥ 1.3)¹⁶.

Biomarkers

Biochemical measurements were performed in a random subgroup of patients with predominantly IAC (n=151) and MAC (n=151). We have selected all subjects with IAC in both the femoral and crural arteries. Of the subjects with MAC, only 120 participants had predominant MAC in both the femoral and crural arteries. Therefore, we added participants with femoral MAC, but absent (n=11) or indistinguishable calcification (n=20) in the crural arteries to this selection. Inflammatory biomarkers (IL-6, IL-8 and TNF- α) were measured in serum with commercially available 4-plex sandwich immunoassay kits (Meso Scale Discovery (MSD), Rockville, MD, US). Calcification markers (osteocalcin, osteonectin and osteopontin) were measured in serum using a multiplex Human Bone Marker Panel

II Kit of Meso Scale Discovery (MSD, Rockville MD, USA). The inter-assay and intra-assay variation coefficients were reasonable: 13.4% and 14.6% for IL-6, 14.1% and 8.6% for IL-8 and 9.8% and 9.0% for TNF- α . For osteocalcin, osteonectin and osteopontin the inter- and intra assay variants were good at 2.7%, 7.1%, 8.3%, 13.6%, 5.7% and 2.9% respectively.

Additionally, markers for vitamin K status were measured. Circulating dp-ucMGP levels were determined in EDTA plasma using the commercially available IVD CE marked chemiluminescent InaKif MGP assay on the IDS-iSYS system (IDS, Boldon, UK). 50 μ L of patient sample or calibrators are incubated with magnetic particles coated with murine monoclonal dpMGP antibody, an acridinium labelled murine monoclonal ucMGP antibody and assay buffer. The magnetic particles are captured using a magnet and a wash step performed to remove any unbound analyte. Trigger reagents are added, the resulting light emitted by the acridinium label is directly proportional to the concentration of dp-ucMGP in the sample. The within-run and total precision of this assay were 0.8 – 6.2% and 3.0 – 8.2%, respectively. The assay measuring range is between 300 – 12,000 pmol/L and was found to be linear up to 11,651 pmol/L. Dp-ucMGP values below 300 pmol/L are considered to be in the normal healthy range. Circulating PIVKA-II (ucFII), or protein induced by vitamin K absence/antagonism II, levels were measured using a conformation-specific monoclonal antibody in an ELISA-based assay¹⁷. Results are expressed as arbitrary units per liter (AU/l) because in states of vitamin K deficiency circulating ucFII may comprise multiple forms of partially carboxylated FII and neither their relative abundance in serum nor their relative affinity for the antibody is known. Using electrophoretic techniques 1 AU is equivalent to 1 μ g of purified ucFII. The detection limit was 0.25 AU/ml ucFII in serum.

Calcification

All participants underwent unenhanced CT-scans, scanning the legs from the femoral head to the feet. Slice thickness was 1 mm with a 0.7 mm increment. A semi quantitative CT score based on annularity, thickness and continuity of the calcifications was used according to a previously developed algorithm¹⁸. The score combined circularity (absent, dot(s), <90°, 90-270° or 270-360°), thickness (absent, $\geq$ 1.5 mm or <1.5 mm), and morphology (indistinguishable, irregular/patchy or continuous), where high degree of circularity, thin and continuous calcifications had a high likelihood of being predominant medial calcification, while low degree of circularity, thick and patchy calcifications had a high likelihood of being predominant IAC. When there was no calcification noticeable, the calcification was scored as “Absent”. When morphology was judged as indistinguishable (mainly in the case of scarce dots of calcification), the calcification was scored as “Indistinguishable”.

In all other cases, points assigned in the scoring system (*Supplementary Table 1*) were summed. Less than 7 points was defined as “Dominant IAC” and 7 or more points was defined as “Dominant MAC”¹⁹. A histogram of this score is shown in *Supplementary Figure 1*. Femoral and crural arteries (anterior tibial artery and posterior tibial artery) were scored separately. All CT-scans were scored by an expert in reading CT-scans (PdJ), blinded for patient’s characteristics.

The algorithm was developed and validated in the intracranial internal carotid artery, showing a reasonable validity. The inter-rater reliability and intra-rater reliability for the algorithm was 0.72 (0.60-0.84) and 0.82 (0.73-0.89), respectively¹⁸. Recently, the calcification score was validated using histological samples of the crural arteries²⁰. 70% of the arteries were correctly classified as no calcification, predominant IAC or predominant MAC, resulting in a reasonable absolute agreement of 0.47 for IAC and 0.42 for MAC²⁰.

Statistical analyses

Baseline characteristics were presented as means with standard deviations (SD) or medians with interquartile ranges (IQR) (in case of non-normally distributed variables) for continuous variables or percentages with number of cases for categorical variables, for SMART and DCS separately. Baseline variables were complete for 95% of the study population. Additionally, baseline characteristics were calculated according to the categories of femoral and crural calcification. Absolute level of agreement between the femoral and crural scores were calculated using a formula described by De Vet *et al*²¹. This formula was used since we wanted to estimate the absolute level of agreement rather than the relative measure, like the Cohen’s Kappa.

Risk factors for the different types of calcification were assessed using multinomial logistic regression analyses, adjusted for age and sex and analyzed in strata of cohort. Presence of diabetes and pack years was assessed in the SMART cohort only. We assessed the risk of predominant IAC, predominant MAC and indistinguishable calcification with no calcification as reference category. To assess whether risk factors for predominant MAC differed from predominant IAC, we additionally performed logistic regression analyses using MAC as outcome measurement with IAC as reference category, adjusted for age and sex and analyzed in strata of cohort.

Furthermore, we calculated means with standard deviations or medians and interquartile ranges for the biomarkers for the group with predominant IAC and MAC. Logistic regression

analyses for the association between the biomarkers and MAC compared to IAC were performed adjusted for age and sex and analyzed in strata of cohort.

All statistical analyses were performed using R, version 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria). Add-on package 'nnet' was used for multinomial regression analyses. A p-value of lower than 0.05 is considered statistically significant.

Results

The SMART cohort consisted of 81% males, with a mean age of 59.7 ± 10.3 years, while 66% was male in the DCS cohort with a mean age of 67.9 ± 9.1 years (*Table 1*). Baseline characteristics according to the calcification categorization are presented in *Supplementary Table 2* (by femoral score) and *Supplementary Table 3* (by crural score).

In the SMART cohort, 39% had a predominant IAC pattern in the femoral arteries, while 26% of the participants were identified as predominant MAC. In the DCS cohort, 35% had predominant IAC calcification, while 34% had predominant MAC in the femoral artery. In both SMART and DCS, 23% had no calcification. So, when femoral calcification is present, 85% of patients have a predominant arterial calcification type throughout the lower extremity. The prevalence of all types of femoral and crural calcification in SMART and DCS are described in *Figure 1*. We observed good absolute agreement across the crural and femoral arteries, as 69% of the patients with predominant IAC in the femoral arteries, also had predominant IAC in the crural arteries. The agreement was the same for MAC.

Table 1. Baseline characteristics of the SMART (n=520) and DCS cohort (n=198).

	SMART (n=520)	DCS (n=198)
Age _(years)	59.7 ± 10.3	67.9 ± 9.2
Male sex	422 (81%)	130 (66%)
BMI _(kg/m²)	27.3 ± 3.9	30.1 ± 5.1
Diabetes (type 1 or 2)	81 (16%)	198 (100%)
Type 2 diabetes	78 (15%)	198 (100%)
Hypertension	264 (51%)	164 (83%)
Hyperlipidemia	70 (14%)	153 (77%)
Systolic blood pressure _(mmHg)	131 ± 16	138 ± 19
Diastolic blood pressure _(mmHg)	78 ± 10	79 ± 8
Smoking		
Current	113 (22%)	23 (12%)
Former	248 (48%)	115 (58%)
Never	158 (30%)	59 (30%)
High ABI _(>1.3)	92 (18%)	51 (26%)
Low ABI _(<0.9)	36 (7%)	10 (5%)
Statin use	430 (83%)	150 (76%)
Manifest cardiovascular disease		
Cerebrovascular disease	98 (19%)	9 (5%)
Coronary artery disease	384 (74%)	24 (12%)
Aneurysma abdominal aorta	24 (5%)	2 (1%)
Peripheral artery disease	31 (6%)	6 (3%)
eGFR _(ml/min/1.73m²)	89 ± 23	75 ± 27
Triglycerides _(mmol/L)	1.3 (1.0-1.8)	1.6 (1.1-2.1)
Total cholesterol _(mmol/L)	4.4 ± 1.1	4.1 ± 1.0
LDL-cholesterol _(mmol/L)	2.5 ± 0.9	2.1 ± 0.8
HDL-cholesterol _(mmol/L)	1.2 ± 0.3	1.2 ± 0.4
HbA1c _(mmol/mol)	38.3 ± 7.6	54.0 ± 12.3
CRP _(mg/L)	1.6 (0.8-3.4)	1.8 (0.9-4.2)

Baseline characteristics are described as mean ± standard deviation, median (interquartile range) or number of participants (%). BMI: body mass index, bp: blood pressure, ABI: ankle brachial index, eGFR: estimated glomerular filtration rate, LDL: low-density lipoprotein, HDL: high-density lipoprotein, CRP: C-reactive protein.

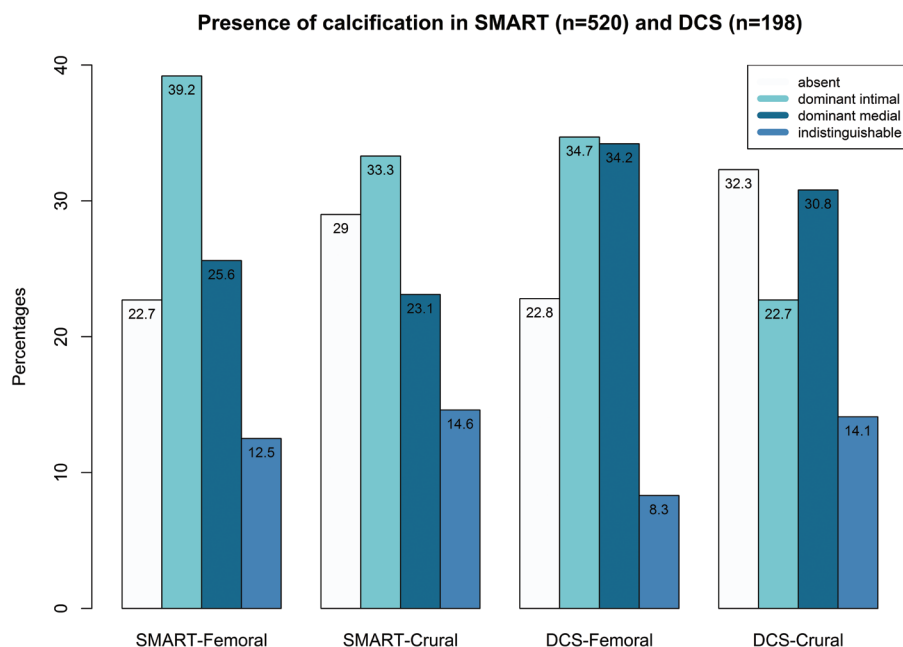


Figure 1. Frequencies of type of calcification in the femoral and crural arteries in the SMART (n=520) and DCS (n=198) cohort.

Risk factors compared to absence of calcification

Risk factors for femoral calcification are presented in *Table 2*. Higher age, male sex, statin use and history of CAD were risk factors for both IAC and MAC compared to absence of calcification. Current smoking was associated with a higher prevalence of predominant IAC (OR 6.47 (3.28;12.79)), but no association was found for MAC. High ABI (≥ 1.3) was associated with less IAC (OR 0.33 (0.18;0.59)), whereas low ABI (≤ 0.9) was associated with higher prevalence of predominant IAC (OR 6.76 (2.04;22.41)). Higher HbA1c (OR 0.72 (0.58;0.90)) and history of CVA (OR 0.43 (0.24;0.79)) was associated with decreased prevalence of IAC.

Comparable results were found for crural calcification (*Supplementary Table 4*). In contrast to the arteria femoralis, higher HDL-cholesterol levels and a history of CVA were not associated with IAC in the crural arteries. However, higher DBP seemed to decrease the prevalence of predominant MAC (OR 0.70 (0.54;0.90) per 10 mmHg increase) in the crural arteries.

Table 2. Risk (OR 95%CI) of predominant intimal, predominant medial or indistinguishable calcification compared to no calcification in the femoral artery.

	Absent (n=162)	Intimal (n=271)	Medial (n=199)	Indistinguishable (n=81)	Media vs. Intimal
Age _(per 10 years)	1	3.02 (2.39;3.82)	3.37 (2.61;4.34)	1.68 (1.28;2.20)	1.03 (0.83;1.29)
Male sex	1	3.71 (2.25;6.12)	4.05 (2.34;7.00)	1.70 (0.94;3.06)	1.16 (0.70;1.93)
BMI _(per kg/m²)	1	0.99 (0.94;1.04)	1.01 (0.96;1.06)	1.01 (0.95;1.07)	1.01 (0.97;1.06)
Diabetes [#] _(type 1 and 2)	1	0.83 (0.40;1.71)	1.59 (0.76;3.33)	0.46 (0.17;1.26)	1.81 (1.03;3.15)
Hypertension _(yes vs no)	1	0.88 (0.56;1.38)	1.18 (0.73;1.93)	0.90 (0.51;1.59)	1.24 (0.83;1.87)
Hyperlipidemia _(yes vs no)	1	0.87 (0.54;1.42)	0.88 (0.52;1.47)	0.86 (0.46;1.59)	0.65 (0.38;1.13)
Systolic blood pressure _(per 10 mmHg)	1	1.01 (0.89;1.16)	1.03 (0.89;1.19)	1.08 (0.91;1.28)	1.00 (0.90;1.12)
Diastolic blood pressure _(per 10 mmHg)	1	0.91 (0.72;1.16)	0.86 (0.67;1.11)	1.17 (0.88;1.55)	0.94 (0.76;1.16)
Smoking _(current vs never)	1	6.47 (3.28;12.79)	0.74 (0.35;1.58)	2.12 (0.96;4.71)	0.11 (0.05;0.21)
Pack years [#]	1	1.04 (1.02;1.06)	1.00 (0.98;1.02)	1.03 (1.00;1.05)	0.96 (0.95;0.98)
High ABI _(≥1.3)	1	0.33 (0.18;0.59)	0.97 (0.56;1.69)	1.07 (0.57;2.04)	2.91 (1.78;4.76)
Low ABI _(≤0.9)	1	6.76 (2.04;22.38)	2.39 (0.63;9.05)	0.58 (0.06;5.45)	0.36 (0.17;0.78)
Statin use _(yes vs no)	1	3.11 (1.79;5.41)	1.97 (1.12;3.46)	1.81 (0.94;3.49)	0.69 (0.39;1.15)
Manifest cardiovascular disease _(yes vs no)					
Cerebrovascular disease	1	0.43 (0.24;0.79)	0.57 (0.31;1.07)	0.57 (0.28;1.18)	1.37 (0.76;2.48)
Coronary artery disease	1	3.31 (2.06;5.31)	2.54 (1.54;4.17)	2.06 (1.16;3.67)	0.93 (0.57;1.52)
Aneurysma abdominal aorta	1	3.05 (0.72;12.90)	1.49 (0.30;7.27)	0.68 (0.07;6.94)	0.52 (0.20;1.37)
Peripheral artery disease	1	2.42 (0.90;6.50)	0.80 (0.23;2.80)	1.28 (0.36;4.59)	0.34 (0.12;0.92)
eGFR _(ml/min/1.73m²)	1	0.98 (0.89;1.09)	0.95 (0.85;1.05)	0.97 (0.86;1.10)	0.95 (0.88;1.03)
Triglycerides _(mmol/L)	1	0.91 (0.76;1.08)	0.87 (0.72;1.06)	0.77 (0.58;1.03)	0.95 (0.80;1.13)
Total cholesterol _(mmol/L)	1	1.01 (0.82;1.22)	0.87 (0.70;1.08)	1.01 (0.79;1.29)	0.88 (0.72;1.06)
LDL-cholesterol _(mmol/L)	1	0.99 (0.78;1.26)	0.82 (0.63;1.08)	1.03 (0.77;1.38)	0.85 (0.67;1.08)
HDL-cholesterol _(mmol/L)	1	2.22 (1.07;4.61)	2.07 (0.95;4.49)	2.75 (1.17;6.44)	0.97 (0.54;1.77)
HbA1c _(mmol/mol)	1	0.72 (0.58;0.90)	0.98 (0.79;1.21)	0.74 (0.55;1.00)	1.37 (1.08;1.73)
CRP _(mg/L)	1	1.00 (0.97;1.04)	0.99 (0.95;1.03)	0.96 (0.90;1.03)	0.99 (0.96;1.02)

Every line of this table represents a separate multinomial model. All models are adjusted for age and sex.

BMI: body mass index, bp: blood pressure, ABI: ankle brachial index, eGFR: estimated glomerular filtration rate,

LDL: low-density lipoprotein, HDL: high-density lipoprotein, CRP: c-reactive protein, AAA: aneurysma Aortae Abdominalis.

[#] assessed in the SMART cohort only (Intimal n=208, medial n=133, indistinguishable n=66).

Risk factors for medial calcification compared to intimal calcification

Compared to the participants with predominant IAC, diabetes (OR 1.81 (1.03;3.15)), HbA1c (OR 1.37 (1.08;1.73)) and an elevated ABI (OR 2.91 (1.78;4.76)) were associated with higher prevalence of predominant MAC in the femoral artery (*Table 2*). Smoking (both status and pack years), low ABI and history of PAD were associated with a lower prevalence of MAC.

In the crural arteries, male sex and BMI were associated with an increased prevalence of MAC compared to IAC (*Supplementary Table 4*). The association between low ABI and increased prevalence of MAC was no longer significant in the crural arteries, as well as the association between history of PAD and MAC. However, history of CAD and statin use were associated with a decreased prevalence of MAC compared to IAC in the crural arteries.

Biomarkers for intimal and medial calcification

The median dp-ucMGP levels were slightly higher in participants with predominant MAC (686 pmol/L (IQR:495-853)) compared to IAC (466 pmol/L (IQR:469-742)) (*Supplementary Table 5*). Age and sex adjusted logistic regression analyses showed that increased dp-ucMGP tended to be associated with higher prevalence of MAC (OR 1.04_{per 100 pmol/L} (0.99; 1.09) (*Table 3*). Increased osteonectin was associated with lower prevalence of MAC compared to IAC in the femoral artery (OR_{per 100ng/ml} 0.91 (0.83; 0.99)). Similar associations were observed in the crural arteries (*Table 3*).

Table 3. Results of multiple biomarkers and risk of medial compared to intimal calcification in the femoral and crural arteries.

	Femoral calcification	Crural calcification
<i>Vitamin K markers</i>		
Dp-ucMGP _(pmol/L)	1.04 (0.99;1.09) ^a	1.05 (1.00;1.11)
PIVKA II >0.25 AU/ml*	1.25 (0.61;2.44)	1.25 (0.61;2.53)
<i>Bone markers</i>		
Osteocalcin _(ng/ml)	1.00 (0.94;1.06) ^b	0.99 (0.93;1.06)
Osteonectin _(ng/ml)	0.91 (0.83;0.99) ^a	0.91 (0.83;1.00)
Osteopontin _(ng/ml)	1.02 (0.98;1.05)	1.01 (0.97;1.05)
<i>Inflammatory markers</i>		
IL-6 _(pg/ml)	1.02 (0.98;1.05)	1.02 (0.98;1.06)
IL-8 _(pg/ml)	1.00 (0.98;1.02)	0.99 (0.97;1.01)
TNF-alfa _(pg/ml)	1.24 (0.94;1.64)	1.22 (0.92;1.60)

Models are adjusted for age and sex.

Dp-ucMGP: desphosphorylated-uncarboxylated Matrix Gla Protein, PIVKA-II: protein induced by vitamin K absence/antagonism II, IL: interleukin

^a per 100 increment, ^b per 5 increment, * PIVKA is measured as detectable/non detectable with a detection limit of 0.25 AU/ml

Discussion

When calcification is present, 85% of the patients have a predominant arterial calcification type (IAC or MAC) throughout the lower extremity. Cardiovascular risk factors appeared to differ between these calcification patterns. If we compared MAC to IAC, diabetes and high ABI were associated with a higher risk of MAC, while smoking and low ABI were associated with a higher risk of IAC. Increased osteonectin levels may result in a higher prevalence of IAC, while increased dp-ucMGP levels may result in higher prevalence of MAC.

Strengths of this study include the concurrent assessment of MAC and IAC and the comprehensive, standardized risk factor assessment performed in all patients included. The method to score intimal and medial calcification was validated histopathologically in the crural arteries, and showed a reasonable agreement²⁰. However, a number of limitations apply to this study. The large proportion of patients that already have a cardiovascular diagnosis (SMART) and type 2 diabetes (DCS) in the study population is inherent to the sampling for this study. Selection conditional on having clinically manifest disease means that risk factors associated with this disease may show different interrelationships when studied amongst this selection compared to amongst the general population. Moreover, the results for the biomarkers are exploratory in nature because of the small sample size, and selection of biomarkers.

Vascular calcification is highly prevalent (77%) and patients have a predominant arterial calcification type throughout the lower extremity (38% had predominant IAC, 28% had predominant MAC). Only two studies investigated the prevalence of IAC and showed a prevalence of 26.7% in patients with diabetes and 37.0% in hemodialysis patients^{6,8}. In previous studies prevalence estimates of MAC in the lower extremities varied between 4.4-15.6% in healthy subjects^{9,10}, and 15.7% - 41.5% for patients with diabetes⁸⁻¹⁰, which is consistent with our findings. MAC is often ignored or considered innocent; however, this calcification type is highly prevalent, especially in diabetes patients.

Patients with type 2 diabetes could be considered as a target population for MAC, while smokers are at risk for IAC^{6,8,10}. In patients with chronic kidney disease (CKD), renal function is a risk factor for MAC⁶. We could not confirm this association; therefore, this might not be a risk factor in patients with cardiovascular disease or patients at high risk of vascular disease. Consistent with our study, a study of patients with type 2 diabetes (n=1,059) showed that advanced age and duration of diabetes were risk factors for both IAC and

MAC in the femoral arteries, whereas patients with IAC were more often smokers compared to patients without IAC⁸. Furthermore, HbA1c levels were higher in patients with MAC compared to patients without MAC⁸. LDL cholesterol is seen as a classic determinant of IAC in CKD patients⁶. However, the previous study including diabetes patients showed lower HDL cholesterol levels in patients with IAC⁸, while we found higher HDL cholesterol levels in patients with femoral IAC. The role of blood lipids and the risk of IAC and MAC need further investigation. A 2003 study examined cardiovascular disease risk factors among end-stage renal disease patients (n=202)⁶. Patients with IAC had higher body mass indexes and smoking history compared to patients without calcification, whereas patients with MAC did not differ from patients without calcification in these aspects⁶. Taken together, aging, male sex, statin use and history of CAD increases both IAC and MAC, while smoking is a specific risk factor for IAC and diabetes a risk factor for MAC; however, these risk factors seems to differ across different study populations.

The mechanisms for IAC and MAC are not entirely known yet. IAC might be a representation of atherosclerotic plaque burden, which might be a result of vascular inflammation, while MAC may have mechanisms similar to bone formation²². To date, no biomarkers have been identified that are specifically associated with IAC or MAC. Our sample for measurement of biomarkers was small and therefore our results are exploratory in nature.

We hypothesized that bone markers, such as osteonectin, may serve as marker of MAC. However, we found that osteonectin was associated with a reduced risk of MAC compared to IAC, which is in line with a previous study identifying osteonectin in atherosclerotic plaques²³. The function of osteonectin is not clear yet; however, our results may suggest that osteonectin could serve as an indicator of IAC. The medial layer of the artery mainly consists of vascular smooth muscle cells (VSMC)²⁴ and an in vitro study showed that VSMC treated with vitamin K antagonists resulted in increased mineralization²⁵. This suggest that vitamin K may specifically be involved in medial calcification, which is supported by MGP knock out mice who suffered from more medial calcification, whereas MGP overexpression reduced medial calcification²⁶. We used dp-ucMGP as a marker of vitamin K status and found similar results compared to previous animal studies. However, further research is necessary to confirm these biomarkers as markers for IAC and MAC.

Our results support the conclusion that MAC and IAC are part of different pathophysiological processes. There is also increasing evidence that links MAC to adverse cardiovascular outcomes^{2,6,7,10}. Intimal calcification may cause lumen narrowing and plaque instability and so appears to be related with acute vascular diseases²⁷, while medial calcification

may increase arterial stiffness, which may result in hypertension, chronic vascular disease such as peripheral arterial disease and heart failure²⁷. In this light, the distinction between MAC and IAC and their respective associated risk factors becomes relevant for the future development of prevention and treatment strategies. We aim to follow these cohorts in a prospective study, to study the clinical consequences of calcification.

In conclusion, there is often a predominant arterial calcification type (IAC or MAC) throughout the lower extremity. These patterns of calcification, predominant IAC, or predominant MAC, appear to have different associated risk factor profiles. Vascular consequences of these patterns need to be investigated in future studies. The distinction between IAC and MAC might be valuable in order to develop prevention and treatment strategies for both pathophysiological mechanisms.

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Disclosures

None.

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Supplementary information

Supplementary Table 1. Calcification score

Aspect		Points
Circularity	Absent	0
	Dot(s)	1
	<90 degrees	2
	90-270 degrees	3
	270-360 degrees	4
Thickness	Absent	0
	Thick ≥ 1.5 mm	1
	Thin <1.5 mm	3
Morphology	Indistinguishable	0
	Irregular/Patchy	1
	Continuous	4

Supplementary Table 2. Baseline characteristics by the femoral calcification score in SMART (n=520) and DCS cohort (n=198).

	<i>Absent</i> (n=162)	<i>Intimal</i> (n=271)	<i>Medial</i> (n=199)	<i>Indistinguishable</i> (n=81)
Age (years)	54.1 ± 11.6	64.7 ± 9.2	65.5 ± 8.7	59.6 ± 8.8
Male sex	100 (62%)	225 (83%)	167 (84%)	58 (72%)
BMI (kg/m ²)	28.1 ± 4.8	27.8 ± 4.2	28.2 ± 4.6	28.3 ± 4.2
Diabetes (type 1 and 2)	54 (33%)	98 (36%)	99 (50%)	23 (28%)
Hypertension	85 (53%)	161 (59%)	133 (67%)	45 (56%)
Hyperlipidemia	50 (31%)	95 (35%)	72 (36%)	25 (31%)
Systolic blood pressure (mmHg)	129 ± 15	134 ± 17	135 ± 19	133 ± 17
Diastolic blood pressure (mmHg)	78 ± 9	78 ± 9	78 ± 9	80 ± 9
Smoking				
Current	30 (19%)	68 (26%)	18 (9%)	18 (22%)
Former	68 (42%)	159 (59%)	94 (48%)	40 (49%)
Never	63 (39%)	42 (16%)	86 (44%)	23 (28%)
High ABI (≥1.3)	36 (22%)	31 (12%)	55 (28%)	21 (26%)
Low ABI (≤0.9)	4 (3%)	32 (12%)	9 (5%)	1 (1%)
Statin use (yes vs no)	107 (66%)	240 (89%)	165 (83%)	65 (80%)
Manifest cardiovascular disease				
Cerebrovascular disease	45 (28%)	26 (10%)	24 (12%)	12 (15%)
Coronary artery disease	62 (38%)	180 (66%)	120 (60%)	46 (57%)
Aneurysma abdominal aorta	3 (2%)	16 (6%)	6 (3%)	1 (1%)
Peripheral artery disease	8 (5%)	20 (7%)	5 (3%)	4 (5%)
eGFR (ml/min/1.73m ²)	82 ± 23	81 ± 19	77 ± 20	88 ± 19
Triglycerides (mmol/L)	1.4 (1.0-2.2)	1.3 (1.0-2.0)	1.4 (1.0-1.8)	1.3 (1.0-1.9)
Total cholesterol (mmol/L)	4.5 ± 1.2	4.3 ± 1.0	4.1 ± 1.0	4.5 ± 1.2
LDL-cholesterol (mmol/L)	2.6 ± 1.0	2.4 ± 0.8	2.2 ± 0.8	2.5 ± 1.0
HDL-cholesterol (mmol/L)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.3 ± 0.4
HbA1c (mmol/mol)	42.3 ± 13.8	41.5 ± 10.2	44.9 ± 11.5	40.6 ± 10.2
CRP (mg/L)	1.5 (0.8-3.9)	1.8 (0.9-4.0)	1.6 (0.8-3.4)	1.4 (0.8-3.3)

Baseline characteristics are described as mean ± standard deviation, median (interquartile range) or number of participants (%).

BMI: body mass index, bp: blood pressure, ABI: ankle brachial index, eGFR: estimated glomerular filtration rate, LDL: low-density lipoprotein, HDL: high-density lipoprotein, CRP: c-reactive protein.

Supplementary Table 3. Baseline characteristics by the crural calcification score in SMART (n=520) and DCS cohort (n=198).

	<i>Absent</i> (n=215)	<i>Intimal</i> (n=218)	<i>Medial</i> (n=181)	<i>Indistinguishable</i> (n=104)
Age (years)	55.4 ± 11.2	64.8 ± 9.0	66.4 ± 8.5	61.8 ± 9.2
Male sex	130 (61%)	178 (82%)	161 (89%)	83 (80%)
BMI (kg/m ²)	28.4 ± 4.8	27.5 ± 4.0	28.5 ± 4.7	27.8 ± 3.7
Diabetes (type 1 and 2)	81 (38%)	71 (33%)	93 (51%)	34 (33%)
Hypertension	116 (54%)	135 (62%)	113 (62%)	64 (62%)
Hyperlipidemia	75 (35%)	67 (31%)	65 (34%)	39 (38%)
Systolic blood pressure (mmHg)	130 ± 16	134 ± 17	135 ± 19	133 ± 15
Diastolic blood pressure (mmHg)	79 ± 10	78 ± 9	77 ± 9	78 ± 8
Smoking				
Current	43 (20%)	52 (24%)	16 (9%)	25 (24%)
Former	99 (46%)	127 (58%)	95 (53%)	42 (41%)
Never	71 (34%)	39 (18%)	69 (39%)	36 (35%)
High ABI (≥1.3)	41 (19%)	26 (12%)	55 (31%)	21 (20%)
Low ABI (≤0.9)	7 (3%)	22 (10%)	13 (7%)	4 (4%)
Statin use	151 (70%)	194 (89%)	150 (83%)	85 (82%)
Manifest cardiovascular disease				
Cerebrovascular disease	50 (23%)	25 (12%)	20 (11%)	12 (12%)
Coronary artery disease	84 (39%)	156 (72%)	114 (63%)	54 (52%)
Aneurysma abdominal aorta	8 (4%)	8 (4%)	6 (3%)	4 (4%)
Peripheral artery disease	11 (5%)	16 (7%)	6 (3%)	4 (4%)
eGFR (ml/min/1.73m ²)	80 ± 23	86 ± 20	87 ± 24	92 ± 28
Triglycerides (mmol/L)	1.4 (1.0-2.0)	1.3 (1.0-1.8)	1.4 (1.0-1.8)	1.4 (1.1-2.0)
Total cholesterol (mmol/L)	4.5 ± 1.2	4.3 ± 1.0	4.1 ± 0.9	4.4 ± 1.2
LDL-cholesterol (mmol/L)	2.5 ± 1.0	2.3 ± 0.8	2.2 ± 0.8	2.4 ± 1.0
HDL-cholesterol (mmol/L)	1.2 ± 0.4	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
HbA1c (mmol/mol)	43.1 ± 13.2	40.7 ± 9.8	45.2 ± 11.9	41.1 ± 9.6
CRP (mg/L)	1.4 (0.7-3.5)	1.6 (0.9-3.9)	1.8 (0.9-3.4)	1.9 (0.9-3.9)

Baseline characteristics are described as mean ± standard deviation, median (interquartile range) or number of participants (%).

BMI: body mass index, bp: blood pressure, ABI: ankle brachial index, eGFR: estimated glomerular filtration rate, LDL: low-density lipoprotein, HDL: high-density lipoprotein, CRP: c-reactive protein.

Supplementary Table 4. Risk (OR 95%CI) of predominant intimal, predominant medial or indistinguishable calcification compared to no calcification in the crural arteries.

	Absent (n=215)	Intimal (n=218)	Medial (n=181)	Indistinguishable (n=104)	Med vs. intima
Age (per 10 years)	1	2.81 (2.23;3.54)	3.55 (2.75;4.59)	1.94 (1.51;2.51)	1.09 (0.85;1.39)
Male sex	1	3.79 (2.33;6.16)	7.30 (4.04;13.20)	3.04 (1.72;5.40)	2.20 (1.20;4.02)
BMI (per kg/m ²)	1	0.95 (0.91;1.00)	1.02 (0.97;1.07)	0.97 (0.92;1.02)	1.06 (1.01;1.11)
Diabetes [#] (type 1 and 2)	1	0.83 (0.40;1.71)	1.59 (0.76;3.33)	0.46 (0.17;1.26)	1.95 (1.08;3.52)
Hypertension (yes vs no)	1	0.96 (0.62;1.49)	0.93 (0.58;1.49)	1.09 (0.65;1.83)	0.81 (0.52;1.26)
Hyperlipidemia (yes vs no)	1	0.55 (0.34;0.88)	0.67 (0.41;1.10)	0.93 (0.55;1.59)	0.63 (0.33;1.22)
Systolic blood pressure (per 10 mmHg)	1	1.01 (0.89;1.15)	1.00 (0.87;1.14)	1.00 (0.86;1.16)	0.97 (0.86;1.09)
Diastolic blood pressure (per 10 mmHg)	1	0.82 (0.65;1.03)	0.70 (0.54;0.90)	0.81 (0.62;1.05)	0.85 (0.68;1.07)
Smoking (current vs never)	1	3.62 (1.89;6.93)	0.62 (0.29;1.30)	1.53 (0.77;3.06)	0.16 (0.08;0.33)
Pack years [#]	1	1.02 (1.01;1.04)	1.00 (0.98;1.01)	1.01 (1.00;1.03)	0.97 (0.96;0.99)
High ABI (>1.3)	1	0.48 (0.27;0.87)	1.50 (0.88;2.58)	0.96 (0.52;1.79)	3.13 (1.84;5.31)
Low ABI (<0.9)	1	3.89 (1.46;10.40)	2.69 (0.91;7.90)	1.41 (0.38;5.19)	0.68 (0.33;1.43)
Statin use (yes vs no)	1	2.69 (1.52;4.76)	1.42 (0.81;2.49)	1.48 (0.81;2.71)	0.53 (0.29;0.97)
Manifest cardiovascular disease (yes vs no)	1				
Cerebrovascular disease	1	0.71 (0.39;1.28)	0.73 (0.38;1.40)	0.64 (0.31;1.31)	1.08 (0.57;2.05)
Coronary artery disease	1	4.60 (2.86;7.38)	2.91 (1.78;4.74)	1.58 (0.95;2.65)	0.81 (0.47;1.42)
Aneurysm abdominal aorta	1	0.62 (0.20;1.87)	0.49 (0.15;1.63)	0.72 (0.20;2.61)	0.92 (0.31;2.73)
Peripheral artery disease	1	2.18 (0.88;5.42)	1.06 (0.34;3.29)	1.01 (0.30;3.42)	0.46 (0.17;1.23)
eGFR (ml/min/1.73m ²)	1	0.93 (0.85;1.03)	0.95 (0.86;1.05)	0.84 (0.75;0.94)	1.00 (0.92;1.10)
Triglycerides (mmol/L)	1	1.00 (0.83;1.20)	0.98 (0.80;1.19)	0.99 (0.79;1.23)	0.96 (0.81;1.14)
Total cholesterol (mmol/L)	1	1.02 (0.84;1.24)	0.91 (0.73;1.14)	1.02 (0.81;1.28)	0.92 (0.74;1.14)
LDL-cholesterol (mmol/L)	1	0.99 (0.78;1.26)	0.89 (0.68;1.16)	1.02 (0.78;1.35)	0.95 (0.73;1.25)
HDL-cholesterol (mmol/L)	1	1.59 (0.83;3.05)	1.10 (0.53;2.29)	1.08 (0.49;2.38)	0.68 (0.34;1.37)
HbA1c (mmol/mol)	1	0.61 (0.48;0.77)	0.95 (0.76;1.17)	0.71 (0.54;0.93)	1.45 (1.13;1.87)
CRP (mg/L)	1	1.00 (0.96;1.05)	1.01 (0.97;1.06)	1.01 (0.96;1.05)	1.01 (0.98;1.04)

Every line of this table represents a separate multinomial model. All models are adjusted for age and sex.

BMI: body mass index, bp: blood pressure, ABI: ankle brachial index, eGFR: estimated glomerular filtration rate, LDL: low-density lipoprotein, HDL: high-density lipoprotein, CRP: c-reactive protein.

[#] assessed in the SMART cohort only (intimal n=208, medial n=133, indistinguishable n=66).

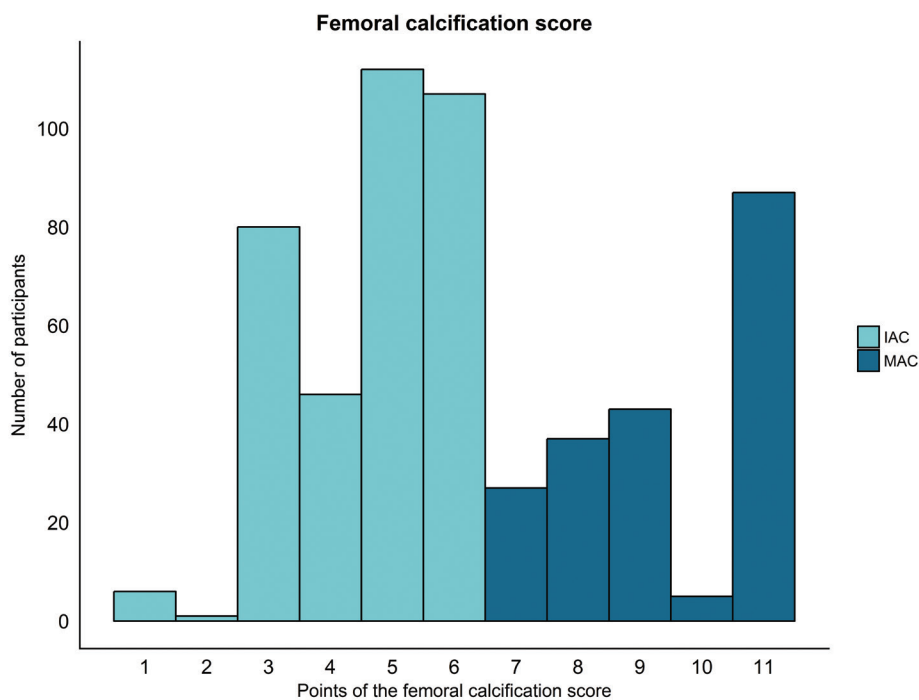
Supplementary Table 5. Biomarkers in participants with predominant Intimal and predominant Medial calcification in the femoral artery.

	Femoral calcification		Crural calcification	
	Dominant Intimal (n=151)	Dominant Medial (n=151)	Dominant Intimal (n=151)	Dominant Medial (n=120)
<i>Vitamin K markers</i>				
Dp-ucMGP _(pmol/L)	466 (469-742)	685.9 (495-853)	567 (469-739)	646.5 (517-882)
PIVKA II _{>0.25 AU/ml*}	19 (6.3%)	22 (7.3%)	19 (7.0%)	18 (6.6%)
<i>Bone markers</i>				
Osteocalcin _(ng/ml)	31 (23-44)	29 (21-40.3)	31 (23-44)	29 (22-40)
Osteonectin _(ng/ml)	1028 (887-1145)	1005 (838-1125)	1026 (886-1143)	1009 (838-1124)
Osteopontin _(ng/ml)	17 (13-22)	16 (13-22)	17 (13-22)	16 (13-22)
<i>Inflammatory markers</i>				
IL-6 _(pg/ml)	0.70 (0.47-1.07)	0.60 (0.43-0.87)	0.71 (0.47-1.08)	0.64 (0.47-0.89)
IL-8 _(pg/ml)	12.4 (8.6-17.5)	12.0 (8.3-20.1)	12.5 (8.6-17.7)	11.9 (8.2-18.5)
TNF-alfa _(pg/ml)	2.05 (1.7-2.5)	2.2 (1.7-2.8)	2.1 (1.7-2.5)	2.3 (1.7-2.8)

Median and interquartile ranges or n (percentage).

Dp-ucMGP: desphosphorylated-uncarboxylated Matrix Gla Protein, PIVKA-II: protein induced by vitamin K absence/antagonism II, IL: interleukin

* PIVKA is measured as detectable/non detectable with a detection limit of 0.25 AU/ml



Supplementary Figure 1. The points of the femoral calcification score described by predominant intimal (IAC) and medial (MAC) calcification.



Bone markers and cardiovascular risk in type 2 diabetes patients

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Abstract

Background: Vascular calcifications are associated with a 3-to 4-fold increased risk of cardiovascular disease (CVD) and are highly prevalent in type 2 diabetes patients. Emerging evidence indicates that vascular calcification is a process of active bone formation regulated by stimulators and inhibitors of calcification. Therefore, this study aimed to evaluate whether six bone markers are associated with CVD risk in patients with type 2 diabetes.

Methods: We used data of a case-cohort study, nested in the EPIC-NL cohort, comprising 134 CVD cases and a random subcohort of 218 participants, all with type 2 diabetes at baseline. Six bone markers (osteocalcin, osteopontin, osteonectin, osteoprotegerin, alkaline phosphatase and sclerostin) were measured in baseline plasma samples with multiplex assays and information on CVD events was obtained. The association of bone makers with CVD risk was evaluated using Cox proportional hazard analyses.

Results: Higher concentrations of plasma osteopontin were associated ($p_{\text{trend}} < 0.01$) with an increased CVD risk with a hazard ratio of 2.00 (95%-CI: 1.20-3.35) for the highest versus the lowest quartile in a multivariable adjusted model. The other bone markers were not associated with CVD risk.

Conclusions: Higher osteopontin concentrations were associated with an increased CVD risk in type 2 diabetes patients. No consistent associations were found for the other five bone markers and risk of CVD in type 2 diabetes patients.

Background

Patients with diabetes are at 2 to 4 fold increased risk of cardiovascular disease (CVD), which cannot be fully explained by traditional risk factors^{1,2}. Vascular calcification was long seen as a passive process of calcium deposition; however, recent evidence indicates it is an active process influenced by several stimulating and inhibiting factors³. Vascular calcification is a process with many similar mechanisms to bone formation, like the mineralization of vascular smooth muscle cells (VSMC)⁴. VSMC differentiate to osteoblast-like cells, due to exposure to a variety of stresses⁵. Expression of multiple bone markers has indeed been found in atherosclerotic plaques⁶. Moreover, hyperglycemia may induce VSMC proliferation, as well as the expression of some bone markers^{7,8}.

Osteocalcin, osteopontin, and osteonectin have been suggested to inhibit mineralization of the VSMC by blocking hydroxyapatite formation³. Studies investigating the association between osteocalcin and CVD risk showed inconsistent results^{9–11}. Osteopontin and osteonectin have been found in calcified vessels^{6,12,13} and one study showed that osteocalcin and osteonectin may predict cardiovascular disease¹⁴. Several studies also showed that osteopontin may be a predictor of CVD risk in patients with type 2 diabetes^{14–16}. However, prospective etiological evidence investigating the association between these bone markers and CVD events is lacking.

Osteoprotegerin, alkaline phosphatase, and sclerostin are suggested to play a role in CVD risk. Osteoprotegerin may inhibit osteoclast differentiation in bone and vascular tissue, and may therefore be considered as a protective factor for vascular calcification and CVD risk³. Alkaline phosphatase is suggested to cause an impaired vascular homeostasis and increase bone metabolism¹⁷. Subsequently, a recent meta-analysis showed that higher levels of alkaline phosphatase were associated with increased CVD risk¹⁷. Finally, sclerostin is a potent inhibitor of bone formation, but the available evidence with CVD risk is limited and inconclusive and mainly studied in patients with an impaired kidney function^{18–22}, since sclerostin levels increase with the progression of kidney function²³.

Presently, etiological studies investigating the association of these different bone markers and CVD incidence are far from conclusive and mainly studied in the general population or patients with chronic kidney disease. Furthermore, most bone markers are not studied prospectively, and none of the bone markers have been studied in patients with type 2 diabetes. Therefore, this study aims to evaluate whether six bone markers

(osteocalcin, osteopontin, osteonectin, osteoprotegerin, alkaline phosphatase, sclerostin) are associated with CVD risk in patients with type 2 diabetes.

Methods

Study population

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 cohorts²⁴. In brief, the Prospect-EPIC study includes 17,357 women 49–70 years of age living in Utrecht and vicinity who participated in the nationwide Dutch breast cancer screening program. The MORGEN-EPIC cohort consists of 22,654 men and women 21–64 years of age selected from random samples of the Dutch population in three different towns. Participants were recruited in both studies from 1993 to 1997. 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). Ascertained cases of diabetes were verified against medical and pharmacy records. Details of the ascertainment sources and verification procedures have previously been described²⁵.

Case-cohort sampling was performed to reduce costs and preserve valuable biological material. The case-cohort design is a method in which a random sample of the cohort at baseline, the subcohort, is used as control group for all cases that occur in the cohort²⁶. From the 40,011 EPIC-NL participants, 526 participants were diagnosed with verified type 2 diabetes at baseline. During follow-up, 172 of the 526 participants with type 2 diabetes had a CVD event, of which 134 had blood samples available at baseline. These 134 participants were included as cases in our analyses. Additionally, a random sample of 218 participants from the diabetes participants was selected to serve as a subcohort in the case-cohort design. Because of the random sampling, this subcohort included 65 CVD cases.

Ethics, consent and permissions

All participants provided written 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 The Netherlands Organization for Applied Scientific Research Nutrition and Food Research (MORGEN).

Bone markers

At baseline, we measured six different bone markers in plasma samples. All bone markers were measured using the human bone marker panel II multiplex assay (Meso Scale Discovery, Rockville MD, USA). Details of this multiplex assay, minimal detection limits and inter- and intra assay coefficients, are described in *Supplementary Table 1*.

Clinical endpoints

Follow-up data on cardiovascular events were obtained from the Dutch Centre for Health Care Information, which holds a standardized computerized register of hospital discharge diagnoses. Follow-up was complete until the first of January 2008. Information on vital status was obtained through linkage with the municipal registries. Causes of death were collected from Statistics Netherlands, and coded according to the ICD-9 and ICD-10. Primary outcome for the present analysis was cardiovascular events (fatal and non-fatal), including coronary heart disease (CHD), congestive heart failure (CHF), peripheral arterial disease (PAD) and stroke. These clinical endpoints included both fatal and non-fatal CVD events. If a participant developed multiple cardiovascular events, the first event was taken into account in the analyses of total cardiovascular events. Since the mechanism behind the association between bone markers and CVD was mainly based on vascular calcification, CHD was analyzed separately. Due to limited statistical power, fatal events were analyzed separately only for total cardiovascular events.

Other measurements

At baseline, participants completed general questionnaires about demographic and lifestyle characteristics and risk factors for chronic diseases. Smoking status was categorized as current, past, or never smoking. Physical activity was measured with the validated Cambridge Physical Activity Index, and was categorized as inactive, moderately inactive, moderately active and active²⁷. Height and weight were measured by trained staff, and Body Mass Index (BMI) was calculated as weight in kilograms divided by squared height in meters. Time since diabetes diagnosis was calculated by subtracting the age of diagnosis from the age at baseline examination and the estimated glomerular filtration rate (eGFR) was calculated based on creatinine with the CKD-EPI formula²⁸. HbA1c was measured in erythrocytes using an immunoturbidimetric latex test. All measurements were performed according to standard operating procedures.

Statistical analyses

Baseline characteristics were summarized as mean $\pm$ SD or median (IQR) as appropriate for continuous variables. In order to understand the possible confounding factors of the different bone markers, baseline characteristics were summarized in quartiles of the different bone markers. Furthermore, the correlation between the different bone markers was tested using the Spearman's rank correlation coefficients. Since only for 6 persons data on eGFR was missing, we imputed these values using single imputation. Cox proportional hazards models were used to estimate Hazard Ratio's (HR) for each individual bone marker and cardiovascular events. Standard errors were calculated using the Prentice weighting method to account for the case-cohort design²⁹. All subcohort members, both cases and non-cases, are weighted equally. Cases outside the subcohort are not weighted before the event. At the time of event, cases have the same weight as the subcohort members, and only then contribute to the risk set. We adjusted for risk factors of chronic diseases in three separate models. Age was the underlying time scale, with entry time defined as the participant's age at recruitment and exit time as age at first fatal or non-fatal CVD event or censoring (whichever came first). The first model was adjusted for sex. The second model was additionally adjusted for risk factors for chronic diseases; BMI, smoking status (never/former/current), prevalent CVD, high sensitive C-reactive protein (hs-CRP), total- and HDL cholesterol. The third model was additionally adjusted for factors indicating the severity of diabetes; the age at onset of diabetes, HbA1c levels and eGFR. Analyses were performed in quartiles and continuously, per 5 or 10 ng/ml, dependent on the scale of the bone markers. P-values for linear trends were calculated over the tertiles by entering the median value of each tertile as a linear covariate in the model.

Osteocalcin, osteopontin and osteonectin are all involved in blocking hydroxyapatite formation³, and may complement each other. Therefore, a risk score was created; osteocalcin, osteopontin, and osteonectin were log transformed, a z-score was calculated for these individual bone markers and these values were summed up. A log-transformed score was used since the bone markers were not normally distributed.

Effect modification by sex was checked for all bone markers by including interaction terms in the model. In case of an interaction term with a p-value below then 0.10, the analyses were stratified. In sensitivity analyses, we excluded the participants with prior CVD. Furthermore, CVD mortality and CHD incidence were analyzed separately. Because of a smaller sample in the sensitivity analyses, we analyzed these associations only

continuously (per 5 or 10 ng/ml). All statistical analyses were performed using R 3.1.1 for Windows and a two-tailed p-value <0.05 was considered statistically significant.

Results

Baseline characteristics of the study population are shown in *Table 1*. In this study population, 82% were women, had an average age of 58 years, mean body mass index of 29.4 kg/m² and a median duration of diagnosed diabetes of 5.3 years. Baseline characteristics of the subcohort according to the quartiles of the bone markers are shown in *Supplementary Table 2*. Osteocalcin and osteopontin levels were correlated ($r=0.58$), and osteoprotegerin and osteopontin levels were moderately correlated ($r=0.28$). The other bone markers were not correlated.

Table 1. Baseline characteristics for participants of the subcohort and cases

	Subcohort (n=218) *	Cases (n=134)
Age _(years)	57.9 ± 6.7	58.6 ± 6.9
Women _{n (%)}	178 (82)	107 (80)
Diabetes duration _(years)	5.3 (2.3-10.2)	6.3 (2.6-12.3)
History of CVD _{n (%)}	30 (14)	40 (30)
Current smoker _{n (%)}	49 (22)	42 (31)
BMI _(kg/m²)	29.4 ± 4.9	30.3 ± 4.9
Systolic blood pressure _(mmHg)	141.7 ± 20.8	145.6 ± 23.3
Total cholesterol _(mmol/l)	5.2 ± 1.2	5.4 ± 1.1
HDL cholesterol _(mmol/l)	1.0 ± 0.3	1.0 ± 0.3
HbA1c _(%)	8.1 ± 1.7	8.2 ± 1.7
eGFR _(ml/min/1.73m²)	107.3 ± 24.1	109.1 ± 19.4
Hs-CRP _(µg/mmol)	3.3 (1.8-6.5)	3.9 (2.5-6.6)
Osteocalcin _(ng/ml)	22.6 (17.1 – 29.2)	21.7 (17.2-28.7)
Osteopontin _(ng/ml)	12.8 (10.1 – 16.1)	13.5 (11.1-17.4)
Osteonectin _(ng/ml)	114.6 (90.0 – 154.8)	124.4 (95.7 – 170.4)
Osteoprotegerin _(ng/ml)	191.9 ± 55.6	201.0 ± 57.4
Alkaline Phosphatase _(ng/ml)	55.8 (46.2-64.2)	56.2 (46.1-70.4)
Sclerostin _(ng/ml)	60.3 (47.6-79.2)	60.8 (49.7-76.0)

Data are shown as frequencies (%), mean ± standard deviation or median (interquartile range). * This subcohort included 65 CVD cases. BMI = body mass index, HDL=high density lipoprotein, eGFR = estimated glomerular filtration, Hs-CRP = high sensitive C-reactive protein.

During the median survival time of 11 years, 134 participants experienced a CVD event (77 CHD, 19 stroke, 30 PAD, 8 CHF), of whom 46 fatal. The associations between the individual bone markers and CVD events are shown in *Table 2*. No association was observed for osteocalcin and osteonectin and CVD risk in the three different models with HR_{Q4vsQ1} 1.17 (0.70-1.93) and HR_{Q4vsQ1} 1.16 (0.72-1.88) for osteocalcin and osteonectin respectively. Higher concentrations of plasma osteopontin were associated with an increased CVD risk in the final models (model 3, HR_{Q4vsQ1} 2.00 (1.20-3.35)), which was adjusted for sex, BMI, smoking status, prevalent CVD, hs-CRP, total cholesterol, HDL cholesterol, age at onset of diabetes, HbA1c levels and eGFR. A significant p for trend was observed in all models (p<0.01). Similar results were observed when we analyzed osteopontin per 5 ng/ml increase with a HR of 1.11 (1.01-1.21), in the multivariable adjusted model (model 3). No associations were observed between osteoprotegerin (model 3, HR_{Q4vsQ1} 1.23 (0.69-2.19)), alkaline phosphatase (model 3, HR_{Q4vsQ1} 1.05 (0.60-1.85)) and sclerostin (model 3, HR_{Q4vsQ1} 0.75 (0.41-1.36)) and CVD risk. Osteonectin, osteocalcin, and osteopontin were combined to one bone marker-score. Increased levels of this score were associated with an increased CVD risk (model 3, HR_{Q4vsQ1} 1.66 (1.03-2.62)).

Table 2. The HR for each individual bone marker and the effect on CVD.

	Q1	Q2	Q3	Q4	P _{trend}	Continuous
Osteocalcin						
(median, ng/ml)	14.22	19.68	25.45	34.75		
Events	33/68	41/80	29/67	31/73		<i>per 5 ng/ml</i>
Model 1	Ref	0.91 (0.58-1.42)	0.74 (0.44-1.25)	0.83 (0.51-1.35)	0.40	1.01 (0.98-1.02)
Model 2	Ref	0.90 (0.59-1.47)	0.94 (0.55-1.60)	1.10 (0.67-1.81)	0.64	1.01 (0.99-1.02)
Model 3	Ref	0.83 (0.51-1.36)	0.92 (0.53-1.59)	1.17 (0.70-1.93)	0.43	1.01 (1.00-1.03)
Osteopontin						
(median, ng/ml)	8.84	11.66	13.78	18.72		
Events	23/63	23/70	41/74	41/81		<i>Per 5 ng/ml</i>
Model 1	Ref	1.18 (0.70-1.97)	1.51 (0.89-2.56)	1.70 (1.03-2.78)	0.02	1.08 (0.97-1.20)
Model 2	Ref	1.34 (0.78-2.33)	1.79 (1.06-3.02)	1.76 (1.08-2.88)	0.02	1.08 (0.98-1.20)
Model 3	Ref	1.36 (0.76-2.44)	1.66 (0.95-2.89)	2.00 (1.20-3.35)	<0.01	1.11 (1.01-1.21)
Osteonectin						
(median, ng/ml)	71.78	103.40	127.9	195.6		
Events	29/70	23/65	41/75	41/78		<i>Per 10 ng/ml</i>
Model 1	Ref	0.83 (0.48-1.44)	1.63 (1.02-2.60)	1.39 (0.85-2.20)	0.08	1.02 (1.00-1.03)
Model 2	Ref	0.77 (0.44-1.32)	1.32 (0.82-2.14)	1.16 (0.72-1.88)	0.30	1.02 (1.00-1.04)
Model 3	Ref	0.74 (0.43-1.27)	1.25 (0.76-2.05)	1.16 (0.72-1.88)	0.30	1.02 (1.00-1.04)

Table 2. continued

	Q1	Q2	Q3	Q4	P _{trend}	Continuous
Osteoprotegerin						
(median, ng/ml)	134.4	168.3	199.0	256.6		
Events	30/69	28/69	32/73	44/77		Per 10 ng/ml
Model 1	Ref	0.88 (0.53-1.46)	0.91 (0.56-1.46)	1.51 (0.91-2.52)	0.07	1.03 (1.00-1.06)
Model 2	Ref	0.82 (0.50-1.36)	0.87 (0.53-1.44)	1.13 (0.65-1.98)	0.46	1.01 (0.98-1.04)
Model 3	Ref	0.88 (0.52-1.47)	0.84 (0.49-1.45)	1.23 (0.69-2.19)	0.35	1.01 (0.98-1.05)
Alkaline Phosphatase						
(median, ng/ml)	42.0	51.39	59.7	74.4		
Events	35/68	31/73	25/68	43/79		Per 10 ng/ml
Model 1	Ref	0.79 (0.49-1.29)	0.67 (0.40-1.14)	1.13 (0.71-1.79)	0.52	1.07 (0.94-1.21)
Model 2	Ref	0.74 (0.45-1.22)	0.67 (0.39-1.16)	1.03 (0.61-1.73)	0.64	1.02 (0.89-1.18)
Model 3	Ref	0.79 (0.47-1.35)	0.68 (0.39-1.17)	1.05 (0.60-1.85)	0.66	1.03 (0.89-1.19)
Sclerostin						
(median, ng/ml)	40.65	53.6	67.8	93.6		
Events	29/65	35/73	42/79	28/71		Per 10 ng/ml
Model 1	Ref	1.07 (0.65-1.78)	1.18 (0.74-1.89)	0.83 (0.49-1.40)	0.42	0.99 (0.97-1.02)
Model 2	Ref	1.05 (0.64-1.73)	1.04 (0.64-1.67)	0.76 (0.44-1.32)	0.27	0.99 (0.96-1.02)
Model 3	Ref	1.15 (0.68-1.93)	1.07 (0.65-1.75)	0.75 (0.41-1.36)	0.24	0.99 (0.96-1.02)

Model 1: Adjusted of sex (age is the underlying time scale).

Model 2: Additionally adjusted for BMI, smoking status, prevalent CVD, hs-CRP, total cholesterol and HDL cholesterol.

Model 3: Additionally adjusted for age at onset of diabetes, HbA1c levels and eGFR.

Sensitivity analyses

The associations between the different bone markers and CHD incidence (n=77) and CVD mortality (n=46) were similar to those with CVD incidence. The hazard ratio (model 3) for the association between osteopontin and CHD and CVD mortality was HR_{per 5 ng/ml} 1.11 (0.97-1.28) and HR_{per 5 ng/ml} 1.13 (0.95-1.33) for CHD incidence and CVD mortality respectively. Excluding participants with a prior CVD event resulted in 237 participants for analyses in whom 94 CVD events occurred during follow up, whereof 45 events occurred in the subcohort. The Hazard Ratio slightly dropped to unity, and no significant association was observed (HR_{per 5 ng/ml} 1.08 (0.97-1.19)). The associations of the other bone markers and CVD events remained similar. No significant interaction was present between any bone markers and sex.

Discussion

The aim of this study was to assess the association between multiple bone markers and CVD risk in patients with type 2 diabetes. We did not observe any consistent associations for bone markers and risk of CVD. Only elevated osteopontin levels were associated with higher CVD risk.

To the best of our knowledge, our study is the first prospective cohort study investigating associations between a variety of bone markers and CVD risk in patients with type 2 diabetes. Nevertheless, some limitations need to be addressed. This study was underpowered to explore the association between these multiple bone markers and specific CVD endpoints such as stroke, PAD and CHF. The sensitivity analyses focusing on CVD mortality, CHD events and in participants without a prior CVD events seem to be underpowered. Additionally, including data on vascular calcification, like coronary artery calcium score, would provide more insights on the underlying mechanism of the association between bone markers and CVD risk.

We hypothesized that multiple bone markers were associated with CVD risk in patients with type 2 diabetes; however, we did not detect an association between these bone markers and CVD risk, except for osteopontin. The available literature is limited and inconsistent, especially cohort studies including diabetes patients are lacking. The evidence linking osteocalcin to CVD events showed contradicting results, showing an inverse association¹¹, null result¹⁰ and positive association^{9,11}. Those studies reporting an association between osteocalcin and CVD risk, only detected an association in elderly (>70 year); however, these associations were in opposite directions^{9,11}. Additionally, one previous study indicated that osteocalcin may be a marker of vascular disease in patients with type 1 diabetes³⁰. Osteonectin was detected in atherosclerotic plaques, but cohort studies focusing on osteonectin and incident CVD risk are lacking, although one study suggested that osteonectin may predict CVD events¹⁴. The bone markers-score, including osteocalcin, osteonectin and osteopontin, was associated with CVD incidence, but this is mainly driven by osteopontin.

Based on previous meta-analyses, we mainly expected to find an association between osteoprotegerin, alkaline phosphatase and CVD risk^{17,31}. A recent meta-analysis showed that elevated osteoprotegerin concentration was associated with increased CVD risk in the general population³¹. Another study showed increased osteoprotegerin levels in

patients with PAD compared to patients without PAD in participants with type 2 diabetes³². We did not detect an association between osteoprotegerin and CVD risk, but we were not able to investigate PAD incidence separately. The meta-analysis focusing on alkaline phosphatase included four prospective studies^{33–36}, of which two studies found an increased CVD risk^{34,36}. These studies focused on CVD risk in men³⁴ and stroke incidence³⁶ and found relative risks comparable to this present study, but the previous studies found significant associations. However, two other large scale prospective studies, focusing on stroke incidence and CVD mortality, did not find any association^{33,35}. The previous cohort studies investigating the association between alkaline phosphatase and CVD risk were all large-scale studies, and our study could be underpowered to detect an association^{33–36}. Sclerostin levels increases with the progression of kidney disease, and is potent inhibitor of bone formation, and therefore suggested to reduce vascular calcification²². Sclerostin is only studied in patients with chronic kidney disease, and the cohort studies showed conflicting results which could be explained by small sample sizes and heterogeneity of the study population²². This study did not detect an association between sclerostin and CVD incidence in patients with type 2 diabetes; however, additional research is necessary to confirm this null result.

The current study is the first prospective study addressing the association between osteopontin and CVD events in patients with type 2 diabetes, and showed that higher osteopontin levels were associated with an increased CVD risk. This association is opposite to what is expected based on osteopontin's function as an inhibitor of calcification. No significant association was present when investigating CHD specific incidence and CVD mortality, and suggests a lack of power (CHD incidence, n=77; fatal CVD, n=46). Excluding participants with a prior CVD event resulted in a lower, non-significant HR, which also could be a power issue (CVD incidence, n=94) or osteopontin might be upregulated due to calcification. Multiple studies suggested that osteopontin levels are elevated because of atherosclerosis^{37,38}. A study using coronary angiography showed that osteopontin levels were indeed associated with the presence and extent of coronary artery disease³⁸. In line with our study, multiple studies also showed that elevated osteopontin may be a predictor of increased CVD risk in patients with diabetes^{14–16}, and osteopontin is elevated in heart failure patients^{39,40}. Furthermore, osteopontin may also be involved in the coronary calcifications process by inhibiting hydroxyapatite formation³. To this end, it has to be elucidated whether osteopontin is a marker of calcification, a protector or an active player in the calcification process; therefore, additional research is necessary to detect osteopontin's role in the process of vascular calcification.

Conclusion

No consistent associations were found for bone markers and risk of CVD in type 2 diabetes patients. Only higher osteopontin concentrations were associated with an increased CVD risk in type 2 diabetes patients. This association attenuated to the null after exclusion of the patients with a prior CVD event.

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Disclosures

None.

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Supplementary information

Supplementary Table 1. Details of the multiplex assay, the minimal detection limit, inter- and intra assay variation coefficients.

	Detection limit (ng/ml)	Inter-assay variation coefficients (%)	Intra-assay variation coefficients (%)
Osteocalcin	0.011	6.6	3.4
Osteopontin	0.027	8.0	4.0
Osteonectin	2.5	6.8	9.9
Osteoprotegerin	0.005	4.9	4.1
Alkaline Phosphatase	8.3	5.7	2.2
Sclerostin	0.003	5.6	5.4

Supplementary Table 2. Baseline characteristics by quartiles of the six bone markers in the subcohort (n=218).

Osteocalcin	Q1	Q2	Q3	Q4	P _{trend}
Age _(years)	56.5 ± 6.9	56.9 ± 6.9	59.5 ± 6.3	58.9 ± 6.2	0.05
Women _{n(%)}	45 (82)	42 (78)	42 (78)	49 (89)	0.38
Diabetes duration _(years)	5.8 (2.7-8.1)	5.5 (1.7-11.9)	3.8 (2.5-14.8)	5.2 (2.4-10.1)	0.99
History of CVD _{n(%)}	9 (16)	9 (17)	8 (15)	4 (7)	0.44
BMI _(kg/m²)	30.9 ± 5.1	29.6 ± 4.4	28.3 ± 4.7	28.8 ± 4.9	0.03
Systolic BP _(mmHg)	141.0 ± 19.2	139.0 ± 17.8	141.6 ± 20.3	145.3 ± 25.2	0.45
Current smoker _{n(%)}	12 (22)	19 (35)	10 (19)	8 (15)	0.19
Total cholesterol _(mmol/L)	5.1 ± 1.2	5.3 ± 1.2	5.3 ± 1.2	5.1 ± 1.3	0.48
HDL _(mmol/L)	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	0.41
HbA1c _(%)	8.4 ± 1.9	8.1 ± 1.7	7.8 ± 1.6	8.0 ± 1.4	0.38
eGFR _(ml/min/1.73m²)	104.3 ± 14.3	106.3 ± 21.8	110.2 ± 29.4	108.4 ± 28.2	0.61
CRP _(µg/mmol)	4.8 (2.7-7.8)	3.3 (1.7-6.3)	2.9 (1.4-5.0)	2.8 (1.5-6.1)	0.07
Osteopontin	Q1	Q2	Q3	Q4	P _{trend}
Age _(years)	56.3 ± 6.8	58.0 ± 5.1	58.5 ± 8.6	59.0 ± 5.5	0.16
Women _{n(%)}	44 (80)	44 (82)	44 (82)	46 (84)	0.97
Diabetes duration _(years)	3.6 (1.7-8.0)	5.9 (2.9-11.0)	6.9 (3.2-14.3)	3.8 (2.2-7.2)	0.51
History of CVD _{n(%)}	10 (18)	6 (11)	7 (13)	7 (13)	0.73
BMI _(kg/m²)	30.0 ± 5.2	29.8 ± 4.2	28.9 ± 5.2	28.9 ± 4.8	0.51
Systolic BP _(mmHg)	140.2 ± 21.3	139.8 ± 16.8	143.9 ± 22.8	143.0 ± 22.1	0.67
Current smoker _{n(%)}	10 (18)	18 (33)	10 (19)	11 (20)	0.15
Total cholesterol _(mmol/L)	5.3 ± 1.2	5.1 ± 1.3	5.3 ± 1.0	5.1 ± 1.1	0.76
HDL _(mmol/L)	1.1 ± 0.3	1.1 ± 0.4	1.0 ± 0.3	1.0 ± 0.3	0.63
HbA1c _(%)	8.3 ± 1.9	8.1 ± 1.7	8.1 ± 1.6	7.8 ± 1.5	0.62
eGFR _(ml/min/1.73m²)	106.0 ± 19.1	101.6 ± 16.5	109.4 ± 22.7	112.1 ± 33.7	0.13
CRP _(µg/mmol)	3.0 (1.9-6.4)	3.5 (1.9-6.6)	3.3 (1.5-5.6)	4.0 (2.0-6.8)	0.82
Osteonectin	Q1	Q2	Q3	Q4	P _{trend}
Age _(years)	58.6 ± 6.2	57.8 ± 5.6	56.4 ± 8.0	59.0 ± 6.3	0.18
Women _{n(%)}	49 (89)	44 (82)	43 (80)	42 (76)	0.36
Diabetes duration _(years)	6.2 (2.6-9.7)	6.4 (3.7-11.2)	4.1 (2.5-10.5)	3.1 (1.4-8.5)	0.14
History of CVD _{n(%)}	10 (18)	5 (9)	10 (19)	5 (9)	0.28
BMI _(kg/m²)	28.9 ± 4.4	29.0 ± 5.0	29.2 ± 4.9	30.4 ± 5.0	0.33
Systolic BP _(mmHg)	142.8 ± 21.5	138.6 ± 18.6	138.7 ± 21.8	146.7 ± 20.7	0.13
Current smoker _{n(%)}	9 (16)	14 (26)	14 (26)	12 (22)	0.90
Total cholesterol _(mmol/L)	5.0 ± 1.2	5.2 ± 1.1	5.3 ± 1.1	5.4 ± 1.3	0.30
HDL _(mmol/L)	1.1 ± 0.4	1.1 ± 0.3	1.0 ± 0.2	1.0 ± 0.3	0.39
HbA1c _(%)	7.9 ± 1.4	8.0 ± 1.7	8.0 ± 1.9	8.4 ± 1.7	0.45
eGFR _(ml/min/1.73m²)	108.6 ± 31.3	104.8 ± 14.3	106.4 ± 24.1	109.3 ± 24.3	0.75
CRP _(µg/mmol)	2.7 (1.3-5.7)	3.1 (1.7-6.6)	4.0 (2.1-6.5)	4.2 (2.2-7.0)	0.25

Data are shown as frequencies (%), mean ± standard deviation or median (interquartile range).

BMI = body mass index, HDL=high density lipoprotein, eGFR = estimated glomerular filtration, Hs-CRP = high sensitive C-reactive protein. Linear trend was tested with ANOVA or Chi-square test, as appropriate.

Skewed variables (diabetes duration and CRP) were loge-transformed prior to analyses.

Supplementary Table 2 (continued). Baseline characteristics by quartiles of the six bone markers in the subcohort (n=218).

Osteoprotegerin	Q1	Q2	Q3	Q4	P _{trend}
Age _(years)	54.6 ± 6.5	56.0 ± 6.1	59.2 ± 6.1	62.0 ± 5.4	<0.01
Women _{(n(%))}	40 (73)	42 (78)	46 (85)	50 (91)	0.07
Diabetes duration _(years)	5.8 (2.2-10.5)	5.8 (2.2-8.7)	5.3 (1.8-10.2)	3.7 (2.6-11.2)	0.91
History of CVD _{(n(%))}	4 (7)	7 (13)	6 (11)	13 (24)	0.08
BMI _(kg/m²)	29.7 ± 4.7	28.4 ± 4.1	30.3 ± 5.5	29.2 ± 5.0	0.24
Systolic BP _(mmHg)	135.8 ± 18.7	138.9 ± 20.9	142.2 ± 18.2	150.0 ± 22.8	<0.01
Current smoker _{(n(%))}	17 (31)	19 (35)	8 (15)	5 (9)	0.01
Total cholesterol _(mmol/L)	5.5 ± 1.1	5.3 ± 1.2	5.0 ± 1.1	4.9 ± 1.2	0.03
HDL _(mmol/L)	1.1 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.0 ± 0.3	0.07
HbA1c _(%)	7.8 ± 1.6	7.6 ± 1.3	8.4 ± 1.7	8.4 ± 1.9	0.01
eGFR _(ml/min/1.73m²)	105.2 ± 13.8	106.8 ± 30.7	109.8 ± 27.3	107.5 ± 21.7	0.80
CRP _(μg/mmol)	3.0 (1.4-5.6)	2.9 (1.3-6.9)	3.1 (1.8-6.2)	4.2 (2.3-6.9)	0.16
Alkaline Phosphatase	Q1	Q2	Q3	Q4	P _{trend}
Age _(years)	56.9 ± 6.7	57.1 ± 7.3	58.9 ± 6.1	58.9 ± 6.3	0.21
Women _{(n(%))}	41 (75)	43 (80)	47 (87)	47 (86)	0.31
Diabetes duration _(years)	6.9 (2.8-11.9)	4.9 (2.7-8.1)	5.3 (1.9-11.9)	4.2 (2.2-9.9)	0.27
History of CVD _{(n(%))}	7 (13)	8 (15)	9 (17)	6 (11)	0.84
BMI _(kg/m²)	29.0 ± 4.5	29.7 ± 4.9	29.5 ± 5.0	29.4 ± 5.2	0.92
Systolic BP _(mmHg)	142.7 ± 19.0	137.2 ± 19.4	142.1 ± 21.3	144.9 ± 23.2	0.26
Current smoker _{(n(%))}	13 (24)	11 (20)	11 (20)	14 (25)	0.97
Total cholesterol _(mmol/L)	5.1 ± 1.2	5.2 ± 0.9	5.4 ± 1.4	5.1 ± 1.2	0.43
HDL _(mmol/L)	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.2	1.0 ± 0.3	0.04
HbA1c _(%)	7.6 ± 1.2	8.1 ± 1.8	8.1 ± 1.7	8.5 ± 1.9	0.04
eGFR _(ml/min/1.73m²)	109.5 ± 28.9	101.8 ± 17.6	104.9 ± 15.8	112.8 ± 29.7	0.08
CRP _(μg/mmol)	2.4 (1.3-4.3)	3.0 (1.7-6.0)	3.1 (1.9-6.2)	6.3 (3.0-10.2)	<0.01
Sclerostin	Q1	Q2	Q3	Q4	P _{trend}
Age _(years)	58.8 ± 6.4	57.5 ± 7.2	57.7 ± 6.4	57.8 ± 6.7	0.75
Women _{(n(%))}	50 (91)	45 (83)	42 (78)	41 (75)	
Diabetes duration _(years)	6.4 (3.3-10.3)	6.1 (2.7-11.2)	5.0 (1.5-9.1)	3.7 (2.2-7.7)	0.19
History of CVD _{(n(%))}	10 (18)	7 (13)	8 (15)	5 (9)	0.57
BMI _(kg/m²)	27.9 ± 4.9	30.2 ± 5.1	29.7 ± 4.3	29.8 ± 4.9	0.06
Systolic BP _(mmHg)	142.3 ± 21.7	141.3 ± 21.7	141.0 ± 21.3	142.3 ± 19.0	0.98
Current smoker _{(n(%))}	16 (30)	11 (20)	13 (24)	9 (16)	0.44
Total cholesterol _(mmol/L)	4.8 ± 1.0	5.0 ± 0.9	5.5 ± 1.3	5.5 ± 1.2	0.98
HDL _(mmol/L)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.3	0.80
HbA1c _(%)	7.8 ± 1.5	7.6 ± 1.4	8.4 ± 1.7	8.4 ± 1.9	0.03
eGFR _(ml/min/1.73m²)	107.6 ± 27.3	107.1 ± 18.4	101.7 ± 16.3	112.7 ± 30.6	0.13
CRP _(μg/mmol)	3.5 (1.0-5.7)	3.1 (1.6-6.6)	3.5 (2.2-7.1)	3.1 (2.0-6.5)	0.73

Data are shown as frequencies (%), mean ± standard deviation or median (interquartile range).

BMI = body mass index, HDL=high density lipoprotein, eGFR = estimated glomerular filtration, Hs-CRP = high sensitive C-reactive protein. Linear trend was tested with ANOVA or Chi-square test, as appropriate. Skewed variables (diabetes duration and CRP) were loge-transformed prior to analyses.



**Osteocalcin is not
associated with the risk of
type 2 diabetes: findings
from the EPIC-NL study**

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Abstract

Objective: To investigate whether total osteocalcin (tOC), uncarboxylated osteocalcin (ucOC) and percentage of uncarboxylated osteocalcin (%ucOC) are related to the risk of type 2 diabetes.

Methods: This nested case control study included 1,635 participants, 833 incident diabetes cases and 802 non-diabetic control participants, aged 21-70 years from the EPIC-NL cohort. Baseline concentrations of tOC, ucOC and %ucOC were assessed. During 10 year follow up, diabetes cases were self-reported and verified against information from general practitioners or pharmacists. The association between the different forms of osteocalcin and diabetes risk was assessed with logistic regression adjusted for diabetes risk factors (waist, age, sex, cohort, smoking status, family history, hypertension, alcohol intake, physical activity and education) and dietary factors (total energy intake and energy adjusted intake of fat, fiber, protein and calcium).

Results: TOC concentration was not associated with diabetes risk, with an odds ratio (OR) of 0.97 (0.91-1.03) for each ng/ml increment after adjustment for diabetes risk factors and dietary factors. No association between ucOC and %ucOC and the risk of diabetes was observed either. In sex stratified analyses ($P_{\text{interaction}}=0.07$), higher %ucOC tended to be associated with an increased risk of type 2 diabetes in a multivariable model in women (OR 1.05 for each increment of 5% ucOC (1.00-1.11), $P_{\text{trend}}=0.08$), but not in men (OR 0.96 for each increment of 5% ucOC (0.88-1.04)). When waist was replaced by BMI, none of the osteocalcin forms were associated with the risk of type 2 diabetes in the final model among both men and women.

Conclusions: Available evidence suggests that TOC, ucOC and %ucOC are not associated with the risk of type 2 diabetes. However, more large scale cohort studies are needed to clarify the presence of any association between the different forms of osteocalcin and the risk of type 2 diabetes.

Background

Osteocalcin is a vitamin K dependent protein, which is primarily known for its role in bone mineralization. Vitamin K is an essential co-factor for carboxylation of osteocalcin and carboxylation of total osteocalcin (tOC) increases the proportion of carboxylated osteocalcin (cOC) and lowers the proportion of uncarboxylated osteocalcin (ucOC)¹. Due to this essential role, percentage uncarboxylated osteocalcin (%ucOC) is used as marker for vitamin K status with high %ucOC indicating a low vitamin K status¹.

In recent years, an accumulating body of evidence has suggested that osteocalcin plays a role in the regulation of glucose metabolism². However, different studies ascribe this effect to different forms of osteocalcin. Mice studies indicate that ucOC is the active form in this respect. To date, different cross-sectional studies have investigated the association between tOC and diabetes risk, showing a pooled risk estimate of 0.23 (0.12-0.46) for the highest quartile compared to the lowest quartile³. However, only three prospective cohort studies were performed on this relation showing inconsistent results regarding the role of tOC⁴⁻⁶. The association between ucOC levels and the risk of type 2 diabetes also remains inconclusive^{2,7}.

Studies on the percentage ucOC are scarce. Only one small scale prospective study investigated the association between the ratio of ucOC with tOC and diabetes incidence in men (n=126), but this study lacked adequate power to detect significant associations⁵. With the exception of this small scale study, all studies measured the absolute level of different osteocalcin forms. But because the differing assays that measure the various forms are not standardized equivalently, they cannot be used to estimate %ucOC¹. The hydroxyl apatite (HAP) binding method first separates the carboxylated and uncarboxylated forms of osteocalcin. A subsequent competitive assay that recognized both cOC and ucOC, equivalently allows the calculation of %ucOC. Only one cross-sectional study used the HAP assay to investigate the association between osteocalcin and diabetes and showed that a higher %ucOC was associated with a higher risk of type 2 diabetes⁷.

To date, no prospective studies are performed investigating the association between %ucOC and incidence type 2 diabetes, using an adequate measurement to determine %ucOC. Furthermore, a recent published meta-analysis showed that, in prospective studies, the association between tOC and type 2 diabetes is inconclusive³. They also stated that large-scale prospective studies are needed to detect whether osteocalcin may be

useful in the prevention of diabetes. Therefore, this study aims to prospectively investigate the association between different forms of osteocalcin, tOC, ucOC and %ucOC as assessed by the HAP assay, and the risk of type 2 diabetes. We will also investigate whether %ucOC is an adequate marker of vitamin K intake.

Methods

Study population

This case-control study was nested within the EPIC-NL cohort, a prospective cohort of the two Dutch contributions (Prospect-EPIC and MORGEN-EPIC) to the European Prospective Investigation into Cancer and Nutrition (EPIC). The cohorts were set up simultaneously in 1993-1997. The Prospect-EPIC study includes 17,357 women between the ages 49 to 70 who were living in Utrecht and vicinity. The MORGEN-EPIC cohort consists of 22,654 adults aged 21-64 years who were selected from random samples of three Dutch towns; Amsterdam, Maastricht and Doetinchem. All participants provided written informed consent before study inclusion. The study complies with the Declaration of Helsinki and was approved by the Institutional Review Board of the University Medical Center Utrecht and the Medical Ethical Committee of TNO Nutrition and Food Research (MORGEN-EPIC). The design and rationale of EPIC-NL are described elsewhere⁸.

A nested case control study is a case control study in which only a subset of controls from the cohort are compared to the incident cases from the cohort. During 10 years follow up, 918 incident type 2 diabetes cases were documented. The control group (n=900) consisted of participants who were free from diabetes at the end of follow up. The control group was randomly drawn from the remainder of the baseline cohort excluding incident cases of type 2 diabetes. For this study, an equal amount of participants was drawn from the Prospect-EPIC as from the MORGEN-EPIC cohort. None of the participants were taking vitamin K antagonist medication. After exclusion of participants with missing osteocalcin levels (n=183), a total number of 1,635 participants remained for analysis (833 incident diabetes type 2 cases and 802 controls).

Osteocalcin

All participants donated a 30 ml non-fasting blood sample at baseline. TOC and ucOC were measured in serum samples with the hydroxyl apatite (HAP) binding assay. This HAP method is based on lower affinity of ucOC for hydroxyapatite compared to the cOC¹. The method has been used extensively and provides discrimination between the carboxylated

and uncarboxylated form. The total amount of osteocalcin was presented as tOC in ng/ml, ucOC in ng/ml and %ucOC. Detailed information of this measurement is described in the supporting information.

Type 2 diabetes

Three different sources were used to ascertain potential diabetes cases: self-report, linkage with hospital discharge registries and a self-administered urine dipstick test. The diagnosis diabetes was self-reported in follow-up questionnaires every three to five years. Participants were asked whether or not diabetes was diagnosed, in what year, by whom and which treatment they received. Secondly, among Prospect participants only, a urinary glucose strip test was sent out with the first follow-up questionnaire for detecting potential diabetes cases with glucosuria. Finally, diabetes was diagnosed based on linkage with the database of the Dutch Center for Health Care Information, which holds a standardized computerized register of hospital discharge diagnoses⁹. Potential diabetes cases detected with one of these methods were verified with questionnaires mailed to the general practitioner or pharmacist of the participant. For 89% of the potential diabetes cases, verification information was available. Finally, 72% were verified as having type 2 diabetes⁹.

Other measurements

At baseline, participants completed general questionnaires about demographic and lifestyle characteristics and risk factors for chronic diseases. Smoking status was categorized as current, past or never smoker. Family history of diabetes was defined as parents without diabetes or at least one parent with diabetes. Education was classified as low, middle and high education. Physical activity was measured with the validated Cambridge Physical Activity Index, and was categorized as inactive, moderately inactive, moderately active and active¹⁰. Hypertension was defined as systolic blood pressure above 140mmHg or diastolic blood pressure above 90 mmHg.

Dietary intake was assessed with a semi-quantitative validated food frequency questionnaire (FFQ). The FFQ estimates the average daily consumption of 178 foods during the year preceding enrolment. The Dutch food composition table (1996) was used to calculate nutrient intake. However, this table does not include information on the vitamin K content of foods. Therefore, the concentrations of phylloquinone and menaquinones in common Dutch foods were measured at the Biochemistry Laboratory,

Maastricht University¹¹. For other foods, published data were used to update the vitamin K database^{11–13}. In total, vitamin K contents of 260 foods were collected.

The FFQ has been validated against 12 monthly 24-hour dietary recalls in 121 individuals. The validity of phylloquinone against these 24-hour recalls was relatively low ($r=0.26$ and 0.19 in men and women respectively). The validity of menaquinones intake was reasonable with correlations coefficients of 0.60 and 0.48 in men and women. The intake of all nutrients was adjusted for total energy intake with the regression residual method¹⁴.

Statistical analyses

Baseline categorical data were presented as percentages and continuous data as means and standard deviation (SD) except for skewed variables, which were presented as medians and interquartile ranges. Since data were missing at random, missing values were multiple imputed with 5 imputations and combined according to Rubin's rules¹⁵. The imputation model included diabetes status, osteocalcin levels and all possible confounders as predictors of missing values.

We used logistic regression analysis to estimate odds ratio (ORs) of type 2 diabetes for quartiles of tOC, ucOC and %ucOC. Exposure variables were also modeled continuously to calculate the OR for type 2 diabetes by one ng/ml change in tOC and ucOC. For ease of interpretation, %ucOC is modelled per 5% increment of %ucOC. The selection for potential confounders was based on literature, and defined as confounder when it changed the association with more than 10%. We adjusted for type 2 diabetes risk factors and dietary factors in three separate models. The first model was adjusted for cohort, sex, age and waist circumference. In the second model we additionally adjusted for other diabetes risk factors: smoking status, physical activity, hypertension (yes/no), alcohol intake measured with the FFQ, family history of diabetes (yes/no) and level of education. In the third model, additional adjustments were made for dietary factors: total energy intake and energy adjusted intakes of saturated fat, mono- and polyunsaturated fat, fiber, protein and calcium.

P-values for linear trends were estimated by including the median value of each quartile as a continuous variable in the model. To explore the presence of non-linear associations, a quadratic term (the square of the median in the model) was added together with the linear term. In certain cases (model 1 of tOC and all models of %ucOC) this term was significant and the shape of the association was further explored using spline regression analyses.

Since spline regression analyses did not confirm a significant non-linear association ($p=0.18$) for any of the variables, we present the results for the quartiles and continuous analyses here. Effect modification by sex was checked for all forms of osteocalcin, by including interaction terms in the model. In case of a significant interaction ($p<0.05$), the analyses were stratified for this variable. Sensitivity analyses were performed to assess whether the associations changed when waist was replaced by BMI, because BMI is an important determinant of bone remodeling¹⁶. Finally, the association between dietary intake of vitamin K, measured with the FFQ, and %ucOC was assessed by Spearman correlation coefficients, to assess whether osteocalcin is a reliable marker for vitamin K intake. Furthermore we investigated the associations between the different forms of osteocalcin with Spearmann correlations (r_s). The software package SPSS (version 20 for Windows) and SAS 9.2 were used for statistical analyses and a two tailed p value of <0.05 was considered to be statistically significant.

Results

Baseline characteristics of diabetes cases and controls are shown in *Table 1*. Participants with diabetes had less favorable metabolic profiles compared to participants without diabetes. At baseline, those who developed diabetes during follow up had a higher BMI, waist circumference and more often had hypertension. Diabetes cases were less active compared with the controls and alcohol intake was higher in the control group compared to the diabetes cases.

Table 2 shows the association between different measures of osteocalcin (tOC, ucOC and %ucOC) and the risk of type 2 diabetes. TOC was not associated with the risk of type 2 diabetes in the three different models with an OR_{Q_4} of 0.97 (0.69-1.36) in the first model and an OR_{Q_4} of 1.02 (0.71-1.48) in the third model. Absolute levels of ucOC were not associated with risk of type 2 diabetes either (model 3, OR_{Q_4} 0.88 (0.61-1.27)). Furthermore, %ucOC was not associated with the risk of type 2 diabetes (model 3, OR_{Q_4} 1.09 (0.76-1.57)).

Because a borderline significant interaction term was observed between %ucOC and sex ($p=0.07$), these analyses were stratified by sex. In women, a higher %ucOC tended to be associated with an increased risk of type 2 diabetes ($P_{trend}=0.08$) with an OR_{Q_4} of 1.36 (0.89-2.09) in the multivariable adjusted model. In continuous analyses, each 5% increment of %ucOC was associated with an increased risk of diabetes with an OR of 1.05 (1.00-1.11). In men, no association between the %ucOC and the risk of type 2 diabetes was found.

Table 1. Baseline characteristics according to diabetes incidence after 10 year follow up of 1,635 Dutch adults participating in the EPIC-NL study.

Characteristics	Diabetes, n=833	Non-diabetes, n=802
Men (%)	20.8	24.9
Age (years)	57.0 (51.8-62.3)	50.5 (41.3-57.1)
BMI (kg/m ²)	29.7 ± 4.5	25.3 ± 3.8
Waist circumference (cm)	96.6 ± 11.4	84.4 ± 11.5
Physically inactive (%)	14.3	8.4
Current smoker (%)	26.2	31.8
Alcohol intake (g/day)	1.5 (0.1-10.5)	5.8 (0.7-16.9)
High education (%)	8.9	21.3
Family history of diabetes (%)	38.4	18.0
Postmenopausal (%)	58.2	36.3
Systolic BP (mmHg)	140.0 ± 21.9	124.7 ± 19.0
Diastolic BP (mmHg)	83.6 ± 10.5	71.0 ± 10.5
Hypertension (%)	68.3	34.3
Cholesterol (mg/ml)	5.9 ± 1.1	5.5 ± 1.0
TOC (ng/ml)	4.0 (2.9-5.5)	4.4 (3.2-5.7)
ucOC (ng/ml)	1.8 (1.2-2.7)	2.0 (1.3-2.9)
%ucOC	47.0 ± 15.8	46.9 ± 14.3
<i>Diet</i>		
Energy (kcal/day)	1815 (1533-2219)	1930 (1610-2375)
Vitamin K (μg/day)	227.0 (167-300)	204.2 (156-574)
Phylloquinone (μg/day)	194.3 (136-266)	173.9 (126-243)
Menaquinones (μg/day)	30.0 (22-39)	27.7 (20-37)
Vitamin C (mg/day)	110.6 ± 45	108.7 ± 46
Calcium (mg/day)	1120.6 ± 397	1058.4 ± 356
Fiber (g/day)	23.8 ± 5	23.4 ± 5
Protein (g/day)	79.4 ± 12	75.4 ± 11

Data are given as mean ± SD or median (interquartile range) or percentages.

All nutrients are measured with the FFQ and all values are energy adjusted except energy intake.

Waist circumference was the strongest confounder in the association between osteocalcin and type 2 diabetes. When waist was replaced by BMI, none of the osteocalcin forms were associated with risk of type 2 diabetes with an OR_{Q4} of 0.92 (0.64-1.32) for tOC, 0.84 (0.59-1.21) for ucOC and 1.05 (0.73-1.50) for %ucOC in the final model. The interaction between sex and osteocalcin was no longer significant (p=0.14).

The correlation between vitamin K intake and %ucOC was -0.05 (p=0.04), with similar results for phylloquinone (r_s = -0.05) and menaquinones (r_s = -0.05). The level of %ucOC was not correlated with tOC levels (r_s = 0.02, p=0.46) while the correlation between ucOC and TOC was 0.8 (p<0.001). The correlation between %ucOC and ucOC was 0.53 (p<0.001).

Table 2. Total osteocalcin, uncarboxylated osteocalcin and percentage uncarboxylated osteocalcin and the risk of type 2 diabetes among 1,635 Dutch men and women

Total osteocalcin						
	Q1	Q2	Q3	Q4	P _{trend}	Per one ng/ml
ng/ml	2.3 ± 0.5	3.7 ± 0.6	4.9 ± 0.4	7.5 ± 2.1		
Model 1	1.0	1.21 (0.87-1.69)	0.58 (0.49-0.69)	0.97 (0.69-1.36)	0.29	0.97 (0.92-1.02)
Model 2	1.0	1.25 (0.88-1.79)	0.60 (0.42-0.85)	0.95 (0.66-1.36)	0.24	0.96 (0.90-1.02)
Model 3	1.0	1.34 (0.93-1.93)	0.60 (0.42-0.86)	1.02 (0.71-1.48)	0.39	0.97 (0.91-1.03)
Uncarboxylated osteocalcin						
	Q1	Q2	Q3	Q4	P _{trend}	Per one ng/ml
ng/ml	0.9 ± 0.4	1.6 ± 0.2	2.3 ± 0.2	3.8 ± 1.2		
Model 1	1.0	0.83 (0.70-0.99)	0.91 (0.76-1.08)	0.83 (0.59-1.16)	0.39	0.96 (0.87-1.06)
Model 2	1.0	0.84 (0.59-1.20)	0.96 (0.67-1.37)	0.84 (0.58-1.20)	0.46	0.95 (0.86-1.06)
Model 3	1.0	0.89 (0.62-1.27)	1.01 (0.70-1.45)	0.88 (0.61-1.27)	0.64	0.97 (0.88-1.08)
Percentage uncarboxylated osteocalcin						
	Q1	Q2	Q3	Q4	P _{trend}	Per 5% increment
%	27.3 ± 6.0	41.8 ± 3.3	52.4 ± 2.9	66.2 ± 7.0		
Model 1	1.0	0.68 (0.57-0.81)	0.72 (0.61-0.86)	1.05 (0.75-1.46)	0.80	1.00 (0.97-1.05)
Model 2	1.0	0.70 (0.49-0.99)	0.76 (0.54-1.09)	1.07 (0.75-1.54)	0.69	1.01 (0.97-1.06)
Model 3	1.0	0.71 (0.50-1.02)	0.78 (0.55-1.12)	1.09 (0.76-1.57)	0.64	1.01 (0.97-1.06)

Data are Odds Ratio's (95%CI), Q= Quartile

Model 1: waist, sex, age and cohort adjusted.

Model 2: additionally adjusted for smoking status, family history, hypertension, alcohol, physical activity and education.

Model 3: additionally adjusted for total energy intake, and energy adjusted intakes of saturated fat, mono- and poly unsaturated fat, fiber, protein and calcium

Discussion

The purpose of this study was to investigate the association between osteocalcin forms and the risk of type 2 diabetes. TOC, ucOC and %ucOC were not associated with the risk of type 2 diabetes. Furthermore, %ucOC was not correlated with vitamin K intake from the FFQ.

Only four prospective studies investigated the association of different osteocalcin forms with diabetes incidence^{2,4-6}. For tOC, three prospective studies investigated the association between tOC and the risk of type 2 diabetes⁴⁻⁶. A meta-analysis of these prospective cohort studies showed a pooled results of 0.89 (0.78-1.01)³. Only one small scale study showed that higher tOC concentrations were associated with lower diabetes incidence (OR 0.90 (0.81-0.99))⁵. The reason for the discrepancy with our results is unclear. Our study is the first that included both men and women. When we stratified this association for sex,

we could not detect an association between tOC and the risk of type 2 diabetes among both women and men as well. It is therefore unlikely that this explains any differences with previous studies. The most likely explanations are the larger sample size and the different assays used. This is the first study using the HAP binding assay to measure osteocalcin levels. The different assays may have resulted in different baseline osteocalcin levels. Furthermore, this is the first large-scale study, while previous studies only consisted of 36 till 90 diabetes patients.

For absolute levels of ucOC, only one previous study of 459 men and women at high risk of cardiovascular disease showed that lower ucOC was associated with an increased risk of type 2 diabetes (OR 1.68 (1.09-2.59) T1 versus T3)². We could not detect an association of ucOC with diabetes risk, which is comparable to the study of Ngarmukos *et al*⁵. The inconsistency may be due to differences in study subjects because the association was only observed in participants at high cardiovascular risk.

However, because of the high correlation between absolute levels of tOC with cOC and ucOC, different assays to measure osteocalcin cannot distinguish the differences between the different carboxylated forms. The study of Ngarmukos *et al.*, of 63 cases and 63 controls explored the relationship between the ratio of ucOC/tOC, reflecting %ucOC, with the risk of type 2 diabetes⁵. However, this study was too small to estimate effects about ucOC/tOC ratio and the risk of type 2 diabetes⁵. This study also used two different assays to measure ucOC and tOC. Because these measurements are not standardized equivalently, it is not an adequate measurement to estimate %ucOC¹. Only the cross sectional study by Yeap *et al.* used the HAP assay and showed a borderline significant association between ucOC/tOC ratio and type 2 diabetes risk (OR 1.11, $p=0.06$)⁷. However, the study of Yeap *et al.* is cross-sectional and only included men. Since our study is the first investigating the association between %ucOC and diabetes risk in women, we are not able to compare the borderline significant association with previous studies.

Several explanations could play a role in the different observations among men and women in our study. Since these results were only apparent adjusted for waist circumference, it could be driven by central adiposity. Waist circumference is a better measure of central obesity compared to BMI and is more strongly associated with diabetes incidence among women than men¹⁷. TOC was moderately associated with waist ($r_s=-0.36$) and BMI ($r_s=-0.23$) in men, but not in women ($r\approx-0.09$). However, %ucOC was not associated with waist circumference or BMI in both men and women ($r\approx-0.05$), which makes it unlikely to explain

our findings. Furthermore, our study consisted of only 22.9% men, giving less power to detect an association in men than women. However, when waist was replaced by BMI, the borderline significant association between %ucOC and diabetes incidence in women disappeared and no association between any of the osteocalcin forms and diabetes incidence was present in both men and women. This makes it likely that the borderline significant association for women could simply be a chance finding.

Previous studies suggested %ucOC as a marker of vitamin K intake, but we could not confirm these findings. The correlation between %ucOC and vitamin K intake in our study was much lower than in previous studies, which showed correlations of -0.21 and -0.33 respectively^{18,19}. However, in these studies vitamin K intake was measured with 3 day food- records before the day of blood examination^{18,19}. The validity of the FFQ may explain the difference between our study and previous studies. The relative validity of menaquinones based on the FFQ was reasonable ($r=0.60$ and 0.48 in men and women), but for phylloquinone the validity was relatively low ($r=0.26$ and 0.19 in men and women respectively). This may explain the low correlation between phylloquinone intake and osteocalcin forms, but not for menaquinones. Another explanation could be genetic variation in vitamin K metabolism. A previous study showed that the correlation between menaquinone-7 intake and ucOC and tOC ratio differed by polymorphisms of the gamma glutamyl carboxylase gene¹⁸.

The strengths of this study include its prospective design, large sample size, and verification of diabetes cases and the use of % ucOC. Most studies do not differentiate between the total and uncarboxylated forms of osteocalcin and use absolute levels. UcOC is highly correlated with tOC ($r=0.8$). Since tOC is dependent of osteoblast activity, it is difficult to differentiate the osteoblastic effect of osteocalcin from the role of the vitamin K dependent carboxylation in these studies. For this reason, %ucOC is preferred as marker for vitamin K intake^{1,21}. Besides the strengths, this study also has some limitations which need to be addressed. We were able to correct for many diabetes risk factors but as in many observational studies, residual confounding may be present. Second, osteocalcin levels and diabetes risk factors are measured at baseline, therefore it was not possible to evaluate whether diabetes risk factors changed overtime.

In summary, this study suggests that TOC, ucOC and %ucOC are not associated with the risk of type 2 diabetes. However, more large scale cohort studies are needed to clarify

the presence of any association between the different forms of osteocalcin and the risk of type 2 diabetes.

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Disclosures

None.

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Supplementary information

The hydroxyl apatite (HAP) method is based on lower affinity of ucOC for hydroxyapatite compared to the cOC. To determine the relative amount of ucOC in an individual serum sample, 100 μ L unknown serum is added to 100 μ L of a 3 mg/mL suspension of hydroxyapatite in Tris buffer. The tubes are shaken vigorously for 1 h at room temperature and then centrifuged for 2 min in a microfuge (10,000 $\times$ g). The supernatant is removed, and the amount of unbound osteocalcin measured by RIA as described below.

TOC and ucOC are then measured by a standard equilibrium radioimmunoassay. Assay buffer consists of 0.01 M NaH_2PO_4 , 0.125 M NaCl, 0.1% Tween, 0.025 M Na_4EDTA , and 0.1% bovine serum albumin, pH 7.4. All assays consist of specific antiserum 1:10,000 final dilution, carrier non-immune serum, 25 μ L of a known concentration of standards ranging from 1 ng/ml to 75 ng/ml in triplicate, unknown serum samples, hydroxyapatite supernatant and quality control samples in duplicate, and 2×10^4 cpm of ^{125}I - osteocalcin in a final volume of 0.50 ml. The mixture is incubated for 18-24 hours at room temperature and terminated by precipitation with donkey anti-rabbit IgG antiserum (Pelfreeze, Rogers AR). Bound fractions are counted in a Beckman automated gamma counter linked to a computer. The B/B_0 ratio of each standard and sample is corrected for non-specific binding. Assay characteristics are computer derived for each assay. Inter- and intra-assay coefficients of variation are 6.7 % and 3.2% respectively. %ucOC is calculated from measured tOC and ucOC.



Circulating phylloquinone concentrations and risk of type 2 diabetes: a Mendelian Randomization study

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Abstract

This study aims to investigate the causal relation between circulating phylloquinone (vitamin K₁) concentrations and type 2 diabetes using a Mendelian Randomization (MR) approach. We used data from three cohorts: the European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct case-cohort study, Diabetes Genetics Replication and Meta-analysis (DIAGRAM) and the UK Biobank, resulting in 69,647 type 2 diabetes cases. We calculated a weighted genetic risk score including four genetic variants previously found to be associated with circulating phylloquinone concentrations. Inverse-variance weighted analysis was used to obtain a risk ratio (RR) for the causal relation between circulating phylloquinone concentrations and risk of type 2 diabetes. Presence of pleiotropy and the robustness of the results were assessed using MR-Egger and weighted-median analyses. Genetically-predicted concentrations of circulating phylloquinone was associated with lower risk of type 2 diabetes with a RR of 0.93 (95% confidence interval: 0.89;0.97) per every ln-nmol/L unit increase in circulating phylloquinone. The MR-Egger and weighted median analyses showed RRs of 0.94 (0.86;1.02) and 0.93 (0.88;0.98), respectively, indicating no pleiotropy. In conclusion, our study supports that higher circulating phylloquinone may be causally related with lower risk of type 2 diabetes, highlighting the importance of sufficient phylloquinone in the human diet.

Background

Vitamin K is a fat-soluble vitamin occurring in two biologically active forms: phyloquinone (vitamin K₁) and menaquinones (vitamin K₂). Phyloquinone is the most common form in circulation, and is predominantly present in green leafy vegetables¹. Observational studies showed that higher phyloquinone intake was associated with a lower risk of type 2 diabetes^{2,3}. Intervention studies also showed improved insulin sensitivity following phyloquinone supplementation, although the results were inconsistent^{4–6}. Moreover, these studies did not use the gold standard (hyperinsulinemic-euglycemic clamp) to measure insulin sensitivity^{4–6}. The underlying mechanism still is unknown, but vitamin K-dependent carboxylation of osteocalcin and a possible anti-inflammatory effect of vitamin K are suggested^{7,8}.

Current evidence thus points towards a beneficial role for phyloquinone the prevention of type 2 diabetes, but the study designs used each have certain limitations. Observational studies may be hampered by reverse causation or confounding⁹, as phyloquinone co-exists with vitamins and phytochemicals of vegetables, which are likely to affect the risk of non-communicable diseases in diverse pathways⁷. Intervention studies are limited by having a short duration and focus on intermediate risk factors for type 2 diabetes, such as insulin sensitivity, instead of risk of type 2 diabetes. Due to these limitations, the causal association between phyloquinone and type 2 diabetes remains debatable.

To date, no study assessed the association between circulating phyloquinone concentrations and type 2 diabetes using a Mendelian Randomization (MR) approach. Therefore, this study aimed to investigate the causal relation between circulating phyloquinone concentrations and type 2 diabetes using a MR approach in which we used a set of genetic variants as an instrumental variable.

Methods

Study populations

For these analyses, we used data from three studies: European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct case cohort study, DIAGRAM and the UK Biobank. Individual participant data was only available in EPIC-InterAct. EPIC-InterAct is a prospective case-cohort study nested within EPIC, involving 8 European countries (Denmark, France, Germany, Italy, the Netherlands, Spain, Sweden and the United

Kingdom)¹⁰. EPIC-InterAct identified 12,403 incident cases of type 2 diabetes between 1991 and 2007 and a randomly selected a subcohort of 16,154 participants¹⁰. After exclusion of individuals with missing genetic data (n=2,332 cases and n=3,115 subcohort members), excluding 1,528 participants from EPIC-Norfolk (which contributes to DIAGRAM), 9,400 cases and 12,182 subcohort members were left for analyses (this subcohort also included a set of 598 incident cases reflecting the case-cohort design). The mean age in the subcohort was 52.4 (SD: 9.2) years, 38.4% were men, and mean BMI was 26.0 kg/m².

Summarized data were included from DIAGRAM and the UK Biobank. The aim of DIAGRAM is to characterize genetic information for type 2 diabetes, mainly in samples of participants of European descent. DIAGRAM included data from 23 studies, which meta-analyzed genetic variants on Metabochip¹¹. DIAGRAM included both men and women, mainly of European ancestry, aged 43 to 72 years with BMIs varying from 22.3 to 33.4 kg/m² among the case-control or cohort participants. Data are publicly available at www.diagram-consortium.org.

The UK Biobank is a large, population-based cohort study established to study the interrelationships between environment, lifestyle and genes. The UK Biobank (www.ukbiobank.ac.uk) recruited over 500,000 men and women between 2006 and 2010 (application number 9161, 9905, 12885, 20684)¹². Age varied between 37 to 73 years, and the mean BMI was 27.5 (SD:4.8). The UK Biobank was approved by the North West Multi-Centre Research Ethics Committee and all participants provided written informed consent to participate in the UK Biobank study. EPIC-InterAct, DIAGRAM and the UK Biobank included up to 69,647 type 2 diabetes cases and 551,336 non-diabetic participants.

Genotyping and construction of weighted genetic risk score

For EPIC-InterAct, DNA was extracted from buffy coat from citrated blood samples. Participants were genotyped with the Illumina Human Core Exome chip and the Illumina Human Quad 660 chip. Missing genotypes were imputed by the Haplotype Reference Consortium v1.0, using IMPUTE v2.3.2, and was performed at the Wellcome Trust Centre for Human Genetics. Genotyping in the UK Biobank was performed using the Affymetrix UK BiLEVE Axiom array and the Affymetrix UK Biobank Axiom Array¹³. DIAGRAM participants are genotyped using different assays, as described previously¹⁴.

We included 4 SNPs selected out of 11 SNPs that were likely to predict circulating phylloquinone (plasma or serum) among European descents in a genome-wide meta-

analysis study ($n=2,138$) (rs964184, rs4645543, rs2199565, rs7018214, rs2108622, rs2192574, rs4852146, rs6862909, rs6862071, rs4122275): these SNPs passed the threshold of $p<5\times 10^{-6}$, but not $p<5\times 10^{-8}$ ¹⁵. Among the 11 SNPs, linkage disequilibrium at $R^2>0.9$ was evident among rs2192574 and rs4852146 (chromosome 2); rs6862909, rs6862071 and rs4122275 (chromosome 5); rs4645543, rs2199565 and rs7018214 (chromosome 8); and rs2108622 and rs12609820 (chromosome 19). Therefore, five SNPs with the strongest association with circulating phylloquinone from each chromosome were initially selected (rs2108622, rs2192574, rs4645543, rs6862071 and rs964184). We further excluded rs964184 with pleiotropic effects on blood lipids and coronary heart disease by searching GWAS catalog and Phenoscanner for the SNPs or its proxies ($R^2>0.8$) and querying associations of the SNPs with variables indicating pleiotropic effects with $p<5\times 10^{-8}$ ^{16,17}. After these exclusions, rs2108622, rs2192574, rs4645543 and rs6862071 were retained for the present study.

In all three cohorts, we calculated a weighted genetic risk score (wGRS) by summing the number of effect alleles coded as 0, 1, or 2, at each individual SNP weighted by the respective allelic effect sizes on circulating phylloquinone concentrations from the previous genome-wide meta-analysis study¹⁵.

Diabetes ascertainment

For EPIC-InterAct, a detailed description of ascertainment and verification of incident diabetes cases has been described previously¹⁰. Briefly, occurrence of type 2 diabetes during follow up was identified through self-report, linkage to primary care registers, secondary care registers, medication use, and hospital admissions and mortality data. Type 2 diabetes was determined when at least two sources identified the diagnosis type 2 diabetes. Diagnosis of type 2 diabetes in DIAGRAM differed per study, as described previously¹⁴. Type 2 diabetes diagnosis in the UK Biobank was self-reported at baseline, as previously described¹⁸.

Statistical analysis

Individual-participant data analysis was performed in EPIC-InterAct. Accounting for its case-cohort design, Prentice-weighted Cox regression¹⁹ was used to quantify the association of the individual SNPs and the wGRS with type 2 diabetes risk. Age was used as an underlying timescale. The associations were evaluated in strata of countries and adjusted for sex, study sites, principal components of ancestry and genetic array. For each of the individual SNPs and the wGRS, we combined the hazard ratios obtained from EPIC-InterAct with odds ratios from DIAGRAM and UK Biobank as risk ratios (RRs)

using a random effect meta-analysis. Then, inverse-variance weighted (IVW) estimates for the causal association between circulating phylloquinone and type 2 diabetes risk were calculated using the formulae reported by Burgess *et al.*²⁰. The ratio estimate of the causal effect of circulating phylloquinone on diabetes risk is calculated as the association between the SNP and diabetes risk divided by the association between the SNP and circulating phylloquinone. The IVW estimate combines the ratio estimates using each variant in a fixed-effect meta-analysis model²¹. We quantified the F-statistic to assess the strength of the genetic instrument, using the proportion of variance explained by the genetic variants, the sample size and number of variants included²².

To assess presence of unbalanced pleiotropy, we performed a MR-Egger and weighted-median analysis in all cohort studies using the ‘Mendelian Randomization’ package in R^{23–25}. Unbalanced pleiotropy was considered present if the intercept significantly deviated from zero ($p < 0.05$). In the subcohort of EPIC-interact only, we assessed potential pleiotropic effects of the wGRS on demographic, lifestyle, and physiological variables using logistic and linear regression analyses in strata of countries and adjusted for sex, center, principal components and genetic array.

Phylloquinone is carried predominantly in triglyceride-rich lipoproteins and in the previous genome-wide association study, beta coefficients for the gene-phylloquinone associations adjusted for fasting triglycerides were also reported¹⁵. Therefore, we performed sensitivity analyses, in EPIC-InterAct data only, using the beta-coefficients adjusted for triglycerides and corrected the association between the wGRS and diabetes incidence for triglycerides and hours of fasting. Missing values on triglycerides (5.1%) and hours of fasting (32.3%) were imputed with predictive mean matching. We used a maximum of 50 iterations and 10 imputations using the R package ‘mice’²⁶. This analysis was also performed in the complete case dataset to assess the influence of the large number of missing values for hours of fasting. Finally, we calculated ORs in EPIC-InterAct and pooled these with the ORs in DIAGRAM and UK Biobank, instead of combining the HR in EPIC-InterAct with OR in DIAGRAM and UK Biobank. All analyses were performed using R Software (version 3.1.1).

Results

For EPIC-InterAct participants only, baseline characteristics are described in *Supplementary Table 1*, and the distributions of the alleles of each phylloquinone-related SNP are shown per country in *Supplementary Table 2*. We did not detect any association

between genetically predicted circulating phylloquinone and possible pleiotropic traits (*Supplementary Table 3*).

The IVW analyses showed that higher genetically-predicted concentrations of circulating phylloquinone were associated with a lower risk of type 2 diabetes in EPIC-InterAct, with a RR of 0.88 (95%-CI: 0.78;0.99) per ln-nmol/L increase in circulating phylloquinone (*Figure 1*). Similar results were observed in DIAGRAM (RR 0.90 (95%-CI: 0.81;1.00)) and UK Biobank (RR 0.95 (95%-CI: 0.91;1.00)). Pooling the three datasets (EPIC-InterAct, DIAGRAM and UK Biobank) resulted in a RR for the wGRS of 0.93 (95%-CI: 0.89;0.97) per ln-nmol/L increase in circulating phylloquinone. There was no heterogeneity between the studies ($I^2=0.3\%$, $p=0.96$). No association was observed for the individual SNPs; however, the directions of genetic associations with the outcome were consistent across the four SNPs, and there was some suggestion of a dose-response relationship in the genetic associations with the risk factor and outcome.

The included SNPs explained 4.7% of the variation in circulating phylloquinone in the GWAS, resulting in an F-statistic of 208.9 in our study. The MR-Egger method indicated no pleiotropic effects (intercept=-0.02, SE=0.01, p-value=0.88) and resulted in a RR of 0.94 (95%-CI: 0.86;1.02) per ln-nmol/L in circulating phylloquinone (*Supplementary Figure 1*). The weighted median method resulted in a RR of 0.93 (95%-CI: 0.88;0.98) per ln-nmol/L in circulating phylloquinone.

In EPIC-InterAct data only, additional adjustments for triglycerides and hours of fasting did not alter the results, and resulted in a HR for the wGRS of 0.87 (95%-CI: 0.78;0.98) in the IVW analyses (*Supplementary Table 4*). Similar results were observed in a complete cases analysis (HR wGRS 0.82 (95%-CI: 0.71;0.95)). Finally, calculating OR in EPIC-InterAct resulted in comparable pooled results (OR wGRS 0.93 (95%-CI: 0.89;0.98)).

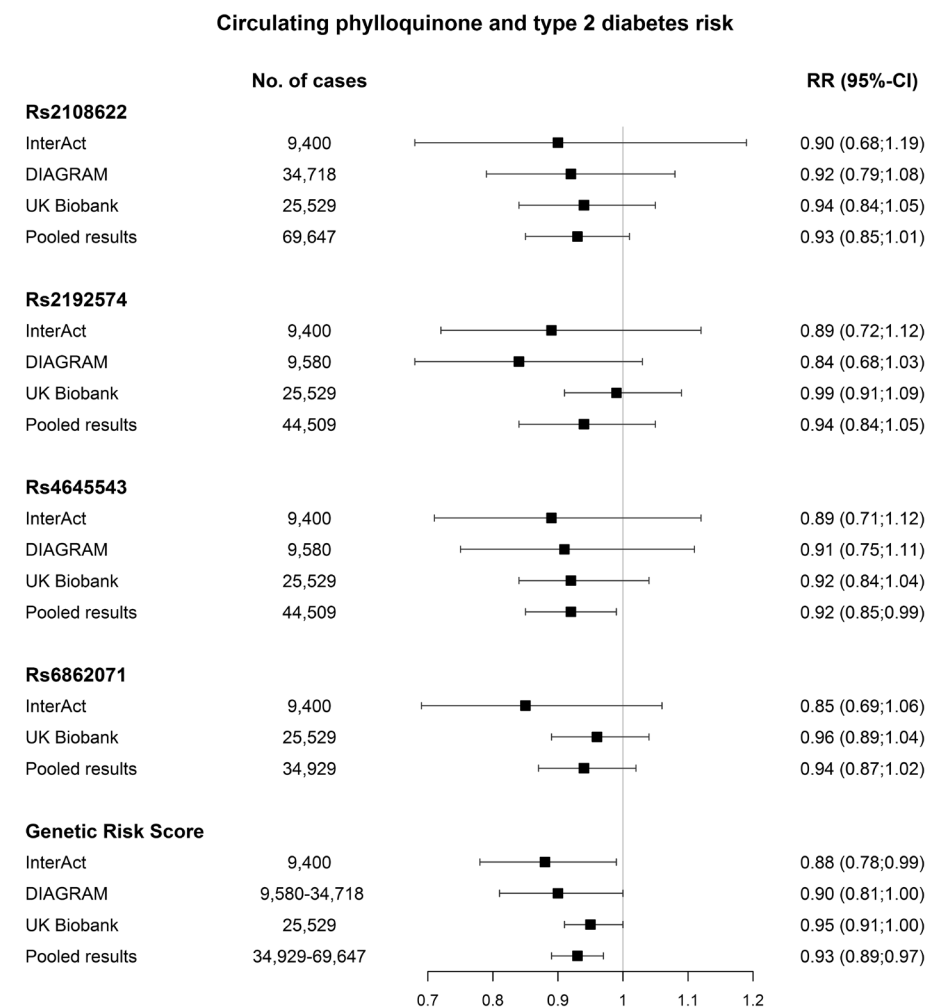


Figure 1. Causal estimates of circulating phylloquinone and diabetes risk: the Mendelian Randomization analyses in EPIC-InterAct, DIAGRAM and UK Biobank. Weighted genetic risk score (wGRS) represents the summary genetic effect of the four variants. Relative risks (RR) (95% confidence interval, CI) were per 1 unit increase in natural log of circulating phylloquinone (nmol/L).

Discussion

Our study supports that higher circulating phyloquinone may be causally related with lower type 2 diabetes risk.

This is the first MR study linking circulating phyloquinone concentrations to the risk of type 2 diabetes. Our results are in line with the current evidence on this association from observational and intervention studies. Two observational studies found that higher phyloquinone intake was associated with a lower type 2 diabetes incidence^{2,3}. Current evidence from trials are not conclusive on the effect of phyloquinone supplementation on insulin sensitivity and insulin resistance⁴⁻⁶. Two studies observed improved insulin sensitivity after phyloquinone supplementation^{4,6}, after 4 weeks intervention⁶ or after 3 years intervention⁴. By contrast, another small-scale study did not find improved insulin sensitivity after 1 year phyloquinone supplementation⁵. While the inconsistency should be acknowledged, altogether, the available evidence from MR, observational studies, and trials supports a causal relation between higher circulating phyloquinone concentrations and a reduced risk of type 2 diabetes. This evidence highlights the need of sufficient phyloquinone in the diet, and supports the further development of phyloquinone requirements. However, the mechanism is still unknown, and it is possible that the causation is due to a biological pathway retaining high phyloquinone concentrations but not due to phyloquinone itself. Furthermore, since phyloquinone concentrations and phyloquinone intake are only modestly correlated ($r=0.51$)²⁷, our results cannot be directly translated to phyloquinone intake. Nonetheless these results highlight the need for further work relating phyloquinone metabolism to the etiology of type 2 diabetes.

MR studies only provide evidence about the causality of an association when three assumptions are met. We will discuss each assumption with its strengths and limitations; the first assumption implies a strong association between the instrumental variable (wGRS) and the risk factor, in our case circulating phyloquinone. None of the SNPs selected for this study reached genome-wide significance in phyloquinone genome-wide meta-analysis study¹⁵. Rs2108622 is a *CYP4F2* variant, which functions as a phyloquinone oxidase²⁸. Previous studies showed that rs2108622 was associated with altered phyloquinone metabolism and warfarin dosage²⁹. Because of the biologically plausible effect of rs2108622 on circulating concentrations of phyloquinone, we considered this functional SNP causally altering circulating phyloquinone. However, this genetic variant did not have a genome wide significant association with phyloquinone concentrations in the GWAS¹⁵.

Presumably, this was due to the relatively small sample size ($n=2,138$) of the meta-analysis and the low minor allele frequency¹⁵. While the F-statistic showed no indication of weak instrument bias (288.2 in EPIC-InterAct) and the included SNPs explained 4.7% of the variation in circulating phylloquinone in the small-scale GWAS, the F-statistic might be an overestimation of the true effect, since there might be an overestimation of the R^2 of the GWAS, due to winners curse resulting in effect size estimates that are upwardly biased³⁰. Although the F-statistic might be an overestimation, there is still no indication of weak instrument since the F-statistic is much higher than the arbitrary threshold of 10. The wGRS has not been validated in an external dataset, because no large scale cohort studies performed a GWAS and assessed circulating phylloquinone levels apart from those included in the GWAS by Dashti *et al.*¹⁵. Because of this lack of validation and because none of the SNPs reached genome wide significance, our findings need to be interpreted with caution. Additional GWAS data on circulating phylloquinone or other markers of vitamin K status are warranted to further investigate causal roles of vitamin K and its metabolic pathway in the development of type 2 diabetes.

The second and third assumption comprises no association between the instrumental variable and outcome via confounders or other mechanisms. The MR-Egger and the linear and logistic regression analyses showed no indication of pleiotropy. Still, we note that rs2108622 is a variant in the *CYP4F2* gene, may play a role in transport and metabolism of vitamin E³¹. Notably, a trial of vitamin E showed no evidence of its effect on type 2 diabetes³², while vitamin E may antagonize vitamin K³³. Whereas we found no evidence of pleiotropy in the genetically predicted effect of vitamin K on diabetes risk, both genetics and the trial support the causal role of vitamin E in vitamin K metabolism and this nutrient-nutrient interaction is of further interest also in etiology of type 2 diabetes. Although we did not find any indication of pleiotropy, circulating phylloquinone may be a biomarker that reflects healthy lifestyle in general instead of phylloquinone specifically.

In conclusion, this study supports that higher circulating phylloquinone concentrations are causally related with lower type 2 diabetes risk, highlighting the importance of sufficient amounts of phylloquinone in the human diet.

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and IS wrote the manuscript. SLB, SB, HD, FI, EF contributed to discussion and reviewed/edited manuscript.

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Disclosures

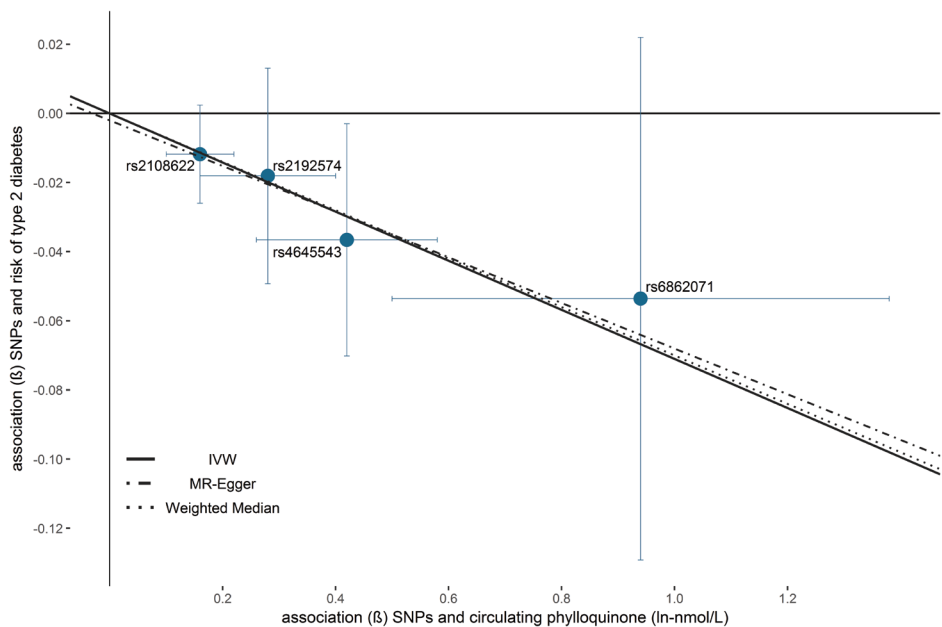
None.

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Supplementary information



Supplementary figure 1. Associations of genetic effects on circulating phylloquinone and genetic effects on risk of type 2 diabetes (β) in EPIC-InterAct, DIAGRAM and the UK Biobank. The dots represent the four SNPs with 95% confidence intervals. Solid line for Inverse-variance weighted (IVW) Mendelian randomization (MR) and MR-Egger were superimposed; and dotted line represent estimate from weighted median MR.

Supplementary table 1. Baseline characteristics of incident type 2 diabetes cases (n=9,400) and a subcohort of random representative participants from the EPIC-InterAct project (n=12,182) of 8 European countries.

	Cases (n=9,400)	Subcohort (n=12,182)
Age at recruitment, years	55.2 ± 7.4	52.0 ± 9.0
Men	50.6	61.8
BMI kg/m ²	29.8 ± 4.8	26.0 ± 4.2
Current smoker	29.1	27.1
Low education level	48.6	60.0
Physical inactivity	38.2	44.3
Hypertension	38.3	18.8
Systolic blood pressure, mmHg	144.1 ± 20.7	132.9 ± 19.9
Diastolic blood pressure, mmHg	87.5 ± 11.2	81.9 ± 11.0
HbA1c, % (mmol/mol)	6.1% ± 3.1 (43.4 ± 10.5)	5.5% ± 2.6 (36.3 ± 5.0)
Hyperlipidaemia	28.0	17.8
Triglycerides, mmol/L	1.7 (1.2-2.4)	1.1 (0.8-1.60)
Total cholesterol, mmol/L	6.2 ± 1.2	5.9 ± 1.1
HDL, mmol/L	1.2 ± 0.4	1.5 ± 0.4
LDL, mmol/L	4.0 ± 1.0	3.8 ± 1.0
Average alcohol intake, grams/day	6.4 (0.7-20.2)	6.7 (1.0-19.2)
Weighted genetic risk score of the phylloquinone-associated SNPs	2.81 ± 0.3	2.82 ± 0.2

Normally distributed variables are shown as mean ± SD, non-normally distributed variables are shown as median (IQR) and categorical variables are shown as %.

Supplementary table 2. Distribution per country of the 4 different phyloquinone-associated SNPs, used for the weighted genetic risk score in EPIC-InterAct.

	Rs2192574				Rs6862071				Rs4645543				Rs2108622			
	CC	CT	TT	AA	AT	TT	CC	CT	TT	CC	CT	TT	TT	TC	TC	CC
France	1.4%	21.8%	76.8%	98.2%	1.8%	0%	91.9%	7.5%	0.6%	10.0%	41.1%	48.9%				
Italy	0.8%	17.2%	82.0%	97.3%	2.8%	0%	94.5%	5.4%	0.1%	12.7%	44.3%	43.0%				
Spain	1.3%	18.8%	79.9%	96.5%	3.5%	<0.1%	94.5%	5.4%	0.1%	12.7%	46.5%	40.8%				
United Kingdom	1.4%	22.1%	76.5%	97.3%	2.7%	<0.1%	90.3%	9.5%	0.2%	8.7%	42.4%	48.9%				
Netherlands	1.5%	21.9%	76.6%	97.6%	2.4%	0%	90.3%	9.6%	0.1%	7.3%	39.8%	52.9%				
Germany	1.4%	21.3%	77.3%	98.1%	1.9%	0%	90.9%	9.1%	<0.1%	6.7%	37.1%	56.2%				
Sweden	1.5%	21.7%	76.8%	98.2%	1.8%	0%	87.8%	11.7%	0.5%	6.7%	38.1%	55.2%				
Denmark	1.7%	22.3%	76.0%	98.3%	1.7%	0%	88.7%	11.0%	0.3%	6.2%	37.4%	56.4%				

Supplementary table 3. Associations of a weighted genetic risk score based on 4 phylloquinone-associated SNPs with baseline covariates in the EPIC-InterAct subcohort.

Variable	Beta (95%CI)
Waist, cm	-0.43 (-1.19;0.34)
BMI, kg/m ²	-0.15 (-0.44;0.14)
Systolic blood pressure, mmHg	-1.39 (-2.89;0.10)
Diastolic blood pressure, mmHg	-0.39 (-1.26;0.48)
Triglycerides, mmol/L	0.02 (-0.04;0.09)
Total cholesterol, mmol/L	0.01 (-0.07;0.08)
HDL cholesterol, mmol/L	0.00 (-0.03;0.03)
LDL cholesterol, mmol/L	0.00 (-0.07;0.07)
Daily alcohol intake, grams/day	-0.45 (-1.69;0.81)
Variable	Odds ratio (95%CI)
Education level (<i>higher vs lower</i>)	1.01 (0.97;1.04)
Physical active (<i>active vs inactive</i>)	1.00 (0.97;1.03)
Hypertension (<i>yes vs no</i>)	0.99 (0.96;1.02)
Hyperlipidemia (<i>yes vs no</i>)	1.00 (0.97;1.04)
Smoking status (<i>current vs former/never</i>)	1.00 (0.97;1.03)

The beta's (95%CI) for the continuous variables are obtained from linear regression.

The odds ratios for the categorical variables are obtained from logistic regression.

Models are adjusted for age, gender, genetic principal components, genetic array, study site and analyzed in strata of country.

The estimates for the variables are per 1 unit increase in natural log of circulating phylloquinone (nmol/L).

Supplementary table 4. Result of the IVW analyses for the association between four SNPs for circulating phylloquinone, the weighted genetic risk score and incident type 2 diabetes in EPIC-InterAct, in the imputed dataset (n=9,400) and complete-case dataset (n=5,806).

	HR (95%-CI) imputed dataset (n=9,400 cases)	HR (95%-CI) complete case (n= 5,806 cases)
Rs2192574	0.96 (0.76 ; 1.20)	0.91 (0.68-1.23)
Rs6862071	0.85 (0.72 ; 1.01)	0.84 (0.66-1.05)
Rs4645543	0.81 (0.62 ; 1.05)	0.77 (0.54-1.10)
Rs2108622	0.78 (0.59 ; 1.04)	0.70 (0.48-1.01)
wGRS	0.86 (0.77 ; 0.96)	0.82 (0.71-0.95)

Adjusted for sex, study sites, principal components, genetic array, triglycerides and hours of fasting. HR (95%CI) represents the association per 1 unit increase in natural log of circulating phylloquinone (nmol/L).



General discussion

This thesis aimed to investigate the role of vitamin K in the progression of vascular calcification, the role of vitamin K in intimal and medial calcification, and whether vitamin K (causally) reduces cardiovascular disease (CVD) and diabetes risk. In this chapter, the findings are presented in a broader context and the implications for future research are discussed.

Findings from this thesis

Observational evidence points towards a beneficial role for menaquinone intake, but not phyloquinone, in reducing CVD risk (*chapter 3*). The effect of menaquinones seems causal according to our Mendelian randomization study (*chapter 4*). We found a causal relationship between dephosphorylated uncarboxylated Matrix Gla Protein (dp-ucMGP) and lower coronary heart disease (CHD) risk, and dp-ucMGP reflects both phyloquinone and menaquinone intake. No relationship was found for circulating phyloquinone, which reflects phyloquinone intake only. Overall these findings may point towards the importance of menaquinones in reducing CVD risk. In contrast, our clinical trial was unable to support the hypothesis that menaquinone-7 supplementation reduces vascular calcification (*chapter 6*), which could partly be explained by the difference in presence of calcification at baseline. This was much higher in the menaquinone-7 than placebo group, despite randomization. In this trial we measured vascular calcification with ^{18}F -NaF PET activity, but its association with CVD risk is unknown.

Vascular calcification is highly prevalent, and there is often a predominant calcification type, intimal or medial calcification, present in the lower extremity. These calcification patterns have different risk factor profiles (*chapter 7*). Medial calcification is suggested to reflect a process of bone formation. In patients with type 2 diabetes, higher osteopontin levels were associated with an increased CVD risk, but no association was found for other bone markers (osteocalcin, osteonectin, osteoprotegerin, alkaline phosphatase and sclerostin) (*chapter 8*).

Vitamin K may also reduce diabetes risk by activating the vitamin K dependent protein osteocalcin. Although we were not able to detect an association between inactive osteocalcin and type 2 diabetes risk (*chapter 9*), our Mendelian Randomization study suggest that high circulating phyloquinone levels are causally associated with a reduced type 2 diabetes risk (*chapter 10*).

Methods to measure vitamin K

Observational studies mostly rely on food frequency questionnaires (FFQ) to estimate dietary vitamin K intake because it is efficient in time and cost. The FFQ is suitable to rank participants in macro-nutrient intake but its value is more limited for single nutrients. For vitamin K, the EPIC-NL FFQ has a relatively low validity to estimate phyloquinone intake (*chapter 2*), while the validity for menaquinone intake is relatively good. However, menaquinones are also produced by gut bacteria, but this intestinal production is not taken into account using dietary questionnaires.

Several biomarkers for vitamin K status exist: circulating phyloquinone responds well to changes in dietary phyloquinone intake, 4-day weighed food records showed a good validity ($r=0.51$) with circulating phyloquinone levels¹. However, the concentration peak is 6-10 hours post-prandially¹⁻³ and thus circulating phyloquinone only reflects recent intakes. Menaquinones are generally not detected in circulation, unless supplements are taken or large quantities of menaquinone-rich foods (such as Natto) are consumed⁴.

Additionally, insufficient vitamin K intake results in inactive fractions of several vitamin K dependent proteins. Uncarboxylated prothrombin, also known as PIVKA-II, responds to dietary vitamin K depletion². The sensitivity is low for detecting normal vitamin K intake and therefore this measurement is not suitable to rank individuals in population studies. Uncarboxylated osteocalcin (ucOC) decreases with a higher vitamin K intake, and may reflect vitamin K status; however, in our study the correlation between ucOC and vitamin K intake was very low (*chapter 9*). Finally, dephosphorylated uncarboxylated Matrix Gla Protein (dp-ucMGP) or inactive MGP, reacts to increased vitamin K intake, such that higher amounts of dp-ucMGP reflect lower vitamin K intake. Although the correlation between dp-ucMGP and vitamin K intake in the Western diet is relatively low (*chapter 2*), this marker reflects both phyloquinone and menaquinone intake. Moreover, dp-ucMGP responds adequately to vitamin K supplementation (*chapter 6*). Dp-ucMGP values below 300 pmol/L are considered to be in the normal healthy range. For the other biomarkers, there is no established threshold that indicates vitamin K insufficiency or deficiency.

Finally, vitamin K can be studied using genetic variants as instrumental variable for vitamin K status to assess causal inferences. For circulating phyloquinone, a small-scale genome wide association study (GWAS) has been performed⁵, while multiple genetic variants for dp-ucMGP have been identified with a small-scale candidate gene study⁶. Including genetic

variants in epidemiological studies could give us more insight in possible mechanisms, and we could assess causality in Mendelian Randomization studies.

Taken together, we can measure vitamin K in multiple ways. The FFQ only estimates intake, of which validity is limited for phylloquinone, and the FFQ does not take into account the bioavailability, absorption, metabolism and the amount of menaquinones produced in the gut. Circulating phylloquinone reflects very short-term intake, and thus far circulating menaquinones cannot be measured. Therefore, dp-ucMGP may be the best method to estimate vitamin K as it represents phylloquinone and menaquinone intake over multiple weeks to months and it has a functional role in reducing CVD risk. However, the results for dp-ucMGP cannot directly be translated to dietary vitamin K intake.

Vitamin K and cardio-metabolic diseases

Cardiovascular disease risk

A limited number of observational studies have shown that high menaquinone intake, but not phylloquinone intake, is associated with improved cardiovascular health⁷⁻¹⁰. Similarly, observational studies indicate that a high vitamin K status, as measured by low dp-ucMGP concentrations, plays a potential role in CVD reduction¹¹. Consistently, menaquinones-7 supplementation lowered arterial stiffness^{12,13}. In contrast to observational studies, phylloquinone supplementation may improve CVD risk by reducing the progression of vascular calcification^{14,15}.

In this thesis, we could confirm the association between high menaquinone intake and reduced CHD mortality (*chapter 3*). However, we were not able to detect a reducing effect of menaquinones-7 supplementation on ongoing vascular calcification in patients with type 2 diabetes. This may partly be explained by the difference in presence of calcification at baseline, which was much higher in the menaquinone-7 than placebo group, despite randomization (*chapter 6*). Therefore, we are unable to draw a conclusion about menaquinone-7 supplementation and vascular calcification based on this study. Our Mendelian Randomization study showed that high circulating phylloquinone was not causally associated with CHD risk, while genetically predicted dp-ucMGP levels, representing both high phylloquinone and menaquinone intake, was associated with a lower CHD risk (*chapter 4*).

Previous studies only found an association between phylloquinone and CVD risk reduction in intervention studies^{14,15}. This suggests that phylloquinone might only be efficient in a higher dose. However, if this was the only explanation, we would have expected to find a causal association in our Mendelian Randomization study. Additionally, phylloquinone is less bio-active and has a shorter half-life compared to menaquinones¹⁶. The trials assessing phylloquinone supplementation only found an effect in patients with chronic kidney disease¹⁴ or with very high baseline calcification levels¹⁵. This might imply that phylloquinone is associated with CVD risk in a critical time period or critical condition and phylloquinone may not alter CVD disease risk in relative healthy participants, which is often the case in cohort studies, and thus in traditional observational and Mendelian Randomization studies¹⁷.

In my opinion, considering the previous studies and the results described in this thesis, the evidence points towards a beneficial role of menaquinones in reducing CVD risk, although we could not support this with our clinical trial. Phylloquinone could be only effective in populations with high vascular calcification levels.

Type 2 diabetes risk

Observational studies showed that phylloquinone intake is associated with a reduced risk of type 2 diabetes^{18,19}, an effect that seems to be causal according to our Mendelian Randomization study focusing on circulating phylloquinone and risk of type 2 diabetes (*chapter 10*). However, intervention studies showed inconsistent results on insulin sensitivity following phylloquinone supplementation^{20–22}. Because the protective association for phylloquinone is mainly found in long-term prospective studies^{18,19} and our Mendelian Randomization study (*chapter 10*), vitamin K might accumulate to prevent diabetes over many years. However, phylloquinone could also be a marker of a healthy lifestyle as phylloquinone is mainly derived from vegetables. The association for menaquinone intake is only assessed in one study, which found results comparable to phylloquinone intake¹⁸.

In summary, the association between vitamin K and diabetes risk is mainly studied for phylloquinone, and the majority of the observational evidence showed that phylloquinone could reduce diabetes risk. Menaquinones may also reduce diabetes risk, but more studies are needed to confirm this association. The mechanism how vitamin K can reduce diabetes risk is not known yet and should be studied in more detail.

Dietary recommendations

In 2017, the European food safety authority defined recommendations for phyloquinone intake only, because the evidence on occurrence, absorption and function for menaquinones is insufficient²³. Phyloquinone is absorbed in the intestine in the presence of dietary fat. Data for the absorption of menaquinone intake is limited and the contribution of gut bacteria is uncertain; therefore, it is not possible to estimate an adequate intake for menaquinones²³. The current adequate intake for phyloquinone is set at 1µg/kg body weight per day. The mean phyloquinone intake in the Netherlands is approximately 150 µg/day (*chapter 3*), indicating a sufficient dietary phyloquinone intake according to these guidelines. For cardiovascular disease risk, phyloquinone supplementation seems only efficient in patients at high risk for cardiovascular disease; therefore, it is debatable whether 1µg/kg body weight is sufficient. If we would consider including menaquinones in the dietary recommendation, it is difficult to translate a recommendation for menaquinone intake to food product recommendations. Menaquinone content varies substantially in products as cheese, since it is highly dependent of fat content and ripening²⁴, and the knowledge gap of the menaquinone metabolism makes it difficult to set dietary recommendations. A better understanding of how and where menaquinone absorption takes place is needed.

Considering that there are still some inconsistencies in the literature regarding vitamin K, we should wait for the ongoing clinical trials assessing the role for phyloquinone and menaquinones in association with cardio-metabolic diseases. Several ongoing trials are investigating the effect of both phyloquinone (5mg, 3 times a week) and menaquinones (varying from 360 µg times a week to 360 µg daily) on arterial stiffness and vascular calcification with a follow up varying from 8 weeks till 24 months.

The majority of these studies are focusing on patients with chronic kidney disease, since vitamin K deficiency is highly prevalent in patients with chronic kidney disease²⁵. If these trials support a beneficial effect of vitamin K in reducing arterial stiffness and vascular calcification, we might consider recommendation of menaquinone-7 to participants with severe vitamin K deficiency, such as chronic kidney disease patients. We should preferably measure vitamin K status in the blood before we start supplementation. Therefore, it is essential that assays measuring vitamin K status, such as dp-ucMGP, are accessible and a threshold indicating a vitamin K insufficiency or deficiency is established.

Evidence from Mendelian Randomization studies

Mendelian Randomization is a method using genetic variants as instrumental variables for modifiable risk factors. Mendelian Randomization analyses are based on multiple underlying assumptions, and violation of these assumptions may lead to biased results. The first assumption of Mendelian Randomization studies is that there is a strong association between the genetic variant and risk factor. For circulating phylloquinone, only one small-scale GWAS has been performed, in which the genetic variants did not meet the criterion for genome wide significance, whereas for dp-ucMGP levels a candidate gene study was performed^{5,6}. Therefore, the Mendelian Randomization studies for vitamin K should be interpreted with caution. The limited sample size in the GWAS might increase the risk of false positive findings²⁶. One genetic variant for circulating phylloquinone is considered a functional variant due to its role in the vitamin K metabolism⁵. We expected stronger effect estimates for this functional variant compared to the other genetic variants, but we observed similar effect estimates in all genetic variants. Since the mechanism by which the other genetic variants influence concentrations of circulating phylloquinone is not clear, it may limit the validity of these genetic variants. Therefore, a large-scale GWAS is essential to verify the genetic variants included, rule out the possibility of false positive findings, and verify the results of our Mendelian Randomization study.

Mendelian Randomization studies become more and more popular since they deal with unmeasured confounding and reverse causation, due to the random assignment of genotypes at conception²⁷. Therefore, we would assume to find consistent results in different studies, since population stratification is not an issue. However, for circulating phylloquinone and CHD risk this was not the case. We found no causal association for circulating phylloquinone (*chapter 4*), while a previous study showed that increased circulating phylloquinone was associated with an increased CHD risk²⁸, in contrast to all observational studies finding the opposite association. This previous study included summary data from the CARDIOGRAMplusC4D consortium, which was also included in our study. The results of this summarized data differed in the different releases (in which slightly different study populations were included). In EPIC-InterAct the genetic risk score was not associated with potential confounders; however, we are not able to check this assumption in CARDIOGRAMplusC4D since we are dealing with summarized data. Because the genetic variants for phylloquinone are rare variants, there is a small risk of imbalance in potential confounders. In our study, we found a consistent null association in EPIC-

InterAct, CARDIOGRAMplusC4D and the UK biobank. Therefore, we should conclude that there is no causal association between circulating phylloquinone and CHD risk.

How should we rank the evidence from Mendelian Randomization studies for vitamin K? Davies and colleagues included Mendelian Randomization studies in the hierarchy of evidence between observational and experimental data²⁹. In my opinion, we should not include Mendelian Randomization studies for vitamin K in the hierarchy of evidence above traditional observational studies, considering the above mentioned limitations. Additional large-scale GWAS data on dp-ucMGP levels or other markers of vitamin K status are essential to further investigate causal roles of vitamin K and cardio-metabolic diseases. Furthermore, our Mendelian Randomization study focusing on type 2 diabetes would be strengthened by including genetic variants for (un)carboxylated osteocalcin levels, since vitamin K may reduce diabetes risk via activation of osteocalcin³⁰. No guidelines should be written based on Mendelian Randomization studies only, but these results should be interpreted in the context of evidence from other studies with different study designs.

Vascular calcification as therapeutic target?

Vascular calcification is associated with a three- to fourfold increased risk of CVD³¹. In the interpretations of the trials assessing the effect of vitamin K on vascular calcification, we assumed that vascular calcification eventually reduce CVD risk. However, it is still unknown whether the calcification itself is a causal factor for the events or whether it only predicts the presence of CVD most accurately. Therefore, vascular calcification as a therapeutic target is still debated. The location, volume and density of vascular calcification may have different effects on clinical outcomes³², which makes it difficult to choose a suitable outcome and interpret effects on such an outcome. For example, a recent study showed that calcification volume is associated with an increased CVD risk, while density is inversely associated with CVD risk³³.

There is an ongoing debate whether we should inhibit vascular calcification, because calcification may contribute to plaque stability³². For example, statin therapy reduces the risk of cardiovascular events, but increase the progression of coronary artery calcium (CAC)³⁴. A recent meta-analysis showed that statin therapy reduces plaque volume and increases CAC progression³⁵, suggesting that it densifies plaques which are then more stable and less prone to rupture.

Vitamin K antagonist treatment is also associated with progression of CAC³⁶. Mice studies showed that warfarin treatment shift atherosclerotic plaque towards a vulnerable plaque by producing micro-calcification and increasing apoptosis³⁷. The results of our trial could also indicate a shift of macro- to micro- calcification following menaquinone-7 supplementation, which resulted in increased ¹⁸F-NaF PET activity (*chapter 6*). In contrast to this, we found that high levels of inactive MGP (reflecting low vitamin K intake), were associated with less plaque hemorrhage, suggesting a higher plaque stability (*chapter 5*). Since active MGP is an inhibitor of vascular calcification, this may suggest that calcification rich plaque may cause more plaque stability, which results in high dp-ucMGP levels. However, our study did not detect an association between dp-ucMGP and calcification, and this study is explorative in nature due to a small sample size.

In summary, we first have to investigate whether calcification is the source of an event, a plaque stabilizer or a predictor for CVD. Therefore, further prospective studies assessing the association between vascular calcification and cardiovascular events are essential before we continue with studies assessing possible inhibitors of vascular calcification.

Intimal and medial calcification

In addition to volume and density of calcification, the location of calcification in the different layers of the vascular wall (intimal or medial calcification) may also influence the prognostic value for risk of CVD. To date, the evidence for these calcification patterns and clinical consequences is still limited. Medial calcification might result in arterial stiffness, while intimal calcification might lead to acute CVD events. Aging, male sex and statin use increases both intimal and medial calcification, while smoking is a specific risk factor for intimal calcification and diabetes is a risk factor for medial calcification (*chapter 7*). This proposes a different pathophysiological mechanism for these calcification types.

The medial layer of the artery mainly consists of vascular smooth muscle cells (VSMC)³⁸ and an in vitro study showed that VSMC treated with vitamin K antagonists resulted in increased mineralization³⁹. This suggests that vitamin K may specifically be involved in medial calcification, which is supported by MGP knock out mice who suffered from more medial calcification, whereas MGP overexpression reduced medial calcification⁴⁰. To the best of our knowledge, no studies have assessed the role of vitamin K in medial calcification in humans. In our study, high dp-ucMGP levels, indicating a low vitamin K

intake, were associated with an increased risk of medial calcification compared to intimal calcification (*chapter 7*).

We made a distinction between intimal and medial calcification based on pattern recognition using non-contrast CT imaging. In my opinion, it is doubtful whether one should make a clear distinction to intimal and medial calcification. Intimal and medial calcification could affect each other, since medial calcification could lower the vessel dilatation which might lead to more plaque formation (intimal calcification). Additionally, the distinction between intimal and medial calcification remains difficult. The absolute agreement between pathology and CT imaging was ~ 0.45 in the crural arteries⁴¹, but this is mainly because histologically observed calcification is not always detectable on CT imaging due to low amount of calcification.

We hypothesized to measure medial calcification with ^{18}F -NaF PET activity in our clinical trial, since medial calcification resembles bone formation and ^{18}F -NaF binds to hydroxyapatite⁴², which is an important component of human bone. We have compared the ^{18}F -NaF activity to intimal and medial calcification, and preliminary results showed that ^{18}F -NaF PET activity was slightly higher in the participants with baseline medial calcification, but this distinction was not very clear.

In summary, the distinction between intimal and medial calcification could result in more insight in the pathophysiological mechanism and treatment or prevention strategies. Vitamin K may be involved in the process of medial calcification, but additional research is necessary to confirm this hypothesis. So far, imaging techniques with pattern recognition are the only available way to disassemble intimal and medial calcification, and we should further develop methods to assess intimal and medial calcification. Before we start developing these methods, we should first address the clinical consequences for intimal and medial calcification to assess whether it is clinically relevant to discriminate between intimal and medial calcification.

^{18}F -NaF PET/CT imaging as measurements of vascular calcification

Vascular calcification is an active process, which could be stimulated and inhibited by different factors. Therefore, measurement of early and dynamic calcification may provide valuable information. To date, the majority of the studies used conventional CT imaging or radiographs to assess vascular calcification, but ^{18}F -NaF PET activity can indicate the presence of micro calcification before they are large enough to be visualized on CT imaging⁴³. Therefore, ^{18}F -NaF PET activity was suggested as a promising technique to detect ongoing vascular calcification due the binding to hydroxyapatite⁴⁴. However, recent literature and results of a recent trial questioned the interpretation of ^{18}F -NaF PET imaging as measurement of vascular calcification^{44,45}. For the same amount of calcification, micro-calcifications have a relatively larger surface area compared to macro calcification, which may result in increased ^{18}F -NaF uptake⁴⁴. So an increase in ^{18}F -NaF uptake may suggest a shift from macro- to micro calcifications but it can also indicate more active vascular calcification.

We measured vascular calcification in our vitamin K trial with ^{18}F -NaF PET activity (*chapter 6*). The data of this trial and the previous trial could be used to enhance the interpretation of ^{18}F -NaF PET imaging, for example by directly comparing ^{18}F -NaF activity and conventional CT imaging. Because ^{18}F -NaF PET activity is detected before it can be visualized on CT imaging, longitudinal studies with multiple CT measurements are essential to establish if ^{18}F -NaF activity results in CT visible calcification in a later stage. However, the radiation dose for these participants may impede the practicality of such studies. More knowledge on this measurement technique is essential before starting new clinical trials using ^{18}F -NaF PET imaging.

Future perspectives

In this thesis, we addressed several questions regarding vitamin K and vascular calcification, CVD and type 2 diabetes risk. The majority of the evidence points towards a beneficial role of vitamin K in human health. Unfortunately, we were not able to support this with the results from our vitamin K trial. Nevertheless, the results from our Mendelian Randomization studies partly confirm the causal relation of vitamin K with cardio-metabolic outcomes. The studies included in this thesis also results in several remaining questions.

The majority of the ongoing clinical trials assessing the role of vitamin K in vascular calcification are focusing on patients with chronic kidney disease. The question whether menaquinone supplementation reduces the progression of vascular calcification in patients with high risk of calcification, such as patients with type 2 diabetes, remains unanswered. If the ongoing clinical trials support a beneficial effect of vitamin K in reducing arterial stiffness and vascular calcification in patients with chronic kidney disease, we might consider conducting another clinical trial in patients with type 2 diabetes. We should conduct a trial using menaquinone-7 supplementation, since this form is proposed more effective compared to phyloquinone and it is easily available. A suitable outcome measure would be arterial stiffness using pulse wave velocity measurements, with a follow up period of 3 years.

Furthermore, to assess whether vitamin K is causally associated with cardio-metabolic health, we have to verify the Mendelian Randomization studies. Considering the above mentioned limitations for Mendelian Randomization studies for vitamin K, large-scale GWAS data on uncarboxylated osteocalcin, dp-ucMGP or other markers of vitamin K status are warranted to further investigate the causal role of vitamin K and cardio-metabolic diseases.

In the past, medial calcification was considered innocent. Recently, multiple studies showed that medial calcification is association with an increased CVD risk^{46–49}. However, these studies have been conducted in selected patients groups, such as patients with chronic kidney disease and diabetes. It is suggested that medial calcification may lead to arterial stiffness⁵⁰, which might result in more chronic CVD such as heart failure and peripheral arterial disease, while intimal calcification may block the arteries and result in acute cardiovascular events such as myocardial infarction. All participants from the study described in chapter 7 are being followed up, and in a couple of years we will be able to assess the cardiovascular consequences of intimal and medial calcification in our cohort study.

Conclusions

In the introduction I presented a figure how vitamin K may activate vitamin K dependent proteins which could reduce cardio-metabolic diseases. In *figure 1*, we included the evidence from this thesis. In summary, menaquinones reduce cardiovascular disease risk, probably via activation of MGP, although we were not able to support this with our

clinical trial. Finally, vitamin K may causally reduce the risk of type 2 diabetes, but we were not able to support the hypothesis that vitamin K reduces type 2 diabetes via activation of osteocalcin.

The role of vascular calcification in reducing CVD risk remains an interesting topic for further research. Additionally, studies using ^{18}F -NaF PET activity as measurement of vascular calcification should be interpreted with caution and the interpretation of ^{18}F -NaF PET activity should be studied in more detail. This thesis also highlights that no guidelines should be written based on one study design, such as Mendelian Randomization studies or clinical trials, but should be interpreted in the context of the available evidence.

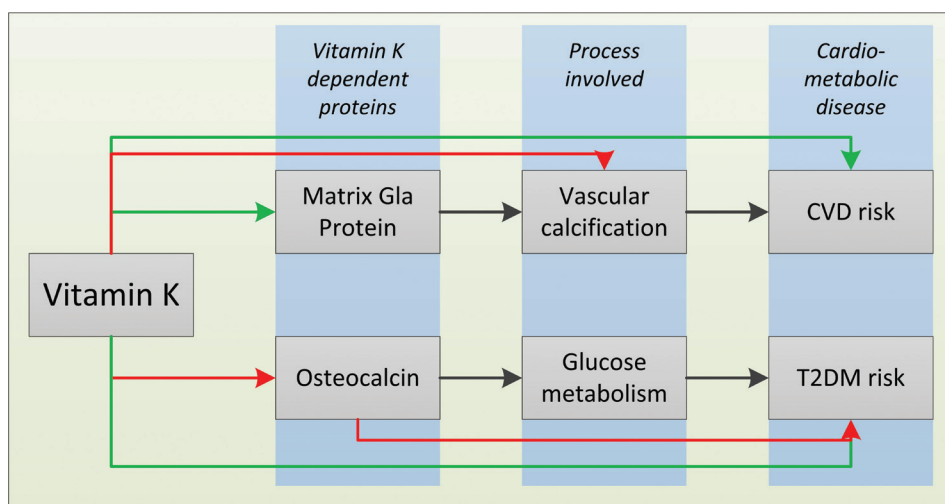


Figure 1. Vitamin K dependent proteins and the cardio-metabolic diseases involved, including the evidence provided in this thesis. The green lines represent the confirmed associations and the red lines represent the null-associations.

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Appendix

Summary

Nederlandse samenvatting

Dankwoord

About the author

Summary

The objectives of this thesis were to investigate the role of vitamin K in the progression of vascular calcification, the role of vitamin K in intimal and medial calcification, and whether vitamin K (causally) reduces cardiovascular disease (CVD) and diabetes risk.

The first chapters of this thesis were focused on vitamin K and CVD risk. In observational studies, vitamin K intake is often measured with a Food Frequency Questionnaire (FFQ). In **chapter 2**, we described the reproducibility and relative validity of the EPIC-NL FFQ to estimate vitamin K intake. The FFQ was reproducible to rank subjects according to their phylloquinone and menaquinone intake. The validity of the FFQ, compared to 24 hour dietary recalls, was relatively good for menaquinones and especially long-chain menaquinones ($r_s=0.60-0.69$). However, the validity to estimate phylloquinone intake was relatively low (women: $r_s=0.28$, men: $r_s=0.40$). Compared to plasma phylloquinone and dephosphorylated uncarboxylated Matrix Gla Protein (dp-ucMGP) levels, the validity of the FFQ was relatively low for both phylloquinone and menaquinones.

With the use of this FFQ, we assessed whether vitamin K intake was associated with all-cause and cause-specific mortality, as described in **chapter 3**. We found no association between both phylloquinone and menaquinones and all-cause mortality, CVD mortality, cancer mortality or mortality from other causes. However, higher intake of long-chain menaquinones seemed to be associated with lower coronary heart disease (CHD) mortality ($HR_{10\mu g} 0.86$, 95%CI: 0.74;1.00).

To assess whether this association between vitamin K and CHD is causal, we investigated the relation between genetically predicted vitamin K status and CHD risk via a two-sample Mendelian Randomization approach in **chapter 4**. Genetically predicted circulating phylloquinone and dephosphorylated uncarboxylated Matrix Gla Protein (dp-ucMGP) were included as instruments, where circulating phylloquinone solely represents phylloquinone intake and dp-ucMGP reflects both phylloquinone and menaquinone intake. Data was used of three European studies resulting in 103,097 CHD cases. Circulating phylloquinone was not causally related to CHD risk (RR 1.00, 95%-CI: 0.98;1.04). However, we found a causal relationship between lower dp-ucMGP levels, representing increased phylloquinone and menaquinone intake, and a lower CHD risk (OR for every 10 $\mu g/L$ decrease 0.96 (95%-CI: 0.93;0.99). In line with previous observational studies, this study also reflects the influence of menaquinones in reducing CHD risk.

In **chapter 5** we aimed to study whether plasma MGP, dp-ucMGP and total-uncarboxylated MGP (t-ucMGP), were associated with MGP levels in plaque, markers of plaque stability and CVD in patients with severe atherosclerotic disease. In this small-scale study (n=100), we found no correlation between plasma MGP levels and plaque uncarboxylated MGP levels. Higher dp-ucMGP levels tended to be associated with less plaque haemorrhage ($OR_{per\ 500nM}$ 0.96, 95%-CI: 0.92;1.00), suggestive of more stable plaques. No association was found between dp-ucMGP and CVD risk (n=20), while higher t-ucMGP levels was associated with a lower CVD risk ($HR_{per\ 500nM}$ 0.79, 95%-CI: 0.62;0.99).

In **chapter 6**, the results of our clinical trial assessing the effect of menaquinone supplementation on vascular calcification in people with type 2 diabetes are presented. Participants were randomly assigned to 360 µg/day menaquinone-7 or placebo during 6 months. Femoral arterial calcification at baseline and 6 months was measured with ^{18}F -NaF PET scans as target-to-background ratios (TBR), which is a promising technique to detect early changes in vascular calcification. Calcification mass on conventional computed tomography (CT) scan was measured as secondary outcome. In linear regression models, TBR tended to increase in the menaquinone-7 group compared to placebo (0.25, 95%-CI: -0.02;0.51). No effect was found on conventional CT, although the effect was directionally concordant with the effect on TBR. The results were in contrast with previous trials, and could partially be explained by the fact that the baseline calcification mass in the vitamin K group was much higher compared to placebo, despite randomization.

Calcification can occur in the intimal or medial layer of the artery. These calcification patterns may represent different mechanisms driven by separate risk factors. In **chapter 7** we studied the risk factors for intimal and medial calcification, measured using CT scans of the legs, in patients at high risk of vascular disease (n=719). Femoral calcification was present in 77% of the participants, of whom 38% had predominant IAC, 28% had predominant MAC and 11% were scored as indistinguishable. Aging, male sex and statin use were risk factors for both intimal and medial calcification compared to absence of calcification. Smoking was associated with a higher risk of intimal calcification, while diabetes was a risk factor for medial calcification. Compared to the patients with intimal calcification, dp-ucMGP may be associated with an increased risk of medial calcification, while osteonectin may be associated with a decreased risk of medial calcification.

Vascular calcification is an active process sharing many features with bone formation. Therefore, we studied six bone markers (osteocalcin, osteopontin, osteonectin,

osteoprotegerin, alkaline phosphatase and sclerostin) and the association with CVD risk in patients with type 2 diabetes (**chapter 8**). This was assessed in a case-cohort study (134 cases and a random subcohort of 218 participants) within the EPIC-NL cohort. Only higher plasma osteopontin levels were associated with an increased CVD risk ($HR_{Q4vs.Q1} 2.00$, 95%-CI: 1.20;3.35). No associations were found for the other five bone markers and risk of CVD.

The last part of this thesis was focused on the association between vitamin K and diabetes incidence. Vitamin K may play a role in the glucose metabolism via activation of osteocalcin. Therefore, we studied the association between total osteocalcin, uncarboxylated osteocalcin and the percentage of uncarboxylated osteocalcin (%ucOC) and its association with type 2 diabetes in **chapter 9**. This nested case control study with 10 years follow up, included 833 incident diabetes cases and 802 non-diabetic control participants from the EPIC-NL cohort. No association was found between total osteocalcin and diabetes risk, while higher %ucOC levels tended to be associated with increased diabetes risk in women ($OR_{per\ 5\%} 1.05$, 95%-CI: 1.00;1.11). The correlation between uncarboxylated osteocalcin and vitamin K intake was very low; therefore, we could not translate these findings to vitamin K intake.

The causal association between circulating phyloquinone and type 2 diabetes, using a two-sample Mendelian Randomization approach, was described in **chapter 10**. Data from three European studies was used, including 69,647 type 2 diabetes cases. Weighted genetic risk scores, with four genetic variants, were used in inverse-variance weighted analyses. Genetically-predicted concentrations of circulating phyloquinone was associated with lower risk of type 2 diabetes with a RR of 0.93 (95%-CI: 0.89;0.97) per every ln-nmol/L unit increase in circulating phyloquinone. This study suggests that circulating phyloquinone is causally related with lower risk of type 2 diabetes.

In **chapter 11**, we discussed the studies presented in this thesis, with its limitations, in light of previous studies. We concluded that menaquinones are considered beneficial in reducing CVD risk, although our clinical trial was not able to support this. Phyloquinone may be effective in reducing CVD risk in high risk populations. If we compare intimal and medial calcification, vitamin K may reduce medial calcification, although additional research is necessary to confirm this hypothesis. Finally, phyloquinone may reduce diabetes risk, but less is known for menaquinones. Overall, this thesis highlights the importance of vitamin K in reducing cardio-metabolic diseases.

Nederlandse samenvatting

De inname van vitamine K in de voeding is in verband gebracht met het voorkomen van hart- en vaatziekten. Vitamine K krijgen we via onze voeding binnen in twee verschillende vormen: phylloquinone (vitamine K₁) en menaquinonen (vitamine K₂). Groene groenten zijn een belangrijke voedingsbron van phylloquinone. Dierlijke producten, zoals vlees en kaas, zijn belangrijke bronnen van menaquinonen. Phylloquinone en menaquinonen verschillen in hun chemische structuur. Als gevolg hiervan zijn menaquinonen langer in het lichaam aanwezig en daardoor effectiever in het activeren van bepaalde eiwitten. In verschillende weefsels in ons lichaam zitten eiwitten waarvan de hormonale werking afhankelijk is van vitamine K: dit worden vitamine K afhankelijke eiwitten genoemd. Matrix Gla eiwit (MGP) en osteocalcine zijn voorbeelden van vitamine K afhankelijke eiwitten. MGP is betrokken in het proces van slagaderverkalking, een voorloper van hart- en vaatziekten, terwijl osteocalcine mogelijk een rol speelt in de glucosehuishouding, wat een rol speelt in het ontstaan van type 2 diabetes. In dit proefschrift hebben we de relatie onderzocht tussen vitamine K en slagaderverkalking en het ontstaan van hart- en vaatziekten en type 2 diabetes.

In wetenschappelijke studies wordt inname van vitamine K vaak gemeten met een voedingsvragenlijst waarin mensen gevraagd worden hoe vaak per week/maand/jaar ze een bepaald voedingsmiddel eten. Deze vragenlijst wordt ook een voedselfrequentievragenlijst genoemd. Voor de interpretatie van een voedingsonderzoek is het noodzakelijk te weten hoe goed de voedselfrequentievragenlijst in staat is om mensen te rangschikken op basis van hun vitamine K inname. De mate waarin deze vragenlijst meet wat hij hoort te meten wordt ook wel validiteit genoemd. In **hoofdstuk 2** hebben we gekeken naar de validiteit van de voedselfrequentievragenlijst in vergelijking met 24-uurs navraag methoden en bloedmarkers voor vitamine K. De 24-uurs navraag methode wordt beschouwd als een betrouwbare methode om onze voedingsinname te meten. De validiteit van de voedselfrequentievragenlijst is goed voor menaquinonen maar relatief laag voor phylloquinone in vergelijking met de 24-uurs navraag methode. De validiteit ten opzichte van de bloedmarkers voor vitamine K status, inactief MGP en circulerend phylloquinone, is relatief laag. Reproduceerbaarheid is het steeds opnieuw kunnen bereiken van een vergelijkbaar resultaat. De reproduceerbaarheid van deze voedselfrequentievragenlijst, gemeten op drie verschillende tijdstippen, is goed voor zowel phylloquinone als menaquinonen.

Deze voedselfrequentievragenlijst is gebruikt in ons onderzoek naar de relatie tussen vitamine K inname en sterfterisico, zoals beschreven in **hoofdstuk 3**. We hebben geen relatie gevonden tussen vitamine K inname, voor zowel phylloquinone als menaquinone inname, en mortaliteit door hart- en vaatziekten en kanker. Wel vonden we dat mensen met een hogere menaquinone inname een lagere kans hebben om te overlijden aan coronaire hartziekten (aandoening in de kransslagaders van het hart).

Op basis van deze studie kunnen we niet aantonen of dit verband causaal is, ofwel dat een hoge menaquinone inname de oorzaak is van het lagere risico op coronaire hartziekten dat we zien. Het is namelijk mogelijk dat de omgevingsfactoren van mensen die veel menaquinonen eten, anders zijn dan de omgevingsfactoren van mensen die weinig menaquinonen eten. Het is ook mogelijk dat mensen die een hoger risico op ziekte hebben door een levenslange lage menaquinone inname, hun voedingsinname kort voor deelname aan de studie hebben veranderd. Daarom hebben we de relatie tussen vitamine K status en kans op coronaire hartziekten onderzocht middels Mendeliaanse Randomisatie (**hoofdstuk 4**). Bij een Mendeliaanse Randomisatie vergelijken we mensen met genetisch bepaalde hoge vitamine K status in het bloed, met mensen met genetisch bepaalde lage vitamine K status in het bloed. Een Mendeliaanse Randomisatie is gebaseerd op het feit dat de genetische varianten bij toeval overgedragen worden van ouder naar kind, en de omgevingsfactoren daarom evenredig verdeeld zijn over dragers en niet-dragers van deze genetische varianten. In drie grote Europese studies, met in totaal meer dan 100.000 mensen met coronaire hartziekten, hebben we gekeken naar de associatie tussen genetisch voorspelde vitamine K status en coronaire hartziekten met twee verschillende Mendeliaanse Randomisatie analyses. In de eerste werd circulerend phylloquinone gebruikt als marker van vitamine K status, welke phylloquinone inname van de afgelopen 6-10 uren representeert. Genetisch voorspelde circulerend phylloquinone was niet causaal geassocieerd met het risico op coronaire hartziekten. Ten tweede is gebruik gemaakt van inactief MGP als marker voor vitamine K status, welke een indicatie geeft van zowel phylloquinone als menaquinone inname van de afgelopen weken tot maanden. Genetisch voorspelde lage inactieve MGP concentraties (wat een hoge vitamine K inname weerspiegelt) resulteerde in een lager risico op coronaire hartziekten. Dit suggereert dat als vitamine K een rol speelt, voornamelijk menaquinonen belangrijk zijn voor het reduceren van coronaire hartziekten.

Menaquinonen kunnen coronaire hartziekten verlagen door activatie van MGP. **Hoofdstuk 5** beschrijft de relatie tussen MGP en de stabiliteit van plaque. Cholesterol en vetten

kunnen zich als plaque ophopen in de binnenkant van de vaatwand. Verkalking van deze plaque wordt ook wel slagaderverkalking genoemd, dit zorgt mogelijk voor stabielere plaques. Actief MGP zou het proces van verkalking kunnen voorkomen of onderbreken. We hebben gekeken naar verschillende vormen van MGP, totaal-ongecarboxyleerd MGP (t-ucMGP) en desfosfo-ongecarboxyleerd MGP (dp-ucMGP). Deze studie toont aan dat hogere concentraties dp-ucMGP (dit betekent een lage vitamine K inname) gerelateerd zijn aan een betere doorbloeding in de plaque, wat leidt tot stabielere plaques. Stabiele plaque verlaagt weer het risico op acute hart- en vaatziekten. Op basis daarvan zou de verwachting zijn dat dp-ucMGP ook tot een lager risico op hart- en vaatziekten leidt. We konden echter geen verband aantonen tussen dp-ucMGP en hart- en vaatziekten, terwijl hogere t-ucMGP concentraties het risico op hart- en vaatziekten zouden kunnen verlagen. We kunnen geen harde conclusies verbinden aan deze resultaten omdat dit een zeer kleine studie betreft van 100 deelnemers.

Een gerandomiseerde gecontroleerde studie naar het effect van menaquinone supplementen en slagaderverkalking bij mensen met type 2 diabetes en hart- en vaatziekten is beschreven in **hoofdstuk 6**. Een gerandomiseerde studie is een manier om iets te kunnen zeggen over causaliteit van de verbanden die zijn gevonden tussen vitamine K en hart- en vaatziekten in andere studies. Door middel van loting werd bepaald of een deelnemer 360µg menaquinone-7 (specifieke vorm van vitamine K) of een placebo (nepmedicijn) kreeg gedurende 6 maanden. Zowel de deelnemers als de onderzoekers wisten niet wie in welke groep ingedeeld was. Tijdens deze studie is gekeken naar de activiteit van slagaderverkalking in de beenvaten. Voor aanvang van de studie werd gedacht dat een hogere activiteit van de slagaderverkalking slechter zou zijn en zo tot een hoger risico op hart- en vaatziekten zou leiden. De activiteit van de aderverkalking werd gemeten met een speciale scan, een zogeheten PET/CT scan. Daarbij kregen de deelnemers een radioactieve vloeistof (NaF) ingespoten welke bindt aan slagaderverkalking. Op een PET/CT scan is de opname van deze vloeistof, doordat zij bindt aan de verkalkingen, zichtbaar. Onze verwachting was dat deelnemers die vitamine K gebruikten, na zes maanden minder NaF opname zouden hebben, wat actievere slagaderverkalking representeert. We zagen echter geen verschil in NaF opname tussen de vitamine K groep en de controle groep. Sterker nog, het leek zelfs alsof de vitamine K groep meer NaF opname had na de interventie periode. Gedurende de uitvoering van deze studie zijn er nieuwe onderzoeken gepubliceerd over deze PET/CT scan, die onze resultaten mogelijk kunnen verklaren. Het blijkt dat er meer NaF opname gemeten wordt door een PET/CT scan als de verkalking afbreekt. De resultaten kunnen ook verklaard worden door het feit dat de deelnemers in

de vitamine K groep bij de start van de studie meer aderverkalking hadden, een verschil wat bij toeval aanwezig was. Kortom, meer onderzoek naar deze PET/CT scan is nodig, om de resultaten van deze studie te kunnen interpreteren.

Slagaderverkalking kan optreden in en tegen de binnenste laag van de wand van de slagader (intima kalk) en in de middelste laag van de vaatwand (media kalk). Intima kalk leidt tot vaatvernauwing en media kalk leidt tot verstijving van de vaten. Eerdere onderzoeken suggereren dat intima en media kalk mogelijk worden veroorzaakt door verschillende risicofactoren. In **hoofdstuk 7** hebben we gekeken naar deze risicofactoren in patiënten met een hoog risico op hart- en vaatziekten. Deelnemers hebben een CT-scan van de benen ondergaan, waarna een radioloog (met behulp van een formule) beoordeelde of dit intima- of media kalk betreft. Het merendeel van de deelnemers had vaatkalk in de bovenbenen (77%), hiervan had 38% intima kalk en 28% media kalk en bij 11% was dit verschil niet te maken. Ouderen, mannen en mensen die statines gebruiken hadden een hoger risico op beide type vaatkalk. Roken was een risicofactor voor intima kalk en diabetes een risico voor media kalk. In een kleine groep van deze deelnemers zijn er bepaalde bloedmarkers gemeten: hierbij zagen we dat hoge dp-ucMGP concentraties, wat een lage vitamine K inname representeert, gerelateerd is aan meer media kalk. Osteonectine, een marker voor botvorming, leidt mogelijk tot minder media kalk ten opzichte van intima kalk.

In een volgend onderzoek hebben we specifiek gekeken naar verschillende botmarkers en de relatie met hart- en vaatziekten bij patiënten met type 2 diabetes (**hoofdstuk 8**). Het proces van slagaderverkalking vertoont overeenkomsten met het proces van botvorming, daarom zouden botmarkers het risico op hart- en vaatziekten kunnen verhogen. Deze botmarkers (osteocalcine, osteopontine, osteonectine, osteoprotegerine, alkalkisch fosfatase en sclerostine) waren gemeten bij 133 deelnemers met hart- en vaatziekten en een willekeurige controlegroep van 218 deelnemers (zowel met *als* zonder hart- en vaatziekten). Een hogere osteopontine concentratie was geassocieerd met een hoger risico op hart- en vaatziekten, maar de andere vijf botmarkers toonden geen verband met het ontstaan van hart- en vaatziekten.

In het laatste deel van dit proefschrift onderzochten we de relatie tussen vitamine K en diabetesrisico. Vitamine K zou het diabetesrisico kunnen verlagen door het activeren van osteocalcine. Osteocalcine is afhankelijk van vitamine K voor activering. Osteocalcine is een hormoon betrokken in het glucose metabolisme. We onderzochten de associatie

tussen totaal osteocalcine en inactief osteocalcine en het risico op type 2 diabetes in **hoofdstuk 9**. In een groep van 833 deelnemers met diabetes en 802 deelnemers zonder diabetes zijn osteocalcine bepalingen uitgevoerd. Totaal osteocalcine is niet gerelateerd aan diabetes risico, maar hogere percentages van inactief osteocalcine lijkt geassocieerd te zijn met een hoger diabetesrisico bij vrouwen. Hogere percentages inactief osteocalcine zou mogelijk veroorzaakt kunnen worden door een lagere vitamine K inname. Ondanks dat dit doet vermoeden dat vitamine K beschermend zou kunnen zijn voor het krijgen van type 2 diabetes, kunnen we de resultaten van deze studie niet direct vertalen naar vitamine K inname.

De causaliteit van de relatie tussen vitamine K en diabetesrisico hebben we onderzocht met de Mendeliaanse Randomisatie methodiek zoals eerder beschreven (**hoofdstuk 10**). Hiervoor hebben we gekeken naar genetische varianten voor phylloquinone concentratie in het bloed. We hebben deze relatie onderzocht in een Europese studie met bijna 70.000 mensen met type 2 diabetes. Genetisch voorspelde phylloquinone concentratie was geassocieerd met een lager risico op type 2 diabetes, wat suggereert dat phylloquinone concentratie in het bloed causaal geassocieerd is met het ontstaan van type 2 diabetes.

Samenvattend laat dit proefschrift zien dat vitamine K het risico op cardiometabole ziekten kan verlagen. De huidige studies laten zien dat menaquinonen het risico op hart- en vaatziekten mogelijk kunnen verlagen. Helaas hebben we deze theorie niet met onze gerandomiseerde studie kunnen onderbouwen. Het verband tussen menaquinonen en risico op type 2 diabetes is nog niet vaak onderzocht, maar phylloquinone kan mogelijk het risico op type 2 diabetes verlagen.

Dankwoord

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About the author

Sabine Zwakenberg was born on the 19th of February 1991 in Zwolle, The Netherlands. She attended secondary school at the Thomas à Kempis College in Zwolle. In 2008, she started the bachelor Nutrition and Dietetics at the Hanzehogeschool for applied sciences in Groningen. Afterwards, she started the master Health Sciences at the Vrije Universiteit in Amsterdam with focus on nutrition and health. As part of the master, she performed research into osteocalcin and type 2 diabetes risk at the Julius Centre for Health Sciences and Primary Care.



In August of 2014, she started to work on the research described in this thesis at the Julius Centre for Health Sciences and Primary Care under supervision of prof. dr. Y.T. van der Schouw and prof. dr. J.W.J. Beulens. During this PhD program, she finished the postgraduate Master in Clinical Epidemiology at Utrecht University and worked as a visiting PhD fellow at Cambridge University in the United Kingdom.

