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Original Article

Vitamin K2 supplementation in haemodialysis patients: a randomized dose-finding study*

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Keywords: haemodialysis, menaquinone, vascular calcification

ABSTRACT

Background. Haemodialysis patients suffer from accelerated vascular calcification. The vitamin K-dependent matrix Gla protein (MGP) is one of the most powerful inhibitors of vascular calcification. Haemodialysis patients have high levels of the inactive form of MGP (desphosphorylated-uncarboxylated-MGP, dp-uc-MGP) and may benefit from pharmacological doses of vitamin K2 (menaquinone) to improve the calcification inhibitory activity of MGP.

Methods. To determine the optimal dose of menaquinone-7 (MK-7) for MGP activation, 200 chronic haemodialysis patients were recruited to randomly receive 360, 720 or 1080 μg of MK-7 thrice weekly for 8 weeks. Dp-uc-MGP was measured at baseline and after 8 weeks. Dietary intake of vitamin K1 (phylloquinone) and menaquinone was estimated based on a detailed questionnaire.

Results. At baseline, dp-uc-MGP was not associated with phylloquinone intake (P = 0.92), but correlated inversely with menaquinone intake (P = 0.023). MK-7 supplementation dose dependently reduced dp-uc-MGP. The levels decreased by 17, 33 and 46% in the respective groups. Drop-outs were mainly due to gastrointestinal side-effects related to the unpleasant smell of the tablets.

Conclusions. Chronic haemodialysis patients have high levels of inactive MGP, possibly related to a low dietary vitamin K intake. Pharmacological doses of MK-7 dose-dependently reduce dp-uc-MGP. Menaquinone supplementation may be a novel approach to prevent vascular calcifications in chronic haemodialysis patients.

INTRODUCTION

Haemodialysis patients are prone to early and accelerated vascular calcification [1, 2]. Both the prevalence and extent of vascular calcification are predictive for cardiovascular morbidity and all-cause mortality in this population [3, 4].

In recent years, the insights into the pathogenesis of vascular calcification in chronic kidney disease (CKD) have changed significantly. Previously, it was considered an entirely passive process associated with an elevated calcium × phosphorus product in the extracellular fluid compartment. However, it is now widely recognized that vascular calcification is an actively regulated process implying death and damage of vascular

smooth muscle cells (VSMCs), phenotypic transformation of VSMCs into osteoblast-like cells and deficiencies in calcification inhibitors [5, 6]. Matrix Gla protein (MGP) is one of the strongest local inhibitors of vascular calcification acting in the vessel wall. The rare Keutel syndrome in humans is caused by mutations in the MGP gene and is characterized by calcifications in vessels and cartilage [7]. Transgenic MGP-deficient mice die within 6 weeks from complications caused by severe calcification [8]. The molecular mechanisms of the inhibitory effect of MGP on calcification have not yet been entirely elucidated. Its Gla residues have a strong affinity for Ca^{++} and mediate MGP's binding to insoluble calcium salts, thereby preventing further growth of hydroxyapatite crystals both in the extracellular matrix and in matrix vesicles of apoptotic VSMCs [9]. Furthermore, MGP inhibits the phenotypic transformation of VSMCs in osteoblast-like cells by blocking the osteo-inductive properties of bone morphogenetic protein-2 [10]. MGP is a small 14-kDa secretory protein consisting of 84 amino acids. The fully maturated protein has undergone two types of post-translational modification: γ-glutamate carboxylation and serine phosphorylation [9]. The process of γ-glutamate carboxylation is vitamin K dependent. Circulating forms of MGP have no known biological function, but reflect the extent of vascular calcification and the availability of vitamin K in the vessel wall [11–14]. Both vitamin K intake and functional vitamin K status (as measured from circulating Gla protein carboxylation) are low in haemodialysis patients [15, 16]. The group of vitamin K vitamins comprises phylloquinone (vitamin K1) and several menaquinones (vitamin K2). Phylloquinone is found in green leafy vegetables, whereas the main dietary source of menaquinones is fermented food such as cheese [17]. The potassium- and phosphorus-restricted diet of haemodialysis patients is poor in both phylloquinones and menaquinones. Pharmacological doses of menaquinone-7 (MK-7) improve the biological activity of MGP in haemodialysis patients [18]. MK-7 belongs to the long-chain menaquinones, which—in contrast with phylloquinones and short-chain menaquinones—are readily incorporated into low-density lipoproteins and transported to extrahepatic tissues such as the arterial vessel wall, where it seems to be a preferred co-factor for vascular carboxylase [19, 20]. However, the optimal dose to achieve this goal is presently unknown, as Westenfeld *et al.* [18] found a dose-dependent effect of MK-7 supplementation on MGP activation without an attenuation of the effect for the highest dose used. This trial intends to investigate whether higher doses of MK-7 supplemented for a longer period of time result in a more pronounced activation of MGP, thereby helping to set up future clinical intervention studies investigating the effect of MK-7 supplementation on vascular calcification and cardiovascular mortality in a population of chronic haemodialysis patients.

MATERIALS AND METHODS

Trial design and patient population

This study was a prospective, randomized, single-blinded intervention study (ClinicalTrials.gov number NCT01675206). All chronic in-centre haemodialysis patients of two tertiary hospitals in a stable medical condition and 18 years or older were screened for inclusion. Warfarin treatment, known intestinal malabsorption, life expectancy of <3 months and inability to provide informed consent were exclusion criteria.

Data for medication, comorbidity and laboratory results were collected from the patients' medical records. Coronary artery disease was defined as a history of myocardial infarction or obstructive coronary artery disease, treated medically or interventionally. Diabetes mellitus was defined as the need for oral anti-diabetics or insulin.

The trial protocol was approved by the ethics committee of both participating hospitals, and written informed consent was obtained from all patients before enrolment.

Interventions

Patients were randomly assigned to one of the three study groups: to be orally administered 360 μg (Group A), 720 μg (Group B) or 1080 μg (Group C) of MK-7 (MenaQ7; NattoPharma, Hovik, Norway) thrice weekly. For reasons of patient compliance, the study medication was administered at the end of the haemodialysis session under the supervision of a nurse. Patient blinding was achieved by the identical appearance of all pills. Blood samples were taken before and after 8 weeks of MK-7 supplementation.

Standardized dietary questionnaires were obtained from patients of both participating hospitals for phylloquinone intake and from one participating hospital also for menaquinone intake. All study participants were asked to complete a 3-day food diary, covering a dialysis day, a non-dialysis week day and a non-dialysis weekend day, including breakfast, lunch, dinner and snacks. They were encouraged to describe the food items, their method of preparation and the quantities in household measurements (teaspoon, tablespoon, cup, pinch, etc.). The dialysis dietician gave each patient instructions on how to fill in the questionnaire and an example of a completed food diary to guide the process.

For each food diary, an inventory was made of the following food items: bread, spread, sweet filling, salt filling, meat/fish, cooking fat, sauce/dressing, vegetables, potatoes/pasta, fruit, dairy products, cookies/cake, liquids and others. The consumed amount in household measurements was converted to grams or millilitre, based on a manual for standardized quantification of food items, provided by the Belgian High Council for HealthCare.

Phylloquinone and menaquinone content of each relevant food item were researched [17, 21, 22]. The average daily phylloquinone and menaquinone intake was calculated based on the sum of the vitamin K content of each individual food item consumed during the 3-day period.

Biochemical measurement and assessment of adverse events

Venous blood samples were taken at the start of dialysis treatment after a long dialysis interval at the dialyser inlet line. Citrated plasma was obtained by 15 min of centrifugation at Rotina 38R and stored at −80°C in 0.5 mL of aliquots until dry ice shipment for analysis (VitaK, Maastricht, the Netherlands).

Circulating desphosphorylated-uncarboxylated-MGP (dp-uc-MGP) was quantified using a previously described dual antibody, enzyme-linked immunosorbent assay [11]. The laboratory staff was blinded to the study set-up. Haemoglobin, calcium, phosphorus, parathyroid hormone and 25-OH-vitamin D levels were measured with the standard laboratory techniques.

Patients were questioned about side-effects by the attending nephrologist during rounds at the haemodialysis unit. Special attention was paid to the possible occurrence of any thrombotic event during the trial.

Statistical analysis

Continuous variables are presented as mean ± standard deviation. Differences between the three study groups were tested using independent one-way analysis of variance and Kruskal–Wallis tests (for normally and non-normally distributed continuous variables, respectively) and χ^2 tests (for categorical variables). Bivariate correlation analysis was performed using the Pearson's and Spearman's correlation coefficient.

A P-value of ≤0.05 was considered to be statistically significant; reported P-values are based on two-tailed tests of statistical significance. All statistical analyses were conducted using Medcalc 12.4, for Windows (www.medcalc.org).

RESULTS

Baseline characteristics and biochemical parameters

From the 200 patients who were included, 26 dropped out: 5 patients died, 6 withdrew early from the study despite initial consent, 13 had inacceptable gastrointestinal side-effects and 2 withdrew because of what they described as general malaise. Three patients were excluded for incompliance. In six patients, sample collection was incomplete. Hence, a complete dataset was obtained from 165 haemodialysis patients (Figure 1).

Analysis of the baseline characteristics did not reveal differences between study groups in terms of demographics, dialysis vintage, K_t/V, comorbidity, biochemical parameters or maintenance medication (Table 1).

Circulating desphosphorylated-uncarboxylated-MGP

Baseline dp-uc-MGP did not differ between study groups (Table 1). A strong correlation between dialysis vintage and baseline dp-uc-MGP was observed ($P < 0.001$, $r = 0.29$). Baseline dp-uc-MGP was not associated with other baseline variables. After 8 weeks of MK-7 supplementation, a significant and dose-dependent decrease in the dp-uc-MGP level with relative reduction rates (the mean of the percentage decrease of each patient) of 17, 33 and 46% was documented in the 360-, 720- and 1080-µg groups, respectively (Table 2 and Figure 2). Absolute reduction rates (the percentage decrease in the means) were in line with these findings (20% for 360 µg thrice weekly; 33% for 720 µg thrice weekly and 46% for 1080 µg thrice weekly). Response rates, with response defined as a decrease in dp-uc-MGP of >10%, were 71% in the 360-µg group, 88% in the 720-µg group and 96% in the 1080-µg group.

Calculation of dietary intake of phylloquinones and menaquinones

Of the 200 study participants, only 125 returned food diaries that allowed a reliable analysis of phylloquinone intake. The mean phylloquinone intake was 122.8 ± 62.5 µg (SD). In a subset of 33 patiënts, an additional calculation of menaquinone intake was performed. The mean menaquinone intake was 15.5 ± 12.7 µg (SD). There was no association between phylloquinone and menaquinone intake ($P = 0.13$), nor

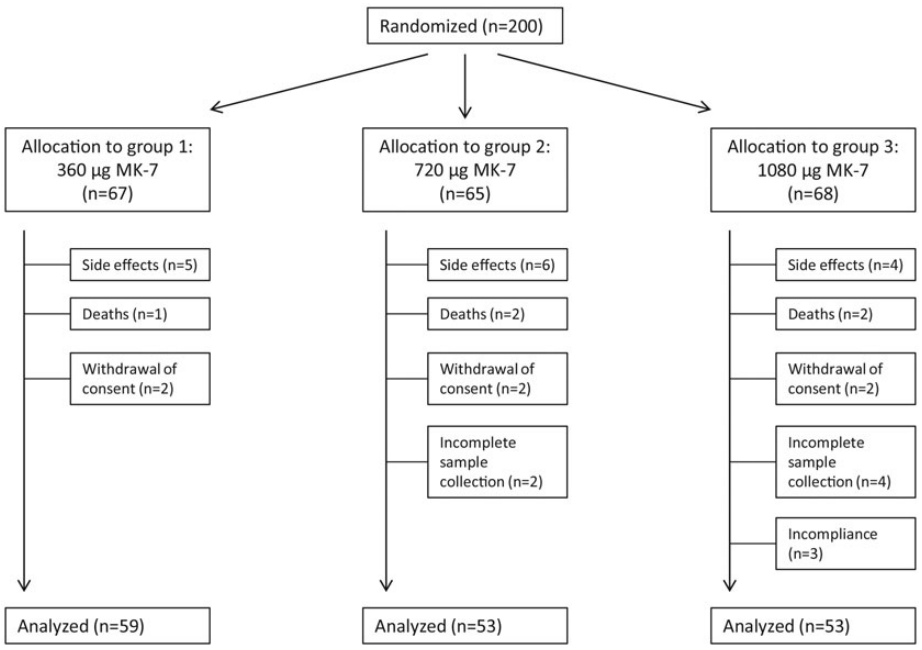

FIGURE 1: Schematic illustration of patient randomization and trial design.

Table 1. Baseline characteristics

	360 µg ($n = 59$)	720 µg ($n = 53$)	1080 µg ($n = 53$)	P-value
Demographics				
Women	31 (52.5%)	23 (43.4%)	29 (54.7%)	0.46
Age (years)	72.2 (38–92)	69.1 (24–93)	71.2 (29–92)	0.44
Dialysis vintage (months)	30.9 (3–117)	41.5 (3–210)	41.9 (1–254)	0.42
K_t/V	1.62 (0.94–2.21)	1.61 (0.82–3.56)	1.66 (0.96–2.29)	0.22
Comorbidity				
Diabetes	21 (35.6%)	22 (41.5%)	18 (34.0%)	0.70
Cerebrovascular disease	9 (15.3%)	8 (15.1%)	5 (9.4%)	0.60
Coronary artery disease	17 (28.8%)	18 (34.0%)	18 (34.0%)	0.79
Peripheral vascular disease	16 (27.1%)	17 (32.1%)	8 (15.1%)	0.11
Biochemical parameters				
25-OH-vitamin D (µg/L)	37.0 (12–69)	35.3 (16–59)	36.7 (15–75)	0.57
PTH (ng/L)	388 (35–1245)	325 (47–793)	331 (49–674)	0.37
Phosphate (mg/dL)	4.7 (1.3–7.9)	4.6 (1.4–8.3)	4.5 (1.8–7.7)	0.76
Calcium (mg/dL)	9.0 (7.2–10.4)	8.8 (4.5–10.6)	8.9 (4.3–11.2)	0.60
Haemoglobin (g/dL)	11.6 (8.8–13.8)	11.6 (7.8–14)	11.4 (8.3–13.7)	0.32
dp-uc-MGP	2872 (123–7539)	2783 (500–7567)	3205 (857–7813)	0.27
Medication				
Cinacalcet (mg/day)	9 (0–90)	8 (0–90)	7 (0–60)	0.93
Alfacalcidol (µg/day)	0.19 (0–1)	0.23 (0–0.86)	0.17 (0–1)	0.28
Calcium acetate (mg/day)	1334 (0–2690)	1533 (128–2938)	1213 (0–2548)	0.45
Lanthanum carbonate (mg/day)	314 (0–4500)	410 (0–3750)	115 (0–3000)	0.31
Sevelamer (mg/day)	1532 (0–7200)	1708 (0–7200)	2083 (0–14 400)	0.63

Categorical variables are given as number (percentage); continuous variables as mean (range). No statistical significant differences between groups were detected in terms of baseline characteristics.
PTH, Parathyroid hormone.

Table 2. Circulating dephosphorylated-uncarboxylated-MGP (pmol/L)

	Treatment group (MK-7) ($N = 165$)		
	360 µg ($n = 59$)	720 µg ($n = 53$)	1080 µg ($n = 53$)
Baseline	2872 (123–7539)	2897 (500–7567)	3206 (857–7337)
After treatment	2306 (105–6618)	1935 (130–6132)	1719 (116–6047)
% Change	17[a]	33[a]	46[a]

Circulating dephosphorylated-uncarboxylated MGP values are presented as mean (range).
[a]$P < 0.001$.

between phylloquinone intake and baseline dp-uc-MGP (P = 0.92) (Figure 3). However, a significant inverse correlation was found between menaquinone intake and baseline dp-uc-MGP (P = 0.023) (Figure 4).

Adverse events and safety

Gastrointestinal side-effects occurred frequently, but were mild and independent of the dose, with 11% of patients complaining of nausea, diarrhoea or abdominal discomfort. The

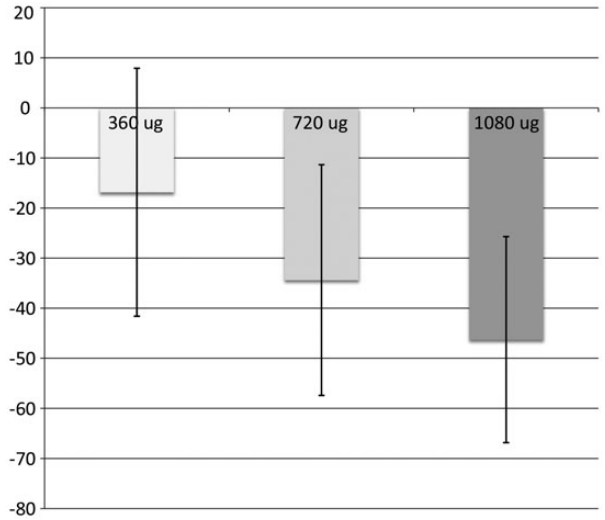

FIGURE 2: Relative decrease (%) in circulating dp-uc-MGP levels after 8 weeks of supplementation with different doses of MK-7. Data represent mean ± standard deviation. The decrease was statistically significant in every treatment group ($P < 0.001$).

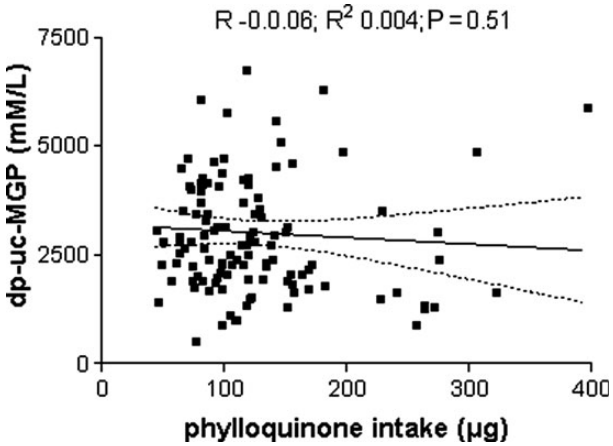

FIGURE 3: Association between phylloquinone intake and baseline dp-uc-MGP. Bivariate correlation analysis was performed using the Pearson's correlation coefficient.

most frequent complaint concerned the unpleasant smell of the tablets. There were five deaths ($n = 1$ in the 360 µg, $n = 2$ in the 720 µg and $n = 2$ in the 1080 µg group), four due to a cardiovascular event in patients with known severe cardiovascular disease, and one due to an opportunistic infection in an immunocompromised patient.

DISCUSSION

The present study was conducted to estimate the required dose of MK-7 to optimize MGP carboxylation in a large and unselected haemodialysis population by supplementing higher doses of MK-7 for a longer period of time than reported earlier [18]. Our data demonstrate a linear and dose-dependent reduction in dp-uc-MGP by supplementing increasing doses of MK-7.

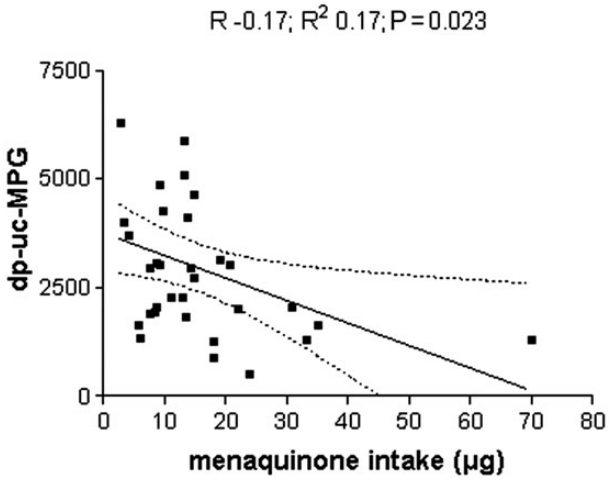

FIGURE 4: Association between menaquinone intake and baseline dp-uc-MGP. Bivariate correlation analysis was performed using the Pearson's and Spearman's correlation coefficient.

Our absolute reduction rates (20% for 360 µg thrice weekly; 33% for 720 µg thrice weekly and 46% for 1080 µg thrice weekly) and relative reduction rates (17% for 360 µg thrice weekly; 33% for 720 µg thrice weekly and 46% for 1080 µg thrice weekly) are fully in line with the absolute reduction rates reported by Westenfeld *et al.* (12% for 45 µg daily; 27% for 135 µg daily and 30% for 360 µg daily). In the latter study, the relative reduction rates are reported to be somewhat higher (18% for 45 µg daily; 37% for 135 µg daily and 61% for 360 µg daily), which may be due to the smaller study sample that gives more importance to outliers. The linear relationship between MK-7 dose and the decrease in dp-uc-MGP and the absence of a plateau phase suggests that even higher doses of MK-7 may be successful aiming at maximal MGP activation.

Side-effects were mild, independent of the dose and can most likely be overcome by coating the pills to prevent discomfort caused by the smell.

MGP requires both carboxylation and phosphorylation for full maturation. However, both processes are generally exerted incompletely. For this reason, various species of MGP can be detected in the circulation. When compared with other forms of MGP, dp-uc-MGP is a very sensitive marker to detect changes in vitamin K status in the vessel wall [11, 14]. Our data confirm a very low functional vitamin K status in haemodialysis patients reflected by high dp-uc-MGP levels when compared with the levels measured in healthy controls by other authors [11, 18].

Vitamin K-dependent proteins, like MGP and coagulation factors, have a limited number of Glu residues capable of γ-carboxylation per molecule, beyond which there can be no further γ-carboxylation or excessive coagulation [23]. This might explain the fact that no increased risk for thrombosis has been documented so far when supplementing phylloquinone or menaquinone.

Vitamin K is a collective term comprising both phylloquinones (vitamin K1) and the menaquinones (vitamin K2), a series of related forms, some of which are synthesized by bacteria and others mainly found in animal products (meat and

dairy products) and fermented food [17]. It can easily be inferred that a typical dialysis diet is insufficient in vitamin K. In the present study, the average phylloquinone and menaquinone intake of haemodialysis patients corresponded with the lowest quartile of intake of the Dutch elderly population (124 μg for phylloquinone and 10 μg for menaquinone) [24]. These data add to the existing evidence for poor vitamin K intake and vitamin K insufficiency in haemodialysis patients [15, 16, 25]. While phylloquinone intake was not associated with baseline dp-uc-MGP, menaquinone intake correlated inversely with baseline dp-uc-MGP levels in our dialysis population. The dialysis diet is a catch 22: in order to prevent vascular calcifications, patients must adhere to a phosphate restriction, leading to a vitamin K deficiency, which promotes vascular calcifications. Our data suggest that minor adaptations of the dietary habits to increase menaquinone intake (e.g. Camembert and goat cheese are low in phosphate and rich in menaquinone, unsalted cream butter does not contain phosphate and is rich in menaquinone) may beneficially affect dp-uc-MGP levels in dialysis patients. However, the strong correlation of dp-uc-MGP with dialysis vintage suggests that other than dietary factors play a role in the poor carboxylation of MGP.

Several large epidemiological studies have demonstrated that a high menaquinone intake is associated with a low risk for coronary heart disease in the general population. The Rotterdam Study was the first to prove the relationship between a high dietary intake of menaquinone and a reduced risk for coronary heart disease in a population of 4807 Dutch men and women aged over 55 years without a history of myocardial infarction [26]. Gast *et al.* [27] highlighted a reduced incidence of coronary heart disease in subjects with a high menaquinone intake from a 16 057 women cohort. Finally, Beulens *et al.* [28] also showed an association between a high menaquinone intake and reduced coronary calcification in a population of 564 post-menopausal women.

Furthermore, a few interventional trials investigated the effect of vitamin K supplementation in non-CKD populations. Braam *et al.* [29] found that a high-dose of phylloquinone (against a background of calcium and vitamin D supplements) preserved the elastic properties of the vessel wall in 181 post-menopausal women over a 3-year period. The effect of phylloquinone supplementation on the progression of coronary artery calcium in older men and women was investigated by Shea *et al.* [30]. They found that especially those with pre-existing coronary artery calcification seem to benefit from phylloquinone supplementation. In this context, it needs mentioning that phylloquinone may be converted to menaquinone-4 up to 25% [31–33].

The lack of a placebo group might be considered a limitation of our study. However, we noticed that dp-uc-MGP levels did not change in nine subjects who withdrew from the study within the first week because of side-effects, but provided consent for the analysis of their blood samples. This fact can be considered a strong indication that dp-uc-MGP levels would remain stable over an 8-week period of time in untreated patients.

In conclusion, our study shows a dose-dependent decrease in dp-uc-MGP by MK-7 supplementation in haemodialysis patients. These data are a *conditio sine qua non* for a large interventional trial examining the effect of vitamin K supplementation on cardiovascular mortality in this population. In such a trial, maximal activation of MGP should be pursued in order to avoid disappointing results. This may be achieved not only by using high-dose MK-7 supplements, but also by implementing dietary changes to improve vitamin K intake.

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CONFLICT OF INTEREST STATEMENT

Cees Vermeer is CEO of VitaK BV.

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