Journal of Thrombosis and Haemostasis, **11**: 1085–1092 DOI: 10.1111/jth.12203

ORIGINAL ARTICLE

Effect of low-dose supplements of menaquinone-7 (vitamin K_2) on the stability of oral anticoagulant treatment: dose–response relationship in healthy volunteers

E. THEUWISSEN,* K. J. TEUNISSEN,* H. M. H. SPRONK,† K. HAMULYÁK,‡ H. TEN CATE,† M. J. SHEARER,¶ C. VERMEER and L. J. SCHURGERS†

**VitaK & Cardiovascular Research Institute Maastricht (CARIM), Maastricht University; †Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University; ‡Division of Hematology, Department of Internal Medicine, Maastricht University Medical Center (MUMC), Maastricht, the Netherlands; and ¶Centre for Haemostasis & Thrombosis, St Thomas' Hospital, London, UK*

To cite this article: Theuwissen E, Teunissen KJ, Spronk HMH, Hamulyák K, ten Cate H, Shearer MJ, Vermeer C, Schurgers LJ. Effect of low-dose supplements of menaquinone-7 (vitamin K_2) on the stability of oral anticoagulant treatment: dose–response relationship in healthy volunteers. *J Thromb Haemost* 2013; **11**: 1085–92.

Summary. *Background and Objective:* Despite the worldwide use of vitamin K antagonists (VKAs), there is limited knowledge of the influence of dietary vitamin K on anticoagulation control. In view of the increasing nutraceutical availability of menaquinone-7 (MK-7; vitamin K_2) and its promotion for bone and cardiovascular health, it is important to determine the posology for the interference of supplemental MK-7 with VKA therapy. *Patients:* Eighteen healthy men and women were anticoagulated for 4 weeks with acenocoumarol, and 15 of them attained a target International Normalized Ratio (INR) of 2.0. In the six subsequent weeks, subjects were given increasing doses of MK-7 (10, 20 and 45 μg day^{-1}) while continuing acenocoumarol treatment at established individual doses. *Results:* Apart from the INR, acenocoumarol treatment significantly increased the levels of uncarboxylated factor II (ucFII), uncarboxylated osteocalcin (ucOC), and desphospho-uncarboxylated matrix Gla-protein (dp-ucMGP), and decreased endogenous thrombin generation (ETP). A daily intake of 45 μg of MK-7 significantly decreased the group mean values of both the INR and ucFII by ~ 40%. Daily intakes of 10 and 20 μg of MK-7 were independently judged by two hematologists to cause a clinically relevant lowering of the INR in at least 40% and 60% of subjects, respectively, and to significantly increase ETP by ~ 20% and ~ 30%, respectively. Circulating ucOC and dp-ucMGP were not affected by MK-7 intake. *Conclusions:* MK-7 supplementation at doses as low as 10 μg (lower than the usual retail dose of 45 μg) significantly influenced anticoagulation sensitivity in some individuals. Hence, the use of MK-7 supplements needs to be avoided in patients receiving VKA therapy.

Keywords: International Normalized Ratio, matrix Gla-protein, menaquinone-7, oral anticoagulants, osteocalcin, thrombin generation.

Correspondence: Elke Theuwissen, VitaK, Maastricht University, Oxfordlaan 70, 6229 EV Maastricht, the Netherlands
Tel.: +31 433885853; fax: +31 433885889.
E-mail: e.theuwissen@vitak.com

Received 13 November 2012
Manuscript handled by: P. de Moerloose
Final Decision: D. Lane, 18 March 2013

Introduction

Despite the worldwide clinical use of oral vitamin K antagonists (VKAs), the influence of dietary vitamin K on anticoagulation control is poorly understood [1]. As recently reviewed [1], several studies have shown associations between dietary vitamin K intake and the sensitivity and stability of anticoagulation during the initiation and maintenance dosing, but nearly all of the dose–response data relate to vitamin K_1 (phylloquinone), the major dietary form of vitamin K. The richest dietary sources of vitamin K_1 are green leafy vegetables and vegetable oils, with contents broadly ranging from 50 to 700 μg (per 100 g of the food item) [2,3]. Lower intakes of longer side-chain menaquinones (MKs; also known as vitamin K_2) may also affect anticoagulation stability, because they have longer half-lives in liver [4] and blood [5]. The richest dietary sources of higher MKs are cheeses (MK-8 and MK-9) in Western diets and natto (MK-7) in Japan. The response to dietary vitamin K during VKA treatment is dependent on the bioavailability from food matrices, which is often low and is influenced by dietary interactions [1,6]. In this context, irregular intakes of over-the-counter supplements containing vitamin K may have a highly detrimental effect on the stability of VKA

therapy, especially as the bioavailability from supplements is much higher than from most foods [7].

There have been only a few systematic dose–response studies of synthetic vitamin K supplements on the stability of anticoagulation therapy, all of them relatively short-term [1]. Whereas earlier studies monitored anticoagulant response with the Thrombotest (with results expressed as a percentage of normal activity), more recent studies have used the International Normalized Ratio (INR). An early study in patients [8] showed that, whereas daily supplementation with 250 μg of synthetic vitamin K_1 caused a significant increase in the Thrombotest values over a period of 1 week, a dose of 100 μg of vitamin K_1 had no effect. A dose-escalation study in healthy volunteers indicated that the threshold dose of vitamin K_1 causing a significant lowering of the INR was 150 μg day^{-1} [7]. In the same study, the daily dose of vitamin K_1 resulting in a significant decrease in the level of uncarboxylated factor II (ucFII) – a sensitive marker of hepatic γ-carboxylation – was 100 μg [7]. On the other hand, vitamin K_1 at 300 μg day^{-1} was needed to cause a significant decrease in the level of uncarboxylated osteocalcin (ucOC) [7], reflecting the known higher vitamin K requirements for Gla-proteins synthesized in extra-hepatic tissues [3,5,6].

In a preliminary study, we showed that daily doses of MK-7 at the high end of commercial availability (100–300 μg) were three to four times more potent on a molar basis than vitamin K_1 in reversing the anticoagulant effect of acenocoumarol in healthy volunteers [5]. In view of the increasing retail availability of MK-7 supplements, it is important to determine the dose range of MK-7 that can be taken without clinically affecting VKA stability. We therefore carried out a dose-escalation study to measure the antidotal potency of lower doses (10, 20 and 45 μg day^{-1}) of MK-7 supplements in healthy volunteers stabilized on acenocoumarol. Apart from conventional INR measurements, we also assessed the effects on thrombin generation (TG) and the γ-carboxylation status of specific Gla-proteins with coagulation and non-coagulation functions.

Materials and methods

Subjects

Healthy men and women aged 18–45 years were recruited from the Maastricht University community. Exclusion criteria were body mass index of > 30 kg m^{-2}, coagulation disorders, metabolic or gastrointestinal diseases, chronic inflammatory diseases, medication that interferes with vitamin K and/or coagulation metabolism, use of supplements containing vitamin K, participation in a clinical trial in the 3 months prior to this study, and soy allergy. On the basis of these exclusion criteria and a prior health check (a questionnaire including an assessment of medical history), 18 volunteers were eligible to participate in this open intervention study. None of the volunteers possessed FV Leiden or prothrombin 20210 variants (data not shown).

This study was conducted according to the Declaration of Helsinki, and approved by the Medical Ethics Committee of Maastricht University. Written informed consent was obtained from all subjects before they entered the study. Trial registration code: clinicaltrials.gov NCT00512928.

Study design: dose-adjustment phase with acenocoumarol

The study design is shown in Table 1. During the first 4 weeks (dose-adjustment phase), all participants were anticoagulated with acenocoumarol (taken with water before evening meals) to attain a stable target INR value of 2.0. The INR was checked three times weekly during the first week, and twice weekly during the next 3 weeks, and the acenocoumarol dosage was adjusted accordingly. The INR was considered to be stable when no dose adjustment was needed for at least three consecutive measurements. Stable anticoagulation (INR, 2.02 ± 0.15 [mean ± standard deviation (SD)]; maintenance acenocoumarol dose, 2.99 ± 1.15 mg day^{-1}) was established in 15 of 18 subjects by the end of week 4. Results of these 15 subjects were therefore used in the statistical analyses. The mean maintenance dose of acenocoumarol in men was higher than that in women (3.40 ± 1.33 mg day^{-1} vs. 2.52 ± 0.72 mg day^{-1}). The established individualized dosage was maintained for each participant throughout the whole study. The acenocoumarol dosage was independent of body weight (data not shown).

Study design: supplementation phase with MK-7

After the acenocoumarol dose-adjustment phase (weeks 1–4), subjects were given increasing doses of MK-7 (weeks 5–10) while continuing to take the same individual doses of acenocoumarol. Over successive 2-week inter-

Table 1 Study design

Study phase and week	Dose of MK-7 (μg day^{-1})*
Dose-adjustment phase with acenocoumarol	
1	0
2	0
3	0
4	0
Supplementation phase with MK-7	
5	10
6	10
7	20
8	20
9	45
10	45
Washout (acenocoumarol stopped)	
11	0

MK, menaquinone.
*Provided as a daily dietary supplement.

vals, the MK-7 dose was increased from 10 μg (one capsule per day) to 20 μg (two capsules of 10 μg per day) to 45 μg (one capsule per day) (Table 1). MK-7 capsules were taken with breakfast. The first day of the supplementation phase began on the morning of the first day of week 5, and fasting blood samples for monitoring the effects of MK-7 were collected on the morning of the first day of successive weeks (weeks 6–10) just before the next weekly MK-7 regimen began. Acenocoumarol and MK-7 were both withdrawn at the end of week 10, and 1 week later a final blood sample was drawn to check the return to baseline of the INR. During both study phases, subjects were asked to consume no more than one alcoholic drink per day, no natto, < 200 g day^{-1} of spinach, broccoli, Brussels sprouts, and kale, and < 50 g day^{-1} of (curd) cheese. Participants were also advised not to perform contact sports during the study period. Compliance was measured by pill counts at the end of each 2-week period; the mean compliance for the MK-7 supplement was 98.9%. Any noticeable changes in health, dietary pattern, physical activity and/or medication use were noted.

Study products

Acenocoumarol tablets (Sintrom Mitis, 1 mg; Novartis Pharma BV, Basel, Switzerland) were provided by the pharmacy of the Maastricht University Medical Center (MUMC). The MK-7 (MenaQ7) capsules were produced in two different MK-7 dosages (10 and 45 μg) by Euro-Pharma Alliance (Wroclaw, Poland) for Nattopharma (Hovik, Norway). The MenaQ7-containing capsules were delivered directly by Nattopharma to VitaK in Maastricht (The Netherlands). The concentrations of MK-7 in the 10-μg and 45-μg capsules were measured immediately before the intervention phase to assure accuracy of dosing.

Blood sampling

Fasting venous blood samples were taken for the preparation of serum and sodium citrate (3.2%) plasma by standard centrifugation techniques. Platelet-poor plasma (PPP) was prepared by a two-step centrifugation process: centrifugation at 2000 × *g* for 15 min, followed by centrifugation at 11 000 × *g* for 10 min.

Acenocoumarol

Plasma concentrations of acenocoumarol were measured by modifying a routine HPLC assay for warfarin, in which pure, synthetic acenocoumarol (obtained as a gift from Ciba-Geigy – now Novartis) was employed as an internal standard [9]. This modification simply involved switching acenocoumarol from internal standard to analyte, with warfarin now being used as the internal standard.

Coagulation

INR measurements were carried out by the Department of Hematology of the MUMC. Prothrombin times (PTs) were determined in citrated plasma with an automated coagulation analyzer (STA model; Diagnostica Stago, Asnières, France), with Thromborel-S (Behringwerke, Marburg, Germany) as the thromboplastin reagent. The INR value was expressed as the ratio of the subject's PT to a normal (control) sample raised to the power of the International Sensitivity Index (ISI); $(PT_{test}/PT_{normal})^{ISI}$. TG in tissue factor-triggered PPP was measured by means of the calibrated automated thrombinography method (Thrombinoscope BV, Maastricht, The Netherlands) [10]. Pooled normal plasma was obtained from 85 healthy volunteers not taking any medication.

ucFII

Plasma species of ucFII, also known as protein induced by vitamin K absence or antagonism, were measured with an in-house (St Thomas' Hospital) ELISA, with a conformation-specific mAb (C4B6) that selectively binds species of ucFII in the presence of calcium ions and does not cross-react with native fully carboxylated FII [7,11,12].

Extrahepatic Gla-proteins

Serum ucOC and carboxylated osteocalcin (cOC) levels were determined with separate immunoassays by use of the respective ELISA kits from Takara Shuzo (Otsu, Shiga, Japan). Plasma desphospho-uncarboxylated Gla-protein (dp-ucMGP) levels were measured with an in-house ELISA kit (VitaK) [13].

Statistical analysis

The number of subjects selected for this study was based on power analysis (α = 5% and power = 80%) with an estimated 20% intrasubject variation and a 15% change in INR, giving a total of 18 subjects (including a 10% dropout rate) [5]. Gender differences were compared by use of the independent samples *t*-test. Within-time effects were analyzed with repeated measures ANOVA. Group effects of VKA therapy or MK-7 supplementation (as compared with baseline) were analyzed with the paired samples *t*-test. Correlation analyses were performed with the Pearson test. Statistical analyses were performed with SPSS for Windows, version 15.0 (SPSS, Chicago, IL, USA). Differences were considered to be statistically significant at $P < 0.05$. In individuals, the effects of MK-7 supplementation on the INR were evaluated in two ways: first, from an algorithm defined as > 20% outside one SD of the mean INR attained after stabilization on acenocoumarol [7], and second, by a clinically relevant lowering of the INR that would have required a theoretical adjustment of the

acenocoumarol maintenance dosage as judged by two independent hematologists. The individual SD and the mean individual INR were calculated from three repeated measurements in the last week of the dose-adjustment phase.

Results

Baseline characteristics and acenocoumarol concentrations

As summarized in Table 2, baseline characteristics were similar for men and women, with the exception of higher ucOC levels and lower endogenous thrombin potential (ETP) and higher lag time in the men. As the ETP value is influenced by the use of oral contraceptives (four women were using oral contraceptives), a higher ETP was to be expected in the women than in the men. When the women using oral contraceptives were excluded, no gender differences in ETP were found. At the start of the study, the INR was within the normal range (0.9–1.1). Stable anticoagulation was reached by the end of week 4 (baseline). As shown in Table 3, the mean interindividual plasma acenocoumarol concentrations measured from the end of week 4 to the end of the MK-7 intervention period did not differ significantly, indicating good compliance. Although the mean acenocoumarol concentrations within each subject over the same period varied widely (22–102 μg L^{-1}), the mean intraindividual coefficient of variation of 16% (range, 4–32%) also indicated good compliance.

INR

After the dose-adjustment phase, the mean (± SD) INR had increased from 1.02 ± 0.05 to 2.02 ± 0.15. Changes in the INR in response to MK-7 supplementation are shown in Table 3. After consecutive doses of 10 and 20 μg of MK-7 for 2 weeks, the group mean INR was not significantly different from the presupplementation INR at week 4. Thereafter, supplementation for 1 week and 2 weeks with 45 μg day^{-1} MK-7 significantly decreased the group mean INR by 20% ($P = 0.002$) and 37% ($P = 0.001$), respectively. After 1-week withdrawal of both acenocoumarol and MK-7 supplements (week 11), the INR values returned to baseline values (1.00 ± 0.04).

Table 4 shows the evaluation of the degree of INR lowering in individuals according to an algorithm or based on the judgement of two independent hematologists (see Methods). It is of note that successive 2-weekly daily intakes of 10 and 20 μg of MK-7 met the dose-adjustment criteria of hematologists in a minimum of seven of 15 (46.7%) and nine of 15 (60.0%) subjects, respectively.

Table 2 Baseline characteristics

	All	Men	Women
Anthropometric data			
No. of subjects	15	8	7
Age (years)	29 ± 8	29 ± 8	30 ± 8
Weight (kg)	72 ± 10	77 ± 10	67 ± 8
BMI (kg m^{-2})	23 ± 3	24 ± 3	22 ± 2
Biochemical markers			
INR	0.99 ± 0.03	1.00 ± 0.00	0.99 ± 0.04
ETP (%)	154 ± 55	126 ± 32	186 ± 59*
Peak (%)	216 ± 102	174 ± 76	264 ± 112
Lag time (min)	5.49 ± 1.89	6.58 ± 1.89	4.22 ± 0.82*
ucFII (ng mL^{-1})	<0.2	<0.2	<0.2
ucOC (ng mL^{-1})	5.12 ± 5.31	7.66 ± 6.28	2.21 ± 1.09*
cOC (ng mL^{-1})	5.33 ± 3.03	5.87 ± 3.43	4.71 ± 2.63
dp-ucMGP (pM)	361 ± 127	382 ± 144	337 ± 111

BMI, body mass index; cOC, carboxylated osteocalcin; dp-ucMGP, desphospho-uncarboxylated matrix Gla-protein; ETP, endogenous thrombin potential; INR, International Normalized Ratio; ucFII, uncarboxylated factor II; ucOC, uncarboxylated osteocalcin.
Values are means ± standard deviations ($n = 15$). ETP and peak are expressed as the ratio to the normal pool value (%).
*Significant gender differences as tested by the independent samples t-test ($P < 0.05$).

TG

Measurements of TG (ETP, peak, and lag time) are shown in Table 3. The mean ETP decreased by 69% after 4 weeks of VKA treatment ($P < 0.001$). ETP was not affected by daily MK-7 doses of 10 μg, but increased significantly by 19% ($P = 0.019$) and 32% ($P = 0.015$), after 20 and 45 μg, respectively. Changes in ETP inversely correlated with changes in INR ($r = -0.568$, $P = 0.027$) during supplementation with 45 μg of MK-7, but not after lower doses.

The peak decreased by 69% after 4 weeks of VKA treatment ($P < 0.001$). Daily MK-7 supplementation at 45 μg significantly increased the peak, by 29% ($P = 0.043$). The two lower doses of MK-7 did not significantly change the peak. Changes in the peak significantly correlated with changes in ETP for all MK-7 doses (10 μg, $r = 0.936$; 20 μg, $r = 0.897$; 45 μg, $r = 0.950$; $P < 0.001$).

The lag time was prolonged by 77% after 4 weeks of anticoagulation treatment ($P < 0.001$). Daily MK-7 supplementation at 45 μg significantly shortened the lag time, by 19% ($P = 0.033$). Both lower doses of MK-7 did not significantly influence the lag time. Changes in the lag time inversely correlated with changes in ETP (10 μg, $r = -0.838$; 20 μg, $r = -0.884$; 45 μg, $r = -0.751$; $P \leq 0.001$) and changes in the peak (10 μg, $r = -0.909$; 20 μg, $r = -0.707$; 45 μg, $r = -0.685$; $P \leq 0.005$) for all three doses of MK-7.

ucFII

As expected, VKA treatment significantly increased the mean circulating ucFII level, by > 100-fold ($P < 0.001$) (Table 3). After 2 weeks of consecutive daily supplementation with 10 and 20 μg of MK-7, respectively, ucFII levels had not significantly decreased, with the exception

Table 3 Effects of acenocoumarol and menaquinone-7 (MK-7) supplementation on markers of coagulation and vitamin K status

		Plasma or serum concentrations or parameter values								
				Thrombin generation			Gla-proteins			
Week	MK-7 daily dose (μg)	Acenocoumarol (μg L^{-1})	INR -	ETP (%)	Peak (%)	Lag time (min)	ucFII (ng mL^{-1})	ucOC (ng mL^{-1})	cOC (ng mL^{-1})	dp-ucMGP (pM)
0	0		1.01 ± 0.05	154 ± 55	216 ± 102	5.48 ± 1.89	< 0.2	5 ± 5	5.33 ± 3.03	361 ± 127
4	0	61 ± 22	2.02 ± 0.15*	47 ± 11*	68 ± 30*	9.72 ± 3.10*	21 ± 6*	28 ± 16*	1.12 ± 0.52*	1111 ± 310*
5	10		1.91 ± 0.43	NM	NM	NM	19 ± 6†	25 ± 16	1.09 ± 0.52	1143 ± 310
6	10	59 ± 20	1.93 ± 0.35	51 ± 12	73 ± 28	9.51 ± 3.51	20 ± 7	27 ± 15	1.19 ± 0.56	1168 ± 313
7	20		1.82 ± 0.47	NM	NM	NM	18 ± 7	25 ± 15	1.17 ± 0.73	1096 ± 263
8	20	61 ± 22	1.83 ± 0.34	56 ± 17†	83 ± 44	10.79 ± 4.83	18 ± 7	26 ± 14	1.16 ± 0.59	1115 ± 255
9	45		1.61 ± 0.27†	NM	NM	NM	16 ± 7†	25 ± 15†	1.11 ± 0.65	1079 ± 300
10	45	63 ± 26	1.47 ± 0.33†	62 ± 22†	88 ± 47†	7.85 ± 2.45†	12 ± 5†	29 ± 15	1.13 ± 0.56	1016 ± 260
11	0		1.00 ± 0.04†	NM	NM	NM	NM	NM	NM	NM

cOC, carboxylated osteocalcin; dp-ucMGP, desphospho-uncarboxylated matrix Gla-protein; ETP, endogenous thrombin potential; INR, International Normalized Ratio; NM, not measured; ucFII, uncarboxylated factor II; ucOC, uncarboxylated osteocalcin.
Blood samples for analyses were taken after 7 days of MK-7 supplementation and thereafter at 7-day intervals. Values are means ± standard deviations ($n = 15$).
*Significant effects of acenocoumarol treatment (as compared with week 0) as tested with the paired samples t-test ($P < 0.05$).
†Significant effects of MK-7 supplementation (as compared with week 4) as tested with the paired samples t-test ($P < 0.05$).

Table 4 Evaluation of lowering of the International Normalized Ratio (INR) in individuals with increasing doses of menaquinone-7 (MK-7)

Method of assessment of INR lowering	Number and proportion of subjects meeting the criteria for INR lowering			
		10 μg of MK-7	20 μg of MK-7	45 μg of MK-7
Calculation from algorithm*	Number after each dose	2	1	1
	Cumulative proportion	2/15	3/15	4/15
Clinical relevance as judged by hematologist 1†	Number after each dose	7	2	4
	Cumulative proportion	7/15	9/15	13/15
Clinical relevance as judged by hematologist 2†	Number after each dose	14	1	0
	Cumulative proportion	14/15	15/15	15/15

*INR lowering defined as > 20% outside the value of one standard deviation of the mean target INR that an individual attained after stabilization on acenocoumarol.
†INR lowering that would have met the criteria of the hematologist for a theoretical change in the dose of acenocoumarol needed to restore the INR to the target of 2.0.

of a significantly lower mean value after the first week on the 10-μg dose ($P = 0.036$). This was probably a chance finding, given that supplementation with 20 μg produced no significant changes. After 1 week and 2 weeks of daily supplementation with 45 μg of MK-7, ucFII levels decreased significantly, by 24% ($P = 0.003$) and 43% ($P < 0.001$), respectively. Changes in circulating ucFII levels significantly correlated with changes in INR after daily MK-7 intakes of 20 μg ($r = 0.804$, $P < 0.001$) and 45 μg ($r = 0.602$, $P = 0.018$), but not for the 10-μg dose. Changes in ucFII levels did not correlate with changes in ETP, peak, or lag time.

Extrahepatic Gla-proteins

After 4 weeks of acenocoumarol treatment, circulating ucOC and dp-ucMGP levels had increased by 460% ($P < 0.001$) and 208% ($P < 0.001$), respectively, and the serum cOC level had decreased by 79% (Table 3). These measures of γ-carboxylation status remained unchanged throughout the 6-week MK-7 supplementation phase. No significant correlations were found between circulating concentrations of these extra-hepatic Gla-proteins and INR, ETP, peak, lag time, or ucFII.

Discussion

This dose-escalation study was undertaken because of the increasing worldwide retail availability of MK-7 supplements intended for bone and cardiovascular health. In Europe, nutraceutical use of MK-7 is set to grow following reviews by the European Food Safety Authority (EFSA) that accepted the safety of menaquinones [14], and the health claim that 'a cause and effect relationship has been established between the dietary intake of vitamin K and the maintenance of normal bone' [15]. Neither of the EFSA panels considered the potential adverse effects of the growing availability of MK-7 supplements on that section of the population who are taking VKAs.

Group analysis of the INR changes showed that there was a gradual fall in mean INR with increasing doses of MK-7, but the mean decrease did not attain statistical significance until the daily dose had increased to 45 μg. However, at the individual level, the 10-μg and 20-μg MK-7 doses were associated with a clinically relevant lowering of INR in at least 40% and 60% of subjects, respectively. This sensitivity to MK-7 is much greater than that for vitamin K_1; daily supplementation with 50 and 100 μg over consecutive weeks produced no clinically relevant decreases in INR, and supplementation with 150 μg day^{-1} in the following week was considered to be clinically relevant in only 25% of subjects [7]. In a preliminary direct comparative study, we showed that a dose of 100 μg of MK-7 had a marked effect on INR as compared with vitamin K_1 in the same subjects [5]. With the daily intakes required to lower the INR from 2.0 to 1.5 as an endpoint, it was calculated that MK-7 was ~ 2.4-fold more potent than vitamin K_1 on a weight basis (130 μg of MK-7 vs. 316 μg of vitamin K_1), and ~ 3.5-fold more potent on a molar basis (200 nmol of MK-7 vs. 700 nmol of vitamin K_1) [5].

Additional insights into the procoagulant effect of MK-7 were provided by serial measurements of TG and ucFII. In the group analyses, the effects of MK-7 on TG mirrored those on the INR, with significant changes in ETP, peak and lag time when the MK-7 dose reached 45 μg day^{-1}, and significant changes in ETP after only 20 μg day^{-1}. Previous cross-sectional analyses have indicated a modest correlation between INR and TG parameters that also differed according to the degree of intensity of the INR [16]. As expected, the ucFII level fell in response to increasing doses of MK-7, reflecting the increasing γ-carboxylation of FII; ucFII has previously been shown to be linearly related to the INR in patients treated with warfarin [17], and to be a candidate marker for monitoring the efficacy of VKA therapy [18].

Our rationale for measuring the effect of MK-7 on the γ-carboxylation status of osteocalcin and matrix Gla-proteins was evidence that VKA usage is associated with impaired bone [19,20] and cardiovascular [21,22] health. A recent study showed that VKA treatment increases calcification of atherosclerotic plaques in humans and plaque instability in mice [23]. Unlike the coagulation Gla-proteins, a fraction of both osteocalcin and matrix Gla-proteins circulate as undercarboxylated species in healthy individuals not taking VKAs. This appears to result from physiologic constraints in the transport of vitamin K to the tissues/cells that synthesize osteocalcin (bone) and matrix Gla-protein (e.g. cartilage, heart, kidney, and vascular smooth muscle cells). It has been postulated that vitamin K_2 is mainly transported to and functions in extra-hepatic tissues, whereas vitamin K_1 is more important for γ-carboxylation of liver-derived vitamin K-dependent coagulation factors [24]. In the human physiologic setting, the preferential transport of MK-7 and MK-9 to extra-hepatic tissues is supported by the high circulating proportions in LDL [25] and, for MK-7, by its enhanced ability to γ-carboxylate osteocalcin [5]. It might therefore be concluded that the combination of VKA with vitamin K_2 would benefit bone and vasculature tissue without affecting VKA activity in the liver. Our finding that, during VKA treatment, MK-7 reverses the abnormal γ-carboxylation of hemostatic Gla-protein before any changes occur in osteocalcin and matrix Gla-protein argues against this concept, and is in keeping with our previous findings for vitamin K_1 [7]. Importantly, MK-7 is more potent than vitamin K_1 in reversing the undercarboxylation of both hepatic and extra-hepatic Gla-proteins [5].

Although this study is the first to provide quantitative information on the agonist properties of low-dose MK-7 during VKA treatment, it may underestimate tissue accumulation during long-term supplementation. In healthy subjects (not on VKAs) who took a daily MK-7 supplement of 0.22 μmol (143 μg) for 42 days, blood MK-7 concentrations did not plateau until ~ 2 weeks, whereas the fraction of γ-carboxylated osteocalcin (cOC/ucOC) continued to rise throughout the entire study period, suggesting that MK-7 was continuing to accumulate in bone [5]. In contrast, after the same 0.22-μmol molar dose (99 μg) of vitamin K_1, blood concentrations and cOC/ucOC values both plateaued within 3 days, and the absolute increase in the fraction of γ-carboxylated osteocalcin was much lower than for MK-7 [5]. On the basis of the present study, any perceived benefits of MK-7 accumulation in bone would be offset by increased liver concentrations, which would necessitate an increased dose of VKA.

One area of current interest is the concept that the stability of VKA treatment might be improved by smoothing out the high variability of dietary intakes of vitamin K [1]. Two intervention trials in which supplements of vitamin K_1 were administered to patients at daily doses of 150 μg [26] and 100 μg [27] have shown promise in improving anticoagulant control. Minimum theoretical criteria for the suitability of a vitamin K compound are that it should be well absorbed and that the removal rate from the liver should be similar to the dosage interval. Current evidence, including that presented here, suggests that, for a daily supplementation regimen, this criterion for relatively fast hepatic turnover applies to vitamin K_1 but not to MK-7. Crucially, a human biopsy study showed that, whereas hepatic vitamin K_1 fell dramatically to 25% of baseline after 3 days of dietary restriction, hepatic MK-7 remained constant, despite a significant decrease in plasma MK-7 [4]. These results support the prediction by Duello and Matschiner [28] and other data [29] that, because MKs with long side chains are very lipophilic, they have a greater affinity for membranes of human liver, and turn over more slowly than shorter-chain analogs such as vitamin K_1.

Other factors that are relevant to the possible use of regular intakes of vitamin K compounds as an aid to

VKA stability are their degradation and excretion. It has been demonstrated that cytochrome P450 (CYP)4F2 is responsible for the initiation of the degradation sequence (ω-oxidation) that leads to the biliary and urinary excretion of vitamin K_1, and that a polymorphism of CYP4F2 (V433M) is associated with warfarin dose, probably by affecting the rate of metabolism of vitamin K_1 [30,31]. A likely physiologic role of CYP4F2 is to limit the accumulation of vitamin K [31]. Many CYPs are induced by their substrates via the activation of nuclear receptors. Isoforms of the CYP3A family have been implicated in the catabolism of supranutritional concentrations of vitamins E and K via activation of the steroid and xenobiotic receptor (SXR), also known as the pregnane X receptor [32,33]. Although there are no data for MK-7, on the basis of findings that the activation of the SXR was enhanced by an unsaturated side chain (e.g. three-fold higher for MK-4 than for vitamin K_1) it is likely that MK-7 is also a potent activator of the SXR [33]. Clearly, the relative ability of different vitamin K compounds to induce CYPs is an important factor if vitamin K is taken regularly with VKAs.

The design of this dose-escalation study was similar to that of our previous studies with vitamin K_1 [7] and higher-dose MK-7 [5]. This was deliberate, because it enabled comparison of the agonist effects of vitamin K_1 and MK-7 with similar protocols. However, a limitation of the present study is that the longer half-life of MK-7 may have prevented a steady state being attained for each dosage level. In part, we allowed for this by giving each dose for 2 weeks, which was the time needed for blood concentrations to plateau after daily administration of the high 143-μg dose of MK-7 [5]. In fact, after 10-μg and 20-μg MK-7 doses, there was no difference between the group mean INR values at the end of the first and second weeks of supplementation (Table 3). Against this, the mean INR did show a further decrease between the first and second weeks of supplementation with 45 μg of MK-7. Clearly, further studies are required to investigate the agonist effect of MK-7 during prolonged supplementation.

We chose a low target INR of 2.0 for two reasons; first, so that the results would be directly comparable to our previous data [5,7]; and second, for the safety of the healthy volunteers. Further studies are required to determine whether the same dose–response relationships are present for higher target INRs such as 2.5–3.0. It is possible that, because our target INR was at the lower end of the therapeutic range, this may have magnified the significance of small changes in the INR and accounted for the partly discordant judgements of clinical relevance by the two hematologists. Another related question is whether the same dose–response relationships would be obtained for other coumarin or indandione VKAs. The molecular target of all VKAs is vitamin K epoxide reductase (VKOR), whereas an NADP(H)-dependent quinone reductase, which is relatively insensitive to VKAs, explains many of the antidotal properties of vitamin K in vivo and in vitro [7]. VKAs such as acenocoumarol, warfarin and phenprocoumon all possess the same 4-hydroxycoumarin ring, which is responsible for their pharmacodynamic effects (i.e. inhibition of VKOR). On the other hand, their different side chain structures dictate disposition and metabolism, and largely account for the differing dose requirements for achieving the same degree of inhibition of VKOR. According to these principles, it is likely that the response to vitamin K may be generalizable to all clinically used VKAs, but this would need to be verified experimentally.

In conclusion, the results presented here confirm that the much higher antidotal potency of MK-7 than of vitamin K_1 extends to even very low doses of MK-7, and serves as a warning to practitioners of VKA therapy of the potential dangers of the increasingly available MK-7 supplements.

Addendum

C. Vermeer and L. J. Schurgers: designed the research; K. J. Teunissen: conducted the research; H. M. H. Spronk, K. J. Teunissen, and M. J. Shearer: responsible for the (biomarker) analyses; E. Theuwissen, H. M. H. Spronk, and L. J. Schurgers: analyzed the data; C. Vermeer, E. Theuwissen, H. M. H. Spronk, M. J. Shearer, and L. J. Schurgers: wrote the article; C. Vermeer: had primary responsibility for the final content. H. ten Cate and K. Hamulyák were the responsible Doctor of Medicine. All authors read and approved the final manuscript.

Disclosure of Conflict of Interest

This work was supported by Nattopharma ASA (Hovik, Norway).

References

1 Holmes MV, Hunt BJ, Shearer MJ. The role of dietary vitamin K in the management of oral vitamin K antagonists. *Blood Rev* 2012; **26**: 1–14.
2 Schurgers LJ, Vermeer C. Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. *Haemostasis* 2000; **30**: 298–307.
3 Shearer MJ, Newman P. Metabolism and cell biology of vitamin K. *Thromb Haemost* 2008; **100**: 530–47.
4 Usui Y, Tanimura H, Nishimura N, Kobayashi N, Okanoue T, Ozawa K. Vitamin K concentrations in the plasma and liver of surgical patients. *Am J Clin Nutr* 1990; **51**: 846–52.
5 Schurgers LJ, Teunissen KJ, Hamulyak K, Knapen MH, Vik H, Vermeer C. Vitamin K-containing dietary supplements: comparison of synthetic vitamin K1 and natto-derived menaquinone-7. *Blood* 2007; **109**: 3279–83.
6 Shearer MJ, Fu X, Booth SL. Vitamin K nutrition, metabolism, and requirements: current concepts and future research. *Adv Nutr* 2012; **3**: 182–95.

7 Schurgers LJ, Shearer MJ, Hamulyak K, Stocklin E, Vermeer C. Effect of vitamin K intake on the stability of oral anticoagulant treatment: dose–response relationships in healthy subjects. *Blood* 2004; **104**: 2682–9.

8 Karlson B, Leijd B, Hellstrom K. On the influence of vitamin K-rich vegetables and wine on the effectiveness of warfarin treatment. *Acta Med Scand* 1986; **220**: 347–50.

9 Shearer MJ. Assay of coumarin antagonists of vitamin K in blood by high-performance liquid chromatography. *Methods Enzymol* 1986; **123**: 223–34.

10 Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smed E, Wagenvoord R, Lecompte T, Beguin S. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb* 2002; **32**: 249–53.

11 Belle M, Brebant R, Guinet R, Leclercq M. Production of a new monoclonal antibody specific to human des-gamma-carboxyprothrombin in the presence of calcium ions. Application to the development of a sensitive ELISA-test. *J Immunoassay* 1995; **16**: 213–29.

12 Widdershoven J, van Munster P, De Abreu R, Bosman H, van Lith T, van der Putten-van Meyel M, Motohara K, Matsuda I. Four methods compared for measuring des-carboxy-prothrombin (PIVKA-II). *Clin Chem* 1987; **33**: 2074–8.

13 Cranenburg EC, Koos R, Schurgers LJ, Magdeleyns EJ, Schoonbrood TH, Landewe RB, Brandenburg VM, Bekers O, Vermeer C. Characterisation and potential diagnostic value of circulating matrix Gla protein (MGP) species. *Thromb Haemost* 2010; **104**: 811–22.

14 Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies on a request from the European Commission on the safety of 'Vitamin K2'. *EFSA J* 2008; **822**: 1–32.

15 Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies on a Request from the European Commission on the substantiation of health claims related to Vitamin K and maintenance of bone pursuant to Article 13(1) of Regulation (EC) No. 1924/20061. *EFSA J* 2009; **7**: 1128–48.

16 Altman R, Scazziota A, Herrera L, Gonzalez C. Relationship between thrombin generation and international normalized ratio in patients receiving oral vitamin K antagonist therapy. *J Thromb Haemost* 2007; **5**: 1552–69.

17 Furie B, Liebman HA, Blanchard RA, Coleman MS, Kruger SF, Furie BC. Comparison of the native prothrombin antigen and the prothrombin time for monitoring oral anticoagulant therapy. *Blood* 1984; **64**: 445–51.

18 Lee W, Chung HJ, Kim S, Jang S, Park CJ, Chi HS, Chun S, Min WK. PIVKA-II is a candidate marker for monitoring the effects of the oral anticoagulant warfarin. *Clin Biochem* 2010; **43**: 1177–9.

19 Rezaieyazdi Z, Falsoleiman H, Khajehdaluee M, Saghafi M, Mokhtari-Amirmajdi E. Reduced bone density in patients on long-term warfarin. *Int J Rheum Dis* 2009; **12**: 130–5.

20 Gage BF, Birman-Deych E, Radford MJ, Nilasena DS, Binder EF. Risk of osteoporotic fracture in elderly patients taking warfarin: results from the National Registry of Atrial Fibrillation 2. *Arch Intern Med* 2006; **166**: 241–6.

21 Koos R, Mahnken AH, Muhlenbruch G, Brandenburg V, Pflueger B, Wildberger JE, Kuhl HP. Relation of oral anticoagulation to cardiac valvular and coronary calcium assessed by multislice spiral computed tomography. *Am J Cardiol* 2005; **96**: 747–9.

22 Chatrou ML, Winckers K, Hackeng TM, Reutelingsperger CP, Schurgers LJ. Vascular calcification: the price to pay for anticoagulation therapy with vitamin K-antagonists. *Blood Rev* 2012; **26**: 155–66.

23 Schurgers LJ, Joosen IA, Laufer EM, Chatrou ML, Herfs M, Winkens MH, Westenfeld R, Veulemans V, Krueger T, Shanahan CM, Jahnen-Dechent W, Biessen E, Narula J, Vermeer C, Hofstra L, Reutelingsperger CP. Vitamin K-antagonists accelerate atherosclerotic calcification and induce a vulnerable plaque phenotype. *PLoS ONE* 2012; **7**: e43229.

24 McCann JC, Ames AB. Vitamin K, an example of triage theory: is micronutrient inadequacy linked to diseases of aging? *Am J Clin Nutr* 2009; **90**: 889–907.

25 Schurgers LJ, Vermeer C. Differential lipoprotein transport pathways of K-vitamins in healthy subjects. *Biochim Biophys Acta* 2002; **1570**: 27–32.

26 Sconce E, Avery P, Wynne H, Kamali F. Vitamin K supplementation can improve stability of anticoagulation for patients with unexplained variability in response to warfarin. *Blood* 2007; **109**: 2419–23.

27 Rombouts EK, Rosendaal FR, van Der Meer FJ. Daily vitamin K supplementation improves anticoagulant stability. *J Thromb Haemost* 2007; **5**: 2043–8.

28 Duello TJ, Matschiner JT. Characterization of vitamin K from human liver. *J Nutr* 1972; **102**: 331–5.

29 Shearer MJ. Vitamin K metabolism and nutriture. *Blood Rev* 1992; **6**: 92–104.

30 Caldwell MD, Awad T, Johnson JA, Gage BF, Falkowski M, Gardina P, Hubbard J, Turpaz Y, Langaee TY, Eby C, King CR, Brower A, Schmelzer JR, Glurich I, Vidaillet HJ, Yale SH, Qi Zhang K, Berg RL, Burmester JK. CYP4F2 genetic variant alters required warfarin dose. *Blood* 2008; **111**: 4106–12.

31 McDonald MG, Rieder MJ, Nakano M, Hsia CK, Rettie AE. CYP4F2 is a vitamin K1 oxidase: an explanation for altered warfarin dose in carriers of the V433M variant. *Mol Pharmacol* 2009; **75**: 1337–46.

32 Landes N, Pfluger P, Kluth D, Birringer M, Ruhl R, Bol GF, Glatt H, Brigelius-Flohe R. Vitamin E activates gene expression via the pregnane X receptor. *Biochem Pharmacol* 2003; **65**: 269–73.

33 Landes N, Birringer M, Brigelius-Flohe R. Homologous metabolic and gene activating routes for vitamins E and K. *Mol Aspects Med* 2003; **24**: 337–44.