Menaquinone-7 supplementation improves arterial stiffness in healthy postmenopausal women

A double-blind randomised clinical trial

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Summary

Observational data suggest a link between menaquinone (MK, vitamin K2) intake and cardiovascular (CV) health. However, MK intervention trials with vascular endpoints are lacking. We investigated long-term effects of MK-7 (180 µg MenaQ7/day) supplementation on arterial stiffness in a double-blind, placebo-controlled trial. Healthy postmenopausal women (n=244) received either placebo (n=124) or MK-7 (n=120) for three years. Indices of local carotid stiffness (intima-media thickness IMT, Diameter end-diastole and Distension) were measured by echotracking. Regional aortic stiffness (carotid-femoral and carotid-radial Pulse Wave Velocity, cfPWV and crPWV, respectively) was measured using mechanotransducers. Circulating desphospho-uncarboxylated matrix Gla-protein (dp-ucMGP) as well as acute phase markers Interleukin-6 (IL-6), high-sensitive C-reactive protein (hsCRP), tumour necrosis factor-α (TNF-α) and markers for endothelial dysfunction Vascular Cell Adhesion Molecule (VCAM), E-selectin, and Advanced Glycation Endproducts (AGEs) were measured. At baseline dp-ucMGP was associated with IMT, Diameter, cfPWV and with the mean z-scores of acute phase markers (APMscore) and of markers for endothelial dysfunction (EDFscore). After three year MK-7 supplementation cfPWV and the Stiffness Index β significantly decreased in the total group, whereas distension, compliance, distensibility, Young's Modulus, and the local carotid PWV (cPWV) improved in women having a baseline Stiffness Index β above the median of 10.8. MK-7 decreased dp-ucMGP by 50 % compared to placebo, but did not influence the markers for acute phase and endothelial dysfunction. In conclusion, long-term use of MK-7 supplements improves arterial stiffness in healthy postmenopausal women, especially in women having a high arterial stiffness.

Keywords

Arterial stiffness, carotid intima-media thickness, menaquinone-7, pulse wave velocity, Stiffness Index β

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Financial support:
This work was supported by Nattopharma (Høvik, Norway). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors, and do not necessarily reflect the view of Nattopharma.

Received: August 14, 2014
Accepted after major revision: December 31, 2014
Epub ahead of print: February 19, 2015

http://dx.doi.org/10.1160/TH14-08-0675
Thromb Haemost 2015; 113: 1135–1144

Introduction

In recent years, there has been a strong focus on the role of arterial stiffness in the development of cardiovascular disease (CVD). Arterial stiffness can be measured directly and non-invasively (1) and has emerged as independent predictor of CV risk (2, 3). Carotid-femoral pulse wave velocity (cfPWV) is considered the gold-standard measurement of regional arterial stiffness and has been demonstrated in epidemiological studies the predictive value of aortic stiffness for CV events (1, 4–6). Measurement of local carotid stiffness may also provide important prognostic information since the carotid artery is a frequent site of atheroma formation (1). Indices of local carotid stiffness include: carotid pulse wave velocity (cPWV) based on carotid diameter and pulse pressure (7), arterial wall thickness (carotid intima-media thickness, IMT), elastic properties of the artery as a whole (Distensibility, Compliance), elastic properties of the arterial wall material (Young's Modulus) and the Stiffness Index β (1, 8). Arterial stiffening accompanying age and other CV risk factors is caused by various phenomena, including breaks in elastin fibres, accumulation of collagen, fibrosis, medial smooth muscle necrosis, diffusion of macromolecules in the arterial wall, inflammation, and calcification (1). Vascular calcification can modify both functional and structural arterial properties, known as arterial remodelling, resulting in increased arterial stiffness.

Observational studies showed lower prevalence of arterial calcification and coronary heart disease mortality in subjects with the highest intake of menaquinones (MKs, vitamin K2) (9–11). Remarkably, no effect was seen for phylloquinone (vitamin K1) in-

take in these studies. This is in line with an earlier publication in which no relation was found between dietary phylloquinone intake and premature coronary calcification (12). Erkkilä et al. found an association between phylloquinone intake and coronary heart disease, but it was lost after adjustment for dietary variables (13, 14). While phylloquinone is primarily found in green leafy vegetables, MKs occur, but to a much lower extent, in meat (menaquinone-4, MK-4) and fermented foods, like cheeses (MK-8 to MK-10) in Western diets and natto (MK-7) in Japan (15). Up to now, only two randomised clinical trials have evaluated the effects of vitamin K supplements on CV health (16, 17) and showed beneficial effects with high daily dosages (0.5–1 mg) of phylloquinone. To date, MK intervention trials with CV endpoints are lacking, despite the increasing retail availability of MK-7 supplements. Its longer half-life and higher efficacy compared with phylloquinone (18) justify the use of much lower doses of MK-7 in trials studying its effects on CV health.

The circulating inactive form of matrix Gla-protein (MGP), i.e. desphospho-uncarboxylated MGP (dp-ucMGP), a marker for vascular vitamin K status (19), has been associated with arterial calcification and CV mortality (20–25). Vitamin K mediates the carboxylation of protein-bound glutamate (Glu) residues into γ-carboxyglutamate (Gla) in MGP resulting in protein functionality, i.e. local calcification inhibition (15). Substantial amounts of dp-ucMGP can be found in the circulation of non-supplemented healthy individuals, indicative of vascular vitamin K insufficiency. Recent work showed that vitamin K supplementation improves MGP carboxylation (26–29), but the question remains whether this beneficially affects CV health. Next to its role in carboxylation, vitamin K may influence arterial stiffness by carboxylation-independent mechanisms. Inflammation plays also a role in arterial remodelling (30), possibly by affecting proliferation of vascular smooth muscle cells, influx of leucocytes, and/or production of proinflammatory markers. *In vitro* studies suggest that vitamin K may suppress inflammation by decreasing gene expression of proinflammatory markers (31). Consistently, observational data showed an inverse association between vitamin K status and proinflammatory markers (26, 32–34). Although phylloquinone supplements (0.5 mg/day) were not effective in healthy elderly (17, 35), three-month MK-4 supplementation (45 mg/day) significantly reduced serum C-reactive protein (CRP) and matrix metalloproteinase-3 (MMP-3) levels in rheumatoid arthritis patients (36).

The aim of this study was to test the hypothesis that MK-7 supplementation has a beneficial effect on the arterial stiffness and on the elastic properties of the carotid artery in healthy postmenopausal women.

Materials and methods

Study design

The study design has been described previously (37). Sample size was determined based on the primary outcome measure, i.e. bone strength. In summary, 244 healthy postmenopausal women aged between 55 and 65 years participated in this randomised, double-blind, placebo-controlled three-year intervention trial (2008–2011). Exclusion criteria were < 2 years postmenopausal, body mass index (BMI) > 30 kg/m2, osteoporotic at baseline (T-score ≤-2.5 SD), coagulation disorders, chronic diseases, metabolic bone diseases, gastrointestinal diseases, medication that interferes with vitamin K and/or blood coagulation, use of corticosteroids, bisphosphonates, or hormone replacement therapy, use of supplements containing vitamin K, participation in a clinical study three months prior to this study, and soy allergy. Participants were randomly assigned to receive placebo capsules (n=124) or capsules containing 180 μg MK-7 (MenaQ7, NattoPharma, Høvik, Norway) (n=120). During the trial were no dietary restrictions. From the 244 volunteers who entered the study, 21 discontinued their participation and were not available for the follow-up measurements. Participants came to the research site (VitaK, Maastricht, The Netherlands) every year for blood sampling and measurements of body weight, height, and vascular parameters. Methods for blood sampling and anthropometrics were described previously (37).

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Medical Ethics Committee of the Maastricht University (Maastricht, The Netherlands). Written informed consent was obtained from all subjects before entering the study. Trial registration code: clinicaltrials.gov NCT00642551.

Study products

Capsules containing 180 μg MK-7 (MenaQ7, Nattopharma, Høvik, Norway) and matching placebo capsules (with the same composition, except for the active component MK-7) were manufactured by EuroPharma Alliance (Wroclaw, Poland) for Nattopharma (Høvik, Norway). They were delivered directly by Nattopharma to VitaK (Maastricht, The Netherlands). One capsule was taken daily either with breakfast or dinner during a period of 36 months. Based on a 3-fold higher efficacy of MK-7 than phylloquinone (18) and the effectiveness of phylloquinone at 500 μg/day (17), supplementing with 180 μg/day of MK-7 was expected to significantly affect CV health. Moreover, the daily amount of 180 μg MK-7 equals ~120 μg phylloquinone on a molar base which is in the range of the dietary reference intake (DRI) of vitamin K (38), and this MK-7 dosage was shown to significantly improve MGP carboxylation (27, 29). No side-effects have been reported for the long-term use of MK-7.

Circulating markers

Total cholesterol and triglyceride (TG) concentrations were determined on a Beckman Coulter LX20 PRO Clinical Chemistry analyser (Beckman Coulter, Fullerton, CA, USA). Assessment of the glucose levels occurred using the Glucose Assay kit (Abcam, Cambridge, MA, USA); intra- and inter assay variations were 2.4 and 5.1 %, respectively. Plasma dp-ucMGP was measured using the ina*K*tif MGP iSYS kit (ImmunoDiagnostic Systems, Boldon, UK), which is a dual-antibody test based on the previously described

Table 1: Definitions, terms, and equations (if applicable).

Definition	Term	Equation
Mean Arterial Pressure in brachial artery	MAP	DBP+(SBP-DBP)/3
Pulse Pressure in brachial artery	Δp	SBP-DBP
Intima-media thickness of the carotid artery	IMT	
Diameter of the carotid artery end-diastole	D	
Distension: change in carotid diameter in systole	Δd	
Change of area from diastole to systole	ΔA	$\pi(2*D*\Delta d+\Delta d^2)/4$
Distensibility Coefficient	DC	$(2*D*\Delta d+\Delta d^2)/(\Delta p *D^2)$
Compliance Coefficient	CC	ΔA/Δp
Young's Elasticity Modulus		(D/IMT)/DC
Stiffness Index β		D * ln(SBP/DBP)/Δd
Local carotid Pulse Wave Velocity	cPWV	$1/\sqrt{(\rho*DC)}$
(ρ = blood density = 1060 kg/m^3)		

The terms D, Δd, DC and CC are only used in the equations in this table. Throughout the text and tables the terms Diameter, Distension, Distensibility and Compliance are used.

sandwich ELISA developed by VitaK (Maastricht, The Netherlands) (19). The intra- and inter-assay variations were 7.6% and 6.8%, respectively, and the sensitivity was 50 pM. Commercial ELISAs were used to determine the acute phase markers Interleukin-6 (IL-6, ultra-sensitive; Invitrogen, Camarillo, CA, USA), high-sensitive C-reactive Protein (hsCRP; DRG International, Springfield, NJ, USA) and Tumour Necrosis Factor-α (TNF-α; R&D Systems Europe, Abingdon, UK) and the markers for endothelial dysfunction E-Selectin (R&D Systems Europe), Vascular Cell Adhesion Molecule (VCAM; IBL International, Hamburg, Germany), and Advanced Glycation End products (AGEs; Cell BioLabs Inc, San Diego, CA, USA). The intra- and inter assay variation and sensitivity were 6.4%, 7.8% and <104 pg/L for IL-6; 7.7%, 8.3% and 0.20 mg/L for hsCRP; 5.4%, 8.3% and 0.11 ng/L for TNF- α; 6.0%, 7.8% and 0.009 µg/L for E-Selectin, 3.1%, 5.2% and 0.6 µg/L for VCAM; 6.0%, 9.2% and 0.5 mg/L for AGEs, respectively.

Local arterial stiffness

Echotracking was performed to determine vascular characteristics of the common carotid artery: diameter and arterial wall thickness (IMT) in end-diastole, change in diameter (Distension) and change in area from diastole to systole (ΔA), elastic properties of the artery as a whole (Distensibility and Compliance), and elastic properties of the arterial wall material (Young's Modulus) (Artlab multiarray echotracking, Esaote Picus Pro, Esaote Europe, Maastricht, The Netherlands) (1, 16). The yearly Quality Control (inter- and intra-operator CVs) assessments for the measurements of the local arterial stiffness showed coefficients of variation of less than 10%.

Arterial blood pressure was recorded in parallel with echotracking at the level of the brachial artery by a semi-automated oscillometric device (Dinamap, KP Medical, Houten, The Netherlands), providing the diastolic (DBP), systolic (SBP) and pulse pressure (Δp). In ▶Table 1 the various characteristics are described with corresponding equation (if applicable).

Regional arterial stiffness

Regional aortic (cfPWV) and arm (crPWV) pulse wave velocity were assessed non-invasively by using mechanotransducers directly applied on the skin (Complior, Artech Medical, Pantin, France) (1). Our yearly Quality Control assessments for the PWV measurements showed coefficients of variation of less than 10%.

Statistical analysis

As described previously, the primary aim of this study was to investigate the effect of MK-7 on bone strength (37). Power calculations were based on this end-point. Normality was tested and data that were not normally distributed were log-transformed before statistical analyses (applicable for IMT, Distensibility, Compliance, IL-6, VCAM, hsCRP, TNF-α, and TG). Independent Samples T test (normally distributed outcomes) and Chi-square test (categorical outcomes) were used to test between-group differences for baseline characteristics. To increase the statistical power mean z-scores were used for the acute phase markers hsCRP, IL-6 and TNF-α (APMscore) and for the markers for endothelial dysfunction E-selectin, VCAM, AGEs (EDFscore). The mean z-score was calculated as the sum of the [(individual value –population mean) / population SD] of each variable divided by the number of markers (i.e. 3).

Correlation analyses at baseline between the parameters of interest were performed using the Pearson test. Multivariate linear regression analysis was performed to study treatment effects on the parameters of interest (adjusted for confounders) and according to the intention-to-treat principle (missing values were replaced by the last observed values). The measure of interest was

used as the dependent variable. The concomitant baseline value and the treatment regimen (placebo/MK-7) were included as independent variables (univariate crude model). Age, BMI, log(TG) were included as potential confounders (base model). Cholesterol-lowering medication (yes/no) and hypertension-lowering medication (yes/no) were used as additional covariates in the base model (multi 1). Multi 2 consisted of the base model with the covariates cholesterol-lowering medication (yes/no), anti-hypertensive medication (yes/no), Heart Rate, SBP and DBP. Data are presented as means with SD (SE in figure). A $p<0.05$ was considered statistically significant. Statistics were performed using SPSS for Windows, version 19 (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics of the study poputalion

Characteristics of the 244 participants included in the trial are presented in ►Table 2. No significant differences were observed between the treatment groups.

Correlations at baseline

cfPWV and Stiffness Index β were positively correlated with the properties of the carotid artery: IMT, Diameter, Heart Rate, Young's Modulus and cPWV, and inversely correlated with Distensibility and Compliance (►Table 3). The Distension showed an inverse correlation with Stiffness Index β, but not with cfPWV. The positive association of cfPWV with the APMscore was due to IL6 (r=0.142, p=0.043) and hsCRP (r=0.154, p=0.030). No associations were found between crPWV and the characteristics of the carotid artery.

Circulating dp-ucMGP (i.e. poor vitamin K status) was associated with IMT, Diameter and cfPWV, but not with Stiffness Index β. A positive correlation was found between dp-ucMGP and the AMPscore, due to IL6 (r=0.297, p<0.0001) and hsCRP (r=0.339, p<0.0001). The separate markers of endothelial dysfunction (VCAM, E-Selectin and AGEs) were not associated with dp-ucMGP. The summarised EDFscore showed a weak association.

Effect of MK-7 on the properties of the carotid artery and on the pulse wave velocity

After three years of treatment the Stiffness Index β in the MK-7 group had decreased significantly after compared to the slight increase in the placebo group (-0.67 ± 2.78 vs +0.15 ± 2.51, respectively, p=0.018). No between-group effects were found during the three-year treatment period on the absolute values nor on the absolute changes of the variables IMT, Diameter, Distension, Distensibility, Compliance, and Young's Modulus.

The total group of postmenopausal women was dichotomised into a group with a low and with a high Stiffness Index β, based on

Table 2: Baseline characteristics.

	Total group (n=244)	Placebo group (n=124)	MK-7 group (n=120)
Age (years)	59.5 ± 3.3	59.3 ± 3.1	59.8 ± 3.5
BMI (kg/m^2)	25.1 ± 3.0	25.1 ± 3.0	25.1 ± 3.1
YSM (y)	8.9 ± 5.5	8.4 ± 5.4	9.4 ± 5.6
Current smoker (%)	13	15	11
Alcohol use (%)	76	78	73
Statin use (%)	10	9	11
Blood pressure medication (%)	22	23	21
Cholesterol (mmol/l)	6.0 ± 1.0	6.0 ± 1.0	6.0 ± 1.0
TG (mmol/l)	1.27 ± 0.66	1.21 ± 0.67	1.33 ± 0.64
Glucose (mmol/l)	4.4 ± 0.6	4.4 ± 0.7	4.4 ± 0.6
Blood pressure			
SBP (mmHg)	126.2 ± 15.8	126.2 ± 16.3	126.2 ± 15.4
DBP (mmHg)	73.5 ± 7.7	73.4 ± 7.8	73.6 ± 7.6
Δp (kPa)	7.0 ± 1.6	7.0 ± 1.6	7.0 ± 1.6
Heart Rate (bpm)	62.8 ± 8.8	62.3 ± 9.0	63.3 ± 8.6
MAP (kPa)	12.1 ± 1.3	12.1 ± 1.3	12.1 ± 1.2
Arterial stiffness			
cPWV (m/s)	8.1 ± 1.5	8.1 ± 1.6	8.2 ± 1.2
crPWV (m/s)	10.2 ± 1.4	10.1 ± 1.4	10.2 ± 1.4
cfPWV (m/s)	9.8 ± 1.8	9.7 ± 1.7	9.9 ± 1.9

Table 2: Continued

	Total group (n=244)	Placebo group (n=124)	MK-7 group (n=120)
Elastic properties of the carotid artery			
IMT (µm)	577 ± 104	572 ± 98	582 ± 110
Diameter (µm)	6986 ± 721	7006 ± 752	6966 ± 690
Distension (µm)	356 ± 108	363 ± 115	350 ± 100
ΔA (mm^2)	4.06 ± 1.52	4.18 ±1.69	3.95 ±1.31
Compliance (mm^2/kPa)	0.59 ± 0.23	0.61 ± 0.27	0.56 ± 0.17
Distensibility (MPa-1)	15.6 ± 5.4	16.0 ± 6.0	15.1 ± 4.8
Young's Modulus (MPa)	0.89 ± 0.39	0.91 ± 0.48	0.88 ± 0.27
Stiffness Index β	11.4 ± 3.8	11.3 ± 4.5	11.4 ± 3.1
Vitamin K status			
dp-ucMGP (pmol/l)	525 ± 266	538 ± 293	511 ± 236
Acute Phase markers			
IL-6 (ng/l)	0.5 ± 1.1	0.6 ± 1.4	0.5 ± 0.7
hsCRP (mg/l)	1.8 ± 2.3	1.9 ± 2.5	1.7 ± 2.0
TNF-α (ng/ml)	1.8 ± 2.3	1.9 ± 2.5	1.7 ± 2.0
Endothelial dysfunction markers			
VCAM (µg/l)	653 ± 167	657 ± 168	649 ± 166
E-Selectin (µg/l)	30.3 ± 10.8	29.5 ± 10.6	31.1 ± 11.1
AGE (mg/l)	23.8 ± 4.9	24.2 ± 5.0	23.5 ± 4.8

Data presented are means with SD or numbers of subjects (percentages). No significant differences were found between the treatment groups (Independent Samples T test and Chi-square test). ΔA, change of area in systole; AGE, advanced glycation end-product; BMI, body mass index; bpm, beats per minute; cPWV, carotid pulse wave velocity; cfPWV, carotid-femoral pulse wave velocity; crPWV, carotid-radial pulse wave velocity; DBP, diastolic blood pressure; dp-ucMGP, desphospho-uncarboxylated matrix Gla-protein; hsCRP, high-sensitive C-reactive protein; IL-6, interleukin-6; IMT, intima-media thickness; MAP, mean arterial pressure; Δp, pulse pressure ; SBP, systolic blood pressure; TNF-α, tumour necrosis factor alpha; VCAM, vascular adhesion molecule.

the 50th percentile (median) of 10.8. At baseline the group with high β (≥10.8) had higher SBP, IMT, Diameter and cPWV values than the group with low β (<10.8). Between group analyses also revealed that the Distension and the elastic properties Distensibility and Compliance were lower in the high β group (►Table 4). During the three-year treatment period the absolute values of the Stiffness Index β in the total group were not affected by MK-7 (►Figure 1A). No effect was observed of MK-7 in the group of women with a low Stiffness Index β (►Figure 1B) after the total group was dichotomised. The group with women having a high Stiffness Index β (i.e. less favourable vascular characteristics), however, did respond to MK-7 (►Figure 1C, p=0.009).

During the first two years of treatment the absolute values of cfPWV in both groups were comparable. After three years cfPWV in the MK-7 group had decreased borderline significantly (p=0.087) compared to the placebo group (►Figure 1D). The absolute changes, however, were -0.36 ± 1.48 m/s in the MK-7 group and +0.021 ± 1.22 m/s in the placebo group after three years (p=0.040). Regarding the women with low Stiffness Index β the cfPWV was comparable for both treatment groups (►Figure 1E), MK-7 tended to decrease cfPWV (p=0.062) in women with high Stiffness Index β (►Figure 1F).

Although crPWV changed to the same extent as cfPWV, the difference was borderline significant (placebo: –0.091 ± 1.51 m/s; MK-7: –0.44 ± 1.42 m/s; p=0.073). The (calculated) cPWV in the MK-7 group had decreased by 0.37 ± 1.15 m/s and in the placebo group by 0.19 ± 1.04 m/s; this difference was not statistically significant, however (p=0.19).

Multivariate linear regression of the effect of MK-7 on the different variables was performed in the total group and in the group with low and with high Stiffness Index β (►Table 5). In the unadjusted crude model, the decrease of the Stiffness Index β and cfPWV in the total group was significant both after adjusting for the potential confounders (age, BMI and log(TG)) in the base model, and after adjusting for the additional covariates in the multivariate model. After adjustment, three-year MK-7 treatment in the group of women with a high Stiffness Index β resulted in a significant improvement (i.e. increase) of the Distension,

Distensibility, Compliance and also in an improved (i.e. decreased) Stiffness Index β, Young's Modulus, cPWV, crPWV. None of these variables responded to MK-7 in the group with a low Stiffness Index β. cfPWV decreased to the same extent in the low- and high group (p-values after adjustment were borderline significant). IMT and Diameter were not influenced by MK-7 treatment, not in the total group nor in groups with a low and high Stiffness Index β.

Effect of MK-7 on circulating markers

MK-7 supplementation significantly decreased circulating dp-ucMGP after three years by ~50% as compared to placebo (MK-7: -32 ± 25%; placebo: 22 ± 45%; p<0.0001). The maximal effect on MGP carboxylation was already reached during the first year (MK-7: –192 ± 137 pmol/l; placebo: +48 ± 157 pmol/l; p<0.0001) and lasted over the next two years of supplementation (after three years MK-7: –188 ± 157 pmol/l; placebo: +74 ± 182 pmol/l; p<0.0001). The change in dp-ucMGP values among women with low or high Stiffness Index β was similar in the MK-7 group (-34 ± 21% and –30 ± 28%, respectively) and placebo group (22 ± 50% and 22 ± 37%, respectively). MK-7 supplementation had no effect on fasting glucose, on the acute phase markers (hsCRP, IL-6 and TNF-α) nor on the markers for endothelial dysfunction (VCAM, E-selectin and AGE).

Table 3: Pearson correlation at baseline in the total group between properties of the carotid artery, mean APM and EDF z-scores with arterial stiffness parameters (PWV and Stiffness Index β) and dp-ucMGP (n=244).

	crPWV	cfPWV	Stiffness Index β	dp-ucMGP
Age	-0.051	0.148*	0.151*	0.176*
BMI	-0.060	0.093	0.126*	0.249#
IMT	0.001	0.212#	0.258#	0.158*
Diameter	-0.035	0.132*	0.175*	0.149*
Distension	0.05	0.081	-0.703#	-0.018
Heart Rate	0.092	0.179*	0.137*	0.206**
SBP	0.282#	0.562#	0.247#	0.078
DBP	0.373#	0.394#	0.107	0.093
Compliance	-0.033	-0.144*	-0.787#	-0.019
Distensibility	-0.024	-0.229**	-0.930#	-0.107
Young's Modulus	0.013	0.141*	0.835#	0.068
Stifness Index β	-0.088	0.073	1	0.08
cPWV	0.028	0.232#	0.946#	0.103
crPWV	1	0.523#	-0.088	0.087
cfPWV	0.523#	1	0.073	0.155*
APMscore	-0.022	0.147*	0.073	0.354#
EDFscore	0.032	0.12	-0.01	0.174*

Pearson correlation: *p<0.05, **p<0.005, #p<0.0001. APMscore, mean z-score of acute phase markers; BMI, body mass index; cPWV, carotid pulse wave velocity; cfPWV, carotid-femoral pulse wave velocity; crPWV, carotid-radial pulse wave velocity; DBP, diastolic blood pressure; dp-ucMGP, desphospho-uncarboxylated matrix Gla-protein; EDFscore, mean z-score of endothelial dysfunction markers; IMT, intima-media thickness; SBP, systolic blood pressure.

Discussion

This is the first study showing that long-term use of MK-7 supplements beneficially affects CV health, thus confirming the population-based studies showing an association between MK intake and coronary heart disease or CV mortality. More particularly, MK-7 supplementation at a nutritional dose significantly improved the parameters for arterial stiffness (cfPWV and the Stiffness Index β). The elastic properties of the carotid artery (Distension, Distensibility, Compliance, Young's Modulus) and also crPWV and cPWV responded to MK-7 in a positive way, but only in the group of women with an increased Stiffness Index β.

cfPWV is generally accepted as the golden standard for central arterial stiffness (39), but thus far it has never been used to study health effects of vitamin K. Whereas the Distensibility is locally quantified (cPWV and the Young's Modulus are calculated from this variable), cfPWV provides a regional measure of the mechanical properties of the aorta. The Distensibility and cfPWV correlate with CV risk and were recently described as representatives of (partly) comparable adverse vascular processes during ageing (39). Also in our study, these vascular measures inter-correlated significantly at baseline. No supplementation effects on the IMT and Diameter were seen, however.

Besides cfPWV, the Stiffness Index β is a marker for local arterial stiffening. Due to the natural log function of SBP/DBP in its equation it is less dependent on the blood pressure. Although both are markers for arterial stiffening, they were not inter-correlated, whereas the Stiffness Index β has strong associations with the measures for elastic properties. Although the response of MK-7 on dp-ucMGP was not depending on the Stiffness Index β, MK-7 improved the elastic properties of the carotid artery only in the group of women who had high values of the Stiffness Index β. This suggests that MK-7 treatment is more effective in subjects with impaired elastic properties than in those with a healthy vasculature.

The Young's Modulus, which takes in account the arterial wall thickness, improved significantly by extra MK-7 intake. Similar results were previously seen in healthy postmenopausal women after high-dose phylloquinone supplementation: beneficial effects on the Young's Modulus without structural changes in IMT (16). The lack of effect on IMT may be explained by the fact that the study populations consisted of healthy postmenopausal women without established CVD. Their IMT values equalled the reference value of healthy women, i.e. ~550 µm whereas an IMT of >900 µm is considered a risk marker for CVD (40). In an early stage of arterial ageing, the relation between functional and structural changes is not fully understood and alterations in the elastic properties of the arterial wall can precede structural alterations. So, to observe substantial effects on IMT a longer supplemental period may be

required. Further, effects of vitamin K supplements on arterial wall thickness may only become manifest among subjects with more advanced atherosclerosis.

Animal and *in vitro* studies have demonstrated that vitamin K is involved in vascular calcification inhibition through its cofactor function in carboxylating MGP (41–43). Decreased MGP carboxylation may decrease the ability of MGP to inhibit arterial calcification which may contribute to accelerated arterial stiffening. Circulating dp-ucMGP is a marker for vascular vitamin K status and has been linked to vascular calcification and mortality (20–24). In a recent study among 2,500 healthy adults with a follow-up period of 15 years it turned out that poor vitamin K status (as concluded from high circulating dp-ucMGP levels) is a strong risk marker for CV mortality, especially in the highest quartile of circulating dp-ucMGP (i.e. above 400 pM) (44). Strongly elevated values of circulating dp-ucMGP are found in subjects at high CV risk such as patients with chronic kidney disease or heart failure (25, 28, 45). MK-7 supplementation significantly improved MGP carboxylation as concluded from the 50% lowering of plasma dp-ucMGP (relative to placebo). No associations were found between improvement of vitamin K status and changes in arterial stiffness and elastic properties of the carotid artery. On the other hand, plasma dp-ucMGP correlated at baseline with several vascular measures (IMT, Diameter and cfPWV). Four different randomised trials have been published, all showing significant decreases in circulating dp-ucMGP levels after phylloquinone (26) and MK-7 (27–29) supplementation. Effective daily doses ranged from 90 µg to 500 µg with effect sizes of 30% to 80%, respectively (26–29). Similar to our study, the phylloquinone-induced changes in circulating dp-ucMGP could not be linked to the beneficial effects on the progression of coronary artery calcification (26). Case-control studies reported higher plasma dp-ucMGP levels among patients at increased CV risk, including patients with aortic valve disease, aortic stenosis, and chronic kidney disease (21). So, the association between MGP and vascular measures may be stronger in less healthy populations.

It should be noted that MK-7 supplementation in our study group lowered serum uncarboxylated osteocalcin (ucOC) levels, a marker for vitamin K status of bone, to a similar extent as circulating dp-ucMGP (37); this is indicative for comparable uptake of MK-7 by bone and arteries. These results support the reported link between CVD and osteoporosis (46, 47). Both vitamin K–dependent proteins play a role in the mineralisation of tissue (vessel wall and bone), but the physiological implications for (maximally) increased carboxylation of MGP and OC are unknown. In this respect it is intriguing that, besides their role in γ-glutamate carboxylation, MKs were found to be involved in a cell-signalling pathway via the steroid and xenobiotic receptor (SXR) on the nuclear membrane of a variety of cells, and may serve as an important transcription factor. This was specifically found for MK-7, which was reported to regulate gene expression in osteoblastic cells (48). It seems at least feasible that MK-7 plays a similar role in regulating gene expression in vascular smooth muscle cell.

Next to arterial calcification, low-grade inflammation and endothelial dysfunction have been associated with arterial stiffness.

Table 4: Characteristics of postmenopausal women with a low (< 10.8) and a high (≥ 10.8) Stiffnesss Index β at baseline.

	Low Stiffness Index β	High Stiffness Index β	P-value
Age (years)	58.9 ± 3.1	60.1 ± 3.4	0.004
BMI (kg/m^2)	24.7 ± 3.0	25.4 ± 3.1	0.065
TG (mmol/l)	1.16 ± 0.61	1.39 ± 0.69	0.002†
Glucose (mmol/l)	4.4 ± 0.7	4.4 ± 0.6	0.87
dp-ucMGP (pmol/l)	505 ± 237	541 ± 293	0.31
SBP (mmHg)	122 ± 15	130 ± 16	< 0.0001
DBP (mmHg)	73 ± 8	74 ± 8	0.12
Heart Rate (bpm)	61.8 ± 7.9	63.5 ± 9.4	0.16
IMT (µm)	560 ± 91	595 ± 114	0.011†
Diameter (µm)	6872 ± 756	7093 ± 672	0.018
Distension (µm)	418 ± 102	297 ± 75	< 0.0001
Compliance (mm^2/kPa)	0.73 ± 0.23	0.46 ± 0.12	< 0.0001†
Distensibility (MPa-1)	19.6 ± 4.7	11.7 ± 2.3	< 0.0001†
Young's Modulus (MPa)	0.67 ± 0.17	1.08 ± 0.27	< 0.0001
cPWV (m/s)	7.1 ± 0.8	9.1 ± 1.0	< 0.0001
crPWV (m/s)	10.4 ± 1.4	9.9 ± 1.4	0.023
cfPWV (m/s)	9.7 ± 1.7	9.9 ± 1.6	0.40
APMscore	-0.049 ± 0.66	0.048 ± 0.80	0.34
EDFscore	-0.023 ± 0.52	0.011 ± 0.53	0.65

Data represent mean values with SD.†: p-values of the log-transformed variables TG, IMT, Compliance, and Distensibility are given. APMscore, mean z-score of acute phase markers; BMI, body mass index; cPWV, carotid pulse wave velocity; cfPWV, carotid-femoral pulse wave velocity; crPWV, carotid-radial pulse wave velocity; DBP, diastolic blood pressure ; EDFscore, mean z-score of endothelial dysfunction markers; IMT, intima-media thickness; SBP, systolic blood pressure; TG, triglycerides.

Changes in these inflammatory processes may be an alternative mechanism by which vitamin K protects against CVD (30). Observational data have shown an inverse association between vitamin K (status) and acute phase markers, including IL-6 and CRP (26, 32–34). We measured acute phase markers (IL-6, hsCRP, TNF-α) and markers for endothelial dysfunction (VCAM, E-selectin, AGEs), but these markers were not influenced by long-term MK-7 supplementation. Remarkably, IL-6 and hsCRP correlated with vascular vitamin K status (i.e. dp-ucMGP) and arterial stiffness (cfPWV) at baseline; no such correlations were however found for the other markers. Our results are in line with those of the long-term phylloquinone study on the progression of coronary artery calcification, i.e. beneficial effects on CV health with no concomitant decrease of circulating cytokines (17, 26, 32). In contrast, Ebina et al. did show that after three-month high-dose MK-4 administration, CRP and MMP-3 levels in female rheumatoid arthritis patients had significantly decreased (36). This implies that patients with inflammatory diseases, such as rheumatoid arthritis

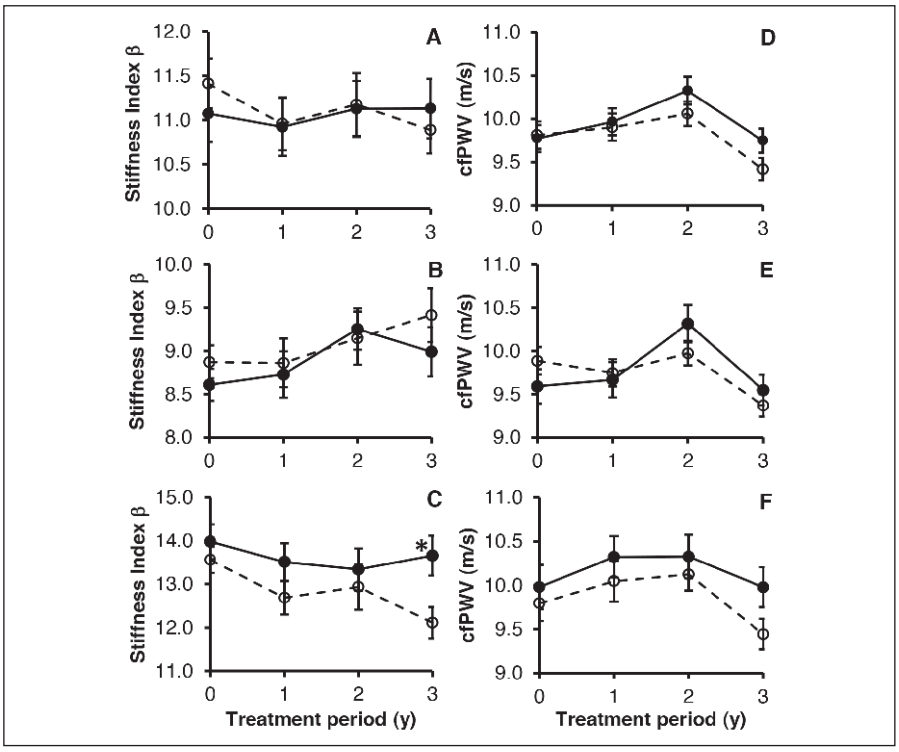

Figure 1: Stiffness Index β (left panels) and cfPWV (right panels) during three-year treatment with placebo (closed symbols) or MK-7 (open symbols) in the total group (A and D), and after dichotomising into low (< 10.8, B and E) and high (≥ 10.8, C and F) Stiffness Index β. Data presented are mean values with corresponding SE. Between group analysis performed with Independent T test; *p< 0.05. cfPWV, carotid-femoral pulse wave velocity.

may be more suitable than healthy elderly to study the efficacy of vitamin K on the inflammatory state. The use of MK-7 supplements in modulating inflammatory measures in such patients merits further investigation.

The additional intake of MK-7 supplements (e.g. 360 µg/day of MK-7) has no effects on haemostasis (29). Such effect is not to be expected, because in healthy subjects the blood coagulation factors (in contrast to MGP and osteocalcin) are fully carboxylated. Hence extra intake of vitamin K will not lead to increased procoagulant activity or thrombosis risk.

What is known about this topic?

- Observational data suggest a link between menaquinone (MK, vitamin K2) intake, but not phylloquinone (vitamin K1), and cardiovascular health.
- MK intervention trials with vascular endpoints are lacking.
- Increased availability of menaquinone-7 (MK-7) supplements marketed for cardiovascular health.

What does this paper add?

- First intervention trial on MK supplements and cardiovascular endpoints.
- Three-year MK-7 supplementation decreased arterial stiffness in healthy postmenopausal women.
- A nutritional dose of natural MK-7 (180 µg/day) was used in this study, as compared to high doses of synthetic phylloquinone in the two previously published studies on this topic.

Our study has some limitations and strengths. The calculated number of participants of this study was based on the predicted changes in our primary outcome measure: bone strength (37). Post-hoc power calculation with cfPWV as primary outcome resulted in a study power less sensitive than for bone strength (p=0.65, rather than p=0.90).We studied effects on non-invasive measures of arterial stiffness and did not include direct measures of calcification. Yet, the parameters we used in our study have prognostic value for CV mortality and are commonly used to estimate arterial stiffness (39). Further, the beneficial effects of MK-7 were shown in healthy postmenopausal women limiting the generalisability. Since cfPWV was described not to differ between men and women (49), similar effects of supplemental MK-7 may be expected in older men. The two main strengths of this study are the novelty of studying effects of MKs, more particular MK-7, on CV health and the use of two different well-established techniques to study effects on arterial stiffness, namely echotracking and mechanotransducers.

Conclusion

In conclusion, three-year vitamin K supplementation as MK-7 improves 1) arterial stiffness in healthy postmenopausal women and 2) the elastic properties of the carotid artery in a subgroup of women having a higher local stiffening of the carotid artery. Confirmatory research is in progress in our institute. In this intervention study subjects (men and women) at elevated risk for vascular stiffening (as concluded from their circulating dp-ucMGP levels) are treated with a higher dose of MK-7 with arterial stiffness (PWV) as the primary outcome.

Table 5: Effect of MK-7 treatment on the local carotid artery and on the regional arterial stiffness properties in the total group, in the women with a low (< 10.8) and with a high (≥ 10.8) Stiffness Index β.

		Total group (n=244)		Stiffness Index β < 10.8		Stiffness Index β ≥ 10.8	
		B (95 % CI)	P-value	B (95 % CI)	P-value	B (95 % CI)	P-value
Stiffness Index β	**crude**	-0.69 (-1.30; –0.07)	**0.028**	0.30 (-0.38; 0.98)	0.38	-1.37 (-2.44; –0.30)	**0.013**
	base	-0.75 (-1.37; –0.13)	**0.019**	0.482 (-0.20; 1.16)	0.16	-1.55 (-2.65; –0.45)	**0.006**
	multi 1	-0.70 (-1.32; –0.07)	**0.029**	0.48 (-0.21; 1.17)	0.17	-1.68 (-2.78; –0.59)	**0.003**
IMT	**crude**	0.001 (-0.012; 0.013)	0.92	-0.008 (-0.025; 0.010)	0.38	0.009 (-0.009; 0.027)	0.32
	base	0.003 (-0.010; 0.016)	0.64	-0.004 (-0.022; 0.013)	0.62	0.010 (-0.009; 0.028)	0.30
	multi 2	0.002 (-0.011; 0.015)	0.72	-0.004 (-0.022; 0.015)	0.69	0.010 (-0.009; 0.028)	0.30
Diameter	**crude**	-19.3 (-100.4; 61.8)	0.64	21.0 (-91.2; 133.1)	0.71	-68.7 (-186.7; 49.4)	0.25
	base	-23.1 (104.4; 58.2)	0.58	27.0 (-85.2; 139.1)	0.64	-93.9 (-212.5; 24.7)	0.12
	multi 2	-23.7 (105.5; 58.2)	0.57	30.6 (-87.2; 148.4)	0.61	-55.8 (-170.3; 58.7)	0.34
Distension	**crude**	5.63 (-11.1; 22.3)	0.51	-8.34 (-34.6; 17.9)	0.53	27.0 (6.5; 47.5)	**0.010**
	base	6.41 (-10.5; 23.4)	0.46	-11.1 (-38.0; 15.8)	0.41	29.1 (7.9; 50.3)	**0.008**
	multi 2	7.24 (-10.2; 24.7)	0.42	-10.2 (-38.4; 17.9)	0.47	30.0 (8.3; 51.7)	**0.007**
Compliance	**crude**	0.007 (-0.017; 0.031)	0.57	-0.017 (-0.050; 0.060)	0.31	0.040 (0.005; 0.075)	**0.024**
	base	0.007 (-0.018; 0.032)	0.59	-0.024 (-0.058; 0.009)	0.15	0.041 (0.006; 0.077)	**0.023**
	multi 1	0.005 (-0.020; 0.031)	0.68	-0.025 (-0.059; 0.009)	0.15	0.040 (0.003; 0.076)	**0.035**
Distensibility	**crude**	0.013 (-0.014; 0.40)	0.36	-0.016 (-0.053; 0.021)	0.40	0.047 (0.007; 0.087)	**0.023**
	base	0.014 (-0.014; 0.41)	0.33	-0.027 (-0.064; 0.010)	0.16	0.053 (0.012; 0.094)	**0.012**
	multi 1	0.012 (-0.016; 0.40)	0.41	-0.027 (-0.065; 0.010)	0.16	0.051 (0.008; 0.094)	**0.020**
Young's Modulus	**crude**	-0.034 (-0.090; 0.021)	0.23	0.027 (-0.045; 0.098)	0.46	-0.142 (-0.235; –0.050)	**0.003**
	base	-0.046 (-0.101; 0.010)	0.11	0.039 (-0.033; 0.112)	0.28	-0.161 (-0.254; –0.068)	**0.001**
	multi 1	-0.039 (-0.095; 0.017)	0.17	0.041 (-0.031; 0.114)	0.26	-0.154 (-0.252; –0.057)	**0.002**
cPWV	**crude**	-0.133 (-0.366; 0.101)	0.26	0.133 (-0.176; 0.442)	0.40	-0.445 (-0.796; –0.095)	**0.013**
	base	-0.158 (-0.394; 0.078)	0.19	0.215 (-0.097; 0.526)	0.17	-0.507 (-0.858; –0.155)	**0.005**
	multi 1	-0.143 (-0.382; 0.097)	0.24	0.221 (-0.092; 0.535)	0.17	-0.482 (-0.848; –0.115)	**0.010**
crPWV	**crude**	-0.244 (-0.547; 0.058)	0.11	-0.055 (-0.459; 0.348)	0.79	-0.464 (-0.924; –0.004)	**0.048**
	base	-0.198 (-0.509; 0.113)	0.21	0.023 (-0.396; 0.442)	0.91	-0.429 (-0.906; 0.049)	0.078
	multi 2	-0.185 (-0.500; 0.130)	0.25	0.100 (-0.313; 0.513)	0.63	-0.477 (-0.982; 0.027)	0.063
cfPWV	**crude**	-0.295 (-0.589; –0.001)	**0.049**	-0.357 (-0.756; 0.032)	0.072	-0.346 (-0.792; 0.099)	0.13
	base	-0.331 (-0.634; –0.029)	**0.032**	-0.391 (-0.802; 0.019)	0.061	-0.397 (-0.858; 0.064)	0.09
	multi 2	-0.352 (-0.661; –0.043)	**0.026**	-0.314 (-0.735; 0.108)	0.14	-0.406 (-0.886; 0.074)	0.10

B: unstandardized coefficient and its 95 % confidence interval (CI) of the effect of MK-7 supplementation on the dependent variable and p-value. Crude: effect of MK-7 after 3 y on the dependent variable compared to the concomitant baseline variable. Base: effect of MK-7 on the dependent variable adjusted for baseline values of age, BMI, log(TG). Multi 1: as Base model with additional confounders cholesterol lowering medication, anti-hypertensive medication. Multi 2: as base model with additional confounders Heart Rate, SBP, DBP, cholesterol lowering medication, anti-hypertensive medication. cPWV, carotid pulse wave velocity; cfPWV, carotid-femoral pulse wave velocity; crPWV, carotid-radial pulse wave velocity; DBP, diastolic blood pressure; IMT, intima-media thickness; SBP, systolic blood pressure; TG, triglycerides.

Acknowledgements

This work was supported by Nattopharma (Høvik, Norway). The financial sponsor did not play a role in the design, execution, analysis, interpretation of data, or writing of the manuscript.

Author contributions

CV: designed research; LAJLMB, MHJK, and NED: conducted research; MHJK, NED, and OB were responsible for analytical measurements; APGH, LAJLMB and MHJK: analysed the data; CV, LAJLMB, and MHJK: wrote the paper; CV had primary

responsibility for the final content. All authors read and approved the final manuscript.

Conflicts of interest
None declared.

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