The present invention relates to a menaquinone preparation for use in the treatment and/or prevention of arterial stiffness.
Menaquinone supplementation and vascular health

Description

The present invention is based on demonstrating that menaquinone-7 supplementation improves vascular properties in healthy postmenopausal women.

Introduction

Matrix Gla-protein (MGP) belongs to the family of vitamin K-dependent proteins; vitamin K mediates the carboxylation of glutamate (Glu) residues into γ-carboxyglutamate (Gla) resulting in protein functionality. MGP is primarily synthesized by vascular smooth muscle cells (VSMC) and functions as an important calcification inhibitor. Vascular calcification can occur in the intimal and/or medial layers of the vessel wall and modifies both functional and structural arterial properties - known as arterial remodeling - which is reflected in increased arterial stiffness. The circulating inactive form of MGP, i.e. desphospho uncarboxylated MGP (dp-ucMGP), has been recognized as marker for vascular vitamin K status and associated with arterial calcification and cardiovascular mortality. 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 significantly improves MGP carboxylation. The question remains, however, whether increased carboxylation of MGP by extra vitamin K intake beneficially affects vascular health.

Observational studies showed a lower prevalence of coronary artery calcification (CAC) and mortality in participants with the highest menaquinone (MK-n, vitamin K2) intake. Remarkably, no effect was seen for phylloquinone (vitamin K1) intake. While phylloquinone is primarily found in green leafy vegetables, menaquinones occur - to a much lower extent- in
meat (MK-4) and fermented foods (MK-7 to MK-10). Up to now, only two randomized clinical trials evaluated the effects of vitamin K supplementation on vascular health and showed beneficial effects with high daily dosages (0.5 - 1 mg) of phylloquinone. We showed that phylloquinone supplementation on top of a background of calcium and vitamin D₃ significantly improved arterial stiffness (measured as carotid compliance, distensibility and Young's modulus) in healthy postmenopausal women. Consistently, phylloquinone intake as part of a multivitamin supplementation program retarded CAC progression in elderly with pre-existing CAC. Unfortunately, the retardation in CAC progression could not be linked to the phylloquinone-induced changes in circulating dp-ucMGP. Menaquinone intervention trials with cardiovascular endpoints are however lacking, despite an increased wholesale of MK-7 supplements marketed for bone and cardiovascular health.

Next to its role in carboxylation, vitamin K may influence vascular health by carboxylation-independent mechanisms. In addition to vascular calcification, inflammation plays a role in arterial remodeling possibly by affecting proliferation of VSMC, 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. Consistently, observational data showed an inverse association between vitamin K status and proinflammatory markers. Although phylloquinone supplements showed no effects in healthy elderly MK-4 supplementation did reduce serum CRP levels in rheumatoid arthritis patients.

The first objective was to investigate vascular effects (vascular parameters and inflammatory markers) of MK-7 supplementation in healthy volunteers. The second objective was to study whether MK-7-induced changes in vascular parameters could be linked to changes in vitamin K status as measured by circulating dp-ucMGP. We hypothesized that long-term supplementation with MK-7 at a nutritionally relevant dosage will beneficially
affect vascular health.

The results of the study described herein demonstrate that MK-7 supplementation significantly improves functional measures such as carotid distensibility (DC) and pulse wave velocity (PWV) as well as structural vascular measures such as Young's modulus (E). Intima-media thickness (IMT) is not changed by MK-7. MK-7 significantly decreases circulating dp-ucMGP by -50% as compared to placebo, indicative for improved vascular vitamin K status. Circulating IL-6 and VCAM are not influenced by MK-7. Circulating dp-ucMGP levels correlate with most vascular measures and IL-6 at baseline. Thus, MK-7 supplements improve vascular properties in postmenopausal women.

Thus, the present invention relates to a menaquinone (MK-n) preparation for use in the treatment and/or prevention of central arterial stiffness and/or peripheral arterial stiffness. Preferred embodiments are described in the appended claims.

The indication "arterial stiffness" is characterized by degradation of elastin fibres within the arterial wall. Arterial stiffness may occur as a consequence of age and/or arteriosclerosis. In one embodiment of the invention the arterial stiffness is age-related arterial stiffness which is distinguished from arteriosclerosis-related stiffness, e.g. by lack of fatty plaque formation within the arteries.

The invention shall be further illustrated by means of the following Figures and experiments.

**Figures**

**Figure 1**: Bar graphs showing changes of structural vascular parameters after 3 years of placebo or MK-7 supplementation. The parameters are **Fig. 1A**: Compliance coefficient (CC), **Fig. 1B**: Distensibility coefficient (DC),
**Fig. 1C:** intima-media thickness (IMT), **Fig. 1D:** Young's Modulus (E), **Fig. 1E:** pulse-wave velocity carotid-femoral (PWV-fem), **Fig. 1F:** disphospho-uncarboxylated matrix GLA protein (dp-ucMGP).

**Figure 2:** Proportional change of the PWF-femoralis radialis, carotis after 3 years of placebo or MK-7 supplementation. Mean values ± SE.

**Figure 3:** The effect of placebo or MK-7 supplementation on the stiffness index $\beta$: absolute values (A), absolute difference (B) and proportional change (C) compared to baseline. Mean values ± SE. *: p<0.05.

**Figure 4:** The effect of 3 years' placebo or MK-7 supplementation on the stiffness index $\beta$, divided into a low (left panel) and high-$\beta$ group (right panel): absolute values (A and B), absolute difference (C and D) and proportional change (E and F) compared to baseline. Mean values ± SE. *: p<0.05; **: p<0.005.

**Figure 5:** The effect of 3 years' placebo or MK-7 supplementation on DC in the low (left panel) and high-$\beta$ group (right panel): absolute values (A and B), absolute difference (C and D) and proportional change (E and F) compared to baseline. Mean values ± SE. *: p<0.05.

**Figure 6:** The effect of 3 years' placebo or MK-7 supplementation on CC in the low (left panel) and high-$\beta$ group (right panel): absolute values (A and B), absolute difference (C and D) and proportional change (E and F) compared to baseline. Mean values ± SE. *: p<0.05.

**Figure 7:** The effect of 3 years' placebo or MK-7 supplementation on Ad in the low (left panel) and high-$\beta$ group (right panel): absolute values (A and B), absolute difference (C and D) and proportional change (E and F) compared to baseline. Mean values ± SE. *: p<0.05.
Figure 8: The effect of 3 years' placebo or MK-7 supplementation on Young's Elasticity Modulus (E) in the low (left panel) and high-β group (right panel): absolute values (A and B), absolute difference (C and D) and proportional change (E and F) compared to baseline. Mean values ± SE.

*: p<0.05; **: p<0.005.

Clinical study on the effects of MK-supplementation

Patients and methods

Study design

The study design has been described elsewhere. Sample size was determined based on the primary outcome measure, i.e. bone strength. Briefly, 244 healthy postmenopausal women aged between 55 and 65 years participated in this randomized, placebo-controlled 3-year trial (2008-2011).

Exclusion criteria were <2 years postmenopausal, 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 (computer-generated random permutation procedure) assigned to receive placebo capsules (n=124) or capsules containing 180 μg MK-7 (MenaQ7, NattoPharma, Hovik Norway) (n=120). From the 244 volunteers who entered the study, 21 discontinued their participation and were not available for the follow-up measurements. The remaining 223 participants completed the study. Participants came to the research site (VitaK, Maastricht, The Netherlands) every year for blood sampling and measurements of body weight, height, and vascular parameters.

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

The capsules were manufactured by EuroPharma Alliance (Wroclaw, Poland) for Nattopharma (Hovik, Norway). The study products, containing 180 pg MK-7 in form of MenaQ7 and matching placebo capsules, were delivered directly by Nattopharma to VitaK in Maastricht (The Netherlands).

Blood sampling

Fasting venous blood was collected once yearly for the preparation of serum and citrated plasma (Vacutainers, Greiner Bio-One BV, Alphen aan de Rijn, The Netherlands). Serum and plasma aliquots were stored at -80°C until analysis. All blood measurements were performed in duplicate.

Circulating markers

Lipid profiles were determined on a Beckman Coulter LX20 PRO Clinical Chemistry analyzer (Beckman Coulter, Fullerton, USA).

Plasma dp-ucMGP levels were measured by an in-house dual-antibody ELISA test. An in-house control pool was run on all ELISA plates.

The soluble form of vascular cell adhesion molecule (VCAM, marker of endothelial dysfunction) was measured in serum by a commercial ELISA test (R&D Systems Europe, Abingdon, UK). Circulating interleukin-6 (IL-6, marker of low-grade inflammation) was measured in serum by a commercial ELISA test (Life Technologies Europe, Bleiswijk, The Netherlands).
**Local arterial stiffness**

Echo-tracking was performed to determine vessel (wall) characteristics of the common carotid artery (Artlab multiarray echo-tracking, Esaote Picus Pro, Esaote Europe, Maastricht, The Netherlands).

Arterial blood pressure (BP) was recorded in parallel with echo tracking at the level of the brachial artery by a semi-automated oscillometric device (Dinamap, KP Medical, Houten, The Netherlands).

The following terms, with definitions and equations (if applicable) were used:

- **IMT** (Intima-media thickness of the artery (pm)).
- **D_{dia}** (Diameter of the artery (adventitia-adventitia) end-diastole (pm)).
- **Ad** (Distension: change in diameter in systole (pm)).
- **D_{sys}** (Diameter of the artery end-systole: D_{dia} + Ad (pm)).
- **D_{mean}** (Mean diameter: (D_{sys} + D_{dia})/2 (pm)).
- **ΔA** (Change of area in systole: π * Ad2 / 4 (mm2)).
- **MAP** (Mean arterial pressure in brachial artery: BP_{dia} + (BP_{sys} - BP_{dia}) / 3 (mmHg)).
- **ΔP_{brach}** (Brachial Pulse Pressure: BP_{sys} - BP_{dia} (kPa)).
- **Ap_{car0id}** (Local Pulse Pressure carotid artery: Ad * [(MAP - BP_{dia}) / (D_{mean} - D_{dia})] (kPa)).
- **DC** (Distensibility Coefficient is the relative change in lumen area during systole for a given pressure change: (2 * D_{dia} * Ad + Ad2) / (Ap_{car0id} * D_{dia2}) (MPa-1)).
- **CC** (Compliance Coefficient is the absolute change in lumen area during systole for a given pressure: ΔA / Ap_{car0id} f_{ad} (mnfVkJPa)).
- **E** (Young’s Elasticity Modulus: (D_{dia} / IMT) / DC (MPa)).
- **β** (Stiffness Index, relatively independent of BP: D_{dia} * ln(BP_{sys} / BP_{dia}) / Ad).
- **cPWV** (Local carotid Pulse wave velocity: 1 / V(p * DC) (m/s); p = blood density 1060 kg/m3).
Regional arterial stiffness

Regional aortic (carotid-femoral pulse wave velocity: cfPWV) and arm (carotid-radial pulse wave velocity: crPWV) stiffness were assessed non-invasive by using mechanotransducers directly applied on the skin (Complior, Artech Medical, Pantin, France). The PWV is directly affected by arterial stiffness.

Statistics

Data, which are not normally distributed were log-transformed (IL-6, IMT, D_dia, D_mean, ΔA DC, CC). Independent Samples T-test and Chi-square test were used to test between-group differences.

The Paired Samples T test and McNemar's test were used to study within-group differences from baseline to year 3. All participants included in the study were used in primary intention-to-treat analyses (n=244). Secondary analyses (n=207, see below) were limited to those with measures of vascular parameters at baseline and year 3 and without heart rhythm disorders; extreme outliers were excluded as well. To investigate the change in arterial stiffness of the carotid artery during the supplementation period of 3 y the 50th percentile of the Stiffness Index β is used to compare the characteristics derived from the carotid artery measurement. Data will be presented as low-β group (<50th percentile) and high β-group (≥50th percentile).

Linear regression was performed to study associations between supplementation of MK-7 after 3y and the measures of interest (β, DC, CC, Ad, E). The measure of interest was used as the dependent variable. The concomitant baseline value and the treatment code were included as independent variables.

Age, years since menopause, BMI, smoking (yes/no), total cholesterol, triglycerides, pulse pressure (ultrasound measurements) or diastolic blood
pressure (PWV), cholesterol-lowering medication (yes/no), blood pressure medication (yes/no), and vascular vitamin K status (baseline dp-ucMGP levels) were included as confounders. Between-group differences in 3-year changes of circulating markers (dp-ucMGP, IL-6, VCAM) were tested by the Independent Samples T test. Correlation analyses at baseline were performed using the Pearson test. In the secondary analyses, missing values were not replaced in the statistical analyses and were excluded analysis-by-analysis. Data are presented as means with SD. A p<0.05 was considered statistically significant. Statistics were performed using SPSS for Windows, version 19 (SPSS Inc., Chicago, Illinois, USA).

From the 244 volunteers who entered the study, 21 discontinued their participation and were not available for the follow-up measurements. Of the remaining 223 participants who completed the study, we excluded subjects with heart rhythm disorders (possible interference with vascular measurements, n=14) or extreme (value <Q1-3*IQ or value>Q3+3*IQ; Q, lower quartile or 25th percentile, IQ, interquartile range, Q3-Q1) outlier values (reliant on the parameter of interest, n=2 to 5) in the secondary analyses.

Results
Baseline characteristics
The baseline characteristics of the total study population and the placebo and MK-7 groups after randomization are presented in table 1. Characteristics of the 207 women used in secondary analyses are presented in table 2. No significant differences were found between the baseline values of both supplementation groups.

At baseline, all vessel wall variables (except Ds100) in the low β-group were statistically different compared to the high β-group (table 3). Triglyceride concentration and BP_systolic were significantly lower in the low-β group, as well as cPWV. However, cPWV was higher in the low β-group, whereas no significance was found for cfPWV.
Table 1. Baseline characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Total (n=244)</th>
<th>Placebo (n=124)</th>
<th>MK-7 (n=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td>60±3</td>
<td>59±3</td>
<td>60±4</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>25±3</td>
<td>25±3</td>
<td>25±3</td>
</tr>
<tr>
<td><strong>Years since menopause (y)</strong></td>
<td>9±6</td>
<td>8±5</td>
<td>10±6</td>
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<td><strong>Current smoker (%)</strong></td>
<td>13</td>
<td>15</td>
<td>11</td>
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<tr>
<td><strong>Alcohol use (%)</strong></td>
<td>76</td>
<td>78</td>
<td>73</td>
</tr>
<tr>
<td><strong>10 Statin use (%)</strong></td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td><strong>Blood pressure medication (%)</strong></td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

**Lipid profile**

- **Cholesterol (mmol/L)**: 6.0±1.0, 6.0±1.0, 6.0±1.0
- **Triglycerides (mmol/L)**: 1.3±0.7, 1.2±0.7, 1.3±0.6

**Blood pressure**

- **Systolic blood pressure (BP.sys)**: 126±16, 126±16, 126±15
- **Diastolic blood pressure (BP.dia)**: 73±8, 73±8, 74±8

**Pulse pressure**

- **ΔP brach (kP)**: 7.0±1.6, 7.0±1.6, 7.0±1.6
- **HR (bpm)**: 64.8±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)**: 8.2±1.5, 8.1±1.7, 8.2±1.2
- **cfPWV (m/s)**: 9.8±1.8, 9.7±1.7, 9.9±1.9
- **crPWV (m/s)**: 10.2±1.4, 10.1±1.4, 10.2±1.4
### Elastic properties of the carotid artery

<table>
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<tr>
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<th>6986±721</th>
<th>7006±752</th>
<th>6966±690</th>
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<tr>
<td>Ddia (μm)</td>
<td>356±108</td>
<td>363±115</td>
<td>350±100</td>
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<tr>
<td>Ad (μm)</td>
<td>7344±758</td>
<td>7371±801</td>
<td>7316±714</td>
</tr>
<tr>
<td>Dsys (pm)</td>
<td>577±104</td>
<td>572±98</td>
<td>582±110</td>
</tr>
<tr>
<td>IMT (pm)</td>
<td>4.06±.52</td>
<td>4.18±.69</td>
<td>3.95±.31</td>
</tr>
<tr>
<td>ΔΛ (mm²)</td>
<td>4.68±.07</td>
<td>4.69±.05</td>
<td>4.68±.07</td>
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<tr>
<td>Δpcarotid (kPa)</td>
<td>0.59±0.23</td>
<td>0.61±0.26</td>
<td>0.56±0.17</td>
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<tr>
<td>CC (mrm²/kPa)</td>
<td>15.5±5.4</td>
<td>15.9±5.9</td>
<td>15.0±4.6</td>
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<tr>
<td>DC (MPa⁻¹)</td>
<td>0.89±0.39</td>
<td>0.91±0.48</td>
<td>0.88±0.27</td>
</tr>
<tr>
<td>P</td>
<td>11.4±3.8</td>
<td>11.3±4.5</td>
<td>11.4±3.1</td>
</tr>
</tbody>
</table>

### Vitamin K status

| dp-ucMGP (pM)       | 525±266  | 538±293  | 511±236  |

### Inflammatory markers

| IL-6 (pg/mL)    | 0.7±3.0 | 0.6±1.4 | 0.8±4.1 |
| VCAM (ng/mL)    | 653±167 | 657±168 | 649±166 |

1 (ΔΛ), Body mass index (BMI), brachial pulse pressure (Apbrach), local pulse pressure of the carotid artery (Apcarotid), compliance coefficient (CC), carotid pulse wave velocity (cPWV), carotid-femoral pulse wave velocity (cfPWV), carotid-radial pulse wave velocity (crPWV), diameter end-diastole (Ddia), diameter end-systole (Dsys), distensibility coefficient (DC), heart rate (HR), intima-media thickness (IMT), mean arterial pressure (MAP), stiffness Index (β), years since menopause (YSM), Young's elasticity modulus (£).

2 Means with SD or % (n=244).

Independent Samples T test and Chi-square test: no significant differences were found between the treatment groups.
Table 2. Vascular parameters at baseline and after 3-years of supplementation with placebo or MK-7\textsuperscript{1,2,3,4}.

<table>
<thead>
<tr>
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<th>Placebo t0 (n=103)</th>
<th>Placebo t3 (n=103)</th>
<th>MK-7 t0 (n=104)</th>
<th>MK-7 t3 (n=104)</th>
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</thead>
<tbody>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>126±1.6</td>
<td>121±14\textsuperscript{**}</td>
<td>126±16</td>
<td>121±1.3\textsuperscript{**}</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>73±8</td>
<td>70±7\textsuperscript{*}</td>
<td>74±8</td>
<td>70±6\textsuperscript{**}</td>
</tr>
<tr>
<td>Pulse Pressure (kP)</td>
<td>7.0±1.5</td>
<td>6.9±1.5</td>
<td>7.0±1.6</td>
<td>6.8±1.5</td>
</tr>
<tr>
<td>Diameter (pm)</td>
<td>6968±741</td>
<td>6969±728</td>
<td>6923±648</td>
<td>6902±669</td>
</tr>
<tr>
<td>Distension (pm)</td>
<td>364±1.12</td>
<td>369±1.19</td>
<td>349±100</td>
<td>367±93\textsuperscript{*}</td>
</tr>
<tr>
<td>CC (mm\textsuperscript{2}kPa)</td>
<td>0.61±0.24</td>
<td>0.62±0.24</td>
<td>0.56±0.16</td>
<td>0.61±0.18\textsuperscript{**}</td>
</tr>
<tr>
<td>DC (MPa\textsuperscript{3})</td>
<td>16.0±5.9</td>
<td>16.5±6.4</td>
<td>15.2±4.6</td>
<td>16.7±4.8\textsuperscript{**}</td>
</tr>
<tr>
<td>IMT (pm)</td>
<td>570±102</td>
<td>609±101\textsuperscript{**}</td>
<td>581±1.10</td>
<td>617±100\textsuperscript{**}</td>
</tr>
<tr>
<td>E (MPa)</td>
<td>0.88±0.35</td>
<td>0.82±0.37\textsuperscript{*}</td>
<td>0.87±0.26</td>
<td>0.75±0.25\textsuperscript{**}</td>
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<tr>
<td>PWV-fem (m/s)</td>
<td>9.7±1.7</td>
<td>9.6±1.4\textsuperscript{'}</td>
<td>10.0±1.7</td>
<td>9.4±1.4\textsuperscript{**}</td>
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<td>PWV-rad (m/s)</td>
<td>10.1±4</td>
<td>9.9±1.4</td>
<td>10.3±1.4</td>
<td>9.8±1.4\textsuperscript{**}</td>
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<tr>
<td>dp-ucMGP (pM)</td>
<td>542±299</td>
<td>617±303\textsuperscript{**}</td>
<td>504±231</td>
<td>325±162\textsuperscript{**}</td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>0.59±1.5</td>
<td>0.52±0.6</td>
<td>0.85±4.3</td>
<td>0.78±2.2</td>
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<tr>
<td>VCAM (ng/mL)</td>
<td>649±161</td>
<td>655±146</td>
<td>645±168</td>
<td>660±170</td>
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</table>

Compliance coefficient (CC), desphospho-uncarboxylated matrix Glaprotein (dp-ucMGP), distensibility coefficient (DC), interleukin-6 (IL-6), intima-media thickness (IMT), pulse wave velocity (PWV), PWV carotid-femoral (PWV-fem), PWV carotid-radial (PWV-rad), vascular adhesion molecule (VCAM), Young's modulus (E).

\textsuperscript{2}Means with SD (n=207). 3 independent Samples T test: no significant differences were found between the treatment groups.

\textsuperscript{4}Paired Samples T test: within-group differences from baseline to year 3(*p<0.05; \textsuperscript{**}p<0.005).
Table 3: Comparison of the vessel wall characteristics at baseline between <50\textsuperscript{th} percentile and >=50\textsuperscript{th} percentile of the Stiffness Index $\beta$ ($\beta = 10.82$).\textsuperscript{1,2}

<table>
<thead>
<tr>
<th></th>
<th>Low- $\beta$</th>
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<th>High- $\beta$</th>
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<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
<td>Mean</td>
<td>sd</td>
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<tr>
<td>Age (y)</td>
<td>58.9</td>
<td>3.1</td>
<td>60.1</td>
<td>3.4</td>
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<tr>
<td>YSM (y)</td>
<td>8.4</td>
<td>5.2</td>
<td>9.4</td>
<td>5.9</td>
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<tr>
<td>Weight (kg)</td>
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<td>69.5</td>
<td>9.5</td>
<td>0.122</td>
<td></td>
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<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>24.7</td>
<td>3.0</td>
<td>25.5</td>
<td>3.1</td>
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<tr>
<td>Waist (cm)</td>
<td>81.1</td>
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<td>83.7</td>
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<td>Hip (cm)</td>
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<tr>
<td>WHR</td>
<td>0.81</td>
<td>0.06</td>
<td>0.84</td>
<td>0.06</td>
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<td>Cholesterol (mmol/l)</td>
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<td>0.9</td>
<td>6.1</td>
<td>1.1</td>
<td>0.082</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.2</td>
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<td>1.4</td>
<td>0.7</td>
<td>0.007</td>
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\textsuperscript{1} ($\Delta A$), Body mass index (BMI), brachial pulse pressure (AP$_{pulch}$), local pulse.
pressure of the carotid artery ($\Delta p_{\text{car}},\text{liq}$), compliance coefficient (CC), carotid pulse wave velocity (cPWV), carotid-femoral pulse wave velocity (cfPWV), carotid-radial pulse wave velocity (crPWV), diameter end-diastole ($D_{\text{di}}$), diameter end-systole ($D_{\text{sys}}$), distensibility coefficient (DC), heart rate (HR), intima-media thickness (IMT), mean arterial pressure (MAP), stiffness Index ($\beta$), years since menopause (YSM), Young's elasticity modulus ($E$).

Independent Samples T test was used. $P<0.05$ is regarded to be statistically significant.

Functional vascular parameters

Ad, DC, and CC increased significantly in the MK-7 group, but remained unaffected in the placebo group. PP, on the other hand, did not change in both groups. In intention-to-treat analyses, there were no differences in functional vascular parameters between the treatment groups. When secondary analyses were restricted to those who completed the study and without heart rhythm disorders and the extreme outliers, the women in the MK-7 group had a significantly improved DC as compared to those in the control group ($p=0.047$, after correction for confounders, Fig 1A). No between-group differences were found with respect to PP, Ad, and CC (Fig 1B).

Structural vascular parameters

E and IMT decreased significantly in both groups. In intention-to-treat analyses, there were no differences in structural vascular parameters between the placebo and MK-7 groups. However, secondary analyses showed a significant between-group difference with respect to E ($p=0.022$, after correction for confounders, Fig 1C). No between-group difference was seen for IMT (Fig 1D).
Effect of MK-7 on pulse wave velocity

After 3y of supplementation crPWV and cfPWV decreased significantly in the MK-7 group compared to the placebo group (p=0.045, p=0.032 respectively). The calculated cpPWV also decreased in the MK-7 group, although the difference compared to the placebo group was not significant (p=0.18) (Figure 2).

A significant decrease was observed in PWV-fem in the MK-7 group, with no change in the placebo group. The PWV-rad did not change in both groups. Intention-to-treat analyses showed no between-group differences. In secondary analyses, PWV-fem was significantly improved by extra MK-7 intake as compared to placebo (p=0.042, after correction for confounders, Fig 1E). No significant between-group difference was found for the PWV-rad.

Circulating markers

MK-7 supplementation significantly decreased circulating dp-ucMGP by 50% as compared to placebo (Fig 1F). The maximal effect on MGP carboxylation was already reached during the first year and lasted over the next two years of supplementation.

MK-7 supplementation had no effects on IL-6 and VCAM.

Correlations at baseline

PWV-fem significantly correlated with PWV-rad (r=0.430, p<0.001) and the vascular parameters PP (r=0.390, p<0.001), DC (r=-0.187, p=0.008), and IMT (r=0.191, p=0.006).

Circulating dp-ucMGP significantly correlated with the vascular measures DC (r=-0.150, p=0.042), IMT (r=0.164, p=0.024), and PWV-fem (r=0.170, p=0.023). Significant correlations were found between log IL-6 and the
vascular measures $DC$ ($r=-0.181$, $p=0.021$) and $E$ ($r=0.177$, $p=0.025$). Log IL-6 also correlated with circulating $dp$-ucMGP ($r=0.255$, $p=0.002$).

Effect of MK-7 treatment on Stiffness Index $\beta$ in total study population

MK-7 supplementation resulted in a steady decrease of the stiffness index $\beta$, whereas the index increased in the placebo group ($p=0.048$) (Fig. 3B). The proportional change, however, did not reach the level of significance.

Effect of MK-7 treatment on arterial stiffness parameters $\beta$, $DC$, $CC$, $Ad$, and $E$

The decrease of the stiffness index during MK-7 treatment in the total group was due to the effect of MK-7 in the high-$\beta$ group (Fig. 4B, D, F). The difference between MK-7 and placebo became significant not earlier than 3y of treatment, considering the absolute values ($p=0.002$), the absolute difference compared to baseline ($p=0.021$) and the proportional change compared to baseline ($p=0.015$). The difference between MK-7 and placebo remained significant after adjusting for confounders ($p=0.001$). In the low-$\beta$ group, both placebo and MK-7 increased. No significant differences were found between placebo and MK-7 in the low-$\beta$ group (Fig. 4A, C, E).

$DC$ (Fig. 5) and $CC$ (Fig. 6) had improved after 3y MK-7 supplementation in the high-$\beta$ group in absolute values of $DC$ and $CC$ ($p=0.014$, $p=0.059$ resp.), as well as in absolute difference ($p=0.032$, $p=0.055$ resp.) and proportional difference ($p=0.031$, $p=0.079$ resp.). After adjustment the difference between MK-7 and placebo was still significant for $DC$ ($p=0.011$), whereas the p-value for $CC$ decreased to 0.047. The distension $Ad$ was significantly increased after 3y MK-7 supplementation in the high-$\beta$ group compared to placebo (absolute values: $p=0.047$, $p=0.003$ after adjustment; absolute difference: $p=0.030$, proportional change: $p=0.040$), whereas in the low-$\beta$ group $Ad$ did not change (Fig. 7).

The Young's Elasticity Modulus $E$ responded to MK-7 supplementation in the high-$\beta$ group, but not low-$\beta$ group. After 3y of supplementation the decrease
in the high-β group was statistically significant compared to the placebo group (p=0.001; Fig. 8B) and remained significant after adjustment with the confounders (p=0.005). Considering the proportional change of E this decrease was borderline significant (p=0.073; Fig. 8F).

No effect of MK-7 treatment was found on IMT and D_{da} (data not shown).

Discussion

This is the first study showing that long-term use of MK-7 supplements beneficially affects vascular health. More particularly, MK-7 supplementation at a nutritional dose significantly improved both peripheral (DC, E) and central measures of arterial stiffness (PWV-fem). PWV-fem is generally accepted as the gold-standard measure for central arterial stiffness and was not used formerly to study vitamin K's health effects. Whereas DC and E can be directly quantified, PWV provides an indirect measure of the mechanical properties of an arterial segment. The functional measures DC and PWV-fem correlate with CVD risk and were recently described as representatives of (partly) comparable adverse vascular processes during ageing. Also in our study, these vascular measures intercorrelated significantly. No significant effects were however seen on IMT. Contrasting, E - which takes in account IMT - did improve significantly by extra MK-7. Similar results were previously seen in healthy postmenopausal women after high-dose phylloquinone supplementation: beneficial effects on E without an effect on IMT. The lack of effect on IMT may be explained by the fact that our study population consisted of healthy postmenopausal women without established vascular disease. Actually, their IMT values equaled the reference value of healthy women, i.e. 550 μm whereas an IMT of >900 μm is considered a risk marker for CVD. It would therefore be interesting to evaluate the effects of vitamin K supplements on arterial wall thickness among subjects at increased risk for CVD. Moreover, substantial changes in IMT may require a longer supplemental period than 3 years.

Animal and in vitro studies have demonstrated that vitamin K is involved in
vascular calcification through its cofactor function in carboxylating MGP. Decreased MGP carboxylation reduces the ability of MGP to inhibit arterial calcification and may contribute to accelerated arterial stiffness. Circulating dp-ucMGP is a recognized marker for vascular vitamin K status and has been linked to vascular calcification and mortality. This marker is detectable in healthy adults, but strongly elevated values are found in subjects at high vascular risk. MK-7 supplementation significantly improved MGP carboxylation, as measured by 50% lowering of plasma dp-ucMGP. No associations were however found between the MK-7-induced changes in circulating dp-ucMGP and vascular parameters. On the other hand, plasma dp-ucMGP correlated cross-sectionally with several vascular measures. Recent work has shown significantly increased MGP carboxylation after MK-7 supplementation in healthy volunteers and patients. Also high-dose phylloquinone supplementation was earlier reported to significantly decrease circulating dp-ucMGP. Similar to our study, the phylloquinone-related changes in circulating dp-ucMGP could not be linked to the beneficial effects on CAC. It should be noted that 3-year use of the MK-7 supplements lowered serum uncarboxylated osteocalcin (ucOC) levels - a marker for vitamin K status of bone - to a similar extent as circulating dp-ucMGP. This is indicative for a comparable efficacy of MK-7 absorption by bone and arteries. As yet, the physiological implications for (maximally) increased carboxylation of circulating dp-ucMGP and ucOC are unknown. In this respect it is intriguing that, besides its role in γ-glutamate carboxylation, menaquinone was found to be involved in a cell signaling pathway via the SXR receptor on the nuclear membrane of a variety of cells, and may serve as an important transcriptional factor. This was specifically found for MK-7, which was reported to regulate gene expression in osteoblastic cells. It seems at least feasible that MK-7 plays a similar role in regulating gene expression in VSMC.

Next to arterial calcification, low-grade inflammation and endothelial dysfunction have been associated with arterial stiffness. Changes in these inflammatory processes may be an alternative mechanism by which vitamin
K protects against CVD. Observational data have shown an inverse association between vitamin K (status) and markers of low-grade inflammation, including IL-6 and CRP. We measured circulating IL-6 and VCAM as markers of low-grade inflammation and endothelial dysfunction, but these markers were not influenced by long-term MK-7 supplementation. Remarkably, however, at baseline IL-6 correlated cross-sectionally with vitamin K status and the peripheral measures of arterial stiffness; no such correlations were however found for VCAM. Our results are in line with those of the long-term phyloquinone supplementation study, i.e. beneficial effects on vascular health with no concomitant decrease of circulating cytokines. In contrast, Ebina et al. did show that after 3-month of high-dose menaquinone (MK-4) administration, CRP and MMP-3 levels in female rheumatoid arthritis (RA) patients had significantly decreased. This implies that patients with inflammatory diseases, such as RA are 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.

Our study has a number of limitations. Firstly, the study power was calculated on the basis of predicted changes in our primary outcome measure bone strength; nevertheless, we were able to see significant changes in vascular parameters. Secondly, we did not include direct measures of calcification, but studied effects on non-invasive measures of arterial stiffness and vascular damage. Yet, the parameters we used in our study have a recognized prognostic value for cardiovascular mortality and are commonly used to estimate arterial stiffness or vascular damage. Thirdly, the beneficial effects of MK-7 were shown in healthy postmenopausal women making it difficult to extrapolate these findings to other population groups, including older men and patients at increased risk for CVD. Clearly, confirmatory studies are warranted in these individuals. Finally, we saw unexpected improvements in a few vascular parameters in the placebo group. This may be explained by a so-called volunteer bias, i.e. people who volunteer are often more health-minded than non-volunteers and may switch
to a more healthy lifestyle because of their participation in a study with health outcomes. Despite this potential bias, we did find significant between-group differences for both functional and structural vascular measures.

**Conclusions**

Low-dose menaquinone supplementation as MK-7 beneficially improved vascular stiffness (as measured by carotid distensibility and pulse wave velocity) over 3 years in healthy postmenopausal women. Also vitamin K status, as measured by circulating dp-ucMGP, was significantly improved by supplemental MK-7. The beneficial changes in vascular measures could not be related to changes in circulating dp-ucMGP. Confirmatory research is needed in other study populations, such as older men and patients at risk for CVD.
References


16. van Bussel BC, Schouten F, Henry RM, Schalkwijk CG, de Boer MR, Ferreira I, Smulders YM, Twisk JW, Stehouwer CD. Endothelial dysfunction and low-grade inflammation are associated with greater


Claims

1. Menaquinone (MK-n) preparation for use in the treatment and/or prevention of central arterial stiffness and/or peripheral arterial stiffness.

2. Menaquinone preparation for use according to claim 1, wherein the menaquinone is menaquinone-4 (MK-4).

3. Menaquinone preparation for use according to claim 1, wherein the menaquinone comprises more than 4 isoprenyl residues (n>4), e.g. 5, 6, 7, 8 or 9 isoprenyl residues.

4. Menaquinone preparation for use according to claim 1 or 3, wherein the menaquinone is menaquinone-7 (MK-7).

5. Menaquinone preparation for use according to any one of the preceding claims, wherein an increased or constant carotid-femoral pulse wave velocity (PWV-fem) at a timepoint $t_{end}$ as compared to a timepoint $t_0$ is indicative for central and/or peripheral arterial stiffness.

6. Menaquinone preparation for use according to any one of claims 1-4, wherein a decreased distensibility coefficient (DC) at a timepoint $t_{end}$ as compared to a timepoint $t_0$ is indicative for central and/or peripheral arterial stiffness.

7. Menaquinone preparation for use according to any one of claims 1-4, wherein an decreased compliance coefficient (CC) at a $t_{end}$ as compared to a timepoint $t_0$ is indicative for central and/or peripheral arterial stiffness.

8. Menaquinone preparation for use according to any one of claims 1-4, wherein an increased Young's elasticity modulus (E) at a $t_{end}$ as
compared to a timepoint $t_0$ is indicative for central and/or peripheral arterial stiffness.

9. Menaquinone preparation for use according to any one of claims 5-8, wherein $t_{end}$ is measured 12 months, preferably 36 months after measurement of $t_0$.

10. Menaquinone preparation for use according to any one of the preceding claims, wherein the preparation is free of vitamin D or analogs thereof.

11. Menaquinone preparation for use according to any one of the preceding claims, wherein the preparation is for administration to healthy subjects.

12. Menaquinone preparation for use according to claim 6, wherein the healthy subjects are postmenopausal women, e.g. of between about 55 and about 65 years of age.

13. Menaquinone preparation for use according to any one of the preceding claims, wherein the preparation is administered as a pharmaceutical or dietary supplement, preferably in the form of powder, tablets or as an emulsion.

14. Menaquinone preparation for use according to any one of the preceding claims, wherein the preparation is administered at least once daily.

15. Menaquinone preparation for use according to any one of the preceding claims, wherein the daily dose of supplemented menaquinone is 10-1000 pg, preferably 50-500 pg, more preferably 150-250 pg and most preferably about 180 pg.

16. Menaquinone preparation for use according to any one of the preceding claims, wherein the preparation is administered for at least 12 months, preferably for at least 36 months, most preferably life-long.
17. Menaquinone preparation for use according to any one of the preceding claims, wherein the preparation is additionally for use in the prevention of bone loss.
Figure 1

3-year change in CC

3-year change in DC

3-year change in IMT

3-year change in E

3-year change in PWV-fem

3-year change in dp-ucMGP
Figure 2

PWV proportional change after 3y

Femorals
Radials
Carotis

Proportional change (%)

Placebo
MK7

p=0.032
p=0.045
p=0.18
Figure 3

A

Stiffness Index $\beta$

- Placebo
- MK-7

B

$\Delta \beta$

- Placebo
- MK-7

C

Proportional change [%]

- Placebo
- MK-7
Figure 5

A. DC (MPa⁻¹) vs. Treatment period (y)
- Placebo
- MK-7

B. DC (MPa⁻¹) vs. Treatment period (y)
- Placebo
- MK-7

C. ΔDC (MPa⁻¹) vs. Treatment period (y)
- Placebo
- MK-7

D. ΔDC (MPa⁻¹) vs. Treatment period (y)
- Placebo
- MK-7

E. Proportional change (%) vs. Treatment period (y)
- Placebo
- MK-7

F. Proportional change (%) vs. Treatment period (y)
- Placebo
- MK-7
Figure 6
Figure 7

A

\[ \text{Ad (\mu m)} \]

\[ 460 \]

\[ 440 \]

\[ 420 \]

\[ 400 \]

\[ 380 \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

Treatment period (y)

----- Placebo

----- MK-7

B

\[ \text{Ad (\mu m)} \]

\[ 360 \]

\[ 340 \]

\[ 320 \]

\[ 300 \]

\[ 280 \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

Treatment period (y)

----- Placebo

----- MK-7

C

\[ \Delta \text{Ad (\mu m)} \]

\[ 20 \]

\[ 10 \]

\[ 0 \]

\[-10 \]

\[-20 \]

\[-30 \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

Treatment period (y)

----- Placebo

----- MK-7

D

\[ \Delta \text{Ad (\mu m)} \]

\[ 50 \]

\[ 40 \]

\[ 30 \]

\[ 20 \]

\[ 10 \]

\[ 0 \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

Treatment period (y)

----- Placebo

----- MK-7

E

Proportional change [%]

\[ 6.0 \]

\[ 4.0 \]

\[ 2.0 \]

\[ 0.0 \]

\[-2.0 \]

\[-4.0 \]

\[-6.0 \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

Treatment period (y)

----- Placebo

----- MK-7

F

Proportional change [%]

\[ 20 \]

\[ 15 \]

\[ 10 \]

\[ 5 \]

\[ 0 \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

Treatment period (y)

----- Placebo

----- MK-7
Figure 8

Graph A: Graph showing the change in stiffness (E in MPa) over the treatment period (y) for Placebo and MK-7.

Graph B: Graph showing the change in stiffness (E in MPa) over the treatment period (y) for Placebo and MK-7.

Graph C: Graph showing the change in ΔE (MPa) over the treatment period (y) for Placebo and MK-7.

Graph D: Graph showing the change in ΔE (MPa) over the treatment period (y) for Placebo and MK-7.

Graph E: Graph showing the proportional change (%) over the treatment period (y) for Placebo and MK-7.
A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/122 A61P9/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 2004/019923 AI (VITAK BV [NL] ; VERMEER CEES [NL]) 11 March 2004 (2004-03-11) page 5, line 10 - page 3, lines 10-14; claim 1; example 1</td>
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See patent family annex.

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Date of the actual completion of the international search: 2 July 2014

Date of mailing of the international search report: 15/07/2014

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Fax: (+31-70) 340-3016

Authorized officer: Cattel, James
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