Plasma phylloquinone, menaquinone-4 and menaquinone-7 levels and coronary artery calcification

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Abstract
Vitamin K is considered to be involved in the pathological mechanisms of coronary artery calcification (CAC). Correlation between CAC and plasma vitamin K levels was studied. A total of 103 patients, with at least one coronary risk factor, were studied. CAC was measured using 64-slice multislice computed tomography (MSCT) and divided into three groups: none (CAC score = 0; n 25), mild to moderate (0 < CAC score < 400; n 52) and severe (CAC score > 400; n 26). Phylloquinone (PK) and menaquinone (MK)-4 and MK-7 were measured by HPLC-tandem MS. Mean age of patients was 64 (SD 13) years, of which 57 % were male. Median CAC score was 57.2. Median levels of PK, MK-4 and MK-7 were 1·33, 0 and 6·99 ng/ml, showing that MK-7 was the dominant vitamin K in this population. MK-7 showed a significant inverse correlation with uncarboxylated osteocalcin (ucOC, \( P = 0.014 \)), protein induced by vitamin K absence of antagonist-2 (PIVKA-2, \( P = 0.013 \)), intact parathyroid hormone (\( P = 0.007 \)) and bone-specific alkaline phosphatase (\( P = 0.018 \)). CAC showed an inverse correlation with total circulating uncarboxylated matrix Gla protein (t-ucMGP, \( P = 0.018 \)) and Hb (\( P = 0.05 \)), and a positive correlation with age (\( P < 0.001 \)), creatinine, collagen type 1 cross-linked N-terminal telopeptide (NTX, \( P = 0.03 \)), pulse wave velocity (\( P < 0.001 \)) and osteoprotegerin (\( P < 0.001 \)). However, CAC did not have a significant correlation with plasma levels of PK, MK-4 or MK-7. In conclusion, plasma MK-7, MK-4 or PK level did not show significant correlation with CAC despite the association between plasma vitamin K levels and vitamin K-dependent proteins such as ucOC or PIVKA-2.

Key words: Vitamin K: Coronary artery calcification: Osteocalcin: Matrix Gla protein

Vitamin K is a cofactor for the enzyme responsible for the conversion of specific glutamyl residues to \( \gamma \)-carboxyglutamyl residues in several blood coagulation factors, bone-related proteins and other molecules. Vitamin K naturally exists in two forms, namely vitamin K\(_1\), also called phylloquinone (PK), and a group designated vitamin K\(_2\), also known as menaquinones (MK). PK is widely distributed in green and leafy vegetables, whereas MK exist preferentially in meats (MK-4), eggs (MK-4), curd (MK-8, MK-9), cheese (MK-8, MK-9) and fermented soyabean products (MK-7). PK is endogenously converted to MK-4\(^{(1)}\).

Arterial calcification is a pathological change found in atherosclerosis but the mechanisms involved have not yet been defined. In mice, gene knockout of the matrix Gla protein...
dietary intake of PK as well as MK reduced coronary artery stroke, myocardial infarction and aortic dissection (8). A high unit correlated clinically with cardiovascular events such as vitamin K. Gene polymorphism of the epoxide reductase subdrug that blocks epoxide reductase, a recycling enzyme of aortic dissection (2). In humans the level of inactive MGP correlated with the risk of CVD and mortality (3) MK (6). A similar relationship between warfarin and arterial calcification in animals which can be reversed by the addition of vitamin K. Gene polymorphism of the epoxide reductase subdrug that blocks epoxide reductase, a recycling enzyme of aortic dissection (2). In humans the level of inactive MGP correlated with the risk of CVD and mortality (3).

We performed an observational study to test the association between CAC and plasma levels of PK, MK-4 and MK-7.

Methods

Patient selection and study protocol

This study, the Vitamin K2 Super Minimizing Mineralization Trial (K2 SUMMIT-1), was performed at three hospitals (Tokai University Hospital, Mitsui Memorial Hospital and Ibaraki Seinan Medical Center Hospital), between 1 May 2008 and 28 December 2012. Patients with at least one coronary risk factor, e.g. hypertension, diabetes mellitus, hypercholesterolaemia, smoking, and a family history of coronary artery disease, were enrolled. Exclusion criteria were patients with an implanted coronary stent or pacemaker. The Institutional Review Board approved the study and all patients gave written informed consent. A medical history that included prior myocardial infarction, prior percutaneous coronary intervention, prior coronary artery bypass graft surgery, prior heart failure, prior stroke, or haemodialysis was obtained from each patient. The correlation between a CAC score and each coronary risk factor was studied.

Cardiac multi-slice computed tomography data acquisition and analysis

A prospective non-enhanced coronary Ca scan was performed with a 64-slice MSCT scanner (Siemens) in all patients. For quantitative assessment of CAC, the Agatston score (9,10) was calculated, using a 3 mm CT slice thickness and a detection threshold of 130 Hounsfld units (HU) involving ≥ 1 mm² area/lesion (three pixels). A total CAC score was determined by summing individual lesion scores from each of four anatomical sites (left main trunk, left anterior descending artery, left circumflex artery, and right coronary artery) (13,14). Patients were divided into three groups according to the clinical grading of CAC level: (1) none (Agatston score = 0); (2) mild to moderate CAC (0 < Agatston score < 400); and (3) severe CAC (Agatston score > 400).

Measurements

Plasma was obtained from patients in the morning after overnight fasting and stored at −30°C. Vitamin K (PK, MK-4 and MK-7) was determined by HPLC tandem MS with atmospheric pressure chemical ionisation (LC-APCI-MS/MS) as described previously (14). Total circulating uncarboxylated MGP (t-ucMGP) measurements were performed by Dr Cees Vermeer’s group (Cardiovascular Research Center, Maastricht University, the Netherlands). Measurement methods and measurement errors have been reported previously (15). Intact parathyroid hormone (PTH), osteocalcin (OC), uncarboxylated OC (ucOC), collagen type 1 cross-linked N-terminal telopeptide (NTX), bone-specific alkaline phosphatase (BAP), high sensitive C-reactive protein, intact PTH, oxidised LDL and protein induced by vitamin K absence of antagonist 2 (PIVKA-2) were measured by SRL Inc. Intra- and inter-assay CV were OC (3–5%), ucOC (1.3–2.0%), NTX (8.8–11.9%), BAP (2.5–2.9%), high sensitive C-reactive protein (0.9–2.3%), intact PTH (0.8–3.0%), oxidised LDL (6.2–5.3%), osteoprotegerin (OPG) (3.0–5.0%) and PIVKA-2 (5.7–6.1%).

Bone density was measured in the lumbar vertebra using a bone densitometer (DSC-900FX; Hitachi-Aloka Medical). Ankle–brachial index and brachial–ankle pulse wave velocity (PWV) were measured using a vascular screening device (BP-203 RPE; Omron-Colin).

Statistics

Continuous variables are presented as means and standard deviations in a normal distribution or as medians and

| Table 1. Patient characteristics (Number of subjects and percentages; mean values and standard deviations) |
|---------------------------------------------------------------|---------------|---------------|
| Patient number                                               | 103           |               |
| Age (years)                                                  |               |               |
| Mean                                                        | 64            | 13            |
| SD                                                          | 9.0           |               |
| Male                                                        | 59            | 57            |
| Height (cm)                                                  | 160.1         | 9.0           |
| Mean                                                        | 60.3          | 13.2          |
| SD                                                          | 23.3          | 3.9           |
| BMI (kg/m²)                                                  |               |               |
| Mean                                                        |               |               |
| SD                                                          |               |               |
| Hypertension                                                | 62            | 60            |
| Hyperlipidaemia                                             | 70            | 68            |
| Diabetes mellitus                                           | 40            | 39            |
| Insulin-treated                                             | 4             | 4             |
| Smoker                                                      | 45            | 44            |
| Prior myocardial infaration                                 | 9             | 9             |
| Prior percutaneous coronary intervention                     | 11            | 11            |
| Prior coronary artery bypass graft surgery                   | 3             | 3             |
| Prior heart failure                                         | 12            | 12            |
| Prior stroke                                                | 6             | 6             |
| Haemodialysis patients                                      | 5             | 5             |
| ACE-inhibitor or ARB                                        | 42            | 41            |
| Antiplatelets                                               | 36            | 35            |
| Statin                                                      | 45            | 44            |

ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker.
Table 2. Baseline blood test, calcification score and pulse wave velocity (Mean values and standard deviations)

|                         | Mean  | SD    |
|-------------------------|-------|-------|
| Hb (g/l)                | 136   | 17    |
| Prothrombin time (INR)  | 0.93  | 0.08  |
| Albumin (g/l)           | 42.4  | 2.7   |
| TAG (mmol/l)            | 1.66  | 1.20  |
| Alkaline phosphatase (IU/l) | 231.1 | 69.9  |
| Estimated GFR (ml/min per 1.73 m²) | 60.4 | 25.7  |
| Phosphate (mmol/l)      | 1.16  | 0.36  |
| Ca (mmol/l)             | 2.28  | 0.23  |
| ucOC (ng/ml)            | 5.8   | 11.9  |
| OC (ng/ml)              | 8.9   | 16.0  |
| ucOC:OC ratio           | 0.73  | 0.96  |
| PIVKA-2 (mAU/ml)        | 23.9  | 11.1  |
| Intact parathyroid hormone (pg/ml) | 55.0 | 82.5  |
| Bone-specific alkaline phosphatase (µg/l) | 17.7 | 9.1   |
| NTX (nmol BCE/ml)       | 18.6  | 13.5  |
| hsCRP (mg/l)            | 1320  | 3707  |
| Osteoprotegerin (ng/ml) | 101.4 | 60.5  |
| Oxidised LDL (µg/l)     | 3.00  | 1.35  |
| t-ucMGP (nmol/l)        | 3315  | 1688  |
| Phylloquinone (ng/ml)   | 1.6   | 1.3   |
| Menaquinone-7 (ng/ml)   | 10.1  | 10.3  |
| Menaquinone-4 (ng/ml)   | 2.1   | 15.9  |
| Coronary artery Ca score (U) | 591 | 1532  |
| Bone density (g/cm²)    | 0.99  | 0.25  |
| % Bone density          | 106.5 | 21.1  |
| ABI                     | 1.14  | 0.11  |
| PWV (cm/s)              | 1682  | 395   |

IU, international units; GFR, glomerular filtration rate; ucOC, uncarboxylated osteocalcin; OC, osteocalcin; PIVKA-2, protein induced by vitamin K absence or antagonist-2; mAU, milli absorbance units; NTX, N-terminal telopeptide; BCE, bone collagen equivalents; hsCRP, high sensitive C-reactive protein; t-ucMGP, total circulating uncarboxylated matrix Gla protein; ABI, ankle-brachial index; PWV, pulse wave velocity.

Results

Baseline patient characteristics are shown in Table 1. The mean age of the study population was 64 (SD 13) years, of which 57% were male. Five patients (4.9%) received haemodialysis. Pathology results for CAC, bone density, ankle-brachial index and PWV are shown in Table 2. The median value of the total Ca score was 57.2.

Plasma levels of PK, MK-4 and MK-7 are shown in Fig. 1. Statistical analysis showed that PK, MK-4 and MK-7 were normally distributed as 1·64 (SD 1·26) ng/ml (median 1·23 ng/ml), 2·1 (SD 1·59) ng/ml (median 0, interquartile range 0–0.04, maximum 157·6 ng/ml) and 10·1 (SD 10·3) ng/ml (median 6·99 ng/ml).

Associations between coronary calcification severity and risk factors are shown in Table 3. Patients with severe calcification were significantly older, and were significantly more likely to have a history of myocardial infarction, stroke or peripheral artery disease. Such patients showed significantly lower Hb levels and poor kidney function; they had significantly higher levels of NTX, OPG and ucOC, and a lower level of t-ucMGP. Patients with severe calcification also had significantly higher PWV values. However, plasma levels of PK, MK-4 or MK-7 did not correlate with the severity of CAC.

MK-7, PK, TAG-adjusted MK-7 and TAG-adjusted PK data and related factors are listed in Table 4. MK-7 was inversely correlated with ucOC, PIVKA-2, creatinine, intact PTH, BAP and NTX, but positively correlated with percentage bone density in a statistically significant manner.
Table 3. Association between the severity of coronary calcification and clinical or blood test factors
(Mean values and standard deviations, or percentages)

|                      | No Ca (n 25): Ca score = 0 | Mild to moderate Ca (n 52): 0 < Ca score < 400 | Severe Ca (n 26): Ca score > 400 | P       |
|----------------------|----------------------------|-----------------------------------------------|---------------------------------|---------|
| Age (years)          | Mean 56 ± 14               | Mean 65 ± 11                                  | Mean 71 ± 12                    | <0·001  |
| History of MI (%)    | 0                          | 8                                             | 19                              | 0·048   |
| History of stroke (%)| 0                          | 4                                             | 15                              | 0·05    |
| Peripheral artery disease (%) | 0                     | 2                                             | 12                              | 0·06    |
| Use of aspirin (%)   | 12                         | 31                                            | 46                              | 0·03    |
| Haemodialysis (%)    | 4                          | 2                                             | 15                              | 0·05    |
| Hb (g/l)             | 141 ± 18                   | 137 ± 14                                      | 129 ± 19                        | 0·03    |
| BMI (kg/m2)          | 24·4 ± 4·0                 | 24·0 ± 4·2                                    | 21·2 ± 1·3                      | 0·004   |
| Albumin (g/l)        | 43 ± 3                     | 42 ± 2                                        | 41 ± 3                          | 0·04    |
| Creatinine (μmol/l)  | 124 ± 230                  | 80 ± 88                                       | 239 ± 398                       | 0·03    |
| Ca (mmol/l)          | 2·3 ± 0·3                  | 2·3 ± 0·2                                     | 2·3 ± 0·1                       | 0·94    |
| Phosphate (mmol/l)   | 1·2 ± 0·5                  | 1·1 ± 0·3                                     | 1·2 ± 0·3                       | 0·94    |
| ALP (IU/l)           | 258 ± 78                   | 213 ± 49                                      | 242 ± 88                        | 0·02    |
| Bone type ALP (μg/l) | 16·1 ± 7·8                 | 17·2 ± 7·2                                    | 20·3 ± 12·9                     | 0·23    |
| Intact PTH (pg/ml)   | 52 ± 58                    | 41 ± 21                                       | 85 ± 148                        | 0·09    |
| Oxidised LDL (mmol/l)| 3·2 ± 1·6                  | 3·1 ± 1·4                                     | 2·7 ± 0·9                       | 0·32    |
| NTX (nmol BCE/l)     | 16 ± 6                     | 17 ± 11                                       | 25 ± 11                         | 0·03    |
| OPG (ng/ml)          | 85 ± 36                    | 85 ± 38                                       | 150 ± 86                        | <0·001  |
| t-ucMGP (nmol/l)     | 3455 ± 1508                | 3659 ± 1843                                   | 2529 ± 1292                     | 0·018   |
| ucOC (ng/ml)         | 4·6 ± 3·6                  | 4·0 ± 3·8                                     | 10·7 ± 22·5                     | 0·056   |
| PIVKA-2 (mAU/ml)     | 21·6 ± 5·3                 | 23·8 ± 8·1                                    | 26·1 ± 18·1                     | 0·36    |
| PK (ng/ml)           | 1·4 ± 1·0                  | 1·8 ± 1·3                                     | 1·6 ± 1·3                       | 0·46    |
| MK-4 (ng/ml)         | 6·3 ± 31·5                 | 0·94 ± 4·8                                    | 0·39 ± 1·9                      | 0·31    |
| MK-7 (ng/ml)         | 8·7 ± 9·1                  | 10·5 ± 10·1                                   | 10·8 ± 11·1                     | 0·72    |
| hsCRP (mg/l)         | 2964 ± 7169                | 918 ± 1076                                    | 523 ± 448                       | 0·03    |
| Bone matrix (g/cm²)  | 1·07 ± 0·23                | 0·99 ± 0·23                                   | 0·95 ± 0·31                     | 0·22    |
| ABI                  | 1·2 ± 0·1                  | 1·2 ± 0·1                                     | 1·1 ± 0·2                       | 0·18    |
| ba-PWV (cm/s)        | 1424 ± 321                 | 1655 ± 281                                    | 2007 ± 453                      | <0·001  |

Mi, myocardial infarction; ALP, alkaline phosphatase; PTH, parathyroid hormone; BCE, bone collagen equivalents; NTX, N-terminal telopeptide; OPG, osteoprotegerin; t-ucMGP, total circulating uncarboxylated matrix GlA protein; ucOC, uncarboxylated osteocalcin; PIVKA-2, protein induced by vitamin K absence or antagonist-2; mAU, milli absorbance units; PK, phylloquinone; MK, menaquinone; hsCRP, high sensitive C-reactive protein; ABI, ankle–brachial index; ba-PWV, brachial–ankle pulse wave velocity.

Discussion

In the studied population, MK-7 was the dominant vitamin K which had inverse correlation with ucOC, PIVKA-2, intact PTH and BAP. CAC showed an inverse correlation with t-ucMGP and Hb, and a positive correlation with age, creatinine, NTX, PWV and OPG. However, CAC did not have a significant correlation with plasma levels of PK, MK-4 or MK-7.

In this study, the medians of PK, MK-4 and MK-7 were found to be 1·33, 0 and 6·99 ng/ml, respectively, with levels of PK and MK-4 similar to those of previous reports(16,17). On the other hand, the plasma MK-7 level was much higher than those of previous reports: a median of 1·43 ng/ml was found by Fusaro et al. and a median of 3·92 ng/ml by Tsugawa et al.(16,17). Such a discrepancy with the published literature may probably exist because this study was conducted in eastern Japan where fermented soya beans known as natto, a food rich in MK-7, is popular. Tsugawa et al. reported that PK and MK-7, but not MK-4, correlated inversely with ucOC(17). In comparison, we observed that MK-7, but not PK or MK-4, correlated inversely with ucOC and suggest this was because MK-7 may be the dominant vitamin K in this studied population. The population with the highest tertile of MK-7 exhibited a high percentage bone density, and low levels of ucOC, PIVKA-2, intact PTH, BAP and NTX. These data suggest that a high intake of MK-7 induced carboxylation to reduce ucOC and PIVKA-2, and to increase bone density with reduced bone destruction.

The group showing severe CAC were older in age, had a high PWV, and a frequent history of myocardial infarction, stroke and peripheral artery disease, indicating severe, whole-body atherosclerosis. As CAC is one of the known forms of atherosclerosis, such a relationship is conceivable(18,19). Furthermore, the group with severe CAC also displayed chronic kidney disease and mineral and bone disorders, as suggested by high creatinine, low Hb and high NTX levels. Chronic kidney disease is also a well-known risk factor for CAC(20,21) as also confirmed in this study.

CAC was also related to high levels of OPG, and low levels of t-ucMGP; these were also previously reported risk factors for arterial calcification(2,3,22–26). The inverse association between t-ucMGP and calcification is generally explained by the tight binding of MGP’s phosphoserine residues to hydroxyapatite, initially reported by Price et al.(25). Despite the inverse correlation between MK-7 and ucOC, we remained puzzled as to the absence of a direct correlation between CAC and plasma vitamin K levels. The plasma vitamin K level reflects food intake over a short period of time but the ucOC level reflects a longer period of vitamin K insufficiency. Time-series studies in which the ucOC level was lowered by...
vitamin K supplementation showed that it remained unchanged at 15 d, but, by 30 d, reduction was observed\(^{26-28}\). Despite a weak association between CAC and ucOC (\(P = 0.056\)), CAC may correlate with the chronic status of vitamin K, but not with the plasma vitamin K level shown by a single measurement.

There are several limitations to this study. First, this study was conducted in eastern Japan where fermented soybeans (natto) are a popular food, thus making the observed plasma MK-7 level higher than in previous studies. Second, as measurements of plasma vitamin K levels were not repeatedly performed, the possibility remains that errors were caused by the ingestion, by patients, of varying food content. Third, the number of patients studied was limited. If patient numbers were increased, a direct correlation between plasma vitamin K levels and CAC may be more readily observed. Finally, we did not measure dephospho-uncarboxylated MGP which is the most sensitive vitamin K marker presently available, and also a risk marker for CVD and mortality.

In conclusion, we could not show a direct correlation between plasma vitamin K levels and CAC. However, CAC correlated with markers of chronic insufficiency of vitamin K such as ucOC. This suggests that the chronic intake of sufficient vitamin K may have an inhibitory effect on CAC.

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### Table 4. Plasma levels of vitamin K and related factors

(Mean values and standard deviations, or medians and ranges)

|                  | Low       | Medium    | High      | P        |
|------------------|-----------|-----------|-----------|----------|
|                  | Mean (ng/ml) | Mean (ng/ml) | Mean (ng/ml) |          |
|                  | n         | Median     | Range     |          |
|                  | 34        | 1.28       | 0–3.12    |          |
|                  | 34        | 5.74       | 3.12–11.8 |          |
|                  | 35        | 20.9       | 11.8–46.4 |          |
| Creatinine (μmol/l) | 221       | 371        | 80–62     | 0.0241   |
| % Bone density   | 103       | 21         | 103–17    | 0.0565   |
| UCOC (mg/ml)     | 10.8      | 20.2       | 4.2–3.4   | 0.0149   |
| PIVKA-2 (mAU/ml) | 28.6      | 16.6       | 22.5–5.7  | 0.0133   |
| Intact PTH (pg/ml) | 89.3   | 135.5      | 46.6–15.6 | 0.0077   |
| BAP (μg/l)       | 21.2      | 12.0       | 16.8–6.1  | 0.0188   |
| NTX (nmol BCE/l) | 23.4      | 21.6       | 16.6–5.3  | 0.0342   |
|                  | MK-7 tertile group | MK-7/TAG tertile group | PK tertile group |          |
|                  | Mean (ng/ml per mmol) | Median     | Range     |          |
|                  | n         | 0.011      | 0.031–0.083 | 0.082–0.584 |          |
|                  | MK-7      | 1.26       | 3.6–2.4   | 3.0       | 2.3       | 0.0080   |
|                  | uOC       | 0.045      | 0.031–0.078 | 0.002–0.300 | 0.0001   |
|                  | PIVKA-2   | 28.9       | 21.8–5.5  | 21.3      | 7.2       | 0.0077   |
|                  | Intact PTH| 89.4       | 42.8–21.2 | 32.1      | 22.3      | 0.0074   |
|                  | BAP       | 21.5       | 15.6–5.9  | 15.9      | 7.4       | 0.0089   |
|                  | PK        | 0.75       | 1.28       | 2.50     |          |
|                  | Hb (g/l)  | 0.25–0.97  | 0.98–1.58  | 1.59–7.76 |          |
|                  | TAG       | 1.3        | 1.8        | 1.8       | 0.7       | 0.0094   |
|                  | BAP       | 22.2       | 15.5–7.1  | 15.6–6.3 | 0.0020   |
|                  | PK/TAG    | 0.0056     | 0.0009     | 0.0020   |          |
|                  | n         | 34         | 34         | 35       |          |
|                  | Age (years) | 61         | 61         | 70       | 8        | 0.0014   |
|                  | TAG (mmol/l) | 2.4         | 1.5–0.7   | 1.1       | 0.6       | <0.0001  |
|                  | BAP (μg/l) | 21.1       | 15.2       | 16.8      | 8.2       | 0.0192   |
|                  | Oxidised LDL (mmol/l) | 3.4         | 2.9        | 2.6       | 0.9       | 0.0320   |

MK, menaquinone; ucOC, uncarboxylated osteocalcin; PIVKA-2, protein induced by vitamin K absence or antagonist-2; mAU, milli absorbance units; PTH, parathyroid hormone; BCE, bone collagen equivalents; BAP, bone-specific alkaline phosphatase; NTX, N-terminal telopeptide; PK, phylloquinone.
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