Original Communication

Maximal dose-response of vitamin-K2 (menaquinone-4) on undercarboxylated osteocalcin in women with osteoporosis

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Abstract: Low concentrations of serum vitamin K accompany high concentrations of undercarboxylated osteocalcin (ucOC) and osteoporotic fractures. Although vitamin K2 (MK-4) is approved as a therapeutic agent for the treatment of osteoporosis in some countries, the dose-response is unknown. The objective of this study was to assess the improvement in carboxylation of osteocalcin (OC) in response to escalating doses of MK-4 supplementation. A nine-week, open-labeled, prospective cohort study was conducted in 29 postmenopausal women who suffered hip or vertebral compression fractures. Participants took low-dose MK-4 (0.5 mg) for 3 weeks (until the second visit), then medium-dose MK-4 (5 mg) for 3 weeks (until the third visit), then high-dose MK-4 (45 mg) for 3 weeks. The mean ± *SD* age of the participants was 69 ± 9 years. MK-4 dose ($p < 0.0001$), but neither age nor other relevant medications (e.g. bisphosphonates) correlated with improvement in %ucOC. As compared to baseline concentrations (geometric mean ± *SD*) of 16.8 ± 2.4, 0.5 mg supplementation halved %ucOC to 8.7 ± 2.2 ($p < 0.0001$) and the 5-mg dose halved %ucOC again (to 3.9 ± 2.2; $p = 0.0002$ compared to 0.5-mg dose). However, compared to 5 mg/day, there was no additional benefit of 45 mg/day (%ucOC 4.6; p = NS vs. 5-mg dose). MK-4 supplementation resulted in borderline increases in γ-carboxylated osteocalcin (glaOC; $p = 0.07$). There were no major side effects of MK-4 supplementation. In postmenopausal women with osteoporotic fractures, supplementation with either 5 or 45 mg/day of MK-4 reduces ucOC to concentrations typical of healthy, pre-menopausal women.

Keywords: Carboxylation of osteocalcin, Dose-finding, Postmenopausal osteoporosis, Vitamin K2 (MK-4), hip or vertebral compression fractures

Introduction

Hip and vertebral fractures are common among postmenopausal women and can prevent independence [1]. Vertebral fractures cause significant back pain and limit functional activity [2]. Hip fractures reduce life expectancy and cost the US $20 billion each year [3, 4]. Given the decline in use of postmenopausal hormonal therapy after pivotal trials [5, 6] and the graying of the population, the frequency of osteoporotic fractures continues to rise [7]. Thus, additional therapies to prevent postmenopausal fractures are needed.

Epidemiologic studies suggest that vitamin K promotes bone health [8, 9]. Low concentrations of circulating vitamin K accompany low bone mineral density [10] and bone fractures [11]. In the Nurses' Health Study, low dietary intake of vitamin K (< 109 μg/d) was associated with hip fractures [12]. The major dietary sources of vitamin K1 are green leafy or cruciferous vegetables, and plant oils (e.g soybean oil). Important dietary sources of vitamin K2 are meat (especially liver), egg yolks, cheese, and the Japanese dish natto. The recommendations for Vitamin K1 are associated with its function in the activation of coagulation factors. However, there is no official recommendation of

vitamin K2 due to lack of sufficient data according to Food and Nutrition Board (www.nationalacademies.org/hmd).

Biochemical studies suggest that vitamin K is beneficial because of its effect on osteocalcin (OC), a regulator of bone mineralization [13, 14]. Specifically, vitamin K functions as a cofactor for the enzyme that catalyzes the post-translational γ-carboxylation of glutamic acid residues on OC and other proteins [15]. With vitamin K deficiency and aging, the proportion of OC that is uncarboxylated (ucOC) rises [16]. Higher concentrations of ucOC are associated with low bone mineral density [17, 18] and hip fractures [19–22]. In young, healthy adults, approximately 7.5% of the OC is ucOC [23].

Vitamin K2 (e.g. menaquinone 4; MK-4) supplementation decreases serum ucOC concentrations within 2 weeks of supplementation [24–33]. Studies of supplementation with vitamin K2 (MK-7) 180 or 360 μg/d, have had variable biochemical benefits [32–39] and suggest that higher doses are necessary to carboxylate OC maximally [40–42]. Some trials of vitamin K supplementation have found a reduction in fractures [37, 43, 44] but others have not [36, 45, 46]. In hemodialysis patients, supplementation with 360 μg of vitamin K2 (MK-7) reduced ucOC by one-third, but lower doses had no significant benefit [41]. In healthy adults (20–60 years of age) supplementation with 90 or 180 μg of synthetic vitamin K2 (MK-7) reduced ucOC by 21% and 29%, respectively, with peak benefits obtained after approximately 3 weeks of supplementation [47]. Given prior research, we wondered what dose of vitamin K2 would maximally reduce ucOC in elderly patients with osteoporosis. Although high doses of vitamin K3 can be toxic [48], doses of vitamin K2 more than100 times the dietary recommendations [49] (e.g. MK-4 up to 45 mg/d are safe [30, 37, 50, 51] and vitamin K2 (MK-7) supplementation does not appear to increase thrombin generation [42]).

The primary objective of this study was to evaluate the biochemical response of escalating doses of vitamin K2 (MK-4) up to 45 mg/d on ucOC. This was an open-labeled prospective cohort study conducted in postmenopausal women with a history of osteoporotic fractures.

Materials and Methods

Study participants

We recruited non-Hispanic, Caucasian women living in the St. Louis area who suffered a non-pathologic hip or vertebral compression fracture that occurred without major trauma. Participants had to be age 50 or older and postmenopausal, with at least 12 months elapsed since their last menses. We excluded women with secondary causes of osteoporosis (i.e., vitamin D deficiency, hyperparathyroidism, malignancy, metabolic bone disease, rheumatoid arthritis, alcoholism, cirrhosis, or chronic kidney disease stage IV or V). We also excluded women who were taking chronic glucocorticoids or warfarin [52]. Recruitment of participants occurred via physician referral located within Washington University Medical Center.

Study design

A nine-week, open-labeled, prospective cohort study was conducted to quantify the biochemical response to oral vitamin K2 (MK-4) supplementation. After the baseline blood draw, participants took low-dose MK-4 (0.5 mg) for 3 weeks (until the second-visit), then medium-dose MK-4 (5 mg) for 3 weeks (until the third-visit), then high-dose MK-4 (45 mg) for 3 weeks. The MK-4 was supplied by Eisai Co., Ltd (Tokyo, Japan) in blister packs. At the end of each 3-week period, participants donated a blood sample. Participants were instructed not to change their eating habits during the study period. In addition to vitamin K2 (MK-4), all participants received standardized doses of elemental calcium, 1200 mg/day, and vitamin D3, 800 IU/day (Citracal™, Bayer HealthCare LLC, New Jersey, USA), divided into two doses per day in accordance with National Osteoporosis Foundation recommendations [53]. Prior to participation, vitamin D deficiency had been corrected and participants had been prescribed calcium as per standard of care.

During each visit, venous blood samples were collected at the General Clinical Research Center or osteoporosis clinics affiliated with Washington University in St. Louis. Blood samples were centrifuged at 1600 g for 15 min at 4 °C. Serum was separated and stored at −80 °C until used. Prior to enrolling, participants provided written informed consent. This study was conducted according to the Declaration of Helsinki and all procedures involving human subjects were approved by the Washington University Human Research Protection Office.

Measurements of ucOC and glaOC

Commercially available kits (Takara Bio Inc., Shuzo, Japan) that utilize sets of monoclonal antibodies highly reactive to ucOC (Cat# MK118) and glaOC (Cat# MK111), were used to measure the different forms of osteocalcin concentrations. The ucOC EIA kit measures 100% of undercarboxylated forms of OC (ucOC) and cross reacts 5% with human bone OC (probably the γ-carboxylated form, glaOC) [54]. Similarly, the glaOC EIA kit specifically measures 100% of γ-carboxylated form of OC with no detectable cross reactivity with undercarboxylated OC [22, 54]. A standard curve was run for every individual plate and the range of detection

Table 1. Dose-dependent changes in OC concentrations following MK-4 administration.

	Baseline concentrations, no MK-4 (n = 29); *geometric mean (SD)*	0.5 mg of MK-4 (n = 28); *geometric mean (SD)*	5 mg of MK-4 (n = 22); *geometric mean (SD)*	45 mg of MK-4 (n = 21); *geometric mean (SD)*
glaOC, (ng/ml)	8.4 (2.3)	9.9 (2.2)	13.9 (1.9)	12.0 (2.2)
ucOC, (ng/ml)	1.9 (2.8)	1.0 (2.5)	0.6 (2.6)	0.6 (2.7)
%ucOC, *[(ucOC/(ucOC + glaOC)) × 100]*	16.8 (2.4)	8.7 (2.2)	3.9 (2.2)	4.5 (3.0)

OC: Osteocalcin, MK-4: Menaquinone 4, ucOC: undercarboxylated osteocalcin, glaOC: γ-carboxylated osteocalcin.

for ucOC EIA kit and glaOC EIA kit were 0.25–8 ng/ml and 0.5–16 ng/ml, respectively. The intra-assay and inter-assay coefficient of variation (CV) for both kits were acceptable: for ucOC, 5.2% and 8.3%, respectively and for glaOC, 3.7% and 1.4%, respectively. All samples were measured in duplicate and averaged.

Statistical analysis

We used the %ucOC for all statistical analysis in this study, since it is a more sensitive marker of vitamin K availability in bone than ucOC concentrations [9, 36]. The %ucOC was calculated as [(ucOC/(ucOC + glaOC)) × 100]. Prior to statistical analysis, the %ucOC values were log-transformed to achieve normal distribution. The effect of different doses of vitamin K2 (MK-4) on ucOC ratio was analyzed using ANCOVA, in a repeated measures model (PROC MIXED in SAS). We adjusted for age and related medication (alendronate, ibandronate, risedronate, calcitonin, and raloxifene). The effect of vitamin K2 (MK-4) was assessed by pair-wise comparisons adjusting for multiple comparisons in Proc MIXED as well as paired t-tests comparing pre-baseline measures to each follow-up measure after the escalating vitamin K2 (MK-4) doses. Two-sided p-values < 0.05 were considered statistically significant.

Results

The mean ± *SD* age of the 29 participants was 69 ± 9.0 years (range 54–84 years). Sixteen patients (55%) took relevant medications (alendronate, ibandronate, risedronate, calcitonin, and raloxifene). Neither age nor medications effect were found as significant predictor of the outcome (%ucOC) in an adjusted model.

Twenty-nine samples were available from the baseline visit, 28 were available from the second visit; 22 from the third-visit and 21 from the fourth visit. Reasons for failure to obtain a sample (and N) were: samples lost or not available (4), patient withdrawal without side effects (3), nausea or bloating (2), treatment for localized cancer (1), distance from medical center (1), development of pruritic rash (1), and fear of thrombosis in patient with history of thrombosis (1). There were no deaths or major side effect of MK-4 supplementation.

The effect of MK-4 was highly significant ($p < 0.0001$) in a repeated measures model (PROC MIXED in SAS). The dose-dependent changes in OC concentrations following escalating dose of MK-4 supplementation were significant (Table I). Pair-wise comparisons revealed that 0.5 mg resulted in significant reduction in %ucOC compared to baseline ($p < 0.0001$) and increasing the dose to 5.0 mg had a significantly greater reduction than 0.5 mg ($p = 0.0002$). However, there was no additional benefit of MK-4 (45 mg/day) compared to 5 mg.

MK-4 significantly lowered %ucOC (Figure 1), primarily because it lowered ucOC concentration ($p < 0.0001$). The geometric mean (*SD*) of baseline %ucOC was 16.8% ± 2.4. At 0.5 mg/day of MK-4 the %ucOC was halved (a 48% reduction) to 8.7% ± 2.2; at 5 mg/day it was halved again to 3.9% ± 2.2 (a 72% reduction vs. baseline) and at 45 mg/day the %ucOC was 4.5% ± 3.0. MK-4 resulted in a borderline increase in glaOC ($p = 0.07$).

Discussion

Similar to studies of vitamin K1 [23] and of MK-7 [33, 41, 42], we found that low-dose MK-4 (0.5 mg/d) prescribed for 3 weeks in elderly women with osteoporotic fractures halved their %ucOC and that higher doses had even greater benefit. Specifically, we found that 5 mg/day of MK-4 reduced %ucOC by 72% (to absolute levels of 3.9%–4.5%). However, the 45 mg/d dose of MK-4 was no more effective than 5 mg/d. Prior studies that prescribed 45 mg/d of MK-4 and found relative reductions of ucOC of 37% to 55% [30, 37, 46, 50]. Thus, there does not appear any biochemical benefit of prescribing 45 mg/d rather than 5 mg/d of MK-4.

Prior studies of MK-7 found that supplementation with (45–360 μg/d) reduced ucOC in a dose-dependent manner [41, 42] with MK-7 doses of 180 to 360 μg/day reducing % ucOC by one-third to one-half, depending on the population [33, 41, 42]. The lower doses of MK-7 necessary to halve the

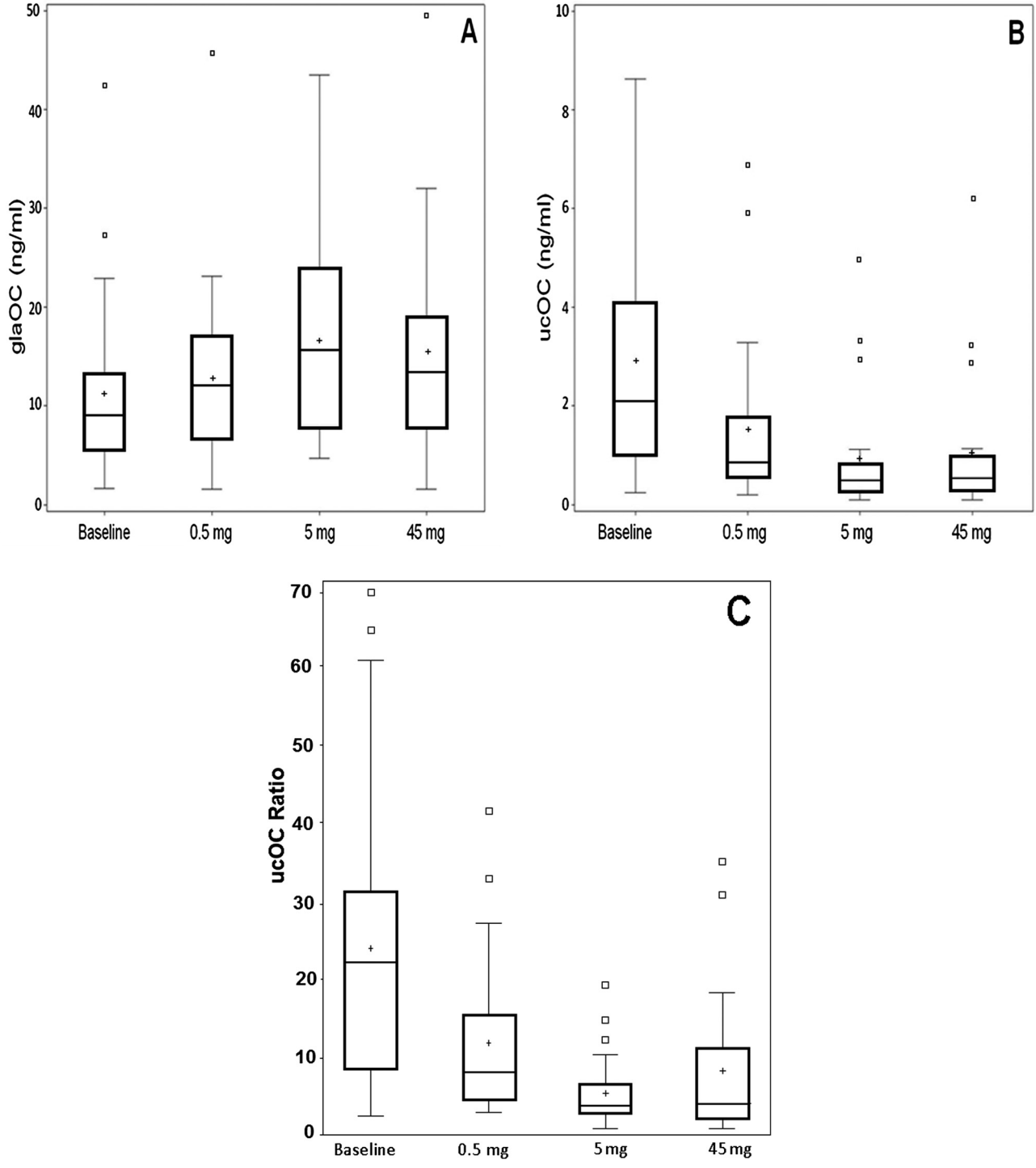

Figure 1. Box plots showing the changes in γ-carboxylated osteocalcin (glaOC) [A]; undercarboxylated osteocalcin (ucOC) [B]; and %ucOC ratio [C] from baseline, following escalating doses of vitamin K2 supplementation. Lines show medians, plus signs show means, whiskers show 1.5 times interquartile ranges, and open squares show outliers. All samples were measured in duplicate and averaged. A nine-week, open-labeled, prospective cohort study was conducted to quantify the biochemical response to oral vitamin K2 (MK-4) supplementation.

%ucOC reflects the greater potency and longer half-life of MK-7 vs. MK-4 [55–57].

The mechanism by which vitamin K may protect against fractures is controversial. In a study of low phylloquinone intake in postmenopausal women, no significant changes in bone and mineral metabolism occurred despite changes in bone markers of vitamin K status [58], but the study duration was too short (84 days) to assess risk of fracture.

A trial (ECKO) [44] of phylloquinone (5 mg/day) reduced fracture risk without improving bone mineral density (BMD). Both vitamin K1 and MK-7 are converted to MK-4 in extrahepatic tissues [59] with MK-7 being a more effective elevator of MK-4 [60]. Knapen et al. suggest that vitamin K2 improves bone geometry [61], perhaps because it allows for carboxylation of osteocalcin, a protein that stimulates bone mineral maturation [62, 63].

This study has strengths and weakness. One weakness is that the short-duration of follow-up did not allow us to assess the effect of MK-4 on rate of fractures. Clinical trials of vitamin K supplementation have been inconclusive, with some trials showing a reduction in fractures [37, 43, 44] and others not [45, 46, 51]. A second weakness is that we did not assess for potential benefits of MK-4 supplementation on bone geometry or density.

One strength is the dose-escalation, which allowed us to quantify the biochemical benefit of escalating doses of MK-4. Prior to this study, the biochemical benefits of higher MK-4 doses were speculated, but required validation because different formulations of vitamin K appear to have different levels of potency [56, 57] and the response to vitamin K supplementation may depend on age or population [23, 28]. For example, a study in 20 elderly Japanese women found a 38% ucOC reduction after 45 mg MK-4 supplementation for 2 weeks [30] – only half the benefit we observed in our Caucasian population.

Prior research suggests that MK-4 is more potent than phylloquinone in the reduction of ucOC concentration [30] and in improving lumbar spine-BMD [64] and we studied MK-4. We found that MK-4 promotes conversion of ucOC to glaOC because the total concentration of OC (ucOC + glaOC) did not rise.

Conclusion

We demonstrated that in postmenopausal women with osteoporotic fractures, supplementation with 5 or 45 mg/day of MK-4 maximally reduced %ucOC to levels typical of young, healthy adults. Although 0.5 mg/d of MK-4 also reduced %ucOC significantly, the higher doses were more effective. In the future, a randomized controlled trial should test the hypothesis that 5 mg/day of MK-4 improves bone health in postmenopausal women with osteoporotic fractures.

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Conflict of interest
The authors declare that there are no conflicts of interest.

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