A high phylloquinone intake is required to achieve maximal osteocalcin γ-carboxylation[1–3]

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ABSTRACT

Background: Dietary vitamin K is usually inadequate to maximize serum osteocalcin γ-carboxylation. Phylloquinone supplementation increases osteocalcin γ-carboxylation; however, the amount required to maximize carboxylation is not known.

Objective: This study assessed the ability of various doses of phylloquinone (vitamin K_1) to facilitate osteocalcin γ-carboxylation.

Design: Healthy adults aged 19–36 y participated in 2 substudies. In an initial dose-finding study (substudy A), 6 women and 4 men received a placebo daily for 1 wk and then phylloquinone daily for 3 wk: 500, 1000, and 2000 μg during weeks 2, 3, and 4, respectively. Osteocalcin and undercarboxylated osteocalcin were measured at baseline and after each week of supplementation. Subsequently, to further delineate the γ-carboxylation response of osteocalcin to various doses of vitamin K, 58 women and 42 men were randomly assigned to receive placebo or phylloquinone supplementation (250, 375, 500, and 1000 μg/d) for 2 wk (substudy B). The percentage of undercarboxylated osteocalcin (%ucOC) was measured at baseline and weeks 1 and 2.

Results: In substudy A, %ucOC decreased with phylloquinone supplementation ($P < 0.0001$); a greater reduction was observed with 1000 and 2000 μg than with 500 μg ($P < 0.05$). In substudy B, %ucOC decreased in all supplemented groups by week 1 (P for the trend < 0.0001), which was sustained through week 2. Phylloquinone supplementation decreased %ucOC dose-dependently; %ucOC was significantly different between the 250-μg and the placebo groups and between the 1000- and 500-μg groups but not between the 250-, 375-, and 500-μg groups.

Conclusion: A daily phylloquinone intake of ≈1000 μg is required to maximally γ-carboxylate circulating osteocalcin.   *Am J Clin Nutr* 2002;76:1055–60.

KEY WORDS   Vitamin K, phylloquinone, osteocalcin, undercarboxylated osteocalcin, γ-carboxylation

INTRODUCTION

Vitamin K is a required cofactor for the carboxylase enzyme that converts glutamyl to γ-carboxyglutamyl (Gla) residues in a small number of proteins (1, 2). With vitamin K inadequacy, less than maximal γ-carboxylation of these proteins is achieved (3–6). Thus, measurement of undercarboxylated proteins, such as osteocalcin, is a sensitive measure of vitamin K status (7).

For the vitamin K–dependent proteins, conversion of glutamyl to γ-carboxyglutamyl conveys the ability to bind calcium ions and is essential for biological activity (8). Because 3 vitamin K–dependent proteins (osteocalcin, matrix Gla-protein, and protein S) are present in bone (9) and may play a role in bone metabolism (10, 11), impaired function could potentially have adverse skeletal consequences. Specifically, subclinical vitamin K insufficiency might contribute to the development of osteoporosis (12). Data to support this possibility include epidemiologic observations that a low vitamin K intake is associated with increased hip fracture risk (13, 14). Additionally, some, but not all, reports find antagonism of vitamin K by clinical use of anticoagulants to be associated with low bone mineral density and increased fracture risk (15–18). Furthermore, less than maximal γ-carboxylation of circulating osteocalcin is common (6, 19–21) and has been reported to correlate with low bone mass (22, 23) and increased hip fracture risk (23–25). However, associations do not necessarily imply causation; alternatively, these indicators of vitamin K insufficiency might simply be surrogate markers of general dietary deficiency (9). Thus, it is not known whether low vitamin K status contributes to bone loss. However, if subclinical vitamin K insufficiency causes adverse skeletal consequences, it is reasonable that this would reflect impaired γ-carboxylation of vitamin K–dependent bone proteins, because the only known role of vitamin K is as a cofactor for the carboxylase enzyme in the synthesis of γ-carboxyglutamyl, ie, γ-carboxyglutamic acid (1, 2). Thus, it is plausible that a higher vitamin K intake might improve bone health by enhancing γ-carboxylation. However, the dose of supplemental vitamin K needed to maximally carboxylate bone proteins, including osteocalcin—the most abundant noncollagenous bone matrix protein—is unknown. Thus, the purpose of this study was to determine the minimum amount of supplemental phylloquinone (vitamin K_1) required to maximize the carboxylation status of serum osteocalcin in healthy young adults as measured by a hydroxyapatite binding assay.

SUBJECTS AND METHODS

Subjects

This study was approved by the University of Wisconsin Health Sciences Institutional Review Board, and informed consent was obtained from all volunteers. All participants were

TABLE 1
Demographic data by substudy group and dose of phylloquinone

Substudy group and phylloquinone dose	Age[1]	BMI[1]	Compliance	No. of subjects replaced
	y	kg/m²	%	
Substudy A (n = 4 M, 6 F)	25.8 (24.2, 27.4)	22.3 (21.1, 23.5)	98	2
Substudy B				
Placebo (n = 9 M, 11 F)	25.8 (24.2, 24.5)	23.3 (21.9, 24.6)	98	1
250 μg (n = 10 M, 10 F)	25.5 (23.6, 27.5)	25.1 (22.6, 27.7)	99	0
375 μg (n = 4 M, 16 F)	26.0 (24.3, 27.7)	22.3 (20.9, 23.7)	100	2
500 μg (n = 10 M, 10 F)	24.5 (23.2, 25.8)	24.1 (22.6, 25.5)	99	0
1000 μg (n = 9 M, 11 F)	26.2 (24.2, 28.3)	25.1 (22.3, 27.8)	97	0

[1] $\overline{x}$; 95% CI in parentheses. There were no significant differences between groups.

healthy volunteers recruited from southern Wisconsin. Eligible subjects were required to have normal screening laboratory values, including a complete blood count, prothrombin time, international normalized ratio, and serum chemistry panel. Volunteers with renal or hepatic disease or a history of malabsorption were excluded. In addition, individuals treated with warfarin or orlistat in the past 4 wk and those consuming foods containing olestra >3 times/wk were excluded.

Six women and 4 men aged 23–31 y ($\overline{x} \pm$ SD: 25.8 ± 2.9 y) were enrolled in the initial dose-finding study (substudy A). Subsequently, 100 subjects (58 women and 42 men) aged 19–36 y (25.6 ± 4.0 y) participated in substudy B.

Study design

Substudy A was conducted to delineate the doses to be used in substudy B. In this 4-wk, open-label study, participants received a placebo daily for the first week followed by doses of 500, 1000, and 2000 μg during weeks 2, 3, and 4, respectively. The placebo and phylloquinone tablets were identical in appearance in both substudy A and substudy B and were supplied in light-shielded containers by Roche Vitamins, Inc (Parsippany, NJ). All tablets were composed of >86% microcrystalline cellulose; magnesium stearate, dry white color, and dry phylloquinone made up the remainder. Because vitamin K is fat soluble, the subjects were instructed to ingest all preparations with their evening meal to maximize absorption. Serum for the measurement of osteocalcin and undercarboxylated osteocalcin (ucOC) concentrations was obtained at baseline and after weeks 1, 2, 3, and 4 of the study.

Substudy B was designed to identify the lowest dose of phylloquinone required to maximally γ-carboxylate osteocalcin. In this single-blind, placebo-controlled, 2-wk trial, the subjects received daily doses of placebo or 250, 375, 500, or 1000 μg phylloquinone. The subjects were randomly assigned to receive 3 tablets containing placebo, 125, or 500 μg phylloquinone to attain their assigned dose. As in substudy A, the participants were instructed to consume the study preparation with their evening meal. Serum was obtained at baseline and at 1 and 2 wk of treatment for the measurement of osteocalcin, ucOC, and phylloquinone concentrations. These specimens were obtained by routine venipuncture between 0800 and 1100 after a fast of ≥8 h. Specimens were allowed to clot for 30 min at room temperature while shielded from light and were then centrifuged (200× g, 15 min, room temperature) and quick-frozen in liquid nitrogen. The samples were subsequently maintained at −80 °C until thawed for analysis.

Subjects in both substudies were asked to provide an estimate of phylloquinone intake by recording in a daily log the number of servings (defined as 0.5 cup) of spinach, lettuce, Brussels sprouts, and broccoli consumed each day. These 4 foods were selected because they provide a large percentage of the total daily phylloquinone intake (26). In addition, to minimize habitual differences in dietary phylloquinone intake, the participants were asked to restrict their diet to one serving or less of the same high vitamin K–containing foods each day. Furthermore, to minimize factors that might impair vitamin K absorption, the subjects were asked to avoid olestra-containing products.

Compliance was calculated weekly by counting tablets; noncompliant subjects (defined as those who ingested <85% of the supplement) were allowed to complete the study; however, the data for these subjects were excluded and replacement subjects were enrolled. This approach was taken to minimize the likelihood of compliance falsification.

Assays

Serum osteocalcin was determined by immunoradiometric assay (ELSA-OSTEO; CisBio International, Gif-sur-Yvette France). ucOC concentrations were determined by using a modification of the hydroxyapatite binding assay (27). Briefly, 0.5 mL serum was treated with 25 mg hydroxyapatite (no. 4280; Mallinkrodt, Inc, Paris, KY) and rotated end over end for 30 min at 4 °C. The samples were then centrifuged at 16 000 × g for 5 min at room temperature. The supernatant fluid was removed and analyzed for osteocalcin by immunoradiometric assay. The percentage of ucOC (%ucOC) was calculated as the ratio of unadsorbed osteocalcin (ie, the amount remaining in the supernatant fluid) to total osteocalcin multiplied by 100. Serum phylloquinone concentrations were determined by HPLC separation with the use of fluorescence detection (28).

Statistical analysis

The groups were defined by substudy (A or B) and treatment (placebo or phylloquinone). The data from subjects in substudy B were analyzed by using a linear regression, with the dose of phylloquinone as a factor. Serum phylloquinone and %ucOC outcomes were transformed to the logarithmic scale to reduce the influence of outliers. Thus, the changes in phylloquinone concentrations and in %ucOC from baseline to week 1 are multiplicative factors rather than differences. The P values were adjusted for multiple comparisons via the Bonferroni method (in this case, multiplied by 4) (29). The test for trend with dose was also performed by fitting a linear regression but with dose considered a continuous predictor. Variables at baseline and after dose increments (substudy A) were examined for significant differences by using a one-way analysis of variance. As above, P values were adjusted by using the Bonferroni method.

TABLE 2
Baseline laboratory values by substudy group and phylloquinone dose[1]

Substudy group and phylloquinone dose	Albumin	Creatinine	ALT	Phylloquinone	ucOC	Estimated phylloquinone intake[2]
	g/L	μmol/L	U/L	nmol/L	%	μg/d
Substudy A (n = 4 M, 6 F)	44 (41.8, 46.2)	79.6 (69.0, 90.2)	20 (12.2, 27.8)	NE	7.5 (6.3, 8.7)	109 (77, 140)
Substudy B						
Placebo (n = 9 M, 11 F)	44 (43.1, 45.6)	79.6 (75.1, 84.9)	20 (17.6, 23.0)	0.70 (0.42, 0.98)	7.2 (6.0, 8.4)	85 (57, 113)
250 μg (n = 10 M, 10 F)	43 (41.3, 44.2)	79.6 (73.4, 83.1)	19 (15.1, 22.6)	0.61 (0.41, 0.82)	7.9 (6.3, 9.6)	77 (59, 96)
375 μg (n = 4 M, 16 F)	43 (41.8, 44.2)	79.6 (70.7, 82.2)	14 (12.7, 15.8)	1.00 (0.61, 1.32)	6.5 (5.5, 7.4)	120 (92, 147)
500 μg (n = 10 M, 10 F)	44 (42.6, 45.3)	79.6 (75.1, 87.5)	22 (16.3, 26.7)	0.83 (0.53, 1.13)	8.7 (7.5, 9.8)	94 (75, 113)
1000 μg (n = 9 M, 11 F)	44 (42.3, 45.5)	79.6 (72.5, 83.1)	17 (15.1, 19.9)	0.81 (0.59, 1.04)	8.3 (6.7, 10.0)	77 (60, 94)

[1] $\bar{x}$; 95% CI in parentheses. ALT, alanine aminotransferase; ucOC, undercarboxylated osteocalcin; NE, not evaluated. There were no significant differences between groups by one-way ANOVA with Bonferroni correction.

[2] Estimated by self-report of spinach, broccoli, Brussels sprouts, and lettuce consumption.

RESULTS

Subjects

The demographic characteristics and laboratory results did not differ significantly between groups (**Tables 1** and **2**). Compliance and adherence were excellent in all groups (Table 1). Five subjects were replaced (2 in substudy A and 3 in substudy B) because of inadequate compliance. No differences in compliance between treatment weeks was observed (data not shown).

Substudy A

The percentage of ucOC decreased ($P < 0.0001$) from 7.48% at baseline to 2.49%, 1.90%, and 1.75% after sequential supplementation for 1 wk with 500, 1000, and 2000 μg phylloquinone/d, respectively (**Figure 1**). Both the 1000- and 2000-μg/d doses reduced %ucOC more ($P < 0.05$) than did the 500-μg dose. However, %ucOC did not differ after supplementation with 1000 or 2000 μg/d. Total serum osteocalcin concentrations were unaffected by phylloquinone supplementation at any dose (data not

shown). On the basis of these data, daily doses of phylloquinone between 250 and 1000 μg were used in substudy B.

Substudy B

An increase in serum phylloquinone concentration was observed from baseline to both weeks 1 and 2 in all phylloquinone-supplemented groups (**Figure 2**). The changes in serum phylloquinone from baseline to week 1 are shown in **Table 3**. Subjects who received 250 μg/d on average had serum phylloquinone concentrations at week 1 that were 3.2 times those at baseline; the true value was likely 2.24–4.57 times the baseline value. Serum phylloquinone concentrations increased with increasing doses of phylloquinone ($P < 0.0001$ for trend); the greatest increase (≈10-fold, from 0.81−0.13 nmol/L at baseline to 7.86−1.0 nmol/L at week 2) was observed in the subjects who received 1000 μg/d. The increase in serum phylloquinone observed with doses of 250, 375, and 500 μg/d did not differ (Table 3).

In addition, the reduction in %ucOC reflected the change in serum phylloquinone (**Figure 3**). Specifically, the change in
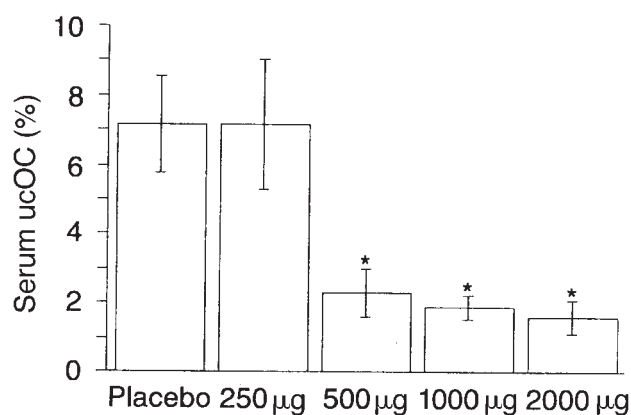

FIGURE 1. Mean percentage of serum undercarboxylated osteocalcin (ucOC) in subjects in substudy A after incremental phylloquinone doses of 250, 500, 1000, and 2000 μg/d. Both the 1000- and 2000-μg/d doses reduced ucOC more than did the 500-μg/d dose ($P < 0.05$). No significant difference was observed between the 1000- and 2000-μg/d doses. Bars indicate 95% CIs. *Significantly different from placebo, $P < 0.0001$ (one-way ANOVA with Bonferroni adjustment). $n = 10$.
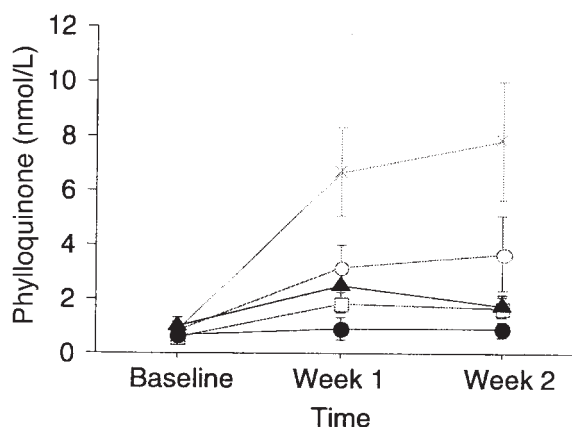

FIGURE 2. Mean serum phylloquinone concentrations in subjects in substudy B after placebo (●) and supplementation with 250 (□), 375 (▲), 500 (○), and 1000 (×) μg phylloquinone/d. Concentrations in all supplemented groups were different from those of the placebo group at week 1 (P for trend ≤ 0.0001), with subsequent stability or further increases in most groups. The greatest increase was observed with 1000 μg/d. Bars indicate 95% CIs. $n = 20$ in all groups.

TABLE 3
Substudy B: changes in serum phylloquinone concentrations from baseline to week 1 by phylloquinone dose[1]

Phylloquinone dose group	Change
Placebo	1.18 (0.85, 1.15)
250 μg	3.20 (2.24, 4.57)[2]
375 μg	2.91 (2.04, 4.16)
500 μg	3.62 (2.53, 5.17)
1000 μg	8.18 (5.73, 11.68)[3]

[1] $\bar{x}$; 95% CI in parentheses. $n = 20$ per group. The changes are multiplicative factors.
[2] Significantly different from placebo, $P < 0.001$ (multiple comparisons with Bonferroni correction).
[3] Significantly different from 500 μg, $P < 0.01$ (multiple comparisons with Bonferroni correction).

TABLE 4
Substudy B: changes in the percentage of undercarboxylated osteocalcin from baseline to week 1 by phylloquinone dose[1]

Phylloquinone dose group	Change
Placebo	0.99 (0.85, 1.15)
250 μg	0.54 (0.46, 0.63)[2]
375 μg	0.45 (0.38, 0.52)
500 μg	0.44 (0.37, 0.51)
1000 μg	0.26 (0.22, 0.30)[3]

[1] $\bar{x}$; 95% CI in parentheses. $n = 20$ per group. The changes are multiplicative factors.
[2] Significantly different from placebo, $P < 0.001$ (multiple comparisons with Bonferroni correction).
[3] Significantly different from 500 μg, $P < 0.01$ (multiple comparisons with Bonferroni correction).

%ucOC from baseline to week 1 are shown in **Table 4**, which depicts changes in %ucOC from baseline to week 1. For example, the subjects who received 250 μg/d had serum %ucOC values at week 1 that were only 0.54 times baseline values; on average, the true value was likely to be 0.46–0.63 times the baseline value. Serum %ucOC decreased with increasing doses of phylloquinone supplementation ($P < 0.0001$ for trend); the greatest reduction was observed with the dose of 1000 μg/d. The reduction in %ucOC observed with the doses of 250, 375, and 500 μg/d did not differ significantly (Table 4). Total serum osteocalcin concentrations were unaffected by phylloquinone supplementation at any dose (**Table 5**).

DISCUSSION

In the current study, circulating osteocalcin was ≈92% γ-carboxylated in the specific assay we used. The absolute number is assay dependent; however, it is comparable with prior findings in healthy young persons (6). As was anticipated, supplementatio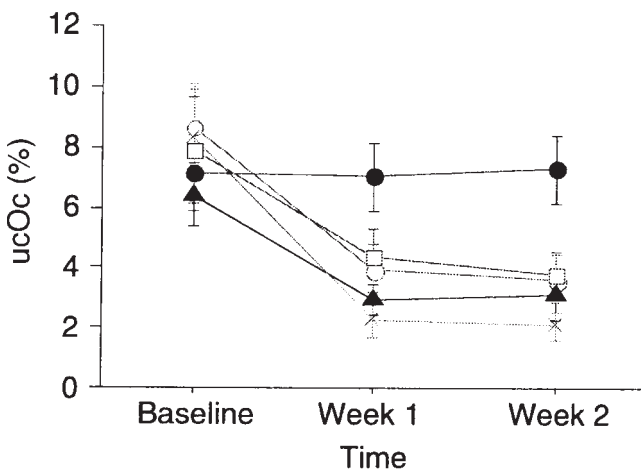n increased circulating phylloquinone concentrations and improved osteocalcin carboxylation, measured as a reduction in %ucOC. Furthermore, whereas daily supplementation with 250 μg phylloquinone increased osteocalcin γ-carboxylation to ≈96%, ≈1000 μg supplemental phylloquinone/d is required to achieve maximal osteocalcin carboxylation.

Although current data are limited, some (30, 31), but not all, (32) studies suggest that the bioavailability of dietary phylloquinone is substantially lower than supplement phylloquinone. As such, to obtain an amount of phylloquinone similar to that supplemented in the current study, ingestion of 2000–5000 μg phylloquinone/d would be required (4, 30). Because current dietary phylloquinone consumption is often in the range of 80–150 μg/d (33), it seems unlikely that the major increase required to achieve maximal osteocalcin γ-carboxylation would be attainable by diet alone. Thus, if maximal osteocalcin carboxylation is found to be beneficial in preventing osteoporosis, routine phylloquinone supplementation will probably be required.

This study suggests that daily doses of 1000 μg phylloquinone may be optimal in studies evaluating the skeletal effects of vitamin K. Previous skeletal studies used supplemental phylloquinone doses of 200 (34), 1000 (20, 35, 36), or 10 000 (37) μg/d. Thus, whereas most previous studies used optimal amounts of phylloquinone, 200 μg/d might be insufficient. In addition, many studies have shown beneficial skeletal effects of high doses (45 000–90 000 μg/d) of vitamin K_2 (menatetrenone) (38–40). These doses are much higher than those required for maximal osteocalcin carboxylation by phylloquinone. Therefore, it seems probable that the vitamin K_2 studies evaluated a pharmacologic effect rather than the physiologic replacement of a deficit. It seems prudent to heed the adage "vitamins taken in large doses

FIGURE 3. Mean percentage of serum undercarboxylated osteocalcin (ucOC) in subjects in substudy B after placebo (●) and supplementation with 250 (□), 375 (▲), 500 (○), and 1000 (×) μg phylloquinone/d. Values decreased (P for trend < 0.0001) in all supplemented groups. The greatest decrease was observed with 1000 μg/d. Bars indicate 95% CIs. $n = 20$ in all groups.

TABLE 5
Substudy B: changes in total osteocalcin concentrations from baseline to week 1 by phylloquinone dose[1]

Phylloquinone dose group	Change
Placebo	1.04 (0.97, 1.11)
250 μg	1.05 (0.98, 1.13)
375 μg	0.93 (0.87, 1.00)
500 μg	0.96 (0.89, 1.03)
1000 μg	0.99 (0.92, 1.06)

[1] $\bar{x}$; 95% CI in parentheses. $n = 20$ per group. The changes are multiplicative factors. There were no significant differences between groups.

should be considered as drugs" (41) rather than assume that vitamin K_2 mediates its effects via improved γ-carboxylation status. Furthermore, these high-dose vitamin K_2 studies are probably not directly applicable to the dietary requirements of phylloquinone.

That this study was limited to healthy young adults must be considered a limitation. Whether maximal osteocalcin γ-carboxylation is achieved with an intake of 1000 μg/d in elderly persons remains unknown. However, in a previous study, a reduction in %ucOC with phylloquinone supplementation was similar in young adults and those aged ≥ 65 y (6). Limiting study to a young adult population may have additionally confounded the assessment of a phylloquinone-induced effect on total osteocalcin concentrations. Specifically, our previous study showed that phylloquinone supplementation resulted in a reduction in total osteocalcin concentration, an observation not replicated in the current study. These divergent results may reflect the large degree of between-subject variability produced by high values in some of the young men in this study.

It should be emphasized that the physiologic importance of maximizing osteocalcin carboxylation is unknown. Whereas it is reasonable that vitamin K might have skeletal effects mediated via γ-carboxylation, it is far from clear that vitamin K insufficiency contributes to bone loss or fracture. Although studies correlate low vitamin K intakes or elevated ucOC with low bone mass and increased hip fracture risk, it is possible that these observations are not causally related; low vitamin K intake may simply be a marker of general dietary inadequacy (5). Finally, even if the undercarboxylation of osteocalcin does produce adverse skeletal effects, it is not known whether increasing carboxylation slightly (in the current study from 92% to 98%) would produce beneficial effects. Thus, routine vitamin K supplementation to optimize osteocalcin carboxylation should not be recommended at this time.

In summary, this study showed that ≈1000 μg phylloquinone is required to maximally γ-carboxylate circulating osteocalcin. Although there is abundant evidence that less than maximal osteocalcin γ-carboxylation is extremely common and that dietary phylloquinone supplementation can improve this situation, it remains unknown whether maximal osteocalcin γ-carboxylation is necessary for optimal skeletal health. Further evaluation of the effect of phylloquinone supplementation is required. Until beneficial skeletal effects are shown by such studies, routine phylloquinone supplementation is unwarranted.

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