Menaquinone-4 in breast milk is derived from dietary phylloquinone

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The effect of maternal phylloquinone supplementation on vitamin K in breast milk was studied to establish: (1) if phylloquinone is the source of menaquinone-4 in breast milk; (2) the dose–effect relationship between intake and obtainable levels. Four groups of lactating mothers with a full-term healthy infant participated and took oral phylloquinone supplements of 0·0 (n 8), 0·8 (n 8), 2·0 (n 8), and 4·0 (n 7) mg/d for 12 d, starting at day 4 post-partum. Milk samples were collected on days 4, 8, 16, and 19. Blood samples were collected on days 4 and 16. Vitamin K and vitamin E concentrations, the latter for reason of comparison, were assayed. Phylloquinone and menaquinone-4 were present in all milk samples: 5·84 (SD 2·31) and 2·98 (SD 1·51) nmol/l (n 31) respectively, in colostrum (day 4 sample). A strong correlation between the vitamers was found (r 0·78, P, 0·001). Breast-milk phylloquinone levels were raised in a dose-dependent manner: 4-, 12-, and 30-fold on day 16 for the 0·8, 2·0, and 4·0 mg group respectively. In addition, menaquinone-4 levels were higher: 2·5- (P, 0·05) and 7-fold (P, 0·001) in the 2·0 and 4·0 mg groups respectively. Plasma of supplemented subjects contained 3-, 5-, and 10-fold higher phylloquinone levels on day 16. Detectable menaquinone-4 was found in ten of thirty-one day 4 plasma samples. All day 16 plasma samples of the 4 mg supplemented group contained the vitamin. There was no correlation between the K-vitamers in plasma. Vitamin E and phylloquinone appear to differ in their distribution in breast milk, milk:plasma concentration ratios were #1 and 3–5 for vitamin E and phylloquinone respectively. The milk:plasma concentration ratio of menaquinone-4 was >10. In conclusion, dietary phylloquinone is a source of menaquinone-4 in breast milk. Phylloquinone supplementation to lactating mothers may be of benefit to the newborn infant, since both phylloquinone and menaquinone-4 are raised by supplementation.

Breast milk: Menaquinone-4: Phylloquinone: Vitamin K: Prophylaxis

Vitamin K belongs to the fat-soluble vitamins. Its biological function is to act as a cofactor in essential post-translational γ-carboxylase reactions wherein substrate-molecule-specific glutamates are converted into γ-carboxyglutamates. Typical substrate molecules are the clotting factors II, VII, IX, and X. Other examples of substrate molecules are bone-gla-protein or osteocalcin and matrix-gla-protein, which play a role in bone maturation. Two molecular forms of vitamin K are known, phylloquinone (vitamin K1), which is present in plants as part of the electron transport in photosynthesis, and the menaquinones. Menaquinones, also known as vitamin K2, are synthesized by bacteria (Conly & Stein, 1992). Instead of the phytol side-chain present in phylloquinone, menaquinones have a polyisoprenoid side-chain. The menaquinones are designated by the number of isoprenoids, i.e. menaquinone-n or MK-n. Major bacterial menaquinones synthesized by, for instance, the gut flora, are MK-7–11 (Ramotar et al. 1984; Kindberg et al. 1987).

Phylloquinone is the main dietary source of vitamin K. Reports on daily phylloquinone intake are mainly from the USA. Estimated mean daily intakes are 60–160 μg/d (Booth & Suttie, 1998). The Food and Nutrition Board of the Institute of Medicine, the National Academies (Washington, DC, USA) has recently reported the adequate intake of vitamin K for adult men and women to be 120 and 90 μg/d respectively (Trumbo et al. 2001). Newborn infants have a higher need for vitamin K. The placenta forms a barrier, and this is the reason why infants are born with a relative vitamin K deficiency (Shearer, 1992; Greer, 1995). Furthermore, vitamin K levels in breast milk are low. Today’s general strategy is to administer 1 mg
phyloquinone at birth and to repeat phyloquinone prophylaxis in the wholly breast-fed child (Cornelissen et al. 1997; Sutor et al. 1999).

The contribution of menaquinones to vitamin K status is considered to be minor (Suttie, 1995). An exception may be MK-4. Several studies have reported that MK-4 is present in animal tissues. MK-4 is not a main bacterial product. Its presence in animal species, particularly domestic-bred species, may be the result of its formation from menadione. Menadione, a pro-vitamin K food additive, is transformed in vivo into MK-4 by the addition of the geranylgeranyl side-chain (Dialameh et al. 1971). Several studies reported on the in vivo synthesis of MK-4 from supplemented phyloquinone. Billeter & Martius (1960), using radiolabelled phyloquinone, were the first to report on the exchange of the phytyl side-chain by the geranylgeranyl group in pigeons. More recently, by using HPLC techniques, the phylloquinone – MK-4 conversion was found to occur in chicks (Will et al. 1992) and rats (Thijssen & Drittij-Reijnders, 1994). In addition, mice are able to synthesise MK-4 from phyloquinone (HHW Thijssen and M-J Drittij, unpublished results). The biological significance of this conversion is not known. Studies in rats suggest that MK-4 may be the preferred substrate for non-hepatic tissues (Thijssen et al. 1996).

Human tissues were also found to accumulate MK-4 (Thijssen & Drittij-Reijnders, 1996). Whether MK-4 in man originates from food products like eggs and chicken-meat that contain limited amounts of MK-4 (Ronden, 1998), or whether human tissue can also convert dietary phyloquinone into MK-4 is not known.

We addressed the question of the phyloquinone – MK-4 conversion in human subjects by studying the concentrations of MK-4 in breast milk. Milk of various species, including that from human subjects, has been found to contain MK-4 (Hiraike et al. 1988; Indyk & Woollard, 1997). Phyloquinone and MK-4 levels in plasma and milk were followed with time after supplementing lactating mothers different daily doses of the vitamin K-containing fraction was dissolved in 0·05 ml 2-propanol. The residue of the vitamin E fraction was dissolved in 0·1 ml 2-propanol.

### Biochemical analysis

**Vitamin K and vitamin E (α- and γ-tocopherol) in plasma.** To 1 ml plasma was added 2 ml ethanol and 0·1 ml 2-propanol containing 0·500 ng 2γ, 3γ-dihydrophyloquinone and 25 μg α-tocopherol nicotinate as the internal standards for vitamin K and vitamin E respectively. The mixture was shaken thoroughly for 5 min, whereupon the vitamins were extracted in 3·5 ml n-hexane. A portion of the hexane fraction (0·3 ml) was used for vitamin E, and a further portion (3 ml) for vitamin K, analysis. The vitamin K fraction was cleaned by adsorption and desorption on silica (Thijssen et al. 1996). The final vitamin K-containing fraction was dissolved in 0·05 ml 2-propanol. The residue of the vitamin E fraction was dissolved in 0·1 ml 2-propanol.

**Vitamin K and vitamin E in milk.** Milk (1·1 ml) was incubated with 0·1 g lipase (type VII; Sigma Chemicals, St Louis, MO, USA) for 2 h at 37°C. A portion (0·1 ml) was separated for glycerol (triacylglycerol) analysis. The remaining 1 ml digested milk was mixed with 1 ml sodium carbonate (100 g/l), 2 ml ethanol and 0·1 ml mixture of internal standards (see earlier). The mixture was shaken for 15 min. Extraction and work-up was done as for plasma samples.

The extracted K vitamins were quantified by fluorescence detection following HPLC separation (Thijssen et al. 1996) and post-column reduction with a Zn-column (Haroon et al. 1986). The E vitamins were quantified by HPLC and u.v. (295 nm) detection using a Nucleosil C18 column (Chrompack, Bergen op Zoom, The Netherlands) with methanol–acetonitrile–water (110:30:7, by vol.) as mobile phase.

### Subjects and methods

Mothers, at term of pregnancy, were recruited from a centre of midwifery. Inclusion criteria were: uncomplicated pregnancy with a healthy full-term (>37 week) infant and breast-feeding. Exclusion criteria were: drug use and/or abuse; gastrointestinal dysfunction of the mother or the infant; low (<50 kg) or high (>90 kg) body weight of the mother. The study had the approval of the Medical Ethical Committee of the University of Maastricht. Informed consent was obtained from all the participants. The study was stratified to contain four groups of eight mothers to be supplemented with either 0·0, 0·8, 2·0, or 4·0 mg phyloquinone/d. Participants were randomly assigned to a group in order of entrance into the study. The study was performed between September 1998 and August 1999.

Mothers gave birth at home. Standard procedures according to the Dutch Society for Pediatrie were followed for vitamin K prophylaxis, i.e. 1 mg phyloquinone orally at birth and 25 μg/d from post-partum day 8 for the wholly breast-fed infant. Phyloquinone supplementation (Kona-kion MM, 10 mg/ml; Roche, Basel, Switzerland) was started at day 4 post-partum to continue until day 16. Participants were instructed to take the vitamin daily in the afternoon. Administration was by drops in a drink (tea or soft drink): two, five and ten drops for the 0·8, 2·0 and 4·0 mg dose respectively. No special dietary advice was given to the mothers. On day 4, between 08.00 and 11.00 hours, a venous blood sample was taken and 5–10 ml milk were collected by means of a manual pump device. Further milk samples were collected on days 8, 16, and 19 before 11.00 hours. Milk was taken from the breast that had not been used for the previous feed. The first 10 ml of the collected milk were discarded and the second 5–10 ml were taken as a sample. A second blood sample was taken on day 16 in the morning. Blood and milk sampling were done by a well-instructed medical female student who visited the mothers at home. Milk and blood samples were kept in the dark and transported as soon as possible (within 4 h) to the laboratory. Plasma and milk were stored at −70°C until analysis.
Menasquinone-4 in breast milk

Mixtures of phylloquinone, MK-4 (range 0·2–200 nmol/l), α- and γ-tocopherol (range 0·2–120 μmol/l) were prepared in 4 g bovine serum albumin/l PBS to construct calibration curves. Extraction efficiency from bovine serum albumin in PBS was >90% for all the vitamins. The CV for the intra-assay accuracy for vitamin K and vitamin E in plasma (two samples, four analyses each) were 7 and 6% respectively. In milk, the CV were 8 and 6%. With each thirty samples three calibration points were included with the assays.

Triacylglycerol concentrations in plasma and milk were assayed using a commercial kit (Sigma Chemicals).

Statistics

The results are presented as means values and standard deviations. Log-transformed data were used (except for the triacylglycerol levels in plasma) for statistical analysis. Applied statistic tests were ANOVA followed by Tukey-Kramer multiple comparison test. Fisher’s Exact test was applied to compare the presence of detectable MK-4 in plasma. The software program Instat (GraphPad Software, San Diego, CA, USA) was used for the calculations.

Results

The planned breast-milk samples were obtained from thirty-one mothers. One mother (4 mg group) withdrew from the study. Three mothers, all belonging to the 0·8 mg group, objected to giving the second blood sample (day 16). Furthermore, results suggested that at least two participants did not fully complete the supplementation schedule. The phylloquinone levels in the day 16 (postpartum day 16) samples of one subject in the 2 mg group and one in the 0·8 mg group were less than 20% of the levels found at day 8. For the former subject this was also reflected in the plasma phylloquinone levels on day 16, which were within normal range. The day 16 and 19 breast-milk results and the day 16 plasma results of these mothers were not included in the analysis. The day 16 plasma sample of one subject in the 4 mg group was lost.

Vitamin K in breast milk

The observed vitamin K concentrations in breast milk are summarized in Table 1. Both phylloquinone and MK-4 were found to be present in breast milk. The colostrum (day 4 samples, n 31) levels of phylloquinone and MK-4 were 5·84 (SD 2·31) and 3·18 (SD 1·53) nmol/l respectively. MK-4 concentrations correlated significantly with phylloquinone (Fig. 1; r 0·78, P<0·001). There were no differences in baseline values between the groups. Phylloquinone concentrations on days 8, 16 and 19 of the control group did not differ from levels on day 4. The MK-4 levels tended to decline, but the differences were not significant. Maternal phylloquinone supplementation significantly increased (P<0·001) the vitamin K levels in breast milk. On day 16 the phylloquinone concentrations were 4·87 (SD 1·43), 24·51 (SD 10·14), 60·64 (SD 31·6) and 139·64 (SD 45·83) nmol/l in the control, the 0·8, 2·0 and 4·0 mg groups respectively. MK-4 was also found to increase after the phylloquinone supplementation. In particular, the MK-4 levels in the days 8 and 16 samples of the 4 mg group was significantly higher (P<0·05 and P<0·001 respectively). The 2 mg group had significantly higher levels at day 16 (Table 1; P<0·05). Again, a highly significant correlation between the concentrations of MK-4 and phylloquinone was found (Fig. 2; r 0·90, P<0·001).

Four days after cessation of the phylloquinone supplementation, breast-milk concentrations (day 19 samples) were significantly lower, 70–80% for phylloquinone (P<0·001), 50–60% for MK-4 (in the 4 mg group, P<0·05).

Higher menaquinones, i.e. MK-6 – MK-8, were found in some of the milk samples. If present the levels were low, and no association was found with phylloquinone supplementation. The epoxide of phylloquinone, but not

![Fig. 1. Relationship between phylloquinone (K1) and menaquinone-4 (MK-4) levels in breast milk at day 4 (colostrum) (r 0·78, P<0·001). ◆, Control; □, 0·8 mg group; △, 2 mg group; ○, 4 mg group. For details of subjects and procedures, see p. 220.](https://www.cambridge.org/core/doi/10.1079/BJN2001505)

![Fig. 2. Relationship between phylloquinone (K1) and menaquinone-4 (MK-4) levels in breast milk after maternal supplementation with phylloquinone for 16 d. (r 0·90, P<0·001). ◆, Control group (n 8), r 0·92, P<0·001; □, 0·8 mg group (n 7), r 0·79, P<0·05; △, 2 mg group (n 7), r 0·89, P<0·01; ○, 4 mg group (n 7), r 0·78, P<0·05. For details of subjects and procedures, see p. 220.](https://www.cambridge.org/core/doi/10.1079/BJN2001505)
Table 1. The effect of maternal phylloquinone (K1) supplementation on menaquinone-4 (MK-4) and phylloquinone levels (nmol/l) in breast milk

(Mean values, standard deviations and 95% confidence intervals)

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All groups for Days 4, 8, 16 and 19

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Mean values were significantly different from those of the control group: * P<0.05, **P<0.01, ***P<0.001.
Mean values were significantly different from those of the 0·8 mg group: †P<0.05, ††P<0.01, †††P<0.001.
Mean values were significantly different from those of the 2·0 mg group: ‡P<0.05, ‡‡P<0.01, ‡‡‡P<0.001.
††For details of subjects and procedures, see p. 220.
of MK-4 nor of the higher menaquinones, was also found to be present in some of the samples.

To see whether the vitamin K status depends also on the fat content, triacylglycerol levels were estimated. Triacylglycerol levels increased significantly with time: 28·1 (SD 8·6), 35·1 (SD 20·1), 40·2 (SD 15·3), and 47·2 (SD 15·7) mmol/l for days 4, 8, 16 and 19 respectively (P, 0·01, days 16 and 19 v. day 4). No correlation between triacylglycerol and vitamin K concentrations was found.

**Vitamin K in plasma**

Plasma vitamin K concentrations are summarized in Table 2. Day 4 plasma phylloquinone concentrations ranged from 0·5 to 7·2 nmol/l (mean value 2·62 (SD 1·91) mmol/l, 95 % CI 1·89, 3·28, n 31). On day 16 plasma phylloquinone concentrations were dose-dependently higher in the supplemented groups, 3-, 5- and 10-fold compared with the non-supplemented group. MK-4 was detected in ten of thirty-one day 4 samples (mean value 0·25 (SD 0·16) nmol/l, 95 % CI 0·14, 0·37). In the day 16 samples MK-4 was found in fifteen of twenty-five samples including all of the day 16 samples (n 6) of the 4 mg group. The presence of detectable plasma MK-4 in the 4 mg group is different from the other groups (P, 0·051, Fisher’s Exact test). The concentrations, however, did not differ significantly from the other groups (Table 2). No correlation was observed between phylloquinone and MK-4 plasma concentrations.

**Vitamin E**

For reason of comparison, the fat soluble vitamin E (α- plus γ-tocopherol) in plasma and milk was estimated. Vitamin E levels in breast milk declined significantly (P<0·001) with lactational period: 26·5 (SD 18·5) (n 31), 13·4 (SD 12·5) (n 29), 7·8 (SD 5·7) (n 28), and 7·5 (SD 5·7) (n 28) μmol/l in the days 4, 8, 16 and 19 samples respectively. Plasma levels of vitamin E on day 16 (27·0 (SD 6·1) μmol/l, n 25) were slightly but significantly lower compared with day 4 (30·4 (SD 6·1) μmol/l, n 31, P, 0·05). There were no differences in vitamin E levels between the groups (results not shown). On day 4 plasma vitamin E correlated with plasma phylloquinone (r 0·378, P, 0·05). No correlation was seen between phylloquinone and vitamin E concentrations in milk.

Plasma triacylglycerol at day 16 was significantly lower compared with day 4 values (3·25 (SD 1·17) and 1·86 (SD 1·04) mmol/l on days 4 and 16 respectively, P<0·05).

**Milk–plasma distribution**

No correlation was found between plasma and milk levels of either vitamin K or vitamin E (as concentration or as concentration:triacylglycerol ratio). Yet, milk:plasma concentration ratios were found to cluster differently for the three vitamins, with vitamin E the lowest and MK-4 the highest ratio (Table 3).

**Discussion**

The primary objective of the present study was to find out if dietary phylloquinone could be a source of tissue MK-4 in man. MK-4 in human tissues has been found to reach, in organs such as brain and kidney, values several-fold higher than phylloquinone (Thijssen & Drittij-Reijnders, 1996).
The present study showed that all the breast-milk samples analysed contained MK-4. In colostrum the amount was 30–40% of the phylloquinone concentrations. The presence of MK-4 in human milk has been reported before. Hiraike et al. (1988) found breast milk of Japanese mothers (n = 6) to contain 5-1 nmol MK-4/l, which was one-fourth the level of phylloquinone. MK-7, a product in fermented soyabeans, was discussed as a possible source of breast-milk MK-4. Our results, however, strongly suggest that breast-milk MK-4 is derived from dietary phylloquinone: (1) phylloquinone and MK-4 concentrations in breast milk were highly correlated; (2) MK-4 levels in milk increased with phylloquinone supplementation. The results do not give a clue on the site of the phylloquinone – MK-4 conversion in the mothers. The high milk:plasma MK-4 concentration ratios may point to conversion in mammary tissue. Efficient uptake of circulating MK-4 by the mammary gland, however, can not be ruled out. We showed previously that, in rats, dietary phylloquinone and MK-4 distribute mainly to liver and heart. MK-4 in other extra-hepatic tissues, however, is mainly present by local synthesis (Thijssen et al. 1996). The pathway of the conversion is not yet clarified. The gut flora seems not to be involved (Davidson et al. 1998; Ronden et al. 1998).

The biological importance of the phylloquinone – MK-4 conversion is still unclear. MK-4 is not the better cofactor in the γ-carboxylation reaction of in vitro liver systems (Buitenhuis et al. 1990). However, the fact that many tissues synthesize and accumulate MK-4 is suggestive of a physiological function. Thus, MK-4 in breast milk may be of special benefit for the newborn infant. Interestingly, the compound has been found in milk of various species (Indyk & Woollard, 1997). We found MK-4 in rat milk increased with phylloquinone supplementation to the dam (HHW Thijssen and M-J Drittij, unpublished results).

It has been shown by others that the phylloquinone content of breast milk can be raised by oral phylloquinone supplements (Greer et al. 1997; Bolisetty et al. 1998). The levels that were achieved after 12 d supplementation were directly related to the supplementation dose. The values for the 2 and 4 mg group are in accordance with those reported by Greer et al. (1997) for 2.5 and 5.0 mg supplements. Thus, to obtain phylloquinone levels in breast milk comparable with levels in infant formulas (50–60 μg/l is equivalent to 100–130 nmol/l) daily oral supplements of 2–4 mg are required. An unknown factor is the bioavailability of phylloquinone in breast milk. It may well be that vitamin K ‘physiologically packed’ in the fat globules of breast milk has a higher bioavailability. Clearly, mammary tissue does not form a store of fat-soluble vitamins and the breast milk content of phylloquinone rapidly declined after ending the supply.

Contrary to Von Kries et al. (1987), we did not see reduced phylloquinone levels with duration of lactation. Fournier et al. (1987) also reported constant phylloquinone levels for colostrum and mature milk. Relative to the triacylglycerol content, however, phylloquinone was lower in transitional (day 12) and mature (days 16 and 19) milk. In agreement with physiological changes encountered after childbirth (Harzer et al. 1983; King, 2000) triacylglycerol in milk (plasma) raised (declined) during lactational period. As reported by others (Chappell et al. 1985; Boersma et al. 1991) vitamin E levels in transitional and mature milk were found to be 50–70% lower compared with day 4 levels. Infant milk intake at day 8 post-partum ranges from 400 to 900 g (Neville et al. 1988) implying that 2–6 mg vitamin E is eliminated. The daily vitamin E intake of young adult women in the Netherlands is approximately 11 mg/d (The Netherlands Nutrition Centre, 1998) explaining the decline of vitamin E in plasma and milk of lactating mothers. Comparably, the elimination of phylloquinone via lactation would be 0.8–2.0 μg/d. The daily phylloquinone intake of young adult women is not known exactly, but the intake by elderly women in the Dutch population has been reported to be 150–250 μg/d (Jie et al. 1995).

Table 3. Effect of maternal phylloquinone supplementation on the milk:plasma ratios of phylloquinone, menaquinone-4 and vitamin E* (Mean values, standard deviations, medians and ranges)

<table>
<thead>
<tr>
<th></th>
<th>Day 4</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean value</td>
<td>SD</td>
</tr>
<tr>
<td>Phylloquinone</td>
<td>3.97</td>
<td>3.11</td>
</tr>
<tr>
<td>Median value</td>
<td>3.27</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.45–10.73</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Menaquinone-4</td>
<td>14.83</td>
<td>8.3</td>
</tr>
<tr>
<td>Median value</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>2.90–28.1</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.97</td>
<td>0.62</td>
</tr>
<tr>
<td>Median value</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.13–2.27</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

* For details of subjects and procedures, see p. 220.
The remarkable difference between phylloquinone and vitamin E in milk: plasma concentration ratios can be explained by their difference in transport in the blood. Phylloquinone is mainly transported in the chylomicron and chylomicron-derived triacylglycerol-rich VLDL fraction (Hagström et al. 1995; Kohlmeier et al. 1996). Both particles are believed to be the main transporters for lipids to the mammary gland (Hachey et al. 1987). Vitamin E is principally transported by LDL and HDL (Meydani, 1995). In conclusion, the present study shows that MK-4 in breast milk is derived from dietary phylloquinone. MK-4 is either synthesized locally or selectively secreted. Its presence may indicate a beneficial role of MK-4 for the postnatal development. Phylloquinone supplementation to lactating mothers therefore might serve two goals: (1) to increase vitamin K levels in breast milk as prophylactic preventer of vitamin K deficiency bleeding of the newborn infant; (2) to increase MK-4 levels in breast milk.

References

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