Vitamin D content in human breast milk: a 9-mo follow-up study

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ABSTRACT
Background: Parents are advised to avoid the direct sun exposure of their newborns. Therefore, the vitamin D status of exclusively breastfed newborns is entirely dependent on the supply of vitamin D from breast milk.

Objectives: We explored concentrations of ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) (vitamin D) and 25-hydroxvitamin D₂ plus D₃ (25(OH)D) in foremilk and hindmilk during the first 9 mo of lactation and identified indexes of importance to the concentrations.

Design: We collected blood and breast-milk samples from mothers at 2 wk (n = 107), 4 mo, (n = 90), and 9 mo (n = 48) postpartum. Blood samples from infants were collected 4 and 9 mo after birth. We measured concentrations of vitamin D metabolites in blood and milk samples with the use of liquid chromatography–tandem mass spectrometry.

Results: Concentrations of vitamin D and 25(OH)D correlated significantly and were higher in hindmilk than in foremilk. Milk concentrations were also correlated with maternal plasma 25(OH)D concentrations. In foremilk and hindmilk, concentrations were a median (IQR) of 1.35% (1.04–1.84%) and 2.10% (1.63–2.65%), respectively, of maternal plasma 25(OH)D concentrations (P < 0.01). Milk concentrations showed a significant seasonal variation. Mothers who were taking vitamin D supplements had higher concentrations than did nonusers. Medians (IQRs) of infant daily intake through breast milk of vitamin D and 25(OH)D were 0.10 μg (0.02–0.40 μg) and 0.34 μg (0.24–0.47 μg), respectively, which were equal to a median (IQR) antirachitic activity of 77 IU/d (52–110 IU/d).

Conclusions: The supply of vitamin D from breast milk is limited. Exclusively breastfed infants received <20% of the daily dose recommended by the Institute of Medicine for infants during the first year of life. This trial was registered at clinicaltrials.gov as NCT02548520.


Keywords: breastfeeding, infants, vitamin D, nutrition, rickets

INTRODUCTION
Vitamin D is essential for calcium absorption and skeletal growth, and a deficiency of vitamin D may cause nutritional rickets (1, 2). Because parents are advised to avoid the sun exposure of their newborns (3), the vitamin D status of exclusively breastfed infants is fully dependent on the vitamin D content of human breast milk (HBM). In a number of studies, a low vitamin D concentration was shown in HBM, which made it difficult to believe that breastfed infants could obtain an adequate intake of 400 IU/d as recommended by the Institute of Medicine (4). However, previous studies have disagreed on a number of conditions that are assumed to be of importance to the vitamin D content of HBM. In some studies, no association was shown between the vitamin D content of HBM and the vitamin D status of lactating mothers (5–7). This lack of an association is a bit peculiar because vitamin D concentrations in HBM, similar to plasma 25(OH)D concentrations, have been shown to vary with the season of the year and to increase in response to the vitamin D supplementation of lactating women (7, 8).

In previous studies, vitamin D [ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃)] and 25-hydroxyvitamin D [25(OH)D] have been shown to be the biological active forms of vitamin D in HBM, which account for >90% of the total vitamin D activity (9). However, discrepant results have been reported on whether the predominant vitamin D metabolite in HBM is vitamin D or 25(OH)D. Although some studies have shown 25(OH)D to be the predominant form (10–12), other investigators have reported vitamin D to be the vitamin D metabolite that is present at highest concentrations in HBM (6, 8, 13–15). It has been argued that the lipophilic environment of HBM favors a higher concentration of vitamin D because 25(OH)D is relatively more hydrophilic than vitamin D is. In contrast, no association has been shown between milk-fat contents and concentrations of vitamin D or 25(OH)D (14), and only a few studies have investigated whether the content of different vitamin D metabolites differs between foremilk and hindmilk. As babies begin nursing, the first part of the milk is relatively low in fat (foremilk), which quenches the thirst of the baby. Later on during nursing, the milk becomes richer in fat (hindmilk), which provides calories for growth. To our knowledge, foremilk and hindmilk were collected systematically in pairs in

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3Abbreviations used: ARA, antirachitic activity; HBM, human breast milk; 25(OH)D, 25-hydroxyvitamin D.

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only one previous study, which showed higher concentrations of 25(OH)D in hindmilk than in foremilk (7). Unfortunately, the study did not report the vitamin D concentrations in milk samples. Thus, it remains uncertain whether the ratio of vitamin D to 25(OH)D differs between foremilk and hindmilk and whether it changes during breastfeeding.

To further elucidate the concentrations of vitamin D in HBM and its predictors, we performed a cohort study in which 107 breastfeeding mothers were followed for 9 mo after giving birth.

METHODS

The design of the study has previously been detailed (16, 17). In brief, we included 107 healthy Caucasian women aged 24–41 y with a normal uncomplicated pregnancy who gave birth to healthy children and had the intention of breastfeeding for 9 mo. Maternal blood and breast-milk samples were collected at 2 wk (15 ± 7 d; visit 1) after birth as well as at 4 mo (129 ± 12 d; visit 2) and 9 mo (280 ± 15 d; visit 3) postpartum. At the day of each visit (or the night before), foremilk (i.e., milk before feeding the child) and hindmilk (i.e., milk after feeding the child) were manually collected by the mothers following directions in handed-out instructions. The milk samples were preserved in a refrigerator at 4°C at home (for a maximum of 18 h) before they were transported to our hospital and stored at −80°C until analyzed. In addition, we collected blood samples from infants 4 and 9 mo after birth. The study was conducted according to the Helsinki Declaration II. The study was approved by the Central Region Committee on Biomedical Research Ethics, Aarhus County (M-2007-0255), and the Danish Data Protection Agency was notified about the study (2008–11-2185). This trial was registered at clinicaltrials.gov as NCT02548520.

Analytic methods

We analyzed plasma 25(OH)D concentrations with the use of isotope-dilution liquid chromatography–tandem mass spectrometry according to a method adapted from Maunsell et al. (18) and described previously in detail (19). The method quantifies both 25(OH)D3 and 25(OH)D2. Calibrators that are traceable to the National Institute of Standards and Technology Standard Reference Material 972 (Chromsystems) were used. CVs for 25(OH)D2 were 6.4% and 9.1% at concentrations of 66.5 and 21.1 nmol/L, respectively, and for 25(OH)D3, CVs were 8.8% and 9.4% at concentrations of 41.2 and 25.3 nmol/L, respectively.

Vitamin D concentrations of breast milk were determined with the use of a method that consisted of an alkaline saponification followed by a heptane extraction and liquid chromatography–tandem mass spectrometry analysis. The detection limit of vitamin D2 was 0.2 nmol/L. CVs for 25(OH)D2 were 7.8% and 4.3% at concentrations of 1.0 and 4.9 nmol/L, respectively, and for 25(OH)D3, CVs were 7.5% and 5.0% at concentrations of 2.7 and 8.8 nmol/L, respectively. As regards the nonhydroxylated compounds, CVs for vitamin D2 was 6.2% at a concentration of 1.0 nmol/L, and for vitamin D3, the CV was 8.3% at a concentration of 0.80 nmol/L.

For the remainder of this article, vitamin D2 and vitamin D3 will be referred to as vitamin D, whereas 25(OH)D will refer to the sum of 25(OH)D2 plus 25(OH)D3. Orally consumed 25(OH)D has been shown to be ~5 times more effective in raising circulating concentrations of 25(OH)D than is an equivalent amount of vitamin D3 (20). Accordingly, we calculated the total antirachitic activity (ARA) in foremilk and hindmilk samples by multiplying the content of 25(OH)D by 5 and adding this to the content of vitamin D as measured in IU. One microgram of vitamin D is equivalent to 40 IU. For milk samples with vitamin D or 25(OH)D concentrations below the detection limit, results are presented with the use of 2 analytic approaches. Data were either analyzed by excluding such samples or by assuming a concentration equal to the detection limit divided by the square root of 2 (equal to 0.14 nmol/L). Seasonal variations were studied by classifying samples collected from May to October as (extended) summertime samples and samples collected from November to April as (extended) wintertime samples (21).

Statistics

We assessed differences between study groups with the use of Fisher’s exact test for categorical variables and a 2-sample t test or Mann-Whitney U test for continuous variables as appropriate. For nonparametric tests, Wilcoxon’s signed rank test and Friedman’s test were used to compare measurements within individuals. Correlations between variables were tested with the use of a bivariate correlation analysis that calculated Spearman’s ρ (r). Multiple linear regression analyses were used to assess associations and adjust for indexes of importance to studied outcomes. Data are reported as means ± SDs or medians with IQRs (25th to 75th percentiles). P < 0.05 was considered statistically significant. We used PASW Statistics 20 software (IBM) for statistical analyses.

RESULTS

Characteristics of the 107 women included in our study are shown in Table 1. The women had a median age of 30.4 y (range: 24.2–41.2 y). All women provided milk samples at visit 1 (shortly after giving birth), whereas samples were available from 90 women at visit 2 (4 mo postpartum) and from 48 women at visit 3 (9 mo postpartum). Plasma 25(OH)D concentrations decreased during follow-up, which was attributable to a decrease in the number of women taking vitamin D supplements and the use of a lower dose during follow-up in women taking supplements. Moreover, the proportion of samples collected at wintertime tended to increase during follow-up (Table 1).

Concentrations of vitamin D metabolites in foremilk and hindmilk

Vitamin D was almost exclusively present as vitamin D3. Vitamin D2 was measured in only one sample, and 25(OH)D2 was present only at low concentrations in 5 samples (3 samples at visit 2 and 2 samples at visit 3).

Vitamin D concentrations were below the detection limit of 0.2 nmol/L in n = 112 (46%) of the total number of foremilk samples and in n = 62 (27%) of hindmilk samples (P < 0.01). In all pairs of samples with undetectable low vitamin D concentrations in hindmilk, vitamin D concentrations were also below the detection limit in foremilk. In pairs of samples with detectable concentrations in hindmilk but not in foremilk, the median concentration in hindmilk was 0.34 nmol/L. In samples with detectable concentrations in foremilk (median: 0.68 nmol/L; IQR: 0.30–1.56 nmol/L), the concentration was
TABLE 1
Characteristics of women giving birth and providing milk samples at the 3 visits

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2 wk (n = 107)</th>
<th>4 mo (n = 90)</th>
<th>9 mo (n = 48)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>30.4 (29.1–34.4)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>69.9 ± 8.9±</td>
<td>66.6 ± 8.1b</td>
<td>64.1 ± 6.7b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma 25-hydroxyvitamin D, nmol/L</td>
<td>73.2 ± 30.6a</td>
<td>64.9 ± 19.8b</td>
<td>50.7 ± 19.0f</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Use of vitamin D supplement, n (%)</td>
<td>84 (78.5)b</td>
<td>63 (70.0)a</td>
<td>26 (54.1)b</td>
<td>0.01</td>
</tr>
<tr>
<td>Dose of vitamin D from supplements in users, µg/d</td>
<td>10.0 (5.0–14.8)a</td>
<td>10.0 (4.3–17.9)a</td>
<td>5.0 (3.8–14.0)b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Time of year of sampling, n (%)</td>
<td>53 (49.5)</td>
<td>43 (47.8)</td>
<td>17 (35.4)</td>
<td>0.15</td>
</tr>
<tr>
<td>Summer</td>
<td>54 (50.5)</td>
<td>47 (52.2)</td>
<td>31 (64.6)</td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>52 (60.5)</td>
<td>37 (74.1)</td>
<td>53 (106.1)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

1For continuous variables that were not normally distributed, P values were calculated with the use of the Kruskal-Wallis test for independent samples and the Mann-Whitney test for post hoc comparisons. For continuous variables with a normal distribution, P values were calculated with the use of an ANOVA and Tukey’s post hoc test. For categorical variables, Fisher’s exact test was used to assess significance. Values that do not share a common superscript letter differ, P < 0.05.

2Median; IQR in parentheses (all such values for continuous variables that were not normally distributed).

3Mean ± SD (all such values for continuous variables with a normal distribution).

Figure 1 shows the concentrations of vitamin D and 25(OH)D in foremilk and hindmilk at the 3 time points of measurements. Because of the high proportion of samples with vitamin D concentrations below the detection limit, Figure 1A shows data with concentrations below the detection limit, while Figure 1B presents data only of samples with vitamin D concentrations above the detection limit. In contrast to vitamin D, 25(OH)D concentrations could be detected in most samples (Figure 1C). 25(OH)D concentrations in foremilk were two-thirds of the concentration in hindmilk (median ratio: 0.65; IQR: 0.51–0.80).

At all 3 time points of measurements, the concentrations of both vitamin D and 25(OH)D were significantly higher in hindmilk samples (1.46 nmol/L; IQR: 0.62–3.40 nmol/L).

Influence of maternal vitamin D status

Maternal plasma concentrations of 25(OH)D correlated significantly with 25(OH)D concentrations in milk samples (Table 3). Overall, median 25(OH)D concentrations in foremilk and hindmilk were 1.35% (IQR: 1.04–1.84%) and 2.10% (IQR: 1.63–2.65%), respectively, of maternal plasma 25(OH)D concentrations (P < 0.01).

Effect of season on milk vitamin D concentrations

Concentrations of vitamin D and 25(OH)D were significantly higher in summertime than in wintertime in both foremilk and hindmilk (Table 4). The proportion of milk samples with vitamin D concentrations below the detection limit was significantly (P < 0.01) lower in summertime (24%) than in wintertime (49%). Maternal plasma 25(OH)D concentrations also varied as a function of the season with significantly higher concentrations in summertime than in wintertime (Table 4). After adjustments for maternal plasma 25(OH)D concentrations, the concentration of 25(OH)D in foremilk and hindmilk no longer differed significantly between summertime and wintertime (data not shown). Figure 2 shows the seasonal variations in maternal plasma concentrations of 25(OH)D and concentrations in foremilk and hindmilk of vitamin D and 25(OH)D.

Effect of use of maternal vitamin D supplements on milk vitamin D concentrations

Women who reported taking vitamin D supplements had significantly higher plasma 25(OH)D concentrations than those of nonusers (Table 5). Compared with nonusers, users of vitamin D supplements had higher 25(OH)D concentrations in their foremilk and hindmilk (Table 5). The daily dose of vitamin D from supplements was significantly associated with the concentration of 25(OH)D in foremilk [β = 0.009 (95% CI: 0.002, 0.016); P < 0.01] and hindmilk [β = 0.010 (95% CI: 0.000, 0.020); P < 0.01]. However, after adjustments for maternal plasma 25(OH)D concentrations, the use of vitamin D supplements and the daily dose of vitamin D from supplements were no longer significantly associated with 25(OH)D concentrations in foremilk or hindmilk (data not shown).

Undetectable low vitamin D concentrations in milk samples were more prevalent in nonusers (47%) than in users (33%) of vitamin D supplements (P < 0.01). If samples with vitamin D concentrations below the detection limit were not accounted for, the concentration of vitamin D in foremilk and hindmilk did not differ significantly between users and nonusers of supplements. However, if it was assumed that the concentration in samples with concentrations below the detection limit was 0.14 nmol/L, concentrations of vitamin D differed significantly between users and nonusers of supplements in hindmilk (P = 0.02), and a trend was shown in foremilk (Table 5). Moreover, within the group of women with milk concentrations of vitamin D above the detection limit, the daily dose of vitamin D from supplements was significantly associated with the concentration...
of vitamin D in foremilk ($\beta = 0.032$ (95% CI: 0.009, 0.055); $P < 0.01$) and hindmilk ($\beta = 0.044$ (95% CI: 0.014, 0.074); $P < 0.01$). Maternal body weight was not associated with concentrations of vitamin D or 25(OH)D in foremilk or hindmilk (data not shown).

### Infant daily intake of vitamin D from breast milk

With the assumption of a daily intake of 750 mL of breast milk with an equal proportion of foremilk and hindmilk, infants’ median daily intake of vitamin D was 0.10 $\mu$g (IQR: 0.02–0.40 $\mu$g), whereas median intake of 25(OH)D was 0.34 $\mu$g (IQR: 0.24–0.47 $\mu$g).

The median ARA was 77 IU/d (IQR: 52–110 IU/d) and did not vary as a function of time (between visits). However, the median ARA was significantly higher in summertime than in wintertime [100 IU/d (IQR: 65–133 IU/d) compared with 62 IU/d (IQR: 43–86 IU/d), respectively; $P < 0.01$] and in users compared with in nonusers of vitamin D supplements [80 IU/d (IQR: 58–114 IU/d) compared with 60 IU/d (41–101 IU/d), respectively; $P < 0.01$].

#### Infant vitamin D status

As previously reported, the median plasma concentrations of 25(OH)D at visits 2 and 3 in samples from the infants were 94.1 ± 24.2 and 82.2 ± 18.9 nmol/L, respectively (17), and infant plasma 25(OH)D concentrations were not associated with concentrations of vitamin D, 25(OH)D or ARA in foremilk or in hindmilk (data not shown).

### DISCUSSION

Our study showed a rather-low vitamin D content in HBM from a cohort of 107 women examined 3 times during the first 9 mo after giving birth. In most samples analyzed, 25(OH)D was present at higher concentrations than vitamin D was. Even after we accounted for the relatively higher biological activity of 25(OH)D from breast milk, the median ARA was less than the effect of 2 $\mu$g vitamin D3/d (80 IU/d). Our data suggest that the vitamin D content of HBM is stable during prolonged breastfeeding (>9 mo) and that the content is highly dependent on maternal vitamin D status. Accordingly, the vitamin D concentration of HBM varies as a function of the season and use of vitamin D supplements.

Conflicting results have previously been reported on associations between plasma and milk concentrations of 25(OH)D. In several studies, no correlation was shown (5–7, 12, 22), whereas a positive correlation has been reported in some studies (8, 23, 24). In the

### TABLE 2

Associations between concentrations in foremilk and hindmilk of vitamin D and 25-hydroxyvitamin D at each of the 3 time points of measurements\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Ratio</th>
<th>r</th>
<th>$\beta$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wk postpartum</td>
<td>45</td>
<td>0.6 (0.4–0.8)(^2)</td>
<td>0.87</td>
<td>1.01 (0.82, 1.20)(^3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4 mo postpartum</td>
<td>47</td>
<td>0.5 (0.3–0.7)(^4)</td>
<td>0.79</td>
<td>0.91 (0.65, 1.17)(^5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>9 mo postpartum</td>
<td>28</td>
<td>0.7 (0.4–0.8)(^6)</td>
<td>0.91</td>
<td>1.98 (1.53, 2.42)(^7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wk postpartum</td>
<td>98</td>
<td>0.7 (0.5–0.8)(^8)</td>
<td>0.78</td>
<td>1.03 (0.81, 1.25)(^9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4 mo postpartum</td>
<td>84</td>
<td>0.6 (0.5–0.8)(^10)</td>
<td>0.59</td>
<td>0.87 (0.57, 1.16)(^11)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>9 mo postpartum</td>
<td>40</td>
<td>0.7 (0.6–0.9)(^12)</td>
<td>0.87</td>
<td>1.24 (0.97, 1.52)(^13)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(^1\)n denotes the number of paired samples, which may not equal the total number of samples because only samples with detectable concentrations of vitamin D and 25-hydroxyvitamin D in foremilk and hindmilk were included. Moreover, on some occasions, women delivered only one of the samples (i.e., either foremilk or hindmilk). The ratio is the concentration in foremilk divided by the concentration in hindmilk. P values were based on a linear regression analysis.

\(^2\)Median; IQR in parentheses (all such values).

\(^3\)Regression coefficient; 95% CI in parentheses (all such values).
study by Hollis et al. (8), the correlation coefficient was 0.42, whereas Hoogenboezem et al. (23) reported a correlation coefficient of 0.62. These values are somewhat similar to our data, because we showed a correlation coefficient in the range of 0.51–0.72, giving support that maternal plasma 25(OH)D concentrations are a major determinant of 25(OH)D concentrations in HBM. This possibility was further supported by our findings of a marked effect of the season and use of vitamin D supplements on the content of vitamin D in HBM, which is also in agreement with the findings from previous observational (7, 25) and randomized (26) studies.

To our knowledge, our study is one of the only studies thus far to investigate the concentrations of vitamin D metabolites in foremilk and hindmilk separately in a cohort of lactating women followed 9 mo after giving birth. In most previous studies, milk samples have been collected as a total sample (12), a mixed foremilk, midmilk, and hindmilk sample (15), a foremilk sample (27), a midmilk sample (28), or unspecified or random samples (6, 8, 10). Only in the study by Ala-Houhala et al. (7) were foremilk and hindmilk analyzed separately. Similar to our findings, this study showed higher concentrations of 25(OH)D in hindmilk than in foremilk. Our findings stress the importance of the use of standardized procedures for collecting HBM because the concentrations of vitamin D metabolites show large variations between foremilk and hindmilk. Differences in sampling procedures likely explain some of the discrepancies in results from previous studies. Although some of our milk samples were collected the night before the visit, and other samples were collected in the morning immediately before the visit, we do not believe that this method affected our results to any major degree because vitamin D is considered stable during storage (29). However, only limited data are available on the diurnal rhythmicity of vitamin D and 25(OH)D concentrations. Sun exposure as well as intake of vitamin D supplements may cause circadian variations in vitamin D concentrations, and we could not exclude that different sampling times may have increased the pre-analytic variability of our measurements (30, 31). Additional studies should aim to assess whether vitamin D and 25(OH)D concentrations in human milk exhibit diurnal variations.

### TABLE 4
Seasonal variations in concentrations of vitamin D and 25-hydroxyvitamin D in foremilk, hindmilk, and maternal plasma1

<table>
<thead>
<tr>
<th>Vitamin D, nmol/L</th>
<th>Winter (n = 118 samples)</th>
<th>Summer (n = 108 samples)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foremilk</td>
<td>0.1 (0.1–0.4)2</td>
<td>0.4 (0.1–1.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hindmilk</td>
<td>0.3 (0.1–0.9)</td>
<td>0.9 (0.3–3.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D, nmol/L</td>
<td>0.8 (0.5–1.1)</td>
<td>0.9 (0.7–1.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Foremilk</td>
<td>1.2 (0.8–1.6)</td>
<td>1.6 (1.1–2.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hindmilk</td>
<td>55.5 ± 22.93</td>
<td>73.6 ± 25.7</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1Samples with concentrations below the detection limit were assigned a value of 0.14 nmol/L (see Methods for explanations). Numbers of samples are minimum numbers (i.e., for some indexes measured, more samples were available for analyses). Statistical tests were performed with the use of the Mann-Whitney U test for nonparametric data or a 2-sample t test for data with a normal distribution. No adjustments were performed for repeated measures (i.e., that some individuals contributed with more than one sample).

2Median; IQR in parentheses (all such values).

3Mean ± SD (all such values).
In several previous studies, vitamin D has been shown to be present at higher concentrations in HBM than is 25(OH)D (6, 8, 13, 15, 22, 24). In the studies of Hollis et al. (8) and Cancela et al. (6), the concentration of vitamin D was almost twice the concentration of 25(OH)D. Because of its hydroxylation in the 25 position, 25(OH)D is more polar (hydrophilic) than is vitamin D. Therefore, it has been suggested that 25(OH)D may pass less readily into the relatively lipophilic environment of the mammary gland. However, this possibility is not supported by our findings because our study showed higher concentrations of 25(OH)D in hindmilk than in foremilk and an almost similar ratio between foremilk and hindmilk of vitamin D and 25(OH)D concentrations. Accordingly, our data do not support that the degree of fat content of the milk is of major importance to the presence of the different vitamin D metabolites. Our results are in agreement with the findings by Greer et al. (13), which showed no correlation between the milk-fat concentration and concentrations of vitamin D or 25(OH)D in HBM. Discrepant results on whether vitamin D or 25(OH)D is present at highest concentrations in HBM may have been due to the lack of control of variations caused by the season and use of supplements. As shown in Figure 2, vitamin D concentrations were 4 times as high in July than during the early winter months.

The presence of relatively high concentrations of 25(OH)D compared with concentrations of vitamin D in HBM is of biological importance because the ARA of 25(OH)D is markedly higher than that of vitamin D (9, 32). However, in accordance with the findings from previous studies (10, 15, 25, 33), we showed that the overall ARA of HBM was rather low with an average supply of vitamin D from breast milk, 80 IU/d. This amount is considerable less than the intake of 400 IU/d recommended by the Institute of Medicine to newborn infants (4). Women included in our study did not suffer from vitamin D insufficiency because their average 25(OH)D concentrations were within the range normally considered as adequate vitamin D status. The fact that vitamin D status declined during follow-up, whereas no effect of time was evident on the vitamin D content of HBM, was most likely due to a lack of power because of the relatively small sample size and the very low proportion of vitamin D in HBM compared with maternal plasma 25(OH)D concentrations. Unfortunately, we did not measure vitamin D concentrations in maternal plasma samples, and therefore, our study did not allow for conclusions on the ratio of vitamin D between plasma and milk samples.

### TABLE 5

<table>
<thead>
<tr>
<th>Use of vitamin D supplements</th>
<th>Nonusers (n = 63 samples)</th>
<th>Users (n = 161 samples)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D, nmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foremilk</td>
<td>0.1 (0.1–0.7)</td>
<td>0.3 (0.1–0.8)</td>
<td>0.08</td>
</tr>
<tr>
<td>Hindmilk</td>
<td>0.3 (0.1–1.3)</td>
<td>0.6 (0.2–1.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D, nmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foremilk</td>
<td>0.7 (0.5–0.9)</td>
<td>0.9 (0.7–1.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hindmilk</td>
<td>1.2 (0.7–1.8)</td>
<td>1.4 (1.0–1.9)</td>
<td>0.01</td>
</tr>
<tr>
<td>Maternal plasma</td>
<td>53.1 ± 22.1</td>
<td>69.2 ± 25.4</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1Samples with concentrations below the detection limit were assigned a value of 0.14 nmol/L (see Methods for explanations). Numbers of samples are minimum numbers (i.e., for some indexes measured, more samples were available for analyses). Statistical tests were performed with the use of the Mann-Whitney U test for nonparametric data or a 2-sample t test for data with a normal distribution. No adjustments were performed for repeated measures (i.e., that some individuals contributed with more than one sample).

2Median; IQR in parentheses (all such values).

3Mean ± SD (all such values).
Our study showed no correlation between infant plasma 25(OH)D concentrations and vitamin D or 25(OH)D concentrations in milk samples. The lack of a correlation in our study was probably attributable to the fact that most of our participants followed the recommendations from the Danish National Board of Health and provided their children with a daily supplement of 400 IU vitamin D₃, which most likely blunted the effect of variations in the milk content of vitamin D metabolites. As previously reported, >95% of the newborns were supplemented at all visits 2 and 3, which may explain the relatively high plasma concentrations of 25(OH)D in samples from infants at visits 2 and 3 (17). In previous studies, a correlation was shown between maternal vitamin D status and infant vitamin D status in exclusively breastfed infants, and high-dose maternal vitamin D supplementation has been shown to improve vitamin D status of breastfed infants (6, 24, 33, 34). Our data also support a marked effect of sun exposure because the vitamin D concentration in breast milk at summertime was 4-fold higher than at wintertime. Because the concentrations of vitamin D metabolites vary with season and use of vitamin D supplements, such approaches seem appropriate. Healthy women, with vitamin D status that is normally considered replete, do not seem to be able to provide their newborns with sufficient amounts of vitamin D through their breast milk.

In conclusion, the vitamin D content of HBM is directly related to the lactating mother’s vitamin D status with higher concentrations in hindmilk than in foremilk. However, the daily supply of vitamin D from breast milk is low. Therefore, it seems appropriate to provide exclusively breastfed infants with vitamin D supplements because, otherwise, they may be at risk of developing nutritional rickets. The authors’ responsibilities were as follows—SvS: conducted the study; SvS, UKM, LH, PV, LM, and LR: designed the study; SvS and LR: analyzed the data and drafted the manuscript; CS and LH: were responsible for the laboratory analyses; and all authors: critically reviewed the manuscript and approved the final manuscript. The Aase og Einar Danielsen fund had no influence on the design, implementation, analysis, or interpretation of data. None of the authors reported a conflict of interest related to the study.

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