The Copenhagen Cohort Study on Infant Nutrition and Growth: breast-milk intake, human milk macronutrient content, and influencing factors\textsuperscript{1-3}

Kim Fleischer Michaelsen, Pia Sauer Larsen, Birthe Lykke Thomsen, and Gösta Samuelson

ABSTRACT  In 91 healthy term infants breast-milk intake was measured at 2, 4, and 9 mo by test weighing and human milk macronutrient content by infrared analysis every 2-4 wk. In infants exclusively breast-fed, mean milk intake was 781 and 855 mL/24 h at 2 and 4 mo, respectively, and correlated positively with the current weight of the infant and negatively with the amount of formula supplement given at the maternity ward. Median daily energy intake was considerably below current recommendations (423 and 381 kJ/kg body wt at 2 and 4 mo, respectively). Protein concentration in the milk was \$\approx8\%\$ higher in primipara. Median daily protein intake was 1.3 and 1.0 g/kg body wt at 2 and 4 mo, respectively. Median fat concentration was 39.2 g/L and was positively associated with pregnancy weight gain. This supports the hypothesis that maternal fat stores laid down during pregnancy are easier to mobilize during lactation than are other fat stores and, if low, may limit milk fat when exhausted.  


KEY WORDS  Lactation, human milk, human milk intake, protein, fat, carbohydrate, energy concentration, pregnancy weight gain, formula supplement

Introduction

The pattern of infant feeding has changed markedly in affluent countries during recent decades, one of the changes being an increase in the prevalence of breast-feeding. In Scandinavia the majority of infants are now being breast-fed for >6 mo and many are breast-fed throughout the second half of infancy. The increase in the prevalence of breast-feeding is probably one of the main causes of the change in growth pattern of infants seen through the last decades (1, 2). Recommendations on when to introduce weaning foods and on the composition of weaning foods must be based on an updated knowledge of the nutritional value of both exclusive and partial breast-feeding. Thus, there is a continuous need to study nutritional aspects of breast-feeding in different populations.

The existing literature on human milk intake and composition is extensive, but the majority of studies are either cross-sectional or based on small selected groups of mothers breast-feeding for a period longer than the population average. To our knowledge there are no studies from industrialized countries in which human milk intake, macronutrient content, and macronutrient intake are followed prospectively in a randomly selected cohort, describing the changes during different stages of lactation, including both mother-infant pairs terminating breast-feeding early and those who continue breast-feeding for an extended period.

The aim of the Copenhagen Cohort Study on Infant Nutrition and Growth is to describe food intake, growth, and several blood indexes in a cohort representing healthy term infants in Copenhagen and the interrelation between these indexes. It is an observational study with no intention to influence the feeding. The aim of the present analysis is to describe the nutritional role of breast-feeding. We wanted to give a detailed description of human milk intake and protein, fat, carbohydrate, and energy contents in human milk, together with an analysis of influencing factors.

Subjects and methods

The infants recruited for this study were all born at Hvidovre Hospital where \$\approx\%50\%\$ of the deliveries from the municipality of Copenhagen take place. During 109 predetermined 24-h periods from October 1987 to February 1988, 251 infants were born who fulfilled the following inclusion criteria: their parents were of Danish origin, they were singleton births, their gestational age was between 37 and 41 wk inclusive, their birth weight for gestational age was between the 10th and 90th percentiles (3), they had no neonatal disease or malformation, and they and their mothers had been admitted to the maternity ward for \(\approx3\) d. The 251 infants were randomly assigned to either a study group (}\textsuperscript{n

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\textsuperscript{2} Supported by a grant from Otto Mønsteds Fond and from the Danish Medical Research Council (12-5957, 12-7735), the Danish Agricultural and Veterinary Research Council (13-4048), the Danish Technical Research Council (16-4338 H), the Danish Natural Research Council (11-7011), and the Research and Development Program for Food Technology (FÔTEK).

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Received May 21, 1992.
Accepted for publication August 25, 1993.

TABLE 1
Characteristics of the 91 infant-mother pairs*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mothers</th>
<th>Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (% boys)</td>
<td>46.2 [91]</td>
<td>64.0 [91]</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3475 ± 379 [91]†</td>
<td>3349 ± 378 [91]</td>
</tr>
<tr>
<td>Given formula supplements in maternity ward (%)</td>
<td>82 [76]</td>
<td>85 [76]</td>
</tr>
</tbody>
</table>

| Age (y)                           | 29 ± 5 [91]      | 65 ± 5 [91]    |
| Weight (kg)                       | 60 ± 9 [90]      | 59 ± 9 [90]    |
| Height (cm)                       | 167 ± 6 [88]     | 160 ± 6 [88]   |
| BMI†                              | 21.3 ± 3.1 [88]  | 21.0 ± 3.1 [88]|
| Parity (%)                        |                  |                |
| 1                                 | 59.3             |                |
| 2                                 | 33.0             |                |
| 3                                 | 6.6              |                |
| >3                                | 1.1 [91]         |                |
| Weight gain during pregnancy (kg) | 12.8 ± 4.5 [77]  | 12.5 ± 4.5 [77]|
| Cesarian delivery (%)             | 8 [91]           |                |
| Smoking 9 mo after delivery (%)   | 38 [84]          |                |

* Number of observations in brackets.
† x ± SD.
‡ ln kg/m³.

 = 139) or a control group (n = 112). Ninety-one of the 139 families (65%) in the study group agreed to participate and 87 infants (96%) continued the study until the age of 12 mo. Table 1 shows infant and maternal characteristics of the study participants.

The control group was included to examine whether participation in this comprehensive study with frequent contacts between families and investigators was influencing the feeding pattern, including duration of breast-feeding. A mailed questionnaire was sent to parents from the control group (n = 112) and to nonparticipants from the study group (n = 48) when the infants were 9 mo old. Seventy percent of the families responded. There were no differences in duration of breast-feeding among the three groups (study participants, study nonparticipants, and control group). Duration of exclusive breast-feeding was slightly lower (≈1 mo) in study participants, but this difference may have been due to a recollection bias because duration of breast-feeding was recorded prospectively in the study participants and retrospectively in the remaining infants (4). The duration of exclusive and partial breast-feeding among the study participants is shown in Figure 1. When study participants were compared with nonparticipants there were no significant differences for the following characteristics: maternal age and body mass index (BMI), parity, weight gain during pregnancy, length of mother's education, and percentage of mothers who were smoking (data not shown). Fewer infants were given formula supplements among nonparticipants (59% vs 82%, P < 0.05) and the distribution in social groups was different, with fewer families in the middle group among nonparticipants (P < 0.05), but with no skewed bias towards higher or lower groups. A more detailed description of the selection procedure and the comparison of characteristics between the group of infants participating and the remaining infants is published elsewhere (4). The study was approved by the Scientific-Ethical Committee of Copenhagen and Frederiksberg.

Breast-feeding status

Current breast-feeding status, distinguishing between exclusive and partial breast-feeding, was registered on monthly food registrations. Exclusive breast-feeding allowed supplements of water or camomile tea with no sugar or milk (commonly used for infants in Denmark) and vitamins. Three infants were getting one or two meals of formula or solids per week during periods when the mother was away but were still included in the exclusively breast-fed group. Infants were classified as partially breast-fed as long as they were breast-feeding at least once a day.

Formula supplements

Formula supplements were given to 82% of the studied infants at the maternity ward during the first 3 d after delivery. The mean ± SD amount of formula received during the 3-d period was 131 ± 133 mL. The amount of formula given was recorded on a special form kept by the mother.

Test weighing of milk intake

The mothers were instructed in the use of an electronic balance (resolution ± 1 g; Sartorius IP 65, Göttingen, Germany) programmed to average 40 weighings taken within 10 s. Test weighings were done at home when the infants were 2, 4, and 9 mo of age, during periods of 2, 2, and 5 d, respectively. At 2, 4, and 9 mo, 76, 62, and 18 mothers completed the test weighing, respectively. The exact ages at the time of test weighings were (x ± SD) 65 ± 4, 126 ± 5, and 283 ± 7 d, respectively. In one, two, and six cases, respectively, the test weighing was not completed satisfactorily, mainly because a meal or two were not included. The sizes of these meals were estimated from the meal pattern during the other test weighing days. At 2, 4, and 9 mo, one, one, and eight, respectively, of the mothers still breast-feeding did not complete the test weighing, in most cases because they were just about to wean their infants completely. The duration in minutes of each feed was registered. This information was not provided
from eight of the test weighings, and we therefore did not correct the individual test weighings for insensible water loss. On the basis of the available data, we have, however, calculated the mean 24-h correction for insensible water loss at 2 and 4 mo by using an estimated insensible water loss of 2 g·kg⁻¹·h⁻¹ (5), and an average body weight of 5.2 and 6.6 kg, respectively. For the 2- or 5-d test-weighing periods, the intake was expressed per 24 h and was calculated as follows: the time the first feeding started and the time of the first feeding after the end of the 2- or 5-d period were recorded and the time span in hours was calculated. This last feeding was not included in the total intake. The total intake was then divided by the time span and multiplied by 24.

The methodological error of the test weighing was evaluated as follows: six mothers weighed their infants twice before and twice after each feed throughout the whole test-weighing period. The difference between the two weighings before and the difference between the two weighings after was calculated. During 12 test-weighing periods a total of 208 differences was obtained. The SD for the test weighings at 2 and 4 mo combined (n = 96) was 6.4 g (SD₁) before the weighing and 4.2 g (SD₂) after, corresponding to an SD of 7.7 g on the measured milk volume

\[ SD = \sqrt{(SD₁^2 + SD₂^2)}. \]

At 9 mo (n = 8) the SD₁ was 44.2 g and the SD₂ was 8.7 g, corresponding to an SD of 45.0 g on the measured milk volume. The estimated SD of a 2-d test weighing performed at the age of 2 or 4 mo, assuming seven feedings per day, is then

\[ 28.6 \text{ g} \times \sqrt{(\text{number of days} \times \text{number of daily feedings}) \times \text{SD}}, \]

corresponding to a CV of 2% assuming a total daily intake of 700 mL. The estimated standard error for a 5-d test weighing at the age of 9 mo, assuming three weighings and a total intake of 300 mL daily is 174 g, corresponding to a CV of 12%. The day-to-day within-subject variability in milk intake was calculated from all test-weighing periods (2 d at 2 and 4 mo, and 5 d at 9 mo) in the study. It corresponded to a CV of 11.2% and 12.0% in exclusively breast-fed infants at 2 and 4 mo, respectively. The corresponding figures were 15.0% and 11.9% in partially breast-fed infants, and at 9 mo, when all infants were partially breast-fed, the CV was 37.2%.

**Milk samples**

Milk samples were collected 4 d after delivery, 14 d after delivery, and then every 2 wk up to 3 mo after delivery and thereafter monthly as long as the mother was breast-feeding. Samples were taken at the first feeding in the morning, after the mother was up. Before feeding the infant, she was asked to take an 8-mL sample of milk from the breast that she intended to give first (foremilk) and another 8 mL when the infant finished that breast (hindmilk). The samples were kept frozen (−20 °C) in the home until analyzed. Fore- and hindmilk samples were analyzed separately for protein, fat, and carbohydrate by infrared (IR) analysis (6). We previously evaluated IR analysis for determination of protein, fat, carbohydrate, and energy and found the method accurate (6). IR protein results had an SE of 0.1 g/L when compared with the Kjeldahl method, and IR fat results had an error of 0.3 g/L when compared with the Roese Gottlieb method (7). Furthermore, the precision was considerably higher than in the reference methods (CV: protein 0.4%, fat 1.0%, carbohydrate 0.2%, and energy 0.1%). The protein result in IR analysis is based on the number of amido groups in the sample and thus, the result represents true protein, excluding the nonprotein nitrogen. Throughout this paper protein concentration denotes true protein. In the calculation of energy concentration and intake, protein values were multiplied by 1.22 to compensate for nonprotein nitrogen (6). The carbohydrate method used measures all carbohydrates, including mono- and oligosaccharides. Gross energy concentration (in joules) was calculated from protein, fat, and carbohydrate by using the following factors: protein 23.6, fat 38.7, and carbohydrate 16.5 (8).

**Blood samples**

Blood samples were taken from the infants by venipuncture at the age of 2 mo. For ethical reasons only one attempt was made; 82% of the attempts were successful. Serum urea nitrogen was measured on a SMAC autoanalyzer (Technicon, Tarrytown, NY).

**Maternal and family characteristics**

The mothers’ heights and prepregnancy weights were self-reported. BMI (wt/ht²) was calculated by using the prepregnancy weight. Weight gain during pregnancy was calculated by subtracting the mother’s weight at the first pregnancy visit from the weight before delivery. Both weights were taken from the hospital’s records. The first pregnancy visit was at a median gestational age of 7.3 wk (10th, 90th percentile: 4.6 wk, 10.9 wk). The weight gain was calculated for mothers with a first pregnancy visit before 12 wk gestation only. Information on maternal education, smoking habits, and social class of the family was taken from a questionnaire filled out by the mother 9 mo after delivery.

**Statistical methods**

The highest possible number of mother-infant pairs was included in each of the analyses. Factors possibly associated with the daily volume of breast milk taken by the infant and protein and fat concentrations in the breast milk were examined by analysis of covariance. The dependence on time of the macronutrient concentration in the milk samples during the period 10 to 161 d after delivery was analyzed by analysis of covariance, allowing an individual regression coefficient for the time covariate. When there was a significant difference between the women, we evaluated the general trends in a random effects model, assuming random variation between women. The tests were based on the usual F test statistic. The mean of the fore- and hindmilk samples, and the differences between them, were analyzed separately. The SPSS/PC+ computer software package (SPSS Inc, Chicago) and the SAS system package (SAS Institute Inc, Cary, NC) were used for statistical analysis.

**Results**

**Milk volume**

The daily intake of human milk for each of the breast-fed infants at 2, 4, and 9 mo is shown in Figure 2. This figure also includes information on breast-feeding status 2 mo after the test weighing, thereby giving a picture of the predictive value of milk volume on later breast-feeding performance. Average values and percentiles for milk volume and milk volume/kg body wt are shown in Table 2. If the mean figures in Table 2 are corrected
for insensible water loss (27 g at 2 mo and 26 g at 4 mo), the average intake was 781 and 855 g/d at 2 and 4 mo, respectively.

Thirty-five infants were exclusively breast-fed at both 2 and 4 mo and their daily milk intake was 781 ± 161 g at 2 mo and 831 ± 138 g at 4 mo (Fig 3). The increase is close to being significant (P = 0.06, paired t test). The daily intake/kg body wt decreased significantly (P < 0.001) from 143 ± 24 to 125 ± 17 g/kg (Fig 3). In these 35 infants the volumes taken by the same infant at 2 and 4 mo were significantly correlated (r = 0.51, P = 0.002), whereas the milk intake/kg body wt was not correlated (r = 0.13, P = 0.44).

In the analysis of factors associated with the volume of human milk taken by exclusively breast-fed infants, the following factors were examined: sex of the infant, current weight of the infant,
TABLE 2
Energy and protein intakes, energy concentration of breast milk, breast milk intake, number of meals, and duration of feedings in exclusively and partially breast-fed infants

<table>
<thead>
<tr>
<th></th>
<th>Exclusively breast-fed</th>
<th>Partially breast-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Percentiles</td>
</tr>
<tr>
<td>Daily energy intake (kJ/kg body wt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mo</td>
<td>428 ± 82 [60]*</td>
<td>299 423 523</td>
</tr>
<tr>
<td>4 mo</td>
<td>381 ± 74 [36]*</td>
<td>270 385 488</td>
</tr>
<tr>
<td>9 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily protein intake (g/kg body wt)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mo</td>
<td>1.31 ± 0.27 [60]</td>
<td>0.99 1.26 1.70</td>
</tr>
<tr>
<td>4 mo</td>
<td>1.01 ± 0.17 [36]</td>
<td>0.83 1.00 1.22</td>
</tr>
<tr>
<td>9 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy concentration of breast milk (MJ/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ages combined‡</td>
<td>3.07 ± 0.44 [96]</td>
<td>2.55 3.09 3.67</td>
</tr>
<tr>
<td>Daily volume (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mo</td>
<td>754 ± 167 [60]</td>
<td>506 765 989</td>
</tr>
<tr>
<td>4 mo</td>
<td>827 ± 139 [36]</td>
<td>645 795 1057</td>
</tr>
<tr>
<td>9 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily volume (g/kg body wt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mo</td>
<td>140 ± 24 [60]</td>
<td>106 140 168</td>
</tr>
<tr>
<td>4 mo</td>
<td>124 ± 17 [36]</td>
<td>103 120 150</td>
</tr>
<tr>
<td>9 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of breast-feedings per day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mo</td>
<td>6.9 ± 1.8 [60]</td>
<td>5.0 6.5 9.0</td>
</tr>
<tr>
<td>4 mo</td>
<td>7.1 ± 1.9 [36]</td>
<td>5.0 6.5 10.5</td>
</tr>
<tr>
<td>9 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of feedings (min/d)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 mo</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* x ± SD; n in brackets.
† Values represent true protein.
‡ No significant differences between ages.
§ Significantly different from exclusively breast-fed group, P = 0.03.
Information missing on some registrations.

and the volume of formula supplement given during the first 3 d after delivery, together with variables concerning the mother (age, height, BMI, education (three levels), social class (five levels), parity (primipara or multipara), smoking status during pregnancy and 9 mo after delivery (smoking or non-smoking and number of cigarettes), and pregnancy weight gain). The current weight of the infant was strongly positively associated at both 2 and 4 mo (Table 3). Forty-two percent and 35%, respectively, of the variation in milk volume could be explained by the infant's current weight alone. Height of the mother was strongly positively associated with milk volume at the age of 2 mo, but not at 4 mo. The height of the mother is usually significantly related to the length of the infant, which was also the case in this study (data not shown). We therefore included the length of the infant in the final regression model to investigate whether this could explain the significance of the mother's height, but the length of the infant was not significant (P = 0.80) although the height of the mother was still significant (P = 0.001). Volume of formula given at the maternity ward was negatively associated with milk volume at both 2 and 4 mo. From the regression equation the magnitude of this association can be estimated. Receipt of a total of 150 mL of formula supplements during the first 3 d was associated with a 5% reduction in milk volume 2 mo after delivery and a 9% reduction 4 mo after delivery. None of the factors examined were associated with milk volume at 9 mo.

Number of feedings per day and the duration of the feedings (min/d) are shown in Table 2. In infants being exclusively breast-fed there was no significant association between number of feedings per day and breast-milk intake (2 mo, P = 0.27; 4 mo, P = 0.46) or between the total duration of the feedings per day and breast-milk intake (2 mo, P = 0.54; 4 mo, P = 0.27).

Macronutrient content
A total of 1382 milk samples, of which 713 were foremilk and 669 were hindmilk, from 88 mothers were analyzed. This is 79% of the 1750 samples that would have been collected had all the mothers collected all scheduled samples for the period they were breast-feeding. Three mothers did not collect any milk samples because they stopped breast-feeding within the first few weeks after delivery. Two mothers collected < 50% of the scheduled samples. In 51 cases only a foremilk or a hindmilk sample was collected.
The decline in protein content with time continued until 6 mo after delivery (Table 4). Four mothers had a marked increase in protein concentration to concentrations >10 g/L during the last 1–2 mo of lactation. In one case this happened before the age of 4 mo, and only data between 14 and 83 d were included in the analysis of protein concentration. In the other three cases it happened 10–12 mo after delivery. Several mothers showed a trend of increasing values during the last months of lactation.

The decrease in protein concentration (x of the fore- and hindmilk samples) with time could be adequately described as a linear relation of log (protein) vs log (time) on the individual level, with no significant curvature (P = 0.25). This is illustrated in Figure 4, which shows the individual courses for five mothers plotted in double logarithmic scale. There was a significant difference (P < 0.0001) between the individual slopes for all the mothers. The difference between the fore- and hindmilk samples varied significantly between mothers (P < 0.0001) around a level significantly different from zero (P < 0.0001), equal to a 4% higher concentration of protein in hindmilk. Urea nitrogen was significantly (P < 0.003) related to protein intake/kg body wt in exclusively breast-fed infants at the age of 2 mo (Figure 5). Protein intake could explain 18% of the variation in urea nitrogen.

**TABLE 3**
Estimated regression parameters (β) for daily breast milk intake at 2 and 4 mo in exclusively breast-fed infants

<table>
<thead>
<tr>
<th></th>
<th>2 mo (n = 51)</th>
<th>4 mo (n = 30)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>786 ± 251</td>
<td>930 ± 42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Current body weight (kg)</td>
<td>141 ± 29</td>
<td>83 ± 35</td>
<td>0.03</td>
</tr>
<tr>
<td>Sex (boys = 0, girls = 1)</td>
<td>-63 ± 30</td>
<td>-103 ± 47</td>
<td>0.04</td>
</tr>
<tr>
<td>Mothers height (cm)</td>
<td>7.5 ± 2.6</td>
<td>0.006</td>
<td>NS</td>
</tr>
<tr>
<td>Formula first 3 d (mL)</td>
<td>-0.26 ± 0.12</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>R²</td>
<td>0.57</td>
<td>0.44</td>
<td></td>
</tr>
</tbody>
</table>

*Mother’s height and current infant weight have been adjusted to 167 cm, 5200 g (at 2 mo), and 6600 g (at 4 mo), respectively.
†β ± SE.

The change with time in the mean fat concentration of the fore- and hindmilk samples, modeled as a linear relation between fat concentration and time, varied significantly between the women (P < 0.0001). However, these slopes varied around a mean that was not significantly different from zero (P = 0.64) and, on an individual level, only a few of the slopes (9 of 75, 4 negative and 5 positive) were significantly different from zero. If the time dependence was disregarded, a highly significant difference be-

**TABLE 4**
Protein, fat, and carbohydrate concentrations of milk

<table>
<thead>
<tr>
<th></th>
<th>10th</th>
<th>50th</th>
<th>90th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ages combined (n = 661)</td>
<td>23.8</td>
<td>39.2</td>
<td>58.7</td>
</tr>
<tr>
<td>Foremilk (n = 713)</td>
<td>12.9</td>
<td>24.5</td>
<td>42.4</td>
</tr>
<tr>
<td>Hindmilk (n = 669)</td>
<td>29.3</td>
<td>53.3</td>
<td>80.8</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ages combined (n = 661)</td>
<td>67.2</td>
<td>71.5</td>
<td>74.2</td>
</tr>
</tbody>
</table>

*All values are means of the fore- and hindmilk sample, except where fore- and hindmilk are specified. Only samples collected within ±4 d of the scheduled collection date are included.
†Values represent true protein.
between the individual fat concentrations was seen \((P < 0.0001)\). The change with time in the difference between the fat concentration of the fore- and the hindmilk varied significantly between the women \((P < 0.0001)\) around an overall slope of 0.08 g·L\(^{-1}·d\)^{-1}, significantly different from zero \((P < 0.002)\). Thus the difference between the fat concentration in the fore- and hindmilk increased in general with time, although not necessarily at the individual level. Disregarding the time dependence we found that the difference between the fore- and hindmilk varied significantly between the mothers \((P < 0.0001)\) around an overall mean that was significantly different from zero \((P < 0.0001)\). On average the fat concentration of the hindmilk was 29 g/L higher than that of the foremilk. Percentiles for fat concentrations for all ages combined, including values for fore- and hindmilk are shown in Table 4. Fat content at different stages of lactation is not shown because there was no significant change with time. However, the median fat content 4 d after delivery (30 g/L) was considerably lower than during later stages of lactation.

Analysis of the change with time in the carbohydrate concentration, modeled as a linear relation between carbohydrate concentration and time, gave analogous results to the fat analysis. Thus, the slopes varied significantly \((P = 0.003)\) between the mothers, around a mean that was not significantly different from zero \((P = 0.23)\), and on the individual level only a few of the slopes were significantly different from zero. If the time dependency was disregarded, a highly significant difference between the individual levels was seen \((P < 0.0001)\). The difference between the carbohydrate concentration of the fore- and the hindmilk did not depend on time \((P = 0.31)\), but the concentration varied significantly between the mothers \((P < 0.0001)\) around an overall mean that was significantly different from zero \((P < 0.0001)\). On average the carbohydrate concentration of the hindmilk was 3 g/L lower than that of the foremilk. Percentiles for carbohydrate concentrations are shown in Table 4.

As a result of the above analysis of the macronutrient concentration we decided to use the following methods to obtain the estimates of protein, fat, and carbohydrate concentrations in the milk of individual mothers 2, 4, and 9 mo after delivery, when a complete food intake was recorded: the analysis showed that the change in protein concentration with time during the period from 10 to 161 d could be estimated as an individual linear relation between log(protein) and log(time). Therefore, the estimates were based on these individual relations between log(protein) and log(time) by using all samples during the period from 10 to 161 d after delivery. For protein at 9 mo and fat and carbohydrate at 2, 4, and 9 mo, the time dependence could not be described adequately in such a simple way. To reduce the day-to-day variation without making a strict assumption about the dependence of the concentration on time, we chose to base the estimates on all available samples during a period from 40 d before until 40 d after the food records. The average of the fore- and hindmilk samples was calculated separately and the macronutrient concentration was estimated as the mean of these two averages. This was done to include samples for which the corresponding fore- or hindmilk sample was missing. Energy and protein intakes from breast-milk at 2, 4, and 9 months calculated according to these methods is shown in Table 2.

![FIG. 4. Protein concentration of foremilk (O) and hindmilk (Δ) against time after delivery for five different mothers representing different patterns: A, high, medium decrease; B, low, medium decrease; C, medium, slow decrease; D, steep decrease; E, steep decrease, late increase. The broken lines connect the means of fore- and hindmilk. The straight lines represent the fitted relation between protein concentration and time. Only values between 10 and 161 d have been included in the regression. Both axes are logarithmic. Symbols are shaded only to distinguish between different patterns within one graph.](https://academic.oup.com/ajcn/article-abstract/59/3/600/4732255/FIG.4.png)

![FIG. 5. Urea nitrogen (serum) at the age of 2 mo in exclusively breast-fed infants according to protein intake. Slope significantly different from zero \((P = 0.003)\).](https://academic.oup.com/ajcn/article-abstract/59/3/600/4732255/FIG.5.png)
For each of the dependent variables (estimated concentration of protein and fat in the milk 2, 4, and 9 mo after delivery), we investigated the following independent variables: 24-h milk volume, breast-feeding status (exclusive vs partial breast-feeding), mother's height, BMI, age, parity, and weight gain during pregnancy.

Parity was the only variable that was associated with protein concentration. At the infant's age of 2 and 4 mo 6% (n = 74, P = 0.03) and 10% (n = 51, P = 0.03), respectively, of the variation in breast-milk protein concentration could be explained by parity. At 2 mo the average protein concentration was 9.7 ± 1.1 g/L in primipara (n = 45) and 9.0 ± 1.3 g/L in multipara (n = 29). At 4 mo the corresponding figures were 8.5 ± 1.0 (n = 31) and 7.8 ± 1.1 g/L (n = 20). Protein concentrations in milk from mothers with a parity of two and three were not significantly different from each other (P > 0.05).

Weight gain during pregnancy was the only variable that was associated with fat concentration in the milk. The association was significant at 4 mo only (P = 0.002), at which time pregnancy weight gain could explain 18% of the variation in fat concentration (Fig 6). Twenty-eight of the mothers fulfilled the following criteria: weight gain during pregnancy recorded, milk samples taken at the nine scheduled times within the first 5 mo after delivery, and no more than two missed samples in the series. These mothers were grouped according to pregnancy weight gain as low (< 11.2 kg, n = 7), medium (n = 14), and high (> 16.8 kg, n = 7), and average fat values according to time are shown in Figure 7. Of the 252 possible milk samples, 3, 6, and 4 samples from the three groups, respectively, were missing.

**Discussion**

The results of this study give a detailed description of milk intake, macronutrient concentration, and intake in a cohort of Copenhagen infants. The cohort can be regarded as being reasonably representative of healthy, full-term Copenhagen infants of Danish origin with a normal birth weight. The data show a large variation in macronutrient concentration of breast milk and thereby a large variation in intake among these healthy infants. Differences in protein intake among exclusively breast-fed infants were reflected in urea nitrogen concentrations in the blood. We found that milk intake was mainly determined by the current weight of the infant, but also by the height of the mother and the sex of the infant. Furthermore, we found a positive association between pregnancy weight gain and milk fat concentration, which has not been described previously, and higher concentration of true protein in the milk of primipara compared with multipara.

**Milk volume**

Test weighing at 2 and 4 mo was successful, with almost all mothers completing the weighing period. The main component of the error in test weighing is the day-to-day within-subject variability. At 2 and 4 mo this was equivalent to a CV of ~12% in exclusively breast-fed infants, which is close to the value in a study from Cambridge (9) and slightly higher than that found in a study from Houston (10). Compared with the within-subject variability the methodological error for test weighing (CV 2%) is small. The accuracy of test weighing decreases when the infant is > 5–6 mo of age, because of increasing physical activity (5). Accordingly, we found a higher methodological error at the age of 9 mo (CV 12%), which was mainly due to a larger error in the weighing before the feeding, when the infant was hungry and therefore likely to be more restless. Furthermore, the day-to-day within-subject variability was high (CV 37%) because of low intakes at this age and several mothers were feeding more during weekends than on working days. Individual results from the test weighings at 9 mo should therefore be interpreted with caution and only results at the group level can be regarded as valid.

The amount and the range of milk intake in exclusively breast-fed infants were close to what has been found in other studies, including the World Health Organization (WHO) collaborative
study (11–16). When correcting for insensible water loss, the
mean volume was slightly above the 750 mL found as an average
in a review of studies performed since 1975 (17).

When the change in milk intake is analyzed with reference to
age in exclusively breast-fed infants, the data are often biased
toward an increase with age, because the number of infants being
studied at each age is decreasing, and mothers with a high milk
volume tend to breast-feed exclusively for a longer period. Con-
trolling for this by only examining infants exclusively breast-fed
at both 2 and 4 mo, we found a small increase (6%) in volume
from 2 to 4 mo, but only with borderline significance.

We found a large variation in the milk intake in the infants
being partially breast-fed. At 2 mo more than one-half of the
infants being partially breast-fed and, at 4 mo, one-third of infants
being partially breast-fed had intakes above the 10th percentile
of those being exclusively breast-fed. Most partially breast-fed
infants with small intakes (<200 g/d) were weaned within 2 mo
(Fig 2).

In the analysis of factors influencing milk intake, the weight
of the infant was found to be the strongest determinant, explain-
ing 42% and 35% of the variation in milk intake at 2 and 4 mo,
respectively. Surprisingly, we found that tall mothers had more
milk 2 mo after delivery than did short mothers, also when infant
length was included in the regression. The association was highly
significant and the magnitude was considerable with a change in
volume of 75 mL (or =10%) if the mother's height differed 10
cm from the average. In the classical study by Hytten (18) there
was a trend noted for tall mothers who had a higher milk yield
at 7 mo after delivery, and in a study by Wardlaw and Dart from
1934 (19), mothers with short stature had significantly smaller
milk volume than the rest. However, the relation between mothers
height and milk volume was not adjusted for infant size in
these studies. We have no explanation for this association be-
tween height and milk volume. A genetic factor influencing both
height and milk volume might be considered. A genetic factor
influencing milk volume was demonstrated in a study by Prentice
et al (17) comparing milk volume in mother-daughter pairs from
The Gambia. The association found in the present study at 2 mo
was, however, not present at 4 mo, and it should be considered
whether maternal height is a proxy for another determinant of
milk volume, which was not included in this study. Boys had an
8–10% higher milk intake than did girls when body weight was
controlled for. However, the finding was only borderline signif-
icant and as it is in contrast to other studies (17, 20) the possibility
of a spurious finding should be considered.

More than 80% of infants in this study received formula sup-
plements during their first days of life, despite the fact that they
were all healthy and born at term. Almost all of these infants
were exclusively breast-fed when discharged from the maternity
ward. The amount of formula received at the maternity ward was
negatively associated with the milk intake at both 2 and 4 mo.
Infants receiving supplements were also breast-fed for a shorter
period (4). Because this is an observational study it is not possible
to determine what is cause and what is effect. It is possible that
early formula supplementation interferes with the establishment
of lactation, resulting in a reduced milk intake throughout lacta-
tion. However, it cannot be excluded that the nursing staff was
able to identify infants who in any case would have had a lower
milk intake, and gave those infants more formula supplements.
In any case, there are no medical reasons for giving healthy full-
term infants formula supplements during the first days of life and
this practice should be abandoned as recommended by the WHO/
UNICEF "baby-friendly hospital" initiative (21).

Macronutrient content

Expression of total 24-h volume or expression of the contra-
lateral breast during each feeding are often regarded as the best
methods for determining the concentration of nutrients in human
milk, especially for determining fat and energy concentrations,
which vary considerably during emptying of the breast and also
during the day. However, these methods also include a significant
element of error at the individual level, because fat concentration
varies with the volume expressed, which is often different from
the volume the infant is usually taking (10, 16, 22). In this study,
in which we wanted frequent milk samples during the whole
period of breast-feeding and in which we wanted as many moth-
ers as possible from our random sample to complete the study,
methods using expression of larger volumes were not feasible.
Several sampling procedures using small samples have been sugg-
ested (23). We chose a simple average of fore- and hindmilk
samples, a method also used by Prentice et al (24). There is no
agreement regarding the time of the day for collecting the milk
sample that best represents the average of the 24-h milk intake
(22, 25). We chose to sample during the first feeding after the
mother was up. Most mothers in this study fed their baby early
in the morning while still in bed, and most of the samples are
thus midmorning samples, which are regarded by some as having
the best correlation to 24-h aliquots with regard to energy con-
tent (22).

To describe accurately the energy or macronutrient concen-
trations in the milk of the individual mother at a given stage of
lactation, it is desirable to reduce the day-to-day variation. By
using the average of samples taken during a limited period, we
eliminated some of the day-to-day variation.

The protein concentrations over time found in this study are
comparable with those in several other studies (26–28) when it
is remembered that our values reflect true protein. Our protein
values should be increased by 22% if they are compared with
protein values based on nitrogen determinations. Likewise, val-
ues for protein intake per kilogram body weight in exclusively
breast-fed infants are comparable with other studies (10, 11, 29).
We found a considerable variation in protein intake, with some
of the infants having a protein intake per kilogram body weight
almost twice as high as those having the lowest intake. Although
this variation is likely to be explained by the error in measure-
ment of protein concentration and milk intake, the large variation
was substantiated by a highly significant correlation between pro-
tein intake and the concentration of urea nitrogen in the blood.
Protein content continued to decrease until 6 mo after delivery,
whereafter the values were stable. The decrease during this pe-
riod could be adequately described in individual mothers as a
straight line after logarithmic transformation of both protein and
time, excluding samples of colostrum and transitional milk. Use
of this method allowed each mother to be characterized by a
protein concentration in early mature milk and a rate of decrease,
with considerable differences between the mothers.

The slightly higher protein concentration in hindmilk in the
present study agrees with the findings of several studies that have
shown small increases during the emptying of a breast (30–33).
The late increase in protein concentration that occurred in several
of the mothers in our study, usually during the last weeks of
lactation, has been described in other studies and is probably
caused by an abrupt decline in volume (28, 34, 35). Our finding of a higher protein concentration in primipara is supported by a recent study from The Gambia (C Kunz and A Prentice, personal communication, 1993), where the findings were based on true protein values as in the present study. Other studies examining protein concentration based on total nitrogen determination did not find a difference between primi- and multipara (36, 37). We have no explanation for this difference in protein concentration, which might offer the first-born infant, whose birth weight is usually somewhat lower, a small advantage in catching-up in growth. Cortisol is the limiting factor of casein gene expression (38) and cortisol concentrations are higher during the first pregnancy compared with later pregnancies (39). However, further investigations are needed to examine whether our findings of a higher protein content in the milk of primipara are caused by hormonal differences.

We found that serum urea nitrogen, a biological marker of protein intake, was significantly correlated with protein intake in exclusively breast-fed infants at the age of 2 mo (Fig 5), which can be taken as a validation of our measurements of protein intake. Because of the large difference in protein intake, formula-fed infants have much higher serum nitrogen values than breast-fed infants (11), but we find it remarkable that the relatively small differences in protein intake among exclusively breast-fed infants in this study were also reflected in the urea nitrogen concentrations.

The mean fat concentration of milk samples differs considerably between different studies and between different populations. Studies from developing countries have shown lower values (12, 20), but there is also a large variation in well-nourished populations. This is likely to be partly due to different sampling techniques, but there is still a considerable variation even in seemingly homogeneous populations examined with the same sampling technique. The median value in our study of 39.2 g/L is in the upper end of the range, although it is not extreme (11, 40, 41). As found in several other studies (27, 35, 37), we found no significant changes in fat concentration over time in the population as a whole, except for lower values during the first weeks after delivery. There were, however, a few mothers with a significant decrease during the period of lactation and a few with a significant increase.

Interestingly, we found a significant positive association between weight gain during pregnancy and fat concentration in the mother’s milk, which to our knowledge has not been described before. Four months after delivery the milk fat concentration in the high-weight-gain mothers had almost doubled compared with the low-weight-gain mothers. The difference was present as a trend from delivery and increased with time (Fig 7). In contrast, Butte et al (37) and Dewey et al (42) found no correlation between weight gain during pregnancy and fat concentration in the milk. They based their calculation of pregnancy weight gain on self-reported prepregnancy weight. Use of the weight recorded at the first pregnancy visit, as in the present study, is more reliable and excludes the weight gain during early pregnancy, which might be caused more by changes in hydration than by fat deposition (43).

The physiological role of the fat stores laid down during pregnancy is to provide an energy reserve for lactation (44). There is some evidence that these fat stores are localized (43, 45) and that after delivery some fat stores are mobilized before others (46, 47). Rebuffé-Scrive et al (48) showed that during pregnancy the lipoprotein lipase concentration is increased in the fat tissue in the femoral region whereas, during lactation, lipolysis is significantly higher in the femoral region compared with the fat tissue in the abdominal region. If fat stores laid down during pregnancy are localized and easier to mobilize during lactation than are other fat stores, we speculate that milk fat concentration will decrease when these lactational fat stores are exhausted.

There was no association in the present study between BMI of the mother and the fat concentration of her milk at 2, 4, and 9 mo. We speculate that the association found between fatness of the mother and milk fat concentration in mothers from marginally nourished populations (20) and in late lactation in well-nourished populations (36) is found only when pregnancy fat stores are depleted or low. The reason that we found no such association at 9 mo, when pregnancy fat stores are likely to be low, might be that the number of mothers still breast-feeding was small and they only produced small volumes.

The median carbohydrate value of 71.5 g/L includes mono- and oligosaccharides, which constitute ≈10% of the total carbohydrate concentration (49). When this is taken into consideration, our estimated lactose values are in accordance with other studies (10, 12). The lower amount of carbohydrate in hindmilk in this study is most likely secondary to the increase in fat concentration, which reduces the aqueous phase in the milk (28, 33).

The average energy concentration of 3.0 mL/L (720 kcal/L) in exclusive breast-feeding is somewhat higher than in other studies (10, 14, 50), reflecting the higher fat concentration found in this study. There is considerable variation between mothers in energy concentration, with the 90th percentile values being ≈50% above the 10th percentile, despite these values being based on the averages of several samples. The variation in energy intake per kilogram body weight in exclusively breast-fed infants is even higher, with the infants at the 90th percentile having an energy intake that is 75–80% above that for infants at the 10th percentile. Although part of this considerable variation is due to the short time variation in the milk intake of individual infants and to the error in measuring the energy intake, it still indicates that there are considerable differences between the energy intakes of individual exclusively breast-fed infants.

We found a gross energy intake in exclusively breast-fed infants of 428 kJ/kg body wt (102 kcal/kg) at 2 mo of age and 381 kJ/kg body wt (91 kcal/kg) at 4 mo of age. These values are almost identical to estimates for energy requirements for young children based on results from the doubly labeled water method (51). According to these estimates the energy requirements per kilogram body weight for infants 2 and 4 mo old are 432 and 385 kJ (103 and 92 kcal) kg body wt, respectively.

The energy intake in the present study is calculated from gross energy factors and thus reflects combustible energy. However, not all of this energy is metabolizable, mainly because the gastrointestinal absorption of protein, fat, and carbohydrate is not complete. If Atwater factors modified for infants fed human milk, (8, 52) are applied, the energy intake of the infants 2 and 4 mo old in this study is ≈7% lower. The current recommendations for energy intake for infants are 470 kJ (112 kcal)-kg⁻¹·d⁻¹ at 2 mo and 424 kJ (101 kcal)-kg⁻¹·d⁻¹ at 4 mo (53). Thus, our data support the need for new recommendations for energy intake in infants.

We thank the families who participated in the study and the laboratory technicians at the Pediatric Department at Hvidovre Hospital, who have all contributed considerably to the study.


