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# Choline status in newborns, infants, children, breast-feeding women, breast-fed infants and human breast milk

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**Abstract**

This study assessed the choline status in newborns, infants, children, breast-feeding women, breast milk, infant formula, breast-fed and formula-fed infants. The serum free choline level was 35.1±1.1 μmol/L at birth and decreased to 24.2±1.6, 18.1±0.8, 16.3±0.9, 14.3±0.8, 12.9±0.6 or 10.9±0.6 μmol/L at 22–28, 151–180, 331–365, 571–730, 731–1095 or 4016–4380 days after birth, respectively. The serum phospholipid-bound choline level was 1997±75 μmol/L at birth and increased gradually to 2315±190 or 2572 ±100 μmol/L at 571–730 or 4016–4380 days after birth, respectively. In breast-feeding women, serum free and phospholipid-bound choline levels were doubled at 12–28 days after birth, they decreased toward the control values with time. Free choline, phosphocholine and glycerophosphocholine were major choline compounds in breast milk. Their concentrations in mature milk were much greater than in colostrum and serum. Choline contents of breast milk varied greatly between mothers, and milk free choline levels were correlated with serum free choline ($r$=.541; $P$<.001), phospholipid-bound choline ($r$=.527; $P$<.001) and glycerophosphocholine ($r$=.299; $P$<.01) concentrations and lactating days ($r$=.520; $P$<.001). In breast-fed infants, serum free choline concentrations were correlated with free choline ($r$=.47; $P$<.001), phosphocholine ($r$=.345; $P$<.002), glycerophosphocholine ($r$=.311; $P$<.01) and total choline ($r$=.306; $P$<.01) contents of breast milk. Serum free choline concentration in formula-fed infants was lower than breast-fed infants. These data show that (a) circulating choline status is elevated during infancy and lactation, (b) choline contents of breast milk vary between mothers and milk free choline contents are influenced by maternal circulating choline status, and (c) the choline contents of breast milk can influence infants' circulating choline status.


*Keywords:* Free choline; Phospholipid-bound choline; Glycerophosphocholine; Phosphocholine; Newborns; Infants; Breast milk; Breast-feeding women

## 1. Introduction

Choline is a vital amine playing a role in structural integrity of cell membranes, methyl group metabolism, transmembrane signaling, lipid-cholesterol transport and metabolism, and normal brain development [1,2]. It is a precursor for biosynthesis of the phospholipids, phosphatidylcholine and sphingomyelin, two phospholipids that are essential components of biological membranes and precursors for intracellular messengers such as ceramide and diacylglycerol [1,2]. Choline is also the precursor of the neurotransmitter acetylcholine and of the two signaling lipids platelet-activating factor and sphingosylphosphorylcholine [1,2].

Choline, a dietary compound present in many foods [3], has been considered as an essential nutrient for humans [4]. An adequate supply of choline is thought to be particularly important during perinatal development when the organism is growing rapidly. The need for choline is also likely to be increased during pregnancy and lactation because large amounts of choline must be delivered to the fetus across the placenta [5] and secreted into breast milk [6] from maternal circulation. Studies in rats have shown that pregnancy and lactation increase sensitivity to dietary choline deficiency [7], and perinatal choline supplementation causes a number of physiological changes, including effects on choline metabolism, alterations in brain physiology and chemistry, and lifelong changes in learning and memory [8–13]. These findings suggest that the availability of choline for brain from circulation during perinatal period may be marginal and is critical for normal brain development.

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Human studies have shown that serum free and phospholipid-bound choline concentrations increase during pregnancy [14] and newborns have higher plasma free choline and lower plasma phospholipid-bound choline than their mothers [14] or other adults have [14–18]. Plasma free choline concentrations remain high for 2 weeks [16] after birth, with considerable decreases by 7 days [16,17], and apparently fall to adult level within the first year of life [18]. Plasma phospholipid-bound choline concentrations elevate considerably, but do not rise to adult levels, within the first year of life [18].

The objectives of the present study were fourfold. First, we sought to determine the changes in circulating free and phospholipid-bound choline levels during the 12-year period (0–4380 days) after birth. A great deal is known about the choline status in newborns from previous studies [14–18], as describe above, but there have been no systematic studies characterizing the changes in the choline concentration in circulation during entire period of infancy and childhood. Secondly, we sought to determine the changes in circulating choline status by measuring serum free choline, phospholipid-bound choline, phosphatidylcholine, sphingomyelin, glycerophosphocholine and phosphocholine concentrations in breast-feeding women during the 6-month period at 0–180 days after birth. To our knowledge, there is no systematic study for characterizing the changes either in serum free choline or in any of these choline compounds during breast-feeding. Thirdly, we sought to determine the changes in the content of free choline and choline compounds in breast milk during the same period. It has been shown that the milk contents of phosphatidylcholine and sphingomyelin remain stable during lactation for a period of 0–85 days after birth [19], while the free choline, glycerophosphocholine and phosphocholine contents of breast milk increase greatly between 7 and 21 days after birth [20]. Since glycerophosphocholine and phosphocholine are major choline compounds in human mature milk [6], it is important to know whether their concentrations vary at different stages of lactation during 6 months of breast-feeding. Fourthly, we sought to determine whether the concentrations of free choline and choline compounds in breast milk, maternal circulation and breast-fed infants are interrelated.

## 2. Subjects and methods

### 2.1. Subjects

All subjects gave written informed consent before participating in the study. Written informed consent for the newborns, infants and children participating in the study was obtained from their parents. The study protocol, the contents of written information sheet and the consent form were approved by the Ethical Committee of the Uludag University Medical School (Bursa, Turkey).

Eight groups of subjects with different ages participated in this study. The first group consisted of 57 full-term [gestational age (weeks)=38±2; weight (g)=3350±50; length (cm)=50±1; boy ($n$)=27; girl ($n$)=30] and 24 preterm [gestational age (weeks)=32±1; weight (g) =1850±100; length (cm)=40±2; boy ($n$)=13; girl ($n$) =11] newborns at the age of 0–2 days. The second group consisted of 380 infants [boy ($n$)=195; girl ($n$)=185] at the age of 3–730 days. The third group consisted of 181 children [boy ($n$)=84; girl ($n$)=97] at 731–4380 days. The fourth group consisted of 100 healthy adults [age (years)=20±1; weight (kg)=68±2; height (cm)=169±3; male ($n$)=50; female ($n$)=50; smoking ($n$)=33]. The fifth group consisted of 116 lactating-breast-feeding women [age (years)=30±1; weight (kg)=66±2; height (cm)=164±1; smoking ($n$)=32] at 0–180 days after giving birth. The sixth group consist of 95 breast-fed infants [boy ($n$)=39; girl ($n$)=46)] at the age of 12–180 days. The seventh group consist of 12 formula-fed infants [boy ($n$)=5; girl ($n$)=7] at the age of 30–150 days. The eighth group consist of 32 nonpregnant and nonlactating women [age (years)=28±2; weight (kg)=57±2; height (cm)=162±3; smoking ($n$)=12].

### 2.2. Collection of blood and breast milk samples

Venous blood samples (about 2 ml) were collected into a plain evacuated glass tube from infants at the age of 181–730 days, children, adults, lactating women, nonlactating and nonpregnant women. From the newborns and the infants at the age of 3–180 days, blood samples (about 0.150 ml) were obtained via heel stick. All blood samples were immediately placed on ice and centrifuged within 30 min at 2000 × $g$ for 10 min at 4 °C. Samples suspected to be hemolysed were excluded. Serum aliquots were extracted (see below) or stored at −20 °C for 24–48 h until analysis.

Breast-feeding women were asked for collection of about 1–2 ml of their breast milk by manual expression prior to the midday feeding of their infant. The milk samples were kept on ice for about 60 min until transferring to laboratory and then extracted (see below) for analysis of contents of free choline and choline-containing compounds.

Infants formulas were purchased locally and prepared according to the given instructions. Commercial powdered formulas were as follows: Aptamil-2 and Aptamil-3 (Milupa-Numil Gıda Urunleri, Istanbul, Turkey); Ulker Hero Baby-2 (Ulker-Avda de Murcia, Hero Gıda Urunleri, Istanbul, Turkey); SMA Plus, SMA Gold and SMA-2 (Wyeth Ilacları, Istanbul, Turkey); Nutricia Nutrilon Soya (Nutricia-Numil Gıda Urunleri, Istanbul, Turkey). Distilled water was used to dilute the formulas. Aliquots (50–500 μl) were extracted (see below) for analysis of contents of free choline and choline-containing compounds.

### 2.3. Extraction and chemical analysis

Free choline, water-soluble choline-containing compounds (e.g., glycerophosphocholine and phosphocholine)

and choline-containing phospholipids (e.g., phosphatidylcholine, sphingomyelin and lysophosphatidylcholine) were extracted from serum, milk and infant formula [20]. Aliquots of serum (25–100 μl) or milk and formula (50–500 μl) were diluted in 1 ml of cold water, mixed with 1.0 ml of methanol and 2.0 ml of chloroform, vortexed vigorously, allowed to stand for about 18 h in a cold room and then centrifuged at 2000×*g* for 10 min at 4 °C. Aliquots of the aqueous phase (upper phase) were dried under vacuum and used for the determination of free choline, phosphocholine and glycerophosphocholine. Aliquots of the organic phase (lower phase) were dried under vacuum and used for the determination of phospholipid-bound choline, phosphatidylcholine and sphingomyelin. The choline-containing individual phospholipid classes, phosphatidylcholine and sphingomyelin, were purified using thin-layer chromatography on silica gel G, with chloroform/ethanol/triethylamine/water (30:34:30:8; v/v) as the mobile phase [21]. The bands that cochromatographed with authentic phosphatidylcholine or sphingomyelin standard were identified and scraped off the plates and the phospholipids separated from the silica by treatment with 1 ml of methanol. After centrifugation for 10 min at 3000×*g*, the aliquots of clear supernatant were dried under vacuum and assayed for phosphatidylcholine or sphingomyelin.

Serum free choline concentrations and the free choline content of the dried aqueous phase of the extracted serum or milk samples were assayed by a modification of the enzymatic radiochemical method [22], as described previously [23].

Serum phospholipid-bound choline concentrations, the phospholipid-bound choline, phosphatidylcholine and sphingomyelin contents of the dried organic phase of extracted milk or serum samples were measured with an enzymatic colorimetric method [24] using a commercially available kit (Roche Diagnostic, Mannheim, Germany), as described previously [14].

Phosphocholine and glycerophosphocholine were first hydrolyzed enzymatically to free choline by using specific enzymes, namely, alkaline phosphatase for phosphocholine [25,26] and glycerophosphocholine phosphodiesterase for glycerophosphocholine [27], and the resulting choline was assayed by high-performance liquid chromatography–electrochemical detection system as described previously [28]. The dried aqueous phase of the extracted serum or milk samples was redissolved in 400 of Tris–HCl buffer (50 mM; pH=8.0) containing 1 mM $MgCl_2$. Three sets of aliquots (100 μl each) were transferred to glass tubes (12×75 cm). Ten microliters of distilled water, 10 μl of alkaline phosphatase solution (5 U) or 10 μl of glycerophosphocholine phosphodiesterase solution (2 U) were added and all the tubes were incubated at 37 °C. After 1 h, 400 μl of boiling distilled water was added to the tubes; they were kept in the water bath at 80 °C for 30 min to stop the enzymatic digestion of phosphocholine or glycerophosphocholine. The tubes were then centrifuged at 5000×*g* for 10 min and aliquots (10–50 μl) of clear supernatant were used for measurement of total free choline content by high-performance liquid chromatography–electrochemical detection system. The phosphocholine and glycerophosphocholine contents of the samples were calculated by taking the difference between the free choline contents of the two paired samples treated with 10 μl of water and with 5 U of the alkaline phosphatase or with 2 U of glycerophosphocholine phosphodiesterase, respectively.

### 2.4. Statistics

Data are expressed as mean±S.E.M. Statistical comparison of the means of two groups was conducted with Student's *t* test (two-tailed). Statistical comparison of the means from different age groups of children were conducted with one-way ANOVA followed by Tukey's multiple comparison procedure. The relations between serum free and bound

Table 1
Serum free and phospholipid-bound choline concentrations in newborns infants, children and adults

| Groups | Age | n | FCh (μmol/L) | PLB-Ch (μmol/L) |
|---|---|---|---|---|
| Preterm infants | 0–2 days | 24 | 33.3±2.9** | 1854±172** |
| Normal infants | 0–2 days | 57 | 35.1±1.1** | 1997±76** |
| | 3–7 days | 38 | 31.5±1.4** | 2131±57** |
| | 8–14 days | 26 | 26.7±1.2** | 2160±97** |
| | 15–21 days | 13 | 23.6±1.8** | 2170±89** |
| | 22–28 days | 12 | 24.2±1.6** | 2134±137** |
| | 31–60 days | 54 | 22.2±0.9** | 2160±105** |
| | 61–90 days | 34 | 20.9±1.1** | 2175±89** |
| | 91–120 days | 24 | 18.2±1.3** | 2143±120** |
| | 121–150 days | 23 | 17.6±1.2** | 2150±88** |
| | 151–180 days | 34 | 18.1±0.8** | 2195±75** |
| | 181–210 days | 13 | 16.3±2.0** | 2154±134** |
| | 211–240 days | 9 | 15.9±1.0** | 2255±111** |
| | 241–270 days | 11 | 16.8±1.0** | 2277±70** |
| | 271–300 days | 13 | 18.8±1.1** | 2255±98** |
| | 301–330 days | 16 | 17.3±0.7** | 2234±143* |
| | 331–365 days | 9 | 16.3±0.9** | 2334±137* |
| | 366–450 days | 21 | 16.1±0.8** | 2311±110* |
| | 451–540 days | 16 | 15.0±1.1* | 2445±107* |
| | 571–730 days | 14 | 14.3±0.8* | 2315±190* |
| Children | 731–1095 days | 38 | 12.9±0.6* | 2285±165* |
| | 1096–1460 days | 13 | 11.7±1.0 | 2302±108* |
| | 1461–1825 days | 22 | 12.0±0.8 | 2382±94* |
| | 1826–2190 days | 21 | 12.9±0.6 | 2472±100* |
| | 2191–2555 days | 14 | 11.7±0.9 | 2326±112* |
| | 2556–2920 days | 21 | 12.0±0.6 | 2397±69* |
| | 2921–3285 days | 7 | 11.5±0.9 | 2472±67* |
| | 3286–3650 days | 15 | 11.4±1.0 | 2342±77* |
| | 3651–4015 days | 18 | 11.0±0.8 | 2520±150 |
| | 4016–4380 days | 12 | 10.9±0.6 | 2572±100 |
| Adults | 20–25 years | 100 | 10.9±0.3 | 2702±68 |

Serum free choline (FCh) and phospholipid-bound choline (PLB-Ch) concentrations were measured in venous blood obtained from preterm infants, normal infants, healthy children and adults. Data are expressed as mean±S.E.M. and were analyzed by using one-way ANOVA followed by Tukey's multiple comparison test. *n*=number of observations.

* $P<.05$ when compared with the values for adults.

** $P<.001$ when compared with the values for adults.

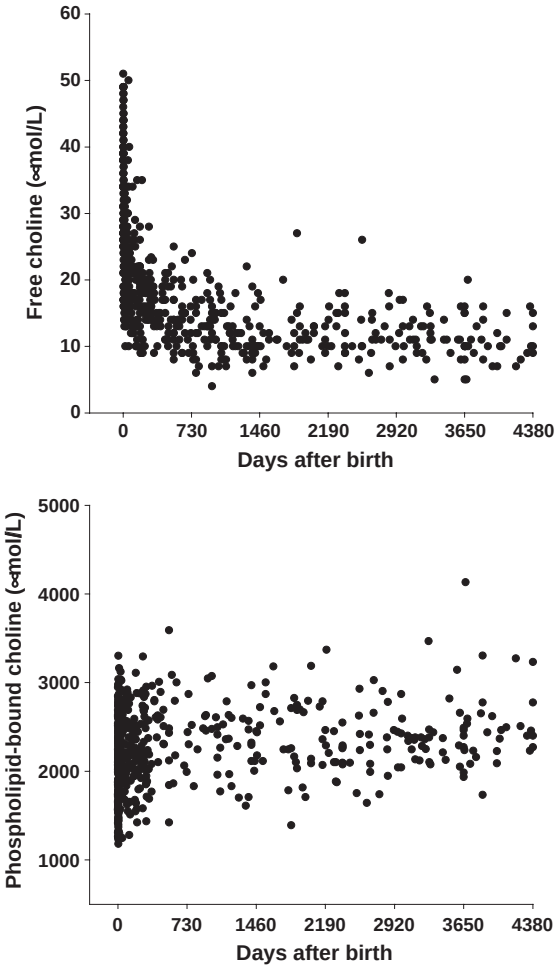


Fig. 1. The relationship between serum free choline and phospholipid-bound choline concentrations and the age of the children as days. Serum free (top) or bound (bottom) choline concentrations in venous blood samples were plotted against to the age (0–4380 days) of children.

choline concentrations and ages of infants and children, and between the concentrations of choline compounds in breast milk and serum free and bound choline concentrations in breast-feeding mothers or in breast-fed infants were determined by Pearson's correlation analysis. $P$ values less than .05 (two-tailed) were considered significant.

## 3. Results

The mean concentrations of serum free choline and phospholipid-bound choline in preterm newborns, full-term newborns, infants, children and adults are shown in Table 1. Preterm newborns had similar serum free choline and slightly less phospholipid-bound choline concentrations than normal newborns (Table 1). In normal infants, serum free choline concentrations were highest at 0–2 days after birth (greater than three times of adult values), decreased rapidly within first 28 days, and then demonstrated a slow and gradual decrease while remaining significantly higher than adult values until after 730 days of age (Table 1). Between 3 and 12 years (1096–4380 days), serum free choline levels remained more or less stable at around 10 μM (Table 1). Conversely, serum phospholipid-bound choline levels were lowest at 0–2 days after birth, remained at these low levels during 3–300 days after birth and then increased gradually toward the adult values during childhood (Table 1). Fig. 1 shows the changes in serum free and phospholipid-bound choline concentrations during the period of 0–4380 days of life (Fig. 1). Regression analysis revealed a significant inverse ($r=-.509$; $P<.001$) or positive correlation ($r=.225$; $P<.001$) between ages of children and serum free or phospholipid-bound choline concentrations, respectively.

The mean concentrations of free choline, phospholipid-bound choline, phosphatidylcholine, syphingomyelin, phosphocholine and glycerophosphocholine in breast-feeding mothers at 0–180 lactating days are shown in Table 2. Lactating women had significantly higher serum free choline [$F(4,78)=20.91$; $P<.001$] and phospholipid-bound choline [$F(4,78)=8.59$; $P<.001$] concentrations than non-pregnant and nonlactating women (Table 2). Serum free and phospholipid-bound choline levels were highest at 12–28 days after birth (lactating days) and decreased gradually with time (Table 2 and Fig. 2). The scattergrams in Fig. 2 show the temporal variation in serum concentrations of free choline and choline compounds in 95 breast-feeding women at 12–180 lactation days. Regression analysis revealed significant and inverse correlation between lactation days and serum concentrations of free choline ($r=-.625$; $P<.001$), total phospholipid-bound choline ($r=-.666$;

Table 2
Concentrations of free choline and choline-containing compounds in serum in control and breast-feeding women

| Groups | n | FCh (μmol/L) | PLB-Ch (μmol/L) | PC (μmol/L) | SM (μmol/L) | GPCh (μmol/L) | PCh (μmol/L) |
|---|---|---|---|---|---|---|---|
| Control women | 32 | 10.6±0.6 | 2554±95 | ND | ND | ND | ND |
| Breast-feeding women | | | | | | | |
| 0–2 days | 21 | 14.7±1.0* | 2920±164* | ND | ND | ND | ND |
| 12–28 days | 14 | 19.2±0.9** | 3481±143** | 1385±54 | 1364±106 | 32± 3 | 2.4±1.2 |
| 75–90 days | 12 | 18.0±0.6** | 3027±94** | 1192±63 | 1244±91 | 35±2 | 2.0±1.0 |
| 165–180 days | 11 | 16.2±0.7** | 2486±98 | 1059±77 | 1145±67 | 33± 4 | 2.2±1.0 |

Free choline (FCh), phospholipid-bound choline (PLB-Ch), phosphatidylcholine (PC), sphingomyelin (SM), glycerophosphocholine (GPCh) and phosphocholine (PCh) concentrations were measured in venous blood samples obtained from the control (nonpregnanat and nonlactating) women and breast-feeding women. Data are expressed as mean±S.E.M. and were analyzed by using one-way ANOVA followed by Tukey's multiple comparison test. $n$=number of observations. ND=No data.

* $P<.05$ compared with the values for control women.

** $P<.001$ compared with the values for control women.

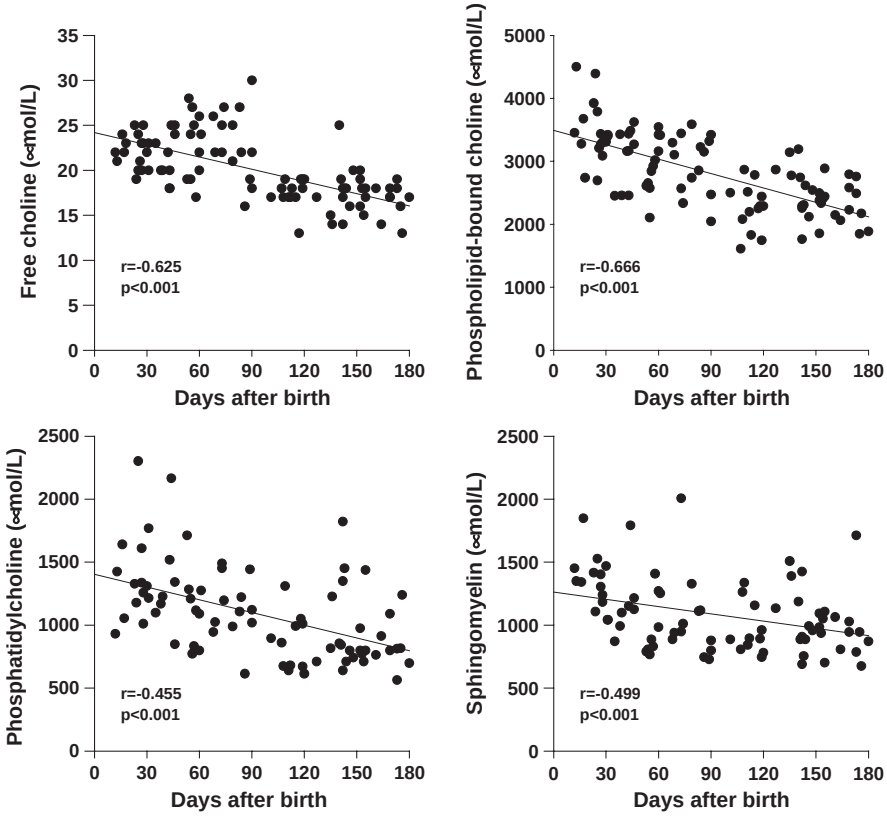


Fig. 2. The relationship between serum concentrations of choline compounds in breast-feeding mothers and the lactation days (days after birth).

$P<.001$), phosphatidylcholine ($r=-.455$; $P<.001$) and sphingomyelin ($r=-.499$; $P<.001$), but not phosphocholine ($r=.046$; $P=.661$) or glycerophosphocholine ($r=.014$; $P=.893$) in breast-feeding lactating mothers (Fig. 2).

The mean concentrations of free choline, phospholipid-bound choline, phosphatidylcholine, sphingomyelin, phosphocholine and glycerophosphocholine in colostrum (breast milk at 0–2 days), mature breast milk (at 12–180 lactating

Table 3
The concentrations of free choline and choline compounds in human breast milk

| Samples | *n* | FCh (μmol/L) | PC (μmol/L) | SM (μmol/L) | GPCh (μmol/L) | PCh (μmol/L) | Total Ch (μmol/L) |
|---|---|---|---|---|---|---|---|
| Colostrums | 21 | 132±21 | 146±18 | 129±13 | 176±13 | 93±26 | 676±35 |
| Mature breast milks | | | | | | | |
| 12–180 days | 95 | 228±10* | 104±11 | 94±9 | 499±16* | 551±33* | 1476±48* |
| 12–28 days | 14 | 299±36* | 103± 9 | 91±14 | 596±83* | 506±42* | 1595±82* |
| 75–90 days | 12 | 286±21* | 155± 21 | 97±26 | 465±40* | 438± 69* | 1441±84* |
| 165–180 days | 11 | 132±15** | 97±23 | 84±18 | 629±135* | 407±48* | 1349±105* |
| Infant formulas | | | | | | | |
| A | 4 | 201±15 | 89±25 | 21±7** | 469±44 | 401±26 | 1173±103 |
| B | 4 | 43±2** | 49±1** | 12±1** | 131±28 | 77±13** | 311±18** |
| C | 4 | 723±12** | 50±4** | 5±1** | 1429±176** | 36±14** | 2241±169** |
| D | 4 | 122±14** | 89±7 | 23±6** | 1355±140** | 773±85 | 2270±174** |
| E | 4 | 56±6** | 78±17 | 8±1** | 712±83 | 368±33 | 1328±113 |
| F | 4 | 172±8** | 128±8 | 20±3** | 914±44** | 836±11** | 2114±132 |
| G | 4 | 521±34** | 50±7** | <5 | 405±55 | <10 | 983±138 |

Colostrum (expressed at 0–2 days after birth) and mature breast milk (expressed at 12–180 days after birth) and infant formulas were assayed for the contents of free choline (FCh), phosphatidylcholine (PC), sphingomyelin (SM), glycerophosphocholine (GPCh) and phosphocholine (PCh). Commercial powdered infant formulas were: A, Aptamil-2 (Milupa-Numil Gıda Urunleri); B, Aptamil-3 (Milupa-Numil Gıda Urunleri); C, Ulker Hero Baby- 2 (Ulker-Avda de Murcia, Hero Gıda Urunleri); D, SMA plus (Wyeth Ilaclari); E, SMA gold (Wyeth Ilaclari); F, SMA-2 (Wyeth Ilaclari); G, Nutricia Nutrilon Soya (Nutricia-Numil Gıda Urunleri). Data are expressed as mean±S.E.M. and were analyzed by using one-way ANOVA followed by Tukey's multiple comparison test. *n* = number of measurements.

* $P<.001$ compared with the values from the colostrum.

** $P<.05-.001$ compared with the values from the mature breast milk at 12–180 days after birth.

days) and in the infant formulas are shown in Table 3. In mature milk, free choline [$F(3,54)=12.35$; $P<.001$], phosphocholine [$F(3,54)=23.73$; $P<.001$] and glycerophosphocholine [$F(3,54)=9.98$; $P<.001$] concentrations were significantly higher than the observed concentrations in colostrums (Table 3). Total phospholipid-bound choline, phosphatidylcholine and sphingomyelin contents of mature milk and colostrum were similar (Table 3). As seen clearly in the scattergrams in Fig. 3, there were considerable variations in total choline (566–3050 μmol/L), free choline (71–521μmol/L), phosphocholine (108–1163 μmol/L), glycerophosphocholine (138–786 μmol/L), phosphatidylcholine (11–329 μmol/L) and sphingomyelin (10–288 μmol/L) contents of human breast milk obtained from different mothers at 12–180 lactating days. Free choline contents in mature breast milk decreased with time and regression analysis demonstrated inverse relationship ($r=-.625$; $P<.001$) between free choline concentrations in breast milk and lactating days of mothers (Fig. 3). There were no significant relationships between the lactation days and total choline, phosphocholine, glycerophosphocholine, phosphatidylcholine or sphingomyelin contents of breast milk (Fig. 3). As seen in Table 3, there were considerable variations in total choline (311–2270 μmol/L), free choline (43–723 μmol/L), phosphocholine (0–836 μmol/L), glycerophosphocholine (131–1429 μmol/L), phosphatidylcholine

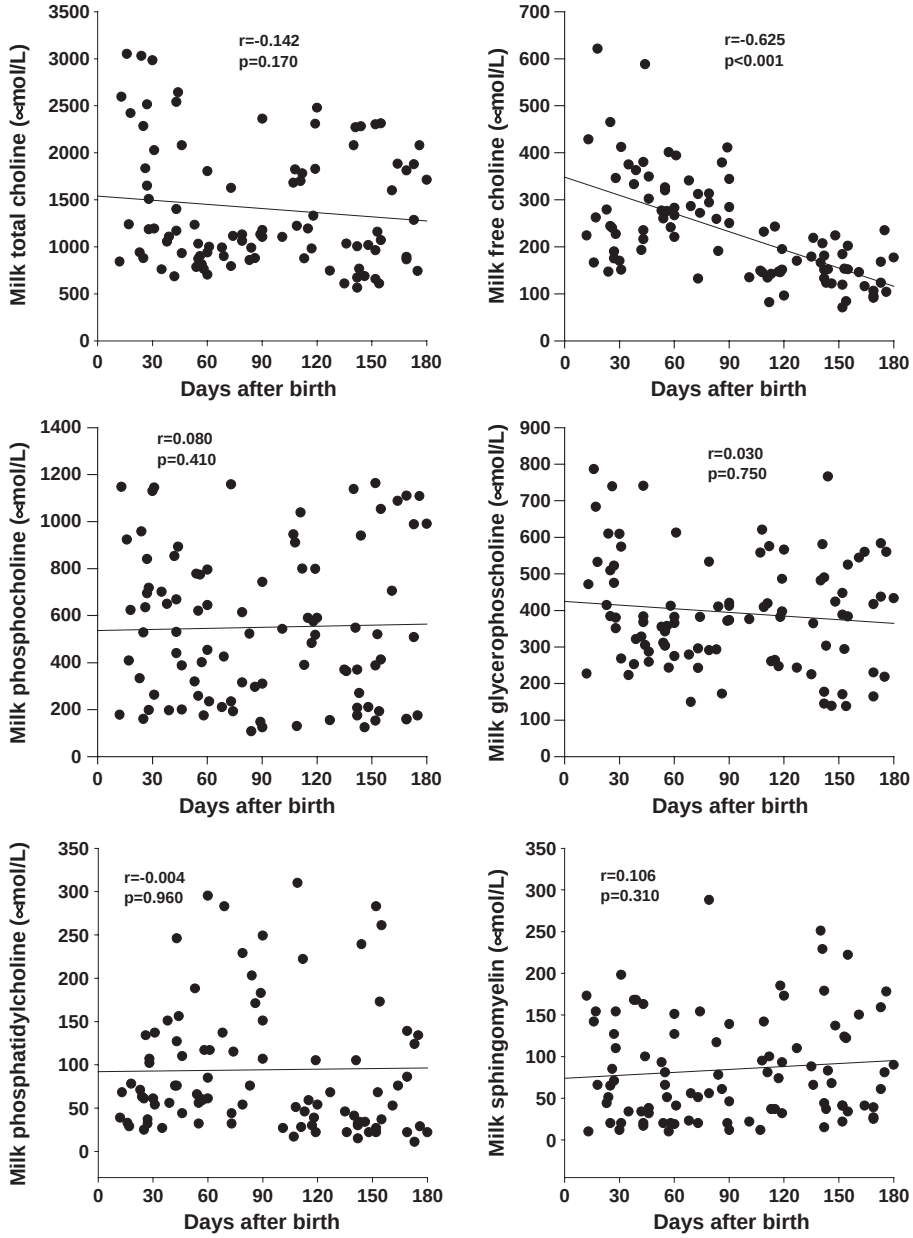


Fig. 3. The relationship between total choline, free choline, phosphocholine, glycerophosphocholine, phosphatidylcholine and sphingomyelin concentrations in breast milk and the lactation days (12–180 days after birth).

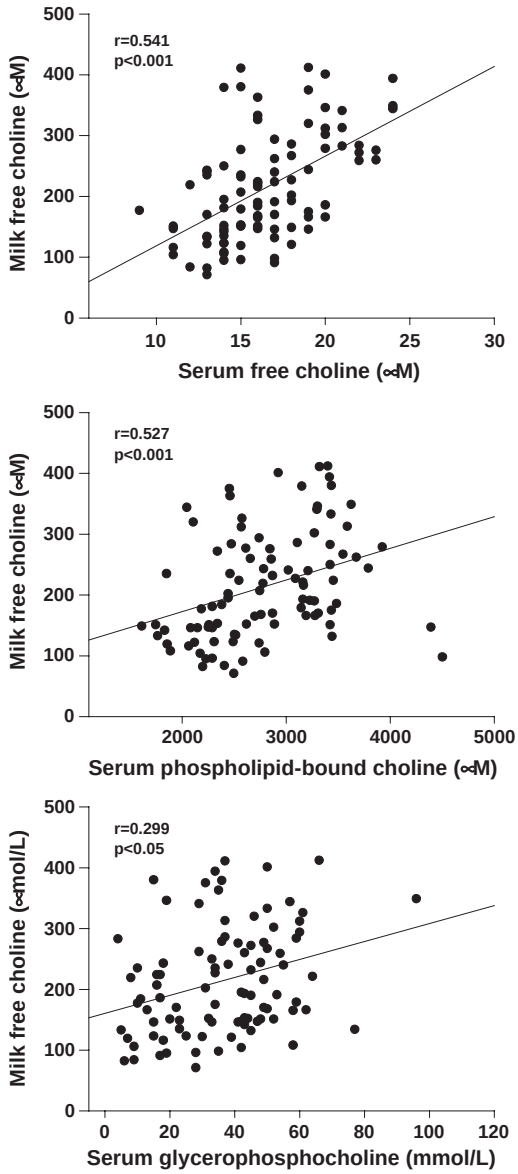


Fig. 4. The relationships in breast-feeding women of serum free choline, phospholipid-bound choline and glycerophoshocholine concentrations with milk free choline content.

(49–128 μmol/L) and sphingomyelin (0–23 μmol/L) contents of the infant formulas obtained from different commercial sources. Also, the choline compositions of the infant formulas and mature breast milk were different (Table 3).

In breast-feeding women, free choline contents in breast milk were positively correlated with serum free choline ($r$=.541; $P$<.001), phospholipid-bound choline ($r$=.527; $P$<.001) and glycerophosphocholine ($r$=.299; $P$<.01) concentrations (Fig. 4). Serum phospholipid-bound choline concentrations were positively correlated with the total choline contents ($r$=.244; $P$<.05) in breast milk. No correlation was found between serum phospholipid-bound choline, phosphatidylcholine, sphingomyelin, phosphocholine and glycerophosphocholine concentrations and breast milk phosphatidycholine, sphingomyelin, phosphocholine and glycerophosphocholine concentrations.

The mean concentrations of free choline, phospholipid-bound choline, phosphatidylcholine, sphingomyelin, phosphocholine and glycerophosphocoline in formula-fed and breast-fed infants at 0–180 days after birth are shown in Table 4. Formula-fed infants had significantly lower serum free choline [$F$(3,45)=21.10; $P$<.001] concentrations than breast-fed infants (Table 4). The serum concentrations of choline compounds were similar in formula-fed and breast-fed infants (Table 4). In breast-fed infants at 12–180 days after birth, serum free choline concentrations were positively correlated with free choline ($r$=.471; $P$<.001), phosphocholine ($r$=.345; $P$<.002), glycerophosphocholine ($r$=.311; $P$<.01) and total choline ($r$=.306; $P$<.01) contents in the consumed breast milk (Fig. 5). Also, serum glycerophosphocholine concentrations in infants were positively correlated with free choline ($r$=.297; $P$<.01) and glycerophosphocholine ($r$=.319; $P$<.01) contents of the consumed breast milk. No correlation was found between serum phospholipid-bound choline, phosphatidylcholine, sphingomyelin, phosphocholine and glycerophosphocholine concentrations and the concentrations of free choline and choline compounds in breast milk.

## 4. Discussion

These data show that choline status is different in newborns, infants, children and breast-feeding women. Serum free choline concentration is highest at birth, remains elevated for 730 days with a rapid decline within 7–28 days and then with a slow and gradual decreases during next 30–730 days. Serum phospholipid-bound choline concentration is lowest at birth, increases slowly and gradually toward the adult values during infancy and childhood. Serum free and phospholipid-bound choline concentrations are elevated in breast-feeding women at 12–180 days after birth, and they both decline significantly over the time during lactation. Total choline, free choline, glycerophosphocholine and phosphocholine contents of human mature breast milk are much higher than in colostrum. Free choline content of mature breast milk declined significantly over the time during 12–180 lactation days. Free choline contents of the mature breast milk are correlated with serum free choline and phospholipid-bound choline concentrations in lactating women. In breast-fed infants at 12–180 days after birth, serum free choline concentrations are positively correlated with free choline, phosphocholine, glycerophosphocholine and total choline contents in their mothers' breast milk.

Previous investigations have found that free choline concentrations are substantially greater in newborns than in adults [14–18]. The free choline concentrations observed in the current study in newborns at 0–2 days after birth were comparable to previously reported values [15,16,18]. It has

Table 4
Concentrations of free choline and choline-containing compounds in serum in formula-fed and breast-fed infants

| Groups | *n* | FCh (μmol/L) | PLB-Ch (μmol/L) | PC (μmol/L) | SM (μmol/L) | GPCh (μmol/L) | PCh (μmol/L) |
|---|---|---|---|---|---|---|---|
| Formula-fed infants | | | | | | | |
| 30–150 days | 12 | 10.8±0.7 | 2100±63 | 1003±85 | 1032±116 | 34±5 | 2.3±1.0 |
| Breast-fed infants | | | | | | | |
| 12–28 days | 14 | 27.1±1.8** | 1998±93 | 1083±105 | 1032±106 | 40±4 | 2.4±0.6 |
| 75–90 Days | 12 | 21.8±2.2** | 2059±126 | 1071±83 | 1042±74 | 36±3 | 2.5±1.2 |
| 165–180 days | 11 | 17.3± 0.3*,*** | 2245±222 | 1121±108 | 1124±116 | 29±5*** | 1.9±0.9 |

Free choline (FCh), phospholipid-bound choline (PLB-Ch), phosphatidylcholine (PC), sphingomyelin (SM), glycerophosphocholine (GPCh) and phosphocholine (PCh) concentrations were measured in blood samples obtained from the formula-fed and breast-fed infants after 12–180 days after birth. Data are expressed as mean±S.E.M. and were analyzed by using one-way ANOVA followed by Tukey's multiple comparison test. *n* = number of observations.

* $P<.05$ compared with the values for formula-fed infants.

** $P<.001$ compared with the values for formula-fed infants.

*** $P<.05$ compared with the value for the 12–28 days of breast-fed infants.

been shown free choline concentrations in infants decrease significantly by 7 days of life [16,17], or by 20 and 60 weeks of life [18]. Our present data are also consistent with these previous reports and show that the decrease in serum free choline concentration continues to a lesser extent over the first 2 years of life (Table 1). This decrease may be related to significant choline requirements for phospholipid synthesis in growing brain, kidney, liver, lung and skeletal muscle. Animal studies have shown that choline may be particularly important for brain development [8–13]. Perinatal choline supplementation and deficiency in rats produces long-lasting changes in brain neurochemistry and cholinergic functions [8–13]. It is well established that choline's diffusion across the blood–brain barrier is facilitated by a bidirectional transport system [29] the net flux of which inward into brain when plasma levels are higher than 14 μM and outward, back into circulation, when circulating levels are lower [30]. In the present study we observed that serum free choline concentrations in infants remained elevated over 14 μM during 0–730 days of life, suggesting that the net uptake of free choline into the brain is also elevated during this period. Maintaining elevated serum free choline during infancy may be the one of the mechanism(s) evolved to ensure enhanced availability of choline to the developing brain.

Serum phospholipid-bound choline concentration has not been previously investigated extensively in newborns or infants. We reported recently that serum phospholipid-bound choline concentration is significantly lower in newborns than their mothers [14]. The present results confirm and extend these previous findings by demonstrating that serum phospholipid concentrations are significantly lower than the adult values at birth and remain at low level during infancy and increase gradually to the adult levels during childhood. The phospholipid-bound choline concentrations observed in the current study in newborns were comparable with the value previously reported from our laboratory [14] but considerably higher than the values reported by Buchman et al. [18]. This difference may result from the difference in method used for the analysis of phospholipid-bound choline in two studies. Total phospholipid-bound choline is measured by quantifying the amount of free choline released from phospholipids — phosphatidylcholine, lysophosphatidylcholine and sphingomyelin — by acidic treatment for 2 h in the study of Buchman et al. [18] or by enzymatically, by phospholipase D, in our study. It is well established that the release of free choline from sphingomyelin is resistant to the acidic treatment and the treatment of sphingomyelin with acid for 2 h unlikely yields a significant amount of free choline [26]. In the present study we found that about half of serum phospholipid-bound choline is present as sphingomyelin (Table 4). It is likely, therefore, that the greater serum phospholipid-bound choline concentrations in newborns and infants in the present study may have reflected complete release of free choline from all of the choline-containing phospholipids present in serum in newborns and infants. Since phospholipids are constituents of plasma lipids and plasma lipid levels in Turkish adults, but not in Turkish newborns and children [31], are significantly different from Europeans and North Americans [32], there is also a possibility that the normal range of serum phospholipid-bound choline is higher in Turkish newborns and infants.

Our present data are the first to demonstrate that serum free and phospholipid-bound choline concentrations are elevated in breast-feeding women. The mechanisms involved in these elevations in serum free and phospholipid-bound choline concentrations are not known, although they could result from increased dietary intake of choline compounds [33–35]. However, the lactating women enrolled in this study did not receive any extra choline (either as free choline or as lecithin) in their diet and/or drug regimes. Thus, an increase in the dietary choline consumption is unlikely to explain the elevation in serum free and phospholipid-bound choline concentrations that occurs during lactation. Since a large amount of choline is accumulated in the breast milk and lost by breast-feeding (see below), it is also unlikely that the elevations in serum free and bound choline concentrations would have resulted from a decreased removal of these choline compounds from circulation in the lactation period. Alternatively, increased hepatic synthesis and release may account for the increased free choline and phospholipid-bound choline observed in

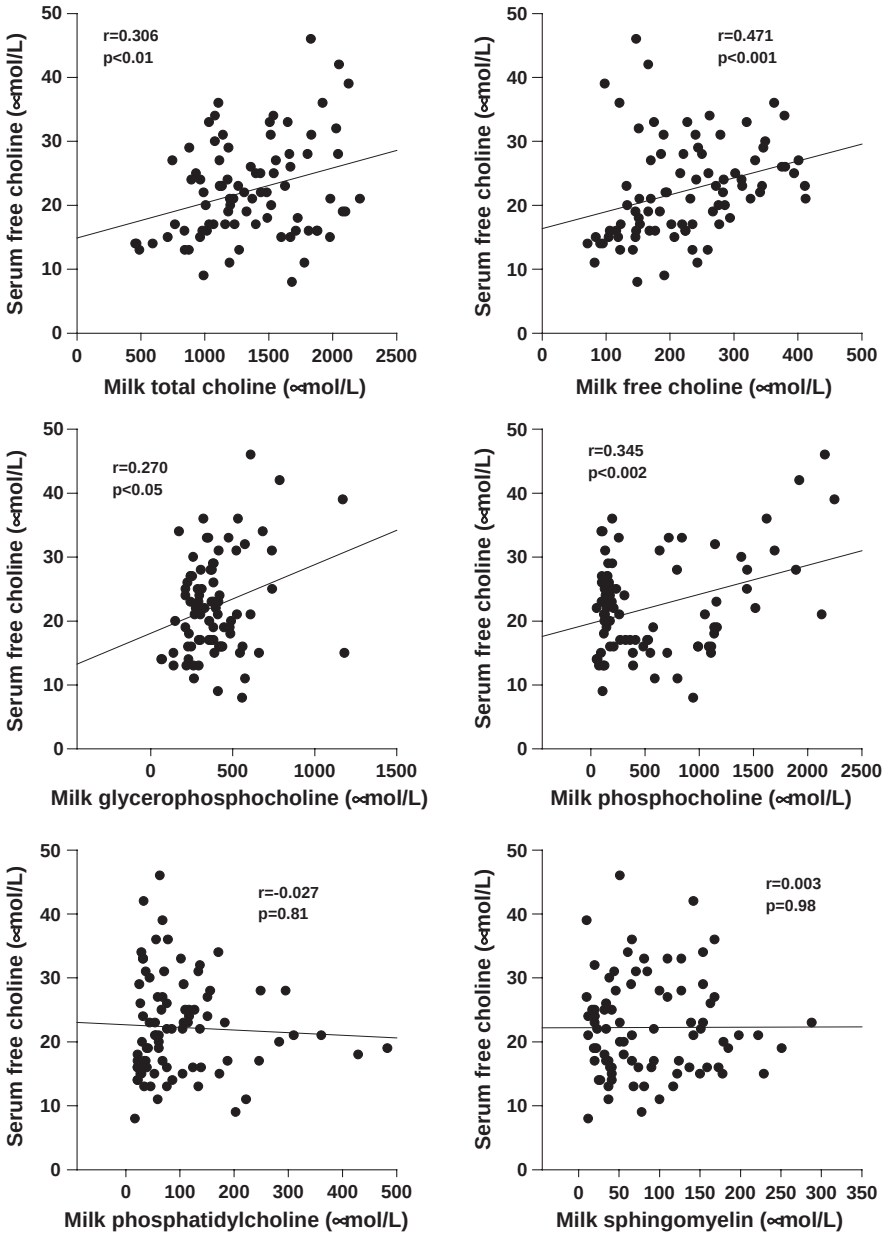


Fig. 5. The relationships between serum free choline concentrations of infants and the total choline, free choline, glycerophosphocholine, phosphocholine, phostatidylcholine and sphingomyelin contents of consumed breast milk.

lactating women. It is likely that the elevated serum choline status in breast-feeding women may be an adaptation mechanism to maintain high choline contents in breast milk.

Human milk contains choline in the form of free choline, phosphocholine, glycerophosphocholine, sphingomyelin and phosphatidylcholine [6]. Our results for the total choline contents of mature milk (1250–1481 μmol/L) agree well with those of Holmes-McNary et al. [6] (1.35 mmol/L) and Holmes et al. [36] (1.28 mmol/L), as do the individual choline components. Our data for the total choline content of the colostrum (706 μmol/L) are also in agreement with those (0.60 mmol/L) of Holmes et al. [36]. Holmes et al. [36] found that the total choline content and levels of free choline, phosphocholine and glycerophosphocholine in human breast milk rise considerably between 7 and 22 days after birth [36]. In accordance with this finding, we found that the levels of free choline, phosphocholine and glycerophosphocholine in breast milk, expressed between 12 and 28 days after birth, were two, three and five times higher than the observed values for the colostrums (0–2 days after birth), respectively. After this initial rise, free choline contents in breast milk decreased significantly over time, while phosphocholine and glycerophosphocholine levels remained constant during 180 days of lactation after birth. PC and SM levels in breast milk remained constant, as shown previously [19], during lactation 0–180 days after birth (Table 3, Fig. 3). Taken together, these data show clearly that the concentrations of free choline and choline

compounds in breast milk are regulated differently during lactation. Phosphocholine in the milk may either be formed by phospholipase C [37] or by choline kinase [38]. Glycerophosphocholine is believed to be generated when phosphatidylcholine is hydrolyzed by phospholipase A [39]. Since metabolic activity of the mammary gland increased considerably between the second and fourth postpartum days, the observed increases in milk phosphocholine and glycerophosphocholine levels suggest that activities of these enzymes also increased during this period of the lactation. Free choline in milk can be derived from maternal circulation via an active transport mechanism [40,41] as well as by de novo synthesis within the mammary gland [42]. The observed significant positive correlations between breast milk free choline levels and serum free choline, phospholipid-bound choline and glycerophosphocholine concentrations indicate that circulating choline status can influence the milk content of free choline.

Previous studies have clearly shown that the choline contents and compositions of infant formulas and mature human breast milk are different [19,36], and oral bioavailability of choline compounds varies considerably [43]. Our present data are in good accordance with these previous findings and show that the serum free choline concentration in formula-fed infants is significantly lower than the serum free choline concentrations observed in breast-fed infants (Table 4). As also noted recently by Zeisel et al. [19] in a small group ($n=8$) of mothers, we demonstrated that the contents of total choline, free choline and choline compounds in human breast milk vary considerably between different lactating breast-feeding mothers. We also demonstrated that the serum free choline concentrations in breast-fed infants significantly correlated with the free choline, phosphocholine, glycerophosphocholine and total choline contents of their mothers' breast milk. Together, these data indicate that the consumption of breast milks and infant formulas with different choline contents and compositions can directly affect serum free choline levels in breast-fed and formula-fed infants. These findings are particularly important because the amount of free choline extracted by the brain varies with the concentrations of free choline in circulation [30] and postnatal choline availability causes significant, life-long alterations in the spatial memory capacity of rats [8–13] associated with altered distribution and morphology of septal neurons. Taken together these data suggest that the ingestion of milks with different choline composition and content could affect brain development in human infants. It is interesting to note that clinical studies show that breast-feeding is associated with significantly higher scores for cognitive development than formula feeding [44]. Since our present data clearly show that breast-fed infants have higher serum free choline concentrations than formula-fed infants, one can suggest that the higher availability of free choline for the developing brain may be one of the contributing factors for the observed higher scores for cognitive development in breast-fed infants. However, the nature of the present study and collected data do not allow us to speculate further on this matter. Firstly, our data are not collected longitudinally in any time period from any particular individual. Thus, the results in the present study are not reflecting the change in serum choline status for any particular individual during a given time period. Secondly, in the present study neither cognitive nor any other developmental scores are determined in infants and/or children. Clearly, further experiments are necessary to determine the relationship between the circulating choline status and developmental scores of infants. Therefore, until we fully understand the choline requirement during infancy, it seems best to encourage breast-feeding and to measure the breast milk choline contents and give supplemental choline to breast-feeding mothers when the choline content in breast milk is low.

In conclusion, the present data establish the normal concentrations of both free and phospholipid-bound choline in newborns, infants and children. Serum free choline concentrations are elevated in newborns, remain elevated with a slow and gradual decrease during infancy and decrease to adult levels in the third year of the life. In contrast, serum phospholipid-bound choline concentrations are low at birth and increase slowly and gradually to the adult levels after the third year of the life. Serum free and phospholipid-bound choline concentrations are significantly higher in lactating women and they both decrease over the time within 12–180 days after birth. The contents of choline compounds (phosphocholine, glycerophosphocholine, phosphatidylcholine and sphingomyelin) in mature breast milks vary considerably from mother to mother independent of the lactating period. The content of free choline in breast milk is changed with the lactating period and its levels in milk significantly correlate with the serum choline status of the breast-feeding woman. Serum free choline concentrations in the breast-fed infants at 12–180 days after birth are correlated with the concentrations of total choline, free choline, glycerophosphocholine and phosphocholine in the consumed breast milk. Serum free choline concentration in formula-fed infants is lower than breast-fed infants.

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