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Fatty acid profiles, antioxidant status, and growth of preterm infants fed diets without or with long-chain polyunsaturated fatty acids

A randomized clinical trial

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Summary Long-chain polyunsaturated fatty acids (LCP) are considered conditionally essential nutrients for the infant born prematurely, and attempts are being made to match fatty acid profiles of formula and breast fed infants. In this double-blind, randomized study we investigated the effects of a formula enriched with both n-6 and n-3 LCP on plasma fatty acid profiles, antioxidant status and growth of premature infants. 29 infants received either a formula devoid of LCP or a LCP supplemented formula (0.5 g/100 g fat linoleic acid metabolites, 0.8 g/100 g fat α-linolenic acid metabolites). 17 breast fed infants served as a control group. At study entry as well as two and four weeks later, plasma and urine samples were collected, growth data obtained and food tolerance was documented. At the end of the four week study period, plasma docosahexaenoic acid (DHA) levels of supplemented infants were significantly higher than those of unsupplemented infants and similar to those of infants fed human milk. Plasma n-6 LCP concentrations including arachidonic acid (AA) were similar between groups. The plasma α-tocopherol levels of breast fed and supplemented infants were similar and tended to be lower than in infants fed the formula devoid of LCP. Urinary malondialdehyde (MDA) excretion of formula fed infants was significantly higher compared to infants fed human milk, but did not differ between the two formula groups. Parameters of growth and milk tolerance did not differ between groups. Our results demonstrate that plasma LCP levels similar to those of breast fed infants can be achieved with the LCP supplemented formula used in this trial, without evidence of adverse effects of the LCP enrichment.

Key words docosahexaenoic acid – omega 3 fatty acids – lipid peroxidation – vitamin E – low birthweight infant nutrition

Abbreviations

AA	arachidonic acid
ALA	α-linolenic acid
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
F	formula devoid of LCP
GLA	γ-linolenic acid
HM	human milk
LA	linoleic acid
LCP	long-chain polyunsaturated fatty acids
LCP-F	formula enriched with LCP
MDA	malondialdehyde
PC	phosphatidylcholine

PCA postconceptional age
PE phosphatidylethanolamine
PUFA polyunsaturated fatty acid

Introduction

Preterm infants miss part of the greatest intrauterine accretion of long-chain polyunsaturated fatty acids (LCP), especially arachidonic acid (AA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) during the last trimester of pregnancy [1, 2]. Due to limited fat stores and the high need for deposition of LCP into rapidly growing tissues, the utilization of LCP may exceed the infant's capability of synthesizing n-6 and n-3 LCP from the precursors linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3) [3, 4]. As a consequence, a rapid depletion of AA and DHA in phospholipids of plasma and erythrocytes occurs in preterm infants fed diets devoid of LCP [5–9]. In term infants fed formula without LCP, lower contents of these fatty acids in brain and retina were found compared to infants fed human milk [10, 11] which contains preformed LCP [12]. While lower AA levels have been correlated with poorer intrauterine [13, 14] and extrauterine [15, 16] growth, a preterm infant formula containing both DHA and AA was reported to enhance growth compared to formulas containing no LCP or only DHA [17]. Inadequate intake of DHA was shown to have functional consequences such as lower scores in tests of visual and cognitive development when compared with infants fed human milk. Supplementation of formulas with DHA improved the performance of preterm infants in tests of visual and cognitive [15, 18–22] functions, matching the performance of breast-fed infants, and LCP are added to European preterm infant formulas [23].

Adverse effects of supplementing preterm infants with LCP have been reported and need to be considered. Supplementation with n-3 LCP alone, especially with oils containing high amounts of eicosapentaenoic acid (EPA, C20:5n-3) relative to DHA, led to a reduction of AA levels and poor growth [15, 16]. Furthermore, the amount of n-6 and n-3 LCP provided with the formula may have an impact on the rate of the infant's endogenous LCP synthesis due to possible product inhibition or competition for the same enzymatic system [24].

Because of the presence of several methylene interrupted double bonds, LCP are subject to oxidative damage, and concerns have been raised that these nutrients may increase the vulnerability of the infant to damage by reactive oxygen species [25]. Preterm infants often are under oxidant stress in the early postnatal period, particularly when exposed to O_2 and ventilation therapy. Oxygen therapy and mechanical ventilation significantly raised malondialdehyde (MDA) excretion in urine to 50–100 % greater levels than those of control premature infants not given therapy [26]. MDA is an end product of lipid peroxidation and has been used as an indicator of oxidative stress in biological models [27]. Elevated MDA levels in plasma of very low birth weight infants have been associated with adverse respiratory and ophthalmological outcome [28].

Tocopherol is an important biological antioxidant, and both preterm and term infants have lower tocopherol levels in plasma and red blood cells compared to adult levels [25, 29]. Preterm infants have low adipose tissue levels and concomitantly low reserves of tocopherol [30]. In addition they may have a poorer ability to absorb fat and vitamin E itself [3, 29], or greater requirements for tocopherol, due to greater oxidative stress [31]. Premature infants surviving respiratory distress syndrome (RDS) had persistent low plasma vitamin E through the first 8 weeks of life, whereas vitamin E gradually increased in control preterms without RDS at similar intakes of vitamin E [32].

In this study, we investigated if feeding a preterm infant formula containing LCP of both the n-6 and the n-3 series, but a somewhat lower n-6/n-3 ratio than typically found in human milk, can match the fatty acid status of preterm infants fed human milk. A control formula fed to a third group of preterm infants contained only traces of LCP. Growth, plasma vitamin E concentrations and urinary excretion of the lipid peroxidation product MDA were determined.

Subjects and methods

Study population

Infants were recruited from the neonatal units of the Pediatric Departments, Zentralklinikum Augsburg, and the University of Frankfurt/Main. Eligible for enrollment were preterm infants in stable clinical conditions with birthweights less than 1800 g. Exclusion criteria were artificial ventilation or an oxygen supply with $FiO_2 > 0.3$ at the time of enrollment and presence of apparent genetic, gastrointestinal or metabolic disorders.

Experimental design

Infants were included in the study within 3 days of established full enteral feeding (≥ 130 ml/kg/d). Prior to enrollment infants received parenteral nutrient supply if required, their own mother's breastmilk or formula without LCP, predominantly an extensive protein hydrolysate (Alfaré, Nestlé, Vevey, Switzerland). After inclusion, the infants were given their own mother's milk when breast milk was available, fortified with a human milk fortifier (5 g/dl milk) (FM 85, Nestlé). Infants whose mothers did not provide breast milk were randomized

in a double-blinded fashion, stratified by birthweight, to receive one of two preterm infant formulas (formula F or LCP-F). While formula F contained only traces of LCP, formula LCP-F contained 0.5 g LA metabolites/100 g fat and 0.8 g ALA metabolites/100 g fat derived from egg lipid extracts, black currant seed oil and low EPA fish oil (Table 1). The formulas were manufactured by Nestlé (Vevey, Switzerland) and their nutrient composition was similar to the standard preterm infant formula Alprem (Nestlé). Formulas contained 2.9 g protein (casein/whey, 30/70), 11.8 g carbohydrates (7.5 g lactose, 3.3 g dextrin-maltose) and 5.2 g fat (30 % medium chain triglyceride oil and vegetable oils) per 100 kcal. Both formulas contained 0.68 mg vitamin E per 100 ml.

Formula-fed infants (group F and LCP-F) received their assigned study formula from study entry for the following 28 days. During this period, the study formula provided at least 90 % of the total energy intake. Correspondingly, breast-fed infants (group HM) received at least 90 % of their energy intake as human milk during the study period.

All measurements were performed by specially trained personnel at the neonatal unit. Growth parameters (weight with an electronic balance, length with a measuring board and head circumference with a validated measuring tape) were documented at birth, on the day of study entry (study day 0) as well as 14 and 28 days afterwards (study days 14 and 28). In order to control for gestational age and to adjust for gender differences, the absolute measurements of weight, length and head circumference were normalized to reference values for German preterm infants as Z scores (standard deviations from the 50th percentile) using data from Brandt [33–35]. Because these data are not available before 31 weeks postconceptional age (PCA), measurements at birth were not normalized.

Additionally, the following parameters were documented at birth and during the study period, respectively: gestational age, special events during pregnancy and birth, Apgar scores at 1, 5, and 10 minutes, duration of artificial ventilation, duration of oxygen supply, type and duration of parenteral feeding, infusion of lipids, presence of disease or complications and therapy. Food intake as well as food tolerance were documented on study days 0, 14 and 28.

Table 1 Major polyunsaturated fatty acids in the study diets (% wt/wt)

	Formula F	Formula LCP-F	Mature human milk (50)
Saturated fatty acids	63.4	52.7	42.93
Monounsaturated fatty acids	21.2	26.9	37.98
n-6 fatty acids			
18:2n-6	14.1	17.7	10.8
18:3n-6	n. d.	0.4	0.2
20:3n-6	n. d.	n. d.	0.3
20:4n-6	0.04	0.1	0.4
n-3 fatty acids			
18:3n-3	1.3	1.2	0.8
18:4n-3	n. d.	0.1	n. d.
20:5n-3	n. d.	0.13	0.04
22:6n-3	n. d.	0.57	0.2
Unsaturation index* (mol double bonds/ 100 mol fatty acids)	39.1	54.0	69.8

* calculated according to (51)
n. d. not detectable

Sample collection

For the analysis of plasma phospholipid fatty acid and vitamin E concentrations, 1.5 ml EDTA-blood were collected on the day of enrollment and on study days 14 and 28. The plasma was immediately separated by centrifugation and stored at –20 °C until further analysis.

For the determination of MDA excretion, 24 hour urine samples were collected by standard infant urine collection bags on study days 0, 14 and 28 and frozen at –20 °C immediately thereafter.

Analytical methods

Plasma phospholipid fatty acids

The fatty acid concentration of plasma phospholipids was determined as described previously [5]. Briefly, lipids were extracted with chloroform/methanol from 0.5 ml plasma after the addition of internal standards. Lipid classes were separated by thin layer chromatography and phospholipid fatty acids transesterified with methanol and hydrochloric acid. Fatty acid methyl esters were analyzed by high resolution capillary gas liquid chromatography with on column injection (HP Series II 5890 A, Hewlett Packard, Böblingen, Germany) [9]. Fatty acid concentrations (mg/l plasma) are reported as median values and interquartile ranges.

Plasma α-tocopherol concentrations

Plasma α-tocopherol concentrations were determined by an HPLC micromethod with UV-detection as described in detail previously [9]. Results are expressed as µg/ml plasma and represent group means with standard deviations. Due to the limited amount of blood available, plasma lipid concentrations and tocopherol/lipid ratios could not be determined.

Urine MDA concentrations

Urinary MDA was measured by high pressure liquid chromatography as described by Schlenzig et al. [26]. Briefly, urinary lipoperoxides were hydrolyzed by boiling in diluted phosphoric acid. The prepared sample was then fractionated by HPLC on an octadecyl silica gel column with a methanol/phosphate buffer and MDA detected spectrophotometrically at 532 nm.

Ethical aspects

The study protocol was approved by the local ethical committees and written informed parental consent obtained.

Statistical analysis

Data were analyzed with SPSS for Windows Release 6.1.3.

Plasma phospholipid fatty acids

Differences between groups on study days 0, 14 and 28 were analyzed for each fatty acid using the Kruskal-Wallis test. If differences between groups were detected, these were further evaluated with Mann-Whitney U tests. Changes over time during the 4-weeks study period within each group were analyzed with the Wilcoxon test.

MDA and α-tocopherol

For MDA and α-tocopherol, the effect of the diet was tested at day 0, 28 and for the difference (day 28–day 0) with a Kruskal-Wallis test. Then a Kruskal-Wallis multiple comparison test with Bonferroni correction was done. The variation of α-tocopherol and MDA over time within groups was tested with a Wilcoxon signed-rank test for differences in medians.

Clinical data

Absolute weight, length and head circumference at birth as well as Z scores of weight, length and head circumference on study days 0, 14 and 28 and changes in Z scores from day 0 to day 28 were compared with ANOVA. When differences were found to be significant, t-tests with Bonferroni correction for multiple comparisons were performed. For all these tests, the assumption of equal group variances was tested with the Levene test for Homogeneity of Variances. For comparisons of frequencies and proportions (number of vomiting, stools and stomach residues per day, gender distribution), Chi-square tests were applied. The level of statistical significance was set at $p < 0.05$.

Results

Subjects

Of the 49 infants enrolled for the study, 19 received human milk, 15 were randomized to formula F and 15 to formula LCP-F. Two infants from the human milk group were excluded from the analysis because their formula intake exceeded 10 % of total energy intake after study day 10. Another infant (group F) was excluded because the final evaluation demonstrated a birth with 38 weeks PCA. From the remaining 46 infants, data or samples from 13 infants were incomplete (human milk: 6, formula F: 3, formula LCP-F: 4) for the following reasons: human milk intake comprised less then 90 % of total energy intake after study day 14 (n = 1), infants were discharged from the hospital after study day 14 (n = 2), parents chose to withdraw their infant from the study after study day 14 (n = 1) or one of the samples was missing (n = 9). Provided that measurements from at least two time points were complete, data from these infants were included in the statistical analysis.

The clinical characteristics of the study population are given in Table 2. Infants of the human milk group were born with higher birthweights than infants from group LCP-F. There was a statistically significant greater number of females in group LCP-F as compared to group HM. There were no differences between groups at birth and during the study period in gestational age, special events during pregnancy and birth, Apgar scores at 1, 5, and 10 minutes, duration of artificial ventilation, duration of oxygen supply, type and duration of par-

Table 2 Characteristics of the study population (mean ± SD)

	Human milk	Formula F	Formula LCP-F
Number of infants	17	14	15
Gender (m/f)	10/7[a]	6/8	2/13[a]
At birth:			
weight (g)	1440 ± 288[b]	1177 ± 344	1145 ± 288[b]
length (cm)	40.0 ± 2.4	38.1 ± 4.6	36.9 ± 3.3
head circumference (cm)	28.4 ± 1.6	26.6 ± 2.5	27.1 ± 2.5
gestational age (wk)	31 ± 2	30 ± 3	30 ± 2
At study entry:			
age (d)	26 ± 14	39 ± 22	39 ± 24
postconceptional age (wk)	35 ± 2	35 ± 2	35 ± 2
Milk intake (ml/kg&day):			
study day 0	145.4 ± 12.6	144.9 ± 15.7	152.4 ± 8.0
study day 14	150.3 ± 17.3	152.3 ± 20.0	153.2 ± 8.1
study day 28	148.1 ± 16.8	153.4 ± 10.9	155.0 ± 16.2

Values bearing identical superscripts ([a, b]) indicate significant differences ($p < 0.05$) between groups ([a] Chi-square test, [b] ANOVA and Bonferroni t-test)

enteral feeding, infusion of lipids, presence of disease or complications and therapy.

Milk intake and tolerance

The mean daily formula or human milk intakes of the three groups on study days 0, 14 and 28 were not different at any of the three time points (Table 2).

All groups showed good feeding tolerances with no significant differences in frequencies of gastric residuals, vomiting or stools per day (data not shown).

Growth

During the study period, all three groups reached similar weight gain. Mean Z scores of weight, length and head circumference did not differ between dietary groups on study days 0, 14 and 28 (Table 3). Furthermore, there was no difference in the changes in Z scores over time during the 4-week period between groups (Table 3).

Plasma phospholipid fatty acids

The concentrations of selected fatty acids in the plasma phospholipid fraction of the three groups of infants at study entry and at the end of the study are shown in Table 4.

Table 3 Z scores (mean ± SD) of weight, length and head circumference of study groups during the 28-day study period

	Human milk (n = 17)	Formula F (n = 14)	Formula LCP-F (n = 15)
Weight:			
study day 0	−1.22 ± 1.21	−1.04 ± 1.41[a]	−1.56 ± 0.82
study day 14	−0.87 ± 1.01	−0.85 ± 1.84	−1.54 ± 1.20
study day 28	−0.83 ± 1.48	−0.49 ± 1.79[a]	−1.30 ± 1.29
ΔZ score (day 0 to day 28)	0.15 ± 0.92	0.65 ± 0.86	0.26 ± 0.66
Length:			
study day 0	−1.16 ± 1.44	−1.55 ± 1.74[b]	−1.82 ± 1.35
study day 14	−0.74 ± 2.22	−1.35 ± 1.52	−1.73 ± 1.32
study day 28	−1.08 ± 1.56	−1.25 ± 1.75[b]	−1.95 ± 1.46
ΔZ score (day 0 to day 28)	0.04 ± 1.38	0.57 ± 0.55	0.02 ± 0.96
Head circumference:			
study day 0	−1.48 ± 0.98	−2.13 ± 1.67[c]	−1.34 ± 1.68
study day 14	−1.52 ± 0.91	−1.51 ± 1.52	−1.28 ± 1.21
study day 28	−1.20 ± 1.23	−1.51 ± 1.35[c]	−1.09 ± 1.33
ΔZ score (day 0 to day 28)	0.02 ± 0.84	0.86 ± 1.13	0.19 ± 0.72

Values with identical superscripts ([a, b, c]) are significantly different (changes over time within groups)

Study entry

Since no formula given to the infants prior to study entry contained LCP, only breast-fed infants received LCP before study day 0. Consequently, formula-fed infants had lower values of AA, DHA, total n-6 LCP and total n-3 LCP in plasma phospholipids in comparison to breast-fed infants at study entry. Breast-fed infants had significantly higher values of trans-fatty acids.

Changes over the study period

n-6 fatty acids. There were only minor changes of n-6 fatty acids over the study period. Significant differences between groups were seen for LA, γ-linolenic acid (GLA) and AA. In breast-fed infants, LA increased significantly from study day 0 to study day 28. There were no significant changes in LA concentrations over time in the formula groups. LA concentrations were already significantly higher in group F compared with group LCP-F on study day 0. This difference remained significant until the end of the study. GLA tended to decline over time in the three groups. On study day 28 GLA was significantly higher in group LCP-F compared to the breast fed group. AA rose significantly in group LCP-F between study entry and study day 14 and was constant thereafter. During the 4 week-study period, AA remained unchanged in group F, while it tended to decline in breast-fed infants (Fig. 1). The initially significant differences between groups were no longer existent two and four weeks after study onset.

n-3 fatty acids. ALA increased significantly over time in groups HM and LCP-F while the increase of ALA in group F did not reach statistical significance. The higher EPA content of formula LCP-F compared to formula F was reflected in significantly higher EPA concentrations in phospholipids of supplemented vs. unsupplemented infants at the end of the study. These levels were similar to those observed in breast fed infants. The concentration of the principal n-3 LCP DHA in the three groups is shown in Fig 2. DHA tended to decrease over the four weeks-study period in breast-fed infants while it remained unchanged in group F. However, there was a highly significant increase ($p < 0.005$) in DHA concentrations from study day 0 to day 14 in group LCP-F reaching equal DHA values to group HM on study day 14. On study days 14 and 28, the mean DHA levels in group HM and group LCP-F were similar and significantly higher than the mean concentration found in group F.

Table 4 Fatty acid concentrations in plasma-phospholipids (mg/l plasma) [Median (interquartile range)]

	Human milk		Formula F		Formula LCP-F	
	study day 0 (n = 15)	study day 28 (n = 13)	study day 0 (n = 13)	study day 28 (n = 12)	study day 0 (n = 12)	study day 28 (n = 14)
Saturated fatty acids (total)	469.81 (191.70)	495.32 (340.12)	445.62 (141.88)	466.86 (232.56)	446.50 (112.80)	424.04 (197.72)
Trans fatty acids (total)	6.37 (2.34)[a]	5.49 (5.26)[b]	2.20 (6.40)	5.43 (7.99)[c]	2.69 (1.96)[a]	1.98 (2.33)[b, c]
Cis-monounsaturated fatty acids (total)	176.95 (81.05)[a]	184.96 (95.42)[b]	140.84 (53.88)[a, *]	118.37 (33.39)[b, *]	165.83 (110.35)	123.07 (73.68)
n-6 PUFA						
C18:2n-6	162.06 (60.48)*	182.56 (105.70)*	201.45 (63.10)[a]	204.24 (84.32)[b]	150.71 (43.63)[a]	183.92 (51.27)[b]
C18:3n-6	1.17 (1.16)	0.70 (1.31)[a]	1.16 (1.58)	1.07 (1.03)	1.86 (1.84)	1.40 (1.28)[a]
C20:2n-6	6.34 (3.04)	5.52 (4.08)	5.41 (2.70)	5.03 (2.67)	4.58 (1.98)	5.47 (1.67)
C20:3n-6	37.72 (20.07)	31.81 (22.17)	30.35 (18.21)	31.45 (14.66)	33.56 (5.90)	37.53 (27.81)
C20:4n-6	113.88 (45.40)[a, b]	90.82 (42.68)	77.28 (20.37)[a]	68.64 (50.95)	66.21 (23.78)[b, *]	75.64 (35.08)*
C22:4n-6	3.80 (2.84)	3.01 (2.73)	3.35 (0.90)	3.13 (1.85)	3.00 (1.40)	3.15 (1.73)
n-3 PUFA						
C18:3n-3	1.19 (1.66)*	1.87 (2.04)*	1.33 (2.44)	1.70 (1.55)	0.62 (2.09)*	1.60 (3.07)*
C20:5n-3	3.95 (1.73)	3.56 (1.48)	2.24 (1.73)	2.61 (1.59)[a]	2.89 (1.24)	4.06 (2.32)[a]
C22:5n-3	3.38 (1.92)	3.05 (1.36)	3.82 (3.31)	3.55 (2.68)	3.68 (4.63)	3.00 (1.93)
C22:6n-3	28.01 (17.31)[a, b]	21.97 (19.15)[c]	15.93 (10.76)[a]	13.95 (10.14)[c, d]	15.02 (9.53)[b, *]	23.90 (12.91)[d, *]
Sums and ratios						
P/S-quotient	0.72 (0.08)	0.77 (0.05)	0.76 (0.10)	0.79 (0.05)	0.69 (0.22)*	0.76 (0.10)*
PUFA	366.78 (136.38)	373.62 (176.43)	340.11 (91.92)	365.18 (125.63)	310.44 (55.48)	346.63 (137.13)
LCP	202.27 (115.99)[a, b]	162.36 (86.79)	144.68 (58.65)[a]	133.95 (81.15)	148.71 (24.30)[b]	154.84 (93.44)
n-3 LCP	34.17 (20.25)[a, b]	27.79 (21.37)	20.62 (9.90)[a]	21.97 (11.90)[a, b]	23.06 (7.32)[b, *]	35.32 (13.76)[c, *]
n-6 LCP	161.35 (89.18)[a, b]	132.30 (67.30)	114.44 (41.24)[a, *]	109.12 (67.66)*	110.17 (21.99)[b]	114.32 (63.77)
n-6/n-3 LCP	4.97 (1.14)	4.63 (1.32)[a]	5.23 (2.02)	5.22 (1.59)[b]	4.87 (2.14)*	3.44 (1.03)[a, b *]

Values bearing identical superscripts ([a, b, c, d]) indicate significant differences ($p < 0.05$) between groups.
* significant differences ($p < 0.05$) over time within groups

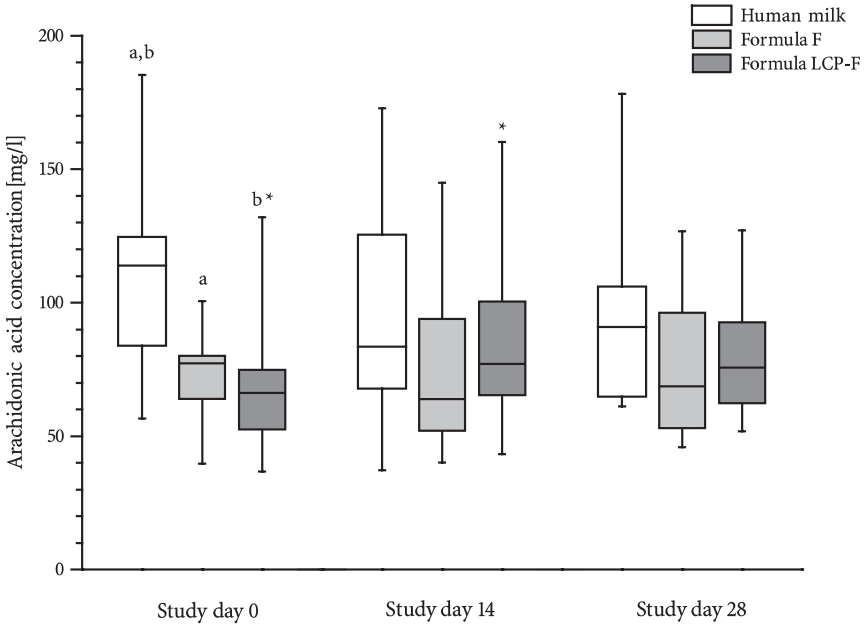

Fig. 1 Arachidonic acid concentrations in plasma phospholipids of the three dietary groups during the study period. Identical letters (**a, b**) indicate significant differences ($p < 0.05$) between groups, * significant differences ($p < 0.05$) over time within groups

α-tocopherol and MDA

Over the 28 day feeding period, there was no change in the plasma α-tocopherol levels in the human milk group, nor in the LCP-F group (Table 5). In infants fed either of the infant formulas at day 28, plasma α-tocopherol levels were similar to those found in infants fed human milk. Compared to HM, the change in α-toco-

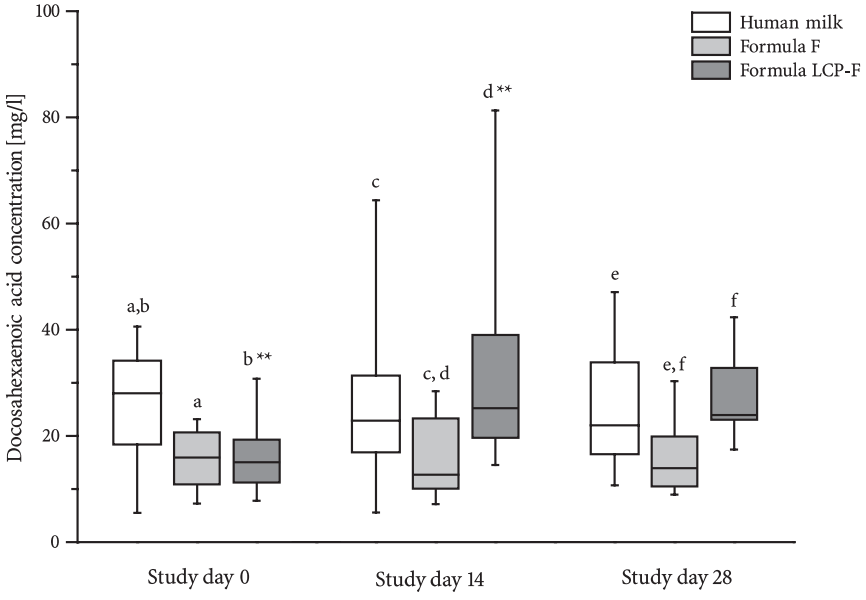

Fig. 2 Docosahexaenoic acid concentrations in plasma phospholipids of the three dietary groups during the study period. Identical letters (**a, b, c, d, e, f**) indicate significant differences ($p < 0.05$) between groups, ** significant differences ($p < 0.005$) over time within groups

Table 5 Plasma α-tocopherol and urinary malondialdehyde (MDA) – median (*interquartile range*)

	Human Milk (n = 9)	Formula F (n = 12)	Formula LCP-F (n = 8)	Probability level[1]
α-tocopherol [mg/l]				
Day 0	3.15 (3.68)	2.72 (2.75)	1.22 (3.19)	0.158
Day 28	3.89 (2.97)	7.81 (7.07)	4.15 (4.03)	0.037
Day 28–day 0	–0.21[a] (1.64)	3.93[a,*] (5.89)	1.20 (3.74)	0.008
MDA [µmol/l]				
Day 0	0.29[a,b] (0.21)	1.94[a] (1.62)	1.62[b] (2.91)	0.001
Day 28	0.40[a,b] (0.58)	1.45[a] (2.39)	1.37[b] (1.83)	0.012
Day 28–day 0	0.16 (0.62)	–0.035 (1.49)	–0.16 (1.14)	0.417

[1] Kruskal-Wallis.
Values bearing identical superscripts ([a, b]) indicate significant differences ($p < 0.05$) between groups.
* Significant differences ($p < 0.05$) over time within groups

pherol level over the 28 days was significantly greater in the standard formula fed group although not in the LCP-F group.

Urinary MDA excretion was similar in the two formula fed groups from day 0 onwards, and was significantly higher than in the infants fed human milk (Table 5). There was no change in this parameter over time.

Discussion

During the study period, the phospholipid contents of the essential fatty acids LA and ALA increased significantly in infants fed human milk, similar to previous observations [5, 7, 9]. In formula fed infants, these fatty acids tended to increase as well, with the increase of ALA in group LCP-F reaching statistical significance. Since most of the infants fed formula received a hydrolysate formula (Alfaré, Nestlé) before study entry, this observation seems to reflect the higher contents of LA and ALA in the two study formulas compared to Alfaré (LA: 18 % vs. 12.1 %, ALA: 1.6 vs. 0.9 %).

At study entry, formula fed infants showed markedly depleted levels of both n-3 and n-6 LCP. Relative to the concentrations found in infants fed human milk, the DHA level in plasma phospholipids at study entry was reduced by 45 % and that of AA by 37 %. This is in agreement with our previous observations of a relatively more marked depletion of DHA as compared to AA [9] and presumably due to the more complex pathway of endogenous DHA synthesis relative to AA formation [36].

In the human milk group, DHA tended to decline from study entry until study day 14 and remained constant thereafter. It has previously been reported that preterm infants fed human milk show falling DHA levels after birth which then tend to stabilize at a lower level [5, 37]. In group F, the most marked decline in DHA apparently occurred before study entry, while DHA levels

remained unchanged over the entire study period. Consequently the unsupplemented infants remained highly depleted in DHA demonstrating only 64% of the DHA-level of breast-fed infants at study end. This reconfirms findings from other studies [5–9, 38] and stresses the importance of dietary DHA supplementation for preterm infants if plasma fatty acid profiles of infants fed human milk shall be matched. This goal was achieved in infants fed the supplemented formula. Despite the low DHA concentrations found in these infants at study entry, values similar to those of breast-fed infants were reached by day 14 and remained stable thereafter.

The level of DHA supplementation used in the present study (0.57% of total fatty acids) was relatively high compared to most other studies in preterm infants [7, 9, 38, 39]. In one previous study with a formula containing 0.1% DHA (of total fatty acids), plasma DHA levels of supplemented infants tended to be lower than those of infants fed human milk [5]. However, other studies using DHA levels of 0.2–0.4% of total fatty acids succeeded in matching plasma DHA levels of supplemented with those of breast-fed infants [7, 9, 38, 39]. In a previous study by our group [9], plasma phospholipid DHA concentrations after 3 weeks of feeding a supplemented formula were very close to those observed at the end of this study (23.9 mg/l vs. 22.4 mg/l) despite different DHA contents of the formulas used (0.3% vs. 0.57%). This might be due to the fact that in the present study formula-fed infants presented with higher LCP depletion at study entry, presumably due to their greater postnatal age. Carlson et al. also found similar percentages of DHA in plasma PE and PC of preterm infants fed formulas containing 0.2 vs. 0.4% DHA for 4 weeks [6]. Since both formulas in Carlson's study were identical except for DHA content, it is unlikely that differences in absorption are the reason for this observation. Thus, other factors may be involved, such as potential inhibition of endogenous DHA synthesis by DHA supplementation [40]. It remains an area of further investigation if a lower DHA intake may enhance endogenous DHA synthesis and contribute to maintaining plasma DHA levels. Studies using stable isotopes are required to elucidate this question [4, 36], since compositional data alone do not allow conclusions on endogenous synthesis rates.

The DHA concentrations of the formulas used in the present study, as well as in the other supplementation studies cited, are all within the range reported for human milk [12]. However, if the plasma and body fatty acid pools of infants fed human milk might be matched with formulas containing less DHA, reducing the concentration would not only provide technological advantages and reduce the costs of the formula but also limit potential risks of DHA supplementation such as increased lipid peroxidation or potential effects on the metabolism of n-6 LCP and growth. With the supplementation used in this study no such adverse effects were observed. Total n-6 LCP levels were stable in both formula groups. In infants fed the supplemented formula, AA even increased significantly from day 0 to day 14. Relative to levels found in infants fed human milk, other studies have found both decreasing [5, 6, 38, 39] and unchanged [7, 9] AA concentrations in preterm infants fed formulas containing LCP. The most marked reductions of n-6 LCP status have been observed with formulas containing n-3 LCP but no n-6 LCP [6, 39], while n-6 LCP levels similar to human milk were achieved with formulas containing LCP of both series [7, 9].

Many current preterm formulas do not only contain AA but also other linoleic acid metabolites including GLA [9], dihomo-γ-linolenic acid (DGLA) [7] or both GLA and DGLA [5]. These fatty acids are thought to be more readily converted to AA than the parent fatty acid LA because the first step in the pathway of AA synthesis, the desaturation of LA to GLA via Δ6-desaturase, has been reported to be rate-limiting both *in vitro* and *in vivo*. So far, isotope studies in infants have not supported this hypothesis [3, 4, 41]. The most frequently used models for studying conversion rates of GLA and DGLA to AA have been rats or rat liver tissues, in which experiments with isotope-labeled GLA demonstrated that rapid conversion to AA [24, 42]. However, the last step in AA synthesis, the conversion of DGLA to AA via Δ5-desaturase, seems much slower in humans than in rats [43, 44] and may be limiting AA synthesis in human infants [4]. Therefore, the true effect of GLA supplementation on AA synthesis in humans remains unclear. Hence, it seems more appropriate to enrich formulas with combinations of both GLA and AA than GLA alone. The supplemented formula fed in the present study contained 0.4% GLA and 0.1% AA (of total fatty acids). With this combination, plasma phospholipid concentrations of total n-6 LCPs were stable and AA levels improved in the supplemented group in spite of the relatively high DHA content of formula LCP-F. Therefore, the n-6 LCP supplementation used here can be regarded as safe and efficient. It remains an area of further investigation to which extent GLA contributed to the AA levels measured in plasma phospholipids of group LCP-F. Further studies in humans using stable isotope-labeled precursors of AA are needed to clarify the role of GLA supplementation on AA status of preterm infants.

Growth was not different among groups in this study which might be related to the stable AA levels observed in all groups. The supplemented formula given here contained only minor amounts of EPA (0.14% of total fatty acids). This low EPA content together with the simultaneous provision of n-6 LCP seems to lead to stable n-6 FA levels and thus normal growth. However, group sizes are too small to draw final conclusions with respect to growth effects. In a trial by Hansen et al. the effects of LCP supplementation on growth were studied in a large

number of preterm infants who were followed until 4 months corrected age. In this study, growth (g/d) was enhanced in infants supplemented with both DHA and AA compared to unsupplemented infants [17] and weight at 2 and 4 months corrected age was higher than in infants fed formulas containing no LCP or only DHA. These results suggest that formulas containing both n-6 and n-3 LCP might promote better growth than formulas devoid of LCP. Still, further research is warranted studying the effects of feeding supplemented formulas for longer periods because an influence on AA levels and growth is not always seen in short-time feeding trials [6].

The LCP content of infant formula may contribute to oxidative stress [45]. Thus the formula must contain sufficient vitamin E both to prevent oxidation of the formula, and once ingested to protect the infant from oxidation reactions within the body with potentially negative health consequences. The European Society for Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends at least 0.9 mg vitamin E/g polyunsaturated fatty acids (PUFA) furnished in the formula [45], and the formulas used in this study contained 2.8 mg vitamin E/g PUFA. These levels appear adequate to support plasma vitamin E since this plasma parameter in infants fed either of the two formulas at day 28 was equivalent to that in infants fed human milk. The change of α-tocopherol in the standard formula fed group was significantly greater than in the HM group over 28 days. This was not the case in the LCP-F group. Since both formulas contained the same level of vitamin E this observation may indicate a greater turnover of vitamin E in the presence of LCP. A previous study from this group showed a tendency for α-tocopherol levels in plasma and erythrocytes in preterm infants to be lower when they were fed fish oil supplemented formulas [25]. In contrast, levels of plasma or RBC α-tocopherol increased after birth in preterm [9, 46, 47] and term [48] infants fed standard or LCP supplemented formulas just as they did with breast feeding. In two of these studies using formulas with egg phospholipids [9, 48], the LCP supplemented group tended to have even higher plasma levels of α-tocopherol than the nonsupplemented group although dietary intakes were not different.

MDA did not change as a function of LCP ingestion, but MDA excretion was higher in infants fed both formulas compared to infants fed breast milk already at day 0, although plasma vitamin E levels were equivalent or higher in these groups compared to breast fed infants. Since MDA can also be formed in the formula before ingestion, this source might have contributed to the urinary MDA found in the formula fed infants. However, we did not measure MDA content of the study formulas during their shelf life.

It seems possible that the added phospholipids in the LCP supplemented formula may enhance oxidative stability. Moreover, Sosenko and coworkers have reported that an LCP supply protects newborn mice against oxygen toxicity and suggested that LCP in the body may protect more important membrane bound fatty acids, if they are not located at critical sites in the cell (e. g., the membrane) and are immediately replaced after autooxidation [49]. This intriguing possibility deserves further investigation.

In conclusion, our trial demonstrates that the LCP enriched formula tested here is effective in improving the plasma LCP status in preterm infants and does not induce appreciable adverse effects.

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