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ADVANCES IN PEDIATRIC RESEARCH

Fatty acid and amino acid content and composition of human milk in the course of lactation

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Abstract

Background: Longitudinal data on the composition of human milk in cohorts of term infants are limited but warranted. In the PreventCD cohort, human milk samples were collected monthly over 6-9 consecutive months enabling longitudinal analysis of macronutrient composition.

Methods: Milk sample series from 25 lactating women were analysed for fatty acid (FA) and amino acid (AA) content and composition; also total N-content was assessed. Mainly mature (i.e. >15 days after delivery) milk samples were collected. Milk content and composition were related to the gender and bimonthly weight accrual of the infant up to 6 months of age.

Results: As sample collection was not standardised for time of day nor for fore- or hindmilk, a large month-to-month variation in fat and protein levels was observed within and between donors. In spite of the quantitative variability, the quality (i.e. composition) of both lipids and protein in the samples obtained was relatively stable. Also, the average content and composition of FA as well as AA remained quite stable over the lactation period. Milk macronutrient content or composition was not different between genders, and bimonthly body weight accrual of the offspring was not associated with milk FA or AA content.

Conclusions: These results show that the inter- and intra-individuality of human milk composition are limited, regardless of sampling procedures. The average composition of the samples showed a stable pattern both in FA and AA profile during lactation.

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Introduction

Infant feeding targets providing an optimal balance of dietary nutrients to support growth and development of the newborn infant. Human milk (HM) provides the natural and best nutrition for a newborn baby for at least the first 6 months of life, as endorsed by both the World Health Organization (WHO) (1) and the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) (2). Epidemiological data support the hypothesis that early feeding strategies have long-

lasting effects on health and performance, and may influence the risk of disease development into adulthood (3, 4): Breastfeeding has been shown to reduce the risk of obesity and to improve cardiovascular health later in life, and there is some evidence that it also confers protection against the development of certain chronic diseases (5-7).

The composition of HM is reported to be variable and adaptive over the course of lactation, during the day (morning vs. evening HM), and even within one nursing session (fore- and hind milk) (8-10). HM protein content is known to decrease rapidly during the first weeks of lactation, but changes little in the period later in lactation (11-15). In contrast to the protein content, HM fat content does not vary as much over the course of lactation (16), although the fat content and fatty acid (FA) composition, particularly the long-chain polyunsaturated FA (LC-PUFA), depend at least in part on maternal dietary intake (17-19).

A wide variety of nutritive and functional proteins in HM contribute to its unique qualities. Whereas nutritive proteins are digested and form an indispensable source of amino acids (AA) for the rapidly growing breastfed infant, functional proteins bring other (additional) benefits such as antimicrobial effects (e.g. immunoglobulins, lysozyme and lactoferrin) or play a pivotal role in the digestion and absorption (e.g. bile salt-stimulated lipase and amylase, lactoferrin and beta-casein) of HM itself.

Understanding HM composition, and its changes during the lactation period, is an important first step in defining the nutritional needs of infants (8, 20). Data are available on HM protein and AA content, especially studied in preterm born infants in their first month of life (21, 22). But research data on longitudinal changes in HM composition in well-documented cohorts of term infants are limited (15, 23). Such studies remain warranted as diet and lifestyles have changed since many of those studies were conducted.

The PreventCD project studied the effect of early diet on Coeliac Disease (CD) development (www.preventcd.com) (24, 25) and followed term-born infants at risk of developing CD from birth onward. As the PreventCD study design included the collection of a monthly HM sample, the study was an opportunity to investigate FA and AA composition of HM prospectively in samples collected from the same donor during 6–9 consecutive months.

Materials and methods

HM sample source

HM samples were collected from 2007–2010 from 25 Dutch mothers participating in the PreventCD project (25). Briefly, term-born infants at risk of developing CD were recruited between 0 and 3 months of age. Included infants had at least one first-degree family member with CD, other than their mother, and were genotyped to be HLA-DQ2 or HLA-DQ8 positive. Mothers were not genotyped. Prematurely born or infants with syndromes associated with an increased risk of CD, such as Down's or Turner's syndrome, were excluded. The infants were randomised to the intervention groups, and received either 200 mg wheat gluten or placebo (200 mg lactose) between 4 and 6 months of age. Infant health status, anthropometric variables (height and weight), and feeding habits (amongst others: breastfeeding, formula feeding, gluten consumption) were assessed periodically using standardised questionnaires (24, 25). CD was diagnosed according to the ESPGHAN criteria (26).

For the analyses reported here, samples were selected from donor mothers without CD, who consumed a normal, gluten-containing diet. Obtained breast milk was checked for and did not contain any gluten (25). The donor's infants, 12 males and 13 females, were genotypically at risk of developing CD, had a first degree relative with CD, but had not developed CD at the age of 5–8 years (at the moment of sample analysis in July 2015). Given the high prevalence (40%) of the genotype associated with CD (i.e. HLA DQ2 and 8) among the general population (24), we consider the 25 mother-infants pairs in the present study to be representative of the general, healthy population. Furthermore, selection was irrespective of the type of intervention given to the infant as part of the PreventCD study; 24 out of the 25 infants had

received the gluten intervention during months 4 and 5 (see (24).

HM samples (10–30 mL, no complete expressions) were collected on an explorative basis. Mothers were asked to express HM manually or by pump once a month, around the same day. The HM samples, collected at home, were kept frozen overnight at -20°C in the home freezer and then transferred on ice to the hospital and stored at -80°C at LUMC (Leiden), until they were transported on dry ice to the laboratory (Nutricia Research) for analysis.

In total, 178 HM samples from 25 donors were analysed, comprising 16 series of monthly samples up to 6 months, and 9 series up to 9 months of lactation. The earliest sampling point was on average at day 28 after delivery (range 7–59 days). Not all longitudinal sampling series were complete, as for some months, samples were missing (Figure 1A and B). For every sample, 1–2 mL aliquots were prepared.

Data on growth and body weight of the 25 infants (48% males) were extracted from the coded PreventCD cohort records. Infants were designated 'fully breastfed' if solely HM fed, and 'mixed fed' if any food other than HM was given to the infant in the time period evaluated.

HM sample analyses

HM samples were thawed overnight at 4°C, whereupon they were gently vortexed and aliquoted. Two 250 µL HM samples were analysed for either AA or FA content and composition by standard methods as described elsewhere (27, 28).

Total lipids were extracted from single HM samples by the Bligh and Dyer method (29) whereupon FA methyl esters (FAME) were prepared and processed according to the Morrison method (28).

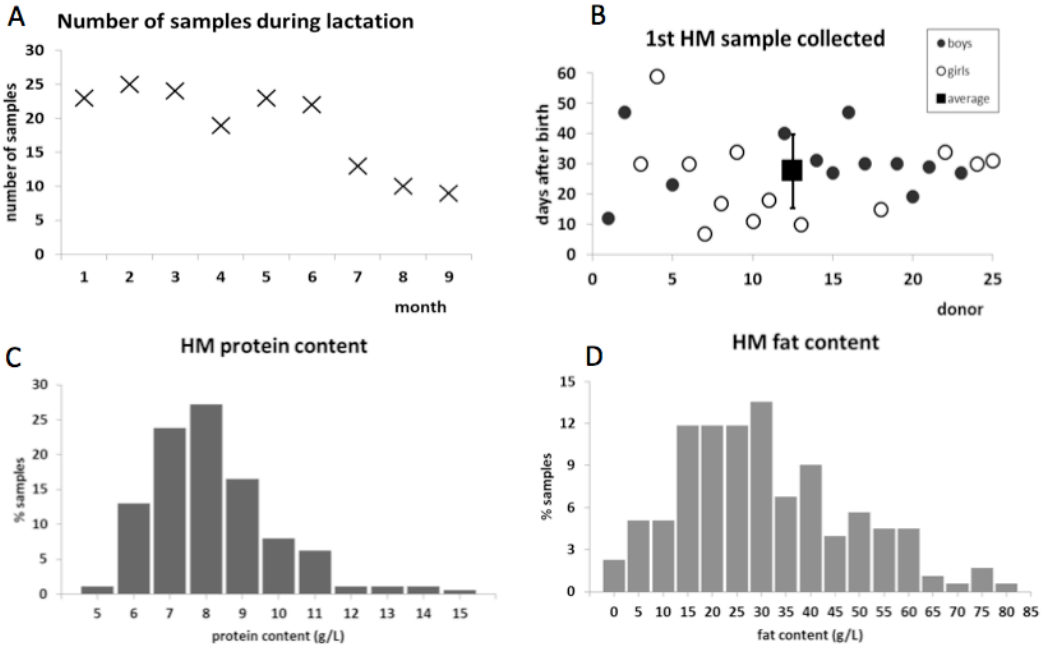

Figure 1. Human milk (HM) samples overview

A: number of samples per time point; B: time point of first sample collected per donor and average time point of first collection; C: distribution of protein content in all samples collected; D: distribution of fat content in all samples collected.

FAME were analysed by gas chromatography (GC; Shimadzu) using a CP-SIL88 column 50 m x 0.25 mm id. (0.22 µm film thickness), and a flame ionization detector (FID). Starting at 150°C for 3.75 min, the temperature was raised to 220°C at 22°C /min, and maintained for 14.07 min.

To enable quantification, HM samples were spiked prior to lipid extraction with C19:0 (Sigma, Zwijndrecht, the Netherlands). FA standards (GLC-461, Nu-Chek Prep Inc. Elysian, MN USA) were used for FA peak identification.

Medium-chain FA (MFCA) group comprised C6-14 FA; the saturated FA (SFA) group included the MCFA species.

The AA analysis method used required total protein hydrolysis (in 6 M HCl) (27), which yielded 15 detectable AA and disabled the detection of Trp, Cys, and Pro. Gln and Glu co-eluted (Glx), as did Asn and Asp (Asx). The AA were analysed by an ultra-fast liquid chromatograph (UFLC) system (Shimadzu) equipped with an Acquity UPLC BEH C18 column (1.7 m, 1002.1 mm) and a fluorimetric detector. AA standards (Sigma) were used for AA peak identification.

Total N-content was measured separately in duplicate in two 200 µL samples according to the Dumas method (30). 'Total protein' present was estimated by multiplying the N-content of the sample by 6.25 (11, 31), and the difference between the 'total protein' and the sum of detectable AA was designated as 'non-protein N' (or NPN). NPN was assumed to comprise amongst others, the three non-detectable AA, taurine, urea, and nucleotides.

Statistics

Data are shown as means ± SD, unless stated otherwise, and are expressed in absolute amounts (e.g. g/L) or relative amounts (e.g. % of total AA, % of total FA).

Data were processed and computed for descriptive statistics using Microsoft Excel and figures show means and SD. Due to the large intra-individual variation between the successive monthly collected HM samples, HM analysis data were only compared as grouped data per collection time point by a two-tailed Student's T-test with $p<0.05$ considered as significantly different. Paired testing was not possible due to missing samples. To determine correlations between FA and AA content in HM and infant growth features (i.e. bimonthly body weight accrual), Pearson's correlation coefficient was used.

Results

Figures 1C and D show the distribution of the protein and fat content of all HM samples collected, respectively. As shown, the fat content distribution (2.86–80.72 g/L) shows a broader range than the protein content (5.95–15.35 g/L).

On average, AA analysis showed that total protein content, derived from the sum of the 15 AA levels, amounted to 8.66 g/L and was quite stable throughout the 9 months of lactation: only the first samples collected (range 7–59 days after delivery) had on average a higher total protein content (Figure 2A and Table 1). The same holds for the group of essential AA (EAA), which comprised 45-46% of total AA, and the group of branched chain AA (BCAA), which comprised about a quarter of total AA (Figure 2A and Table 1). Also the relative AA composition (% of total AA, %AA) remained relatively stable over the lactation period, with the exception of the first samples for some AA species, mainly non-EAA (NEAA; see Figure 2C). As shown in Table 1, the main AA fraction in HM is the combined Glu+Gln moiety (Glx, 20.5–22.5 %AA), followed by Leu (11.3–11.8 %AA) and the combined Asp+Asn fraction (Asx, 10.6–11.2 %AA). The lowest AA level in the HM samples is Met (<2 %AA). Figure 2D summarizes the total protein content based on the N-content according the Dumas method ('protein'), and was found always to exceed the total sum of AA: this apparent 'non-protein N' fraction (or 'NPN') amounted on average to 22–24%.

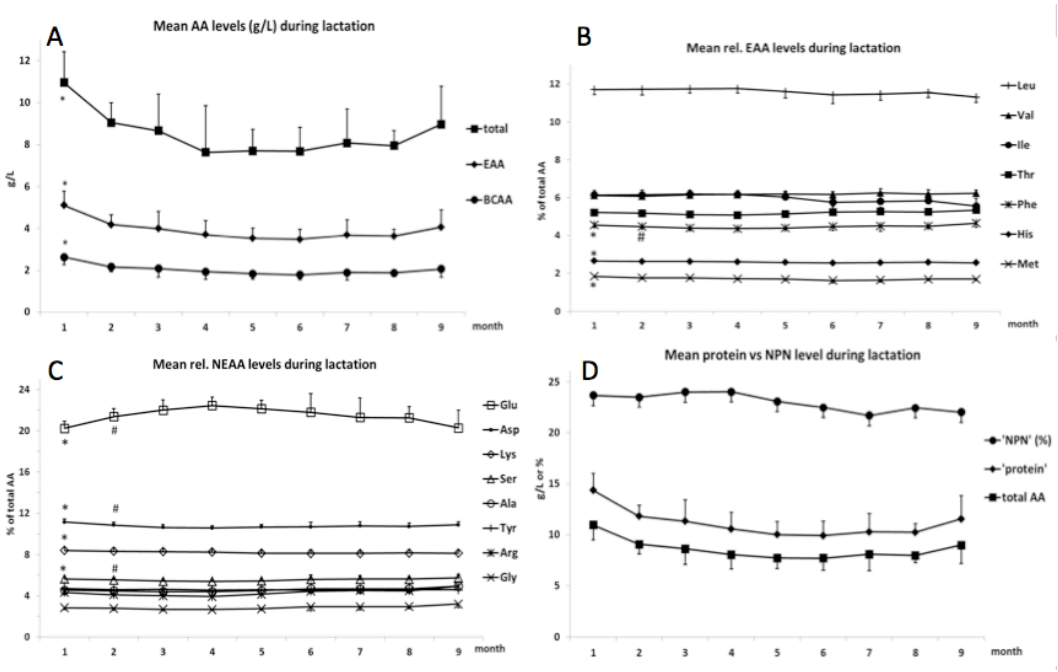

Figure 2. HM protein content overview

A: mean AA content (total, EAA, BCAA); B: mean relative EAA content; C: mean relative NEAA content; D: mean protein and NPN content. Data shown are means ± SD, n per time point as shown in Figure 1A; *p<0.01; # p<0.05 vs. later time points. AA, amino acids; EAA, essential AA; BCAA, branched chain AA; NEAA, non-essential AA; NPN, non-protein nitrogen.

The average fat content in HM, as derived from the quantified sum of all FA peaks discriminated, amounted to 33.21 g/L throughout lactation (Figure 3A), and no significant level changes were observed. The composition/quality of the HM fat (Figure 3B, Tables 2 and 3) remained quite stable over time as can be derived from the relative contribution of several FA groups, such as saturated FA (SFA), medium chain FA (MCFA), and mono-unsaturated FA (MUFA). Also the relative content of n-3 and n-6 FA was very constant throughout lactation (Figure 3C+D, Table 3). The main saturated FA were palmitic acid (PA, C16:0) and stearic acid (SA, C18:0) which consistently contributed 22–24 %FA and 7–8 %FA, respectively, throughout lactation (Table 2). The main mono-unsaturated FA was the n-9 FA oleic acid (OA, C18:1) which was present at stable levels of around 34–38 %FA in all HM samples assessed (Table 2). N-3 polyunsaturated FA (PUFA) add up to a total of about 1.5 %FA, made up of mainly α-linolenic acid (ALA; mean 0.97 %FA, range 0.35–1.64 %FA) and docosahexaenoic acid (DHA; mean 0.17 %FA, range 0.09–0.75 %FA) and small amounts of other n-3 PUFA, like eicosapentaenoic acid (EPA; mean 0.05 %FA, range 0.02–0.21 %FA).

We observed no sex differences in milk AA or FA content or composition.

The HM total FA or total AA content showed no consistent ratio or relation. Using these compositional data, we found no linear correlations between (net) body weight accrual of the infants from birth to 6 months (or per 2 months) and HM total FA or AA content (Figure 4B and C). Also, the FA or AA composition did not correlate with the bodily weight increase observed over the first 6 months (or intervals thereof). Despite an equal birth weight (3586 ± 445 g), weight accrual over the first two months of life was higher in boys than girls (Figure 4A). No other sex differences were observed in the (net) body weight increases in the first half year, irrespective of whether the infants were 'fully breastfed' or not.

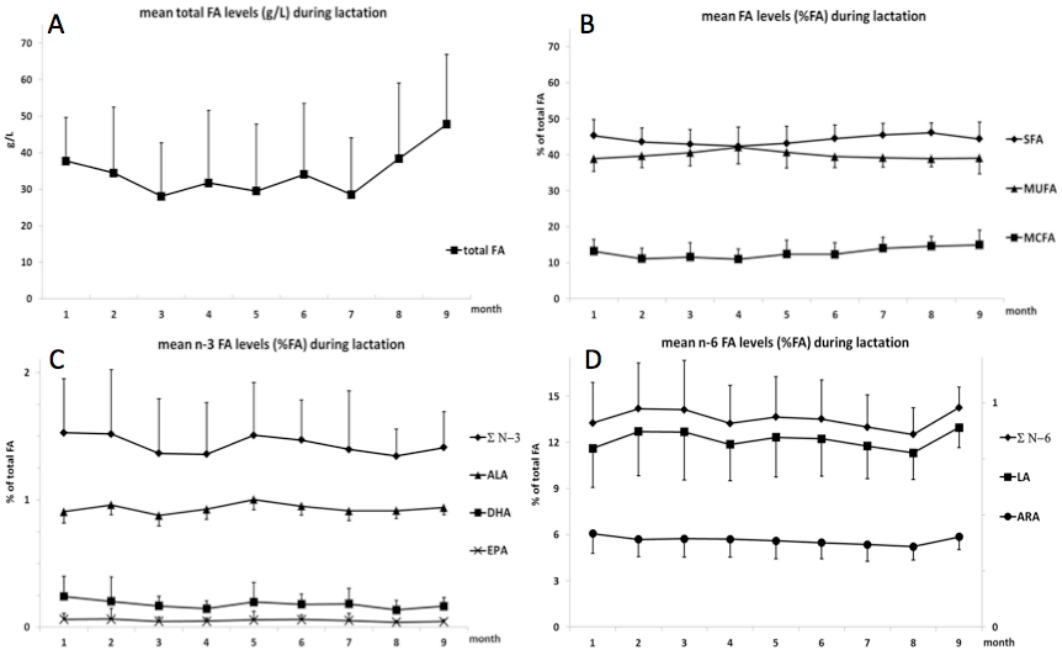

Figure 3. HM fat content overview

A: mean total fat content; B: mean rel. SFA, MUFA and MCFA content; C: mean relative n-3 FA content; D: mean relative n-6 FA content, use right Y-axis for ARA. Data shown are means ± SD, n per time point as shown in Figure 1A. ARA, arachidonic acid; FA, fatty acids; SFA, saturated FA; MUFA, mono-unsaturated FA; MCFA, medium-chain FA.

Next to the average data reported, also two examples of individual series of samples are shown, subject 069 (female infant, first sample at day 11) and 110 (male infant, first sample at day 30), to illustrate the large intra-donor variance in AA and FA levels (quantity) and composition (quality) in the series of monthly samples collected up to 9 months of lactation (Figures 5 and 6). Both donors clearly show higher total AA levels early in lactation (panels A), but a very stable HM AA composition throughout lactation (panels B). This is even more apparent in their HM FA composition which, in spite of quite some variability in the total FA content (panels C), remains qualitatively very stable (panels D).

Discussion

HM composition varies from mother to mother, from day to day, between different time points of the day, and even during one single nursing (32, 33). Yet, HM is likely to be adjusted to the individual infant's requirements and is thus regarded as the gold standard, although data on HM production, and on intake in relation to feeding habits of the breastfed infant over the course of lactation are scarce and/or may require updating (8, 10, 12, 16). In the past, many papers reported on randomly or cross-sectionally collected HM samples, which also yielded insights in longitudinal development and changes in HM composition during lactation. Still, a need for continued research on the longitudinal composition of HM exists.

Here we report the lipid and protein content and composition of 25 HM sample series collected at consecutive monthly intervals up to 9 months after birth in term born healthy infants. Our results show that, on average, both the quantity and quality of lipid and protein, i.e. the content and composition of FA and AA, remain quite stable throughout lactation.

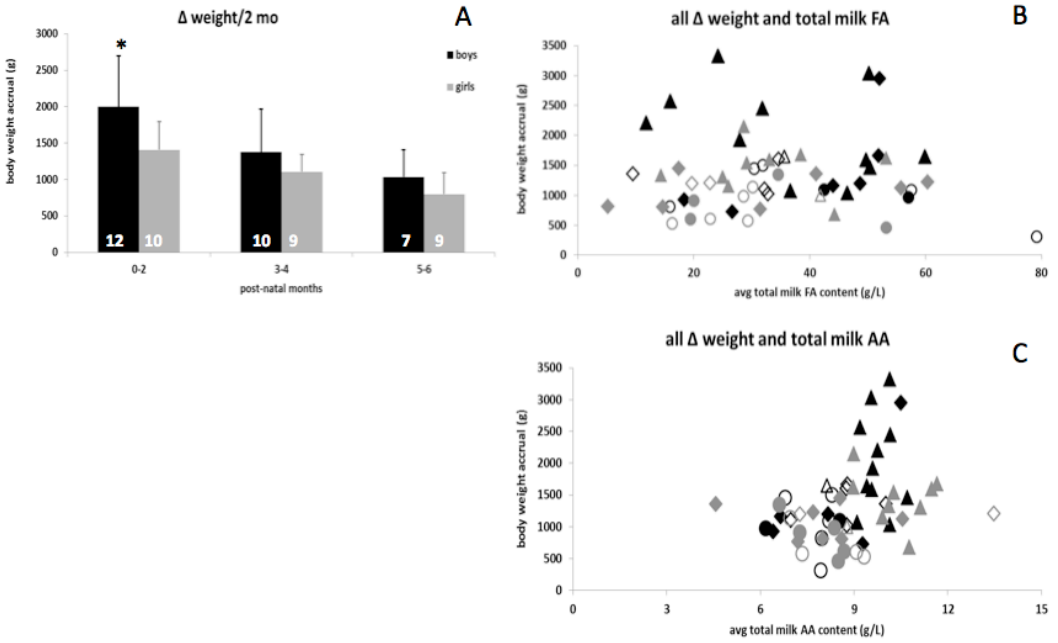

Figure 4. Body weight gain and human milk composition

A: Bimonthly body weight gain per sex; B: Bimonthly body weight gain vs. milk total FA content; C: Bimonthly body weight gain vs. milk total AA content. Data are means ± SD, n in bars; *p<0.05; Δ 0–2 mo, ◊ 3–4 mo, O 5–6 mo; black symbols for boys, grey symbols for girls; closed symbols for fully breastfed, open symbols for mixed fed infants. FA, fatty acids; AA, amino acids.

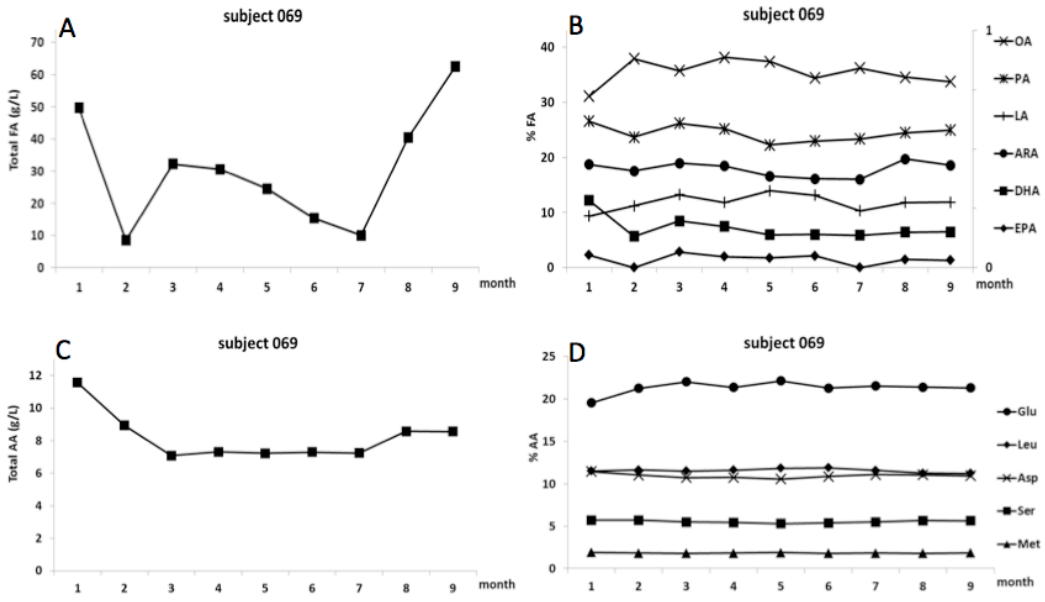

Figure 5. FA and AA content and composition overview of subject 069 (female infant, first sample at day 11 after delivery).

A: total AA content; B: AA composition (in %AA); C: total FA content; D: FA composition (in %FA), use right Y-axis for ARA, DHA and EPA. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; AA, amino acids.

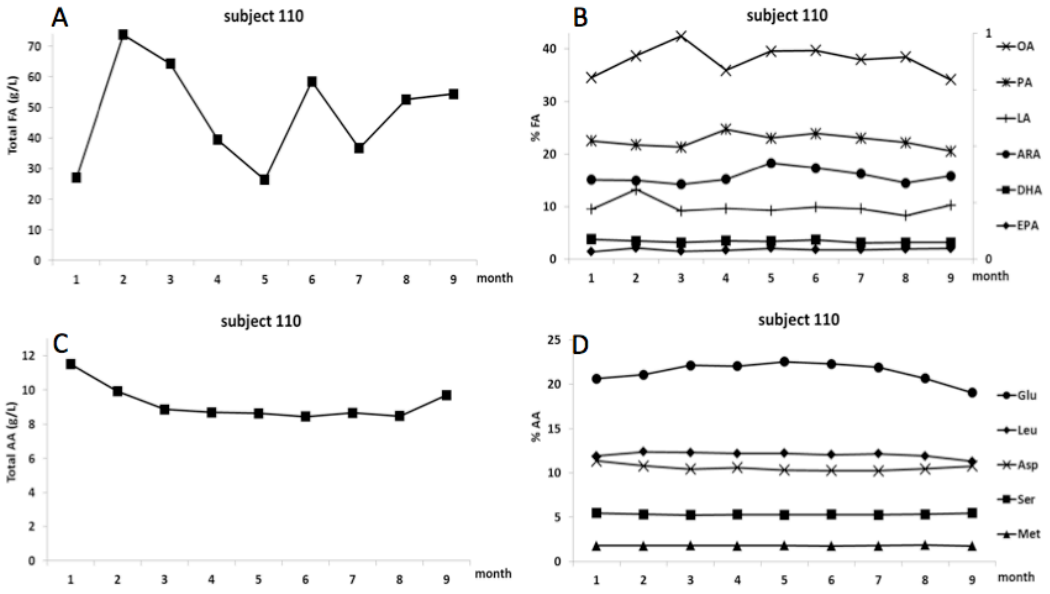

Figure 6. FA and AA content and composition overview of subject 110 (male infant, first sample at day 30 after delivery).

A: total AA content; B: AA composition (in % AA); C: total FA content; D: FA composition (in %FA), use right Y-axis for ARA, DHA and EPA. FA, fatty acids; AA, amino acids; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Typically, three types of HM are discerned (34, 35): colostrum (0–5 d), transitional (6–15 d), and mature milk (>15 d). The earliest sample in our study was obtained on postnatal day 7, followed by three first collections between day 10 and 12, but all other donors started at or after day 15. So, by definition, no colostrum, some transitional HM, and mainly mature HM was collected. The composition of mature HM is known to be much less variable compared to the earlier stages of lactation, and confirmed by our study, which found mean protein levels in line with previous reports (10, 11, 36, 37).

The protein content of HM decreases rapidly during the first month of lactation and declines much more slowly thereafter (10-12, 35, 37), as also confirmed by our data. We did not assess whey to casein ratios, also known to change rapidly during early lactation. As we report the outcome based on the sum of 15 AA levels assessed, we clearly underestimated the 'true' protein content in the samples, confirmed by the total N-content measurements. In agreement with previous reports (11, 35, 37) we found an average NPN content of 20–25%. The contribution of individual AA to total AA calculations observed in our study (with Glu/Gln as highest contributor) confirmed previous reports (35, 38); however, we do not know how much of the AA were present in free form.

The diet of the mother has a major impact on her milk composition: HM EPA and DHA content constitute the highest variability of all FA, and are shown to be closely linked to maternal dietary EPA and DHA intake (17-19, 34, 39, 40). The total levels of DHA and arachidonic acid (ARA) detected in our HM samples confirm findings worldwide (41), showing a relatively low contribution of these LC-PUFAs to total PUFA content. However, no data was collected on maternal diet in our study.

Although previously suggested (42, 43), we found no sex-bias or differences in HM composition related to the sex of the infant, or to the (bimonthly) body weight accrual of the offspring. Regarding the observed higher weight accrual in boys during the first 2 months of life, in line with others (42) and the WHO growth curve, we speculate that boys respond differently to the same macronutrient (i.e. protein) content than girls, or at least are able to use the available nutrients in a more efficient way.

Table 1. Mean (and *SD*) levels of 15 AA, total AA, and defined AA groups in HM samples for month 1 to 9, expressed in absolute amounts (mg/L) and relative amounts (%AA). Also shown is the number of observations per time point (n). Asx= Asp+Asn; Glx= Gln+Glu; EAA, essential AA; NEAA, non-essential AA; BCAA, branched-chain AA.

mg/L	AA	Asx	Glx	Ser	His	Gly	Thr	Arg	Ala	Tyr	Val	Met	Phe	Ile	Leu	Lys	total AA	n	EAA	NEAA	BCAA
month	1	1221.2	2213.5	616.9	290.6	308.9	571.8	475.6	496.5	516.2	671.0	201.8	499.1	670.7	1282.1	918.2	10954.0	23	5105.2	5848.8	2623.8
		173.8	*274.5*	*89.0*	*39.2*	*46.1*	*79.5*	*85.4*	*72.5*	*75.8*	*99.7*	*29.1*	*71.2*	*89.8*	*170.3*	*116.1*	*1468.6*		*683.6*	*789.5*	*357.1*
	2	982.7	1930.9	501.2	237.5	250.1	468.4	370.7	404.4	414.0	549.7	159.4	405.1	555.6	1061.7	753.5	9044.9	25	4190.9	4854.0	2166.9
		282.1	*489.5*	*164.9*	*53.1*	*82.0*	*122.2*	*135.4*	*113.0*	*102.5*	*132.3*	*33.3*	*104.2*	*108.2*	*235.3*	*176.7*	*2074.3*		*466.8*	*492.4*	*242.0*
	3	922.7	1895.9	470.9	227.0	233.1	442.8	349.8	381.4	399.3	532.7	153.4	381.5	534.1	1016.4	716.7	8657.7	24	4004.6	4653.1	2083.2
		331.3	*622.9*	*172.1*	*58.3*	*65.0*	*114.1*	*102.3*	*98.1*	*103.8*	*131.6*	*39.2*	*97.4*	*138.0*	*257.9*	*189.4*	*2139.6*		*816.0*	*923.4*	*413.7*
	4	850.9	1797.7	433.8	209.7	214.1	408.0	317.0	353.4	365.6	495.9	138.6	351.3	495.9	946.3	662.7	8041.1	19	*3708.5*	*4332.6*	*1938.2*
		153.7	*271.0*	*76.1*	*38.1*	*38.1*	*72.6*	*64.7*	*63.4*	*70.3*	*92.5*	*27.5*	*64.7*	*91.8*	*174.0*	*118.3*	*1400.7*		*675.0*	*728.2*	*357.1*
	5	821.4	1702.7	419.3	198.3	210.8	395.2	320.6	346.7	353.3	477.1	131.3	338.7	465.0	893.7	625.9	7699.9	23	3525.1	4174.8	1835.7
		113.8	*203.1*	*58.3*	*29.1*	*30.7*	*53.9*	*52.4*	*46.7*	*50.9*	*66.6*	*21.7*	*48.3*	*67.4*	*128.2*	*88.2*	*1020.4*		*495.7*	*530.5*	*260.3*
	6	823.8	1658.9	433.2	195.4	227.0	404.3	347.2	362.5	349.6	473.4	124.2	345.2	438.1	874.6	622.7	7680.2	22	3478.0	4202.2	1786.2
		149.0	*160.6*	*96.5*	*27.6*	*62.0*	*79.8*	*112.2*	*84.0*	*60.4*	*76.0*	*17.3*	*67.6*	*47.6*	*114.9*	*91.6*	*1151.0*		*495.1*	*664.5*	*227.2*
	7	875.0	1699.8	457.1	206.2	239.8	428.2	373.1	379.5	373.8	507.4	132.5	366.8	464.1	925.5	653.8	8082.5	13	3684.5	4398.0	1897.0
		355.2	*614.8*	*191*	*63.0*	*97.6*	*143.8*	*162.6*	*145.0*	*121.8*	*161.0*	*39.7*	*131.2*	*127.2*	*276.4*	*210.1*	*2533.9*		*727.0*	*894.5*	*354.4*
	8	853.6	1685.2	446.1	205.6	235.3	417.0	357.2	371.9	363.3	492.9	134.7	357.4	463.6	917.5	646.8	7947.9	10	3635.5	4312.4	1874.0
		95.2	*100.3*	*48.4*	*18.8*	*35.1*	*45.9*	*55.0*	*47.2*	*36.3*	*53.5*	*14.7*	*36.9*	*46.4*	*79.1*	*54.0*	*711.6*		*332.3*	*386.1*	*174.8*
	9	979.0	1795.7	519.0	228.4	290.1	481.6	450.3	447.5	411.3	560.4	152.0	420.9	492.6	1010.2	728.7	8967.6	9	4074.7	4892.9	2063.1
		256.7	*535.3*	*138*	*62.7*	*75.3*	*124.6*	*119.3*	*116.4*	*109.3*	*147.7*	*39.8*	*106.9*	*150.2*	*283.0*	*199.3*	*2397.8*		*819.5*	*994.0*	*387.8*

%AA	AA	Asx	Glx	Ser	His	Gly	Thr	Arg	Ala	Tyr	Val	Met	Phe	Ile	Leu	Lys		n	EAA	NEAA	BCAA
month	1	11.1	20.2	5.6	2.7	2.8	5.2	4.3	4.5	4.7	6.1	1.8	4.6	6.1	11.7	8.4		23	46.6	53.4	24.0
		0.3	*0.7*	*0.2*	*0.0*	*0.2*	*0.1*	*0.4*	*0.2*	*0.1*	*0.1*	*0.1*	*0.1*	*0.2*	*0.3*	*0.3*			*0.5*	*0.5*	*0.5*
	2	10.9	21.4	5.5	2.6	2.8	5.2	4.1	4.5	4.6	6.1	1.8	4.5	6.1	11.7	8.3		25	46.3	53.7	23.9
		2.4	*5.4*	*1.1*	*0.5*	*0.6*	*1.1*	*1.0*	*0.9*	*0.9*	*1.3*	*0.3*	*0.9*	*1.2*	*2.4*	*1.7*			*0.7*	*0.7*	*0.6*
	3	10.6	22.0	5.4	2.6	2.7	5.1	4.0	4.4	4.6	6.1	1.8	4.4	6.2	11.7	8.3		24	46.2	53.8	24.1
		3.8	*5.6*	*3.9*	*0.5*	*0.6*	*1.1*	*0.9*	*0.9*	*1.0*	*1.3*	*0.4*	*0.9*	1.2	*2.4*	*1.8*			*0.5*	*0.5*	*0.5*
	4	10.6	22.5	5.4	2.6	2.7	5.1	3.9	4.4	4.5	6.2	1.7	4.4	6.2	11.8	8.2		19	46.1	53.9	24.1
		0.2	*0.8*	*0.1*	*0.0*	*0.1*	*0.1*	*0.2*	*0.2*	*0.1*	*0.1*	*0.1*	*0.1*	0.2	*0.2*	*0.2*			*0.6*	*0.6*	*0.5*
	5	10.7	22.2	5.4	2.6	2.7	5.1	4.2	4.5	4.6	6.2	1.7	4.4	6.0	11.6	8.1		23	45.7	54.3	23.8
		0.3	*0.8*	*0.2*	*0.1*	*0.2*	*0.1*	*0.4*	*0.2*	*0.1*	*0.1*	*0.1*	*0.1*	0.2	*0.3*	*0.3*			*0.8*	*0.8*	*0.6*
	6	10.7	21.8	5.6	2.5	2.9	5.2	4.4	4.7	4.5	6.2	1.6	4.5	5.8	11.4	8.1		22	45.3	54.7	23.3
		0.4	*1.8*	*0.4*	*0.1*	*0.4*	*0.3*	*0.8*	*0.4*	*0.1*	*0.2*	*0.1*	*0.2*	0.5	*0.5*	*0.3*			*0.9*	*0.9*	*1.0*
	7	10.8	21.3	5.6	2.6	2.9	5.3	4.5	4.6	4.6	6.2	1.6	4.5	5.8	11.5	8.1		13	45.6	54.4	23.5
		2.9	*6.6*	*1.9*	*0.7*	*0.9*	*1.5*	*1.3*	*1.3*	*1.3*	*1.7*	*0.5*	*1.3*	1.7	*3.2*	*2.3*			*0.7*	*0.7*	*0.8*
	8	10.7	21.3	5.6	2.6	2.9	5.2	4.5	4.7	4.6	6.2	1.7	4.5	5.8	11.6	8.1		10	45.7	54.3	23.6
		0.3	*1.1*	*0.2*	*0.1*	*0.2*	*0.2*	*0.4*	*0.2*	*0.1*	*0.2*	*0.1*	*0.1*	0.3	*0.3*	*0.3*			*0.6*	*0.6*	*0.6*
	9	10.9	20.3	5.8	2.6	3.2	5.3	4.9	4.9	4.6	6.2	1.7	4.7	5.5	11.3	8.1		9	45.4	54.6	23.1
		3.5	*6.6*	*1.8*	*0.8*	*0.9*	*1.7*	*1.5*	*1.5*	*1.5*	*2.0*	*0.5*	*1.4*	1.9	*3.7*	*2.6*			*0.54*	*0.54*	*0.64*

Table 2. Table showing means (and *SD*) of FA levels assessed in HM samples for month 1 to 9, expressed in absolute amounts (mg/L) and relative amounts (%FA). n= number of observations per time point.

mg/L	FA	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1 n-x	C15:0	C16:0	C16:0 dma	C16:1 n-7	C16:1 n-7 tr	C17:0	C18:0	C18:1 n-7	C18:1 n-9	C18:1 tr	C18:2 n-6	C18:3 n-3	C18:3 n-6	C18:4 n-3
month	1	0.0	46.3	502.0	2188.7	2350.6	86.0	120.7	9160.1	6.2	818.7	29.8	107.6	2628.0	661.1	12839.0	220.1	4378.8	347.3	49.5	44.9
		0.0	*21.9*	*192.7*	*965.0*	*967.2*	*37.7*	*44.7*	*3003.1*	*4.3*	*324.4*	*15.6*	*36.6*	*1118.8*	*207.0*	*3931.5*	*121.7*	*1773.9*	*187.6*	*28.2*	*15.7*
	2	0.0	37.8	394.7	1621.6	1875.1	76.7	110.8	8212.9	3.0	709.9	33.3	100.1	2486.4	600.7	12073.4	232.7	4435.1	355.1	46.3	40.0
		0.0	*29.2*	*228.5*	*989.7*	*1191.4*	*51.3*	*68.9*	*4480.5*	*3.6*	*365.7*	*25.3*	*54.8*	*1346.8*	*334.5*	*6393.5*	*181.9*	*2533.3*	*244.7*	*32.2*	*24.6*
	3	0.0	28.1	306.9	1358.5	1479.0	54.0	82.0	6371.1	1.5	570.7	23.7	76.8	2074.1	481.8	10270.0	166.1	3644.8	251.8	39.3	31.3
		0.0	*18.1*	*166.7*	*915.7*	*924.4*	*27.7*	*40.8*	*3193.8*	*3.1*	*281.5*	*14.5*	*37.4*	*1322.1*	*252.7*	*5683.2*	*123.5*	*2372.5*	*169.8*	*22.3*	*15.9*
	4	0.0	32.9	339.4	1452.3	1663.5	71.1	101.0	7401.3	1.9	714.6	29.9	90.7	2307.5	565.2	11896.3	190.8	3724.1	301.5	46.2	33.2
		0.0	*27.4*	*204.7*	*1008.9*	*1143.6*	*46.5*	*64.6*	*4732.1*	*3.2*	*493.1*	*23.0*	*56.1*	*1415.2*	*382.9*	*7487.4*	*152.5*	*2727.5*	*238.3*	*32.9*	*20.8*
	5	0.8	30.5	336.5	1581.9	1713.6	54.3	81.6	6567.4	1.9	567.0	22.6	77.5	2034.2	538.9	10767.9	210.7	3780.1	294.8	38.0	28.7
		3.6	*26.9*	*221.4*	*1245.2*	*1334.4*	*30.5*	*43.5*	*3880.0*	*3.0*	*345.2*	*15.8*	*41.5*	*1223.7*	*375.0*	*6723.3*	*242.3*	*2849.1*	*202.2*	*27.6*	*15.9*
	6	0.6	34.2	366.2	1858.2	2045.0	68.7	104.6	8150.9	2.1	705.3	29.8	98.9	2752.6	577.3	12124.7	231.0	4021.6	318.8	39.3	35.1
		2.7	*28.4*	*212.1*	*1312.5*	*1490.2*	*51.4*	*74.6*	*5268.8*	*3.6*	*508.9*	*26.2*	*67.1*	*1887.0*	*386.7*	*7128.3*	*195.3*	*2071.5*	*186.3*	*26.9*	*20.6*
	7	0.0	30.0	352.6	1787.0	1976.2	56.7	84.6	6536.9	2.0	523.5	19.0	81.7	2199.9	457.0	9968.0	165.9	3246.2	270.1	26.9	29.7
		0.0	*20.1*	*191.6*	*1089.5*	*1257.6*	*38.3*	*50.9*	*3655.2*	*3.1*	*322.9*	*13.0*	*51.7*	*1314.1*	*249.5*	*5450.8*	*118.6*	*1718.6*	*168.3*	*17.2*	*19.1*
	8	0.0	44.1	473.9	2398.3	2900.4	83.6	124.7	9137.5	2.9	678.5	29.6	112.2	2913.4	574.7	13283.8	255.4	4090.4	339.5	37.8	41.7
		0.0	*29.2*	*258.9*	*1416.9*	*1909.2*	*58.5*	*81.3*	*5209.8*	*3.6*	*380.3*	*23.2*	*70.5*	*1758.8*	*278.5*	*6941.6*	*237.8*	*2005.2*	*186.5*	*22.3*	*26.7*
	9	0.0	52.0	558.5	3072.2	3593.2	74.8	125.1	10247.5	7.2	812.0	25.1	127.6	3236.1	748.0	16997.7	235.5	6196.9	428.5	46.3	46.8
		0.0	*30.9*	*260.3*	*1537.7*	*2010.8*	*37.5*	*57.6*	*4185.6*	*5.5*	*343.3*	*12.5*	*56.7*	*1249.0*	*335.0*	*7318.7*	*190.2*	*2635.3*	*194.4*	*19.2*	*22.8*

%FA	FA	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1 n-x	C15:0	C16:0	C16:0 dma	C16:1 n-7	C16:1 n-7 tr	C17:0	C18:0	C18:1 n-7	C18:1 n-9	C18:1 tr	C18:2 n-6	C18:3 n-3	C18:3 n-6	C18:4 n-3
month	1	0.0	0.1	1.3	5.7	6.1	0.2	0.3	24.3	0.0	2.2	0.1	0.3	6.9	1.8	34.3	0.6	11.6	0.9	0.1	0.1
		0.0	*0.0*	*0.3*	*1.6*	*1.4*	*0.1*	*0.1*	*2.1*	*0.0*	*0.6*	*0.0*	*0.0*	*1.5*	*0.3*	*3.1*	*0.2*	*2.5*	*0.3*	*0.1*	*0.0*
	2	0.0	0.1	1.1	4.6	5.3	0.2	0.3	24.0	0.0	2.2	0.1	0.3	7.5	1.8	35.2	0.6	12.7	1.0	0.1	0.1
		0.0	*0.0*	*0.2*	*1.5*	*1.3*	*0.1*	*0.1*	*2.6*	*0.0*	*0.6*	*0.0*	*0.1*	*1.4*	*0.3*	*2.9*	*0.3*	*2.9*	*0.3*	*0.0*	*0.0*
	3	0.0	0.1	1.1	4.9	5.4	0.2	0.3	23.1	0.0	2.1	0.1	0.3	7.3	1.7	36.2	0.7	12.7	0.9	0.1	0.1
		0.0	*0.0*	*0.3*	*2.1*	*1.7*	*0.1*	*0.1*	*2.5*	*0.0*	*0.6*	*0.0*	*0.1*	*1.2*	*0.3*	*3.4*	*0.4*	*3.1*	*0.4*	*0.0*	*0.0*
	4	0.0	0.1	1.1	4.6	5.2	0.2	0.3	23.1	0.0	2.2	0.1	0.3	7.3	1.8	37.6	0.6	11.9	0.9	0.1	0.1
		0.0	*0.0*	*0.2*	*1.4*	*1.4*	*0.1*	*0.1*	*2.8*	*0.0*	*0.6*	*0.0*	*0.0*	*1.4*	*0.4*	*4.4*	*0.3*	*2.4*	*0.3*	*0.1*	*0.0*
	5	0.0	0.1	1.2	5.3	5.8	0.2	0.3	22.8	0.0	2.0	0.1	0.3	7.1	1.8	36.4	0.6	12.3	1.0	0.1	0.1
		0.0	*0.0*	*0.3*	*2.0*	*1.8*	*0.1*	*0.1*	*2.6*	*0.0*	*0.6*	*0.0*	*0.1*	*1.6*	*0.3*	*4.3*	*0.4*	*2.6*	*0.3*	*0.0*	*0.0*
	6	0.0	0.1	1.1	5.4	5.8	0.2	0.3	23.4	0.0	1.9	0.1	0.3	7.9	1.7	35.4	0.7	12.2	1.0	0.1	0.1
		0.0	*0.0*	*0.2*	*1.4*	*1.5*	*0.1*	*0.1*	*1.9*	*0.0*	*0.6*	*0.0*	*0.0*	*1.4*	*0.3*	*2.6*	*0.3*	*2.4*	*0.2*	*0.0*	*0.0*
	7	0.0	0.1	1.2	6.1	6.6	0.2	0.3	22.8	0.0	1.7	0.1	0.3	7.7	1.6	35.4	0.6	11.8	0.9	0.1	0.1
		0.0	*0.1*	*0.2*	*1.6*	*1.6*	*0.1*	*0.1*	*1.7*	*0.0*	*0.4*	*0.0*	*0.0*	*1.2*	*0.2*	*2.5*	*0.3*	*2.1*	*0.2*	*0.0*	*0.0*
	8	0.0	0.1	1.2	6.1	7.1	0.2	0.3	23.2	0.0	1.7	0.1	0.3	7.4	1.6	35.2	0.7	11.3	0.9	0.1	0.1
		0.0	*0.0*	*0.1*	*1.0*	*1.8*	*0.1*	*0.1*	*2.2*	*0.0*	*0.3*	*0.0*	*0.0*	*1.0*	*0.2*	*2.1*	*0.5*	*1.7*	*0.2*	*0.0*	*0.0*
	9	0.0	0.1	1.2	6.3	7.4	0.2	0.3	21.6	0.0	1.8	0.1	0.3	6.9	1.6	35.3	0.5	13.0	0.9	0.1	0.1
		0.0	*0.0*	*0.2*	*1.5*	*2.4*	*0.1*	*0.1*	*2.5*	*0.0*	*0.4*	*0.0*	*0.0*	*1.0*	*0.4*	*4.1*	*0.3*	*1.3*	*0.3*	*0.0*	*0.0*

Table 3. Table showing mean (SD) of levels of defined FA groups in HM samples for month 1 to 9, expressed in absolute amounts (mg/L) and relative amounts (%FA). Also shown is the ratio of n-3: n-6 PUFA and LA:ALA, and the number of observations per time point (n). MCFA, medium-chain fatty acids (C6-C14); SFA, saturated FA; MUFA, mono-unsaturated FA; n-3 PUFA, n-3 polyunsaturated FA; n-6 PUFA, polyunsaturated FA; LA, linoleic acid; ALA, α-linolenic acid.

mg/L	FA	MCFA	SFA	MUFA	n-3 PUFA	n-6 PUFA	n-6/n-3	LA/ALA	n
month	1	5087.7	17255.9	14531.2	579.1	4995.3	9.0	12.2	25
		2116.4	*6035.0*	*4408.2*	*245.9*	*1933.4*	*1.8*	*3.5*	
	2	3929.1	14970.9	13561.0	554.3	4949.4	10.0	12.1	25
		2395.6	*8168.4*	*7119.9*	*344.1*	*2785.4*	*2.9*	*4.2*	
	3	3172.5	11884.9	11464.8	393.3	4056.3	11.0	12.3	24
		1991.3	*6078.1*	*6250.7*	*241.9*	*2575.9*	*3.0*	*5.2*	
	4	3488.0	13501.4	13320.6	443.0	4150.2	10.5	11.9	22
		2349.9	*8434.8*	*8386.0*	*326.1*	*2991.5*	*3.8*	*4.6*	
	5	3663.2	12531.6	12018.8	442.2	4167.8	9.6	11.9	23
		2801.7	*7698.3*	*7454.9*	*272.7*	*3061.7*	*2.5*	*3.9*	
	6	4304.2	15534.9	13561.9	486.1	4446.4	9.4	13.1	21
		3030.3	*10173.3*	*8070.7*	*259.2*	*2279.7*	*1.7*	*2.8*	
	7	4145.8	13158.8	11086.5	431.0	3608.7	10.4	13.9	11
		2529.0	*7470.9*	*6086.0*	*264.5*	*1903.0*	*4.1*	*3.6*	
	8	5816.6	18239.2	14704.3	514.3	4550.0	9.4	15.3	10
		3586.9	*10434.6*	*7666.5*	*288.4*	*2231.6*	*1.4*	*1.9*	
	9	7275.9	21185.2	18769.7	645.8	6810.7	10.8	14.4	8
		3781.5	*8848.6*	*7928.5*	*252.7*	*2871.9*	*3.6*	*2.6*	

%FA	FA	MCFA	SFA	MUFA	n-3 PUFA	n-6 PUFA			n
month	1	13.2	45.4	38.9	1.5	13.3			25
		3.3	*4.5*	*3.5*	*0.4*	*2.6*			
	2	11.1	43.6	39.7	1.5	14.2			25
		2.9	*3.9*	*3.3*	*0.5*	*3.0*			
	3	11.6	43.0	40.6	1.4	14.1			24
		3.9	*4.0*	*3.7*	*0.4*	*3.2*			
	4	11.0	42.4	42.1	1.4	13.2			22
		2.9	*5.3*	*4.6*	*0.4*	*2.5*			
	5	12.4	43.2	40.7	1.5	13.7			23
		3.9	*4.7*	*4.4*	*0.4*	*2.6*			
	6	12.3	44.6	39.5	1.5	13.5			21
		3.3	*3.7*	*3.0*	*0.3*	*2.5*			
	7	14.0	45.5	39.2	1.4	13.0			11
		3.0	*3.2*	*2.6*	*0.5*	*2.1*			
	8	14.6	46.2	38.9	1.3	12.5			10
		2.7	*2.7*	*2.2*	*0.2*	*1.7*			
	9	14.9	44.4	39.1	1.4	14.3			8
		4.1	*4.6*	*4.4*	*0.3*	*1.3*			

In contrast to other studies, HM sample collection in the PreventCD cohort was optional and not strictly supervised (10, 12, 36, 44). This probably supported study participation and retention, at the expense of a well-protocolled sample collection. Factors known to greatly impact HM nutrient (fat) content include, amongst others, the milk amount removed at preceding and current breast-feed bouts, and the interval length between breast-feeds, variable that were not controlled for in this study. HM samples could be collected by donors throughout the day at their own convenience, from either breast, before or after a feeding session, and could comprise fore- or hind milk (or both). This resulted in a high inter- and intra-individual sample variability, which may be particularly relevant for the measurement of fat content as illustrated by the data (see Figures 5 and 6). Despite this, FA and AA composition appeared to be rather stable throughout lactation, as also noted for fat in HM before (11, 40, 44, 45).

The high inter-individual variability in HM macronutrient levels might have resulted in different daily intakes in infants of the same age which could have affected their growth rate. However, we did not find any correlation between the fat or protein content of the individual monthly HM samples and growth (i.e. body weight accrual) of the infants, either exclusively breastfed or not. As we did not collect any data on HM yield, the amount of HM transferred to the suckling infant per breast-feed, or frequency of breastfeeds during the day, we were unable to calculate daily protein and fat intake, which might be a more logical and relevant entity to relate to growth (12). On the other hand, Mitoulas (36) could not find any relationship between growth and HM macronutrient concentration or intake during the first 6 months of life when using controlled measures as described, but does conclude that a 'normal' amount of HM intake was the first prerequisite for healthy growth. In addition, in a recent, more extensive study on 614 mother-infant pairs (46), only the composition of hind milk samples collected 1–2 months after delivery were designated 'relevant', as these correlated positively with the infant's growth. In contrast, the caloric and fat content of HM samples collected between 3 and 12 months showed a wide variation and were found to negatively associate with weight accrual. This suggests that particularly in the early phase of lactation, there might be a correlation between macronutrient levels and growth velocity, although this assumption is not supported by our data.

A strength of our study includes the longitudinal and monthly frequency of HM sampling, which provides on average a reliable reflection of the infant's nutritional supply throughout lactation, although we have no information on volume intakes.

The best source of nutrition for a newborn infant is HM from its (healthy) mother. Whether the composition of the HM is tailored to her infant remains unknown. It is likely that a combination of maternal factors, such as health condition and diet, and specific traits of her infant (including, but not limited to genetic factors, and pregnancy conditions impacting gestational age and birth weight) may affect this relationship (8, 10, 40, 46). Hence, assessing HM composition is only a first step in defining the nutritional needs for optimal growth of healthy term babies (8, 20, 34). Different aspects of weight and length gain should be taken into consideration, as should functional outcomes (e.g. visual, IQ, immune, and psychomotor development). Although we realize that the nutritional requirements for growth should provide a base to inform the content and composition of (new to develop) infant formula concepts, knowledge on the (longitudinal changes in) HM macronutrient content and composition as well as other bioactive constituents, and their functionality may provide valuable new insights into the relation between growth characteristics and nutrient supply in early life.

Conclusions

This paper shows that the inter- and intra-individuality of HM composition are limited: On average HM composition shows a stable pattern both in fatty and amino acid profile during lactation.

Ethical approval

The PreventCD study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC), was compliant to Good Clinical Practice guidelines and regulations (ICH-GCP), and was conducted according to the guidelines laid down in the Declaration of Helsinki. The PreventCD trial was registered as ISRCTN74582487. Participants gave their written informed consent before study entry; HM samples were donated monthly.

Author contribution

MLM and MWS designed, conducted and supervised the clinical study; BJMvdH and BS conducted and supervised the sample analyses; BJMvdH wrote the paper; BS, MLM, EMvdB and EHHMR gave input and critically reviewed the paper. All authors read and approved the final manuscript.

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