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Original article

Longitudinal human milk macronutrients, body composition and infant appetite during early life

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SUMMARY

Background & aims: Breastfeeding is the gold standard infant feeding. Data on macronutrients in relation to longitudinal body composition and appetite are very scarce. The aim of this study was to investigate longitudinal human milk macronutrients at 1 and 3 months in association with body composition and appetite during early life in healthy, term-born infants. We hypothesized that infants receiving higher caloric human milk would have more body fat mass and satiate earlier.

Methods: In 133 exclusively breastfed infants (Sophia Pluto Cohort), human milk samples at 1 and 3 months were analyzed for macronutrients (fat, protein, carbohydrate) by MIRIS Human Milk Analyzer, with appetite assessment by Baby Eating Behavior Questionnaires. Fat mass (FM) and fat-free mass (FFM) were measured by PEA POD and DXA, and abdominal FM by ultrasound.

Results: Milk samples showed large differences in macronutrients, particularly in fat content. Protein and energy content decreased significantly from 1 to 3 months. Fat and carbohydrate content tended to decrease ($p = 0.066$ and 0.081). Fat (g/100 ml) and energy (kcal/100 ml) content at 3 months were associated with FM% at 6 months (β 0.387 and 0.040, resp.) and gain in FM% from 1 to 6 months (β 0.088 and 0.009, resp.), but not with FM% at 2 years. Carbohydrate content at 3 months tended to associate with visceral FM at 2 years (β 0.290, $p = 0.06$). Infants receiving higher caloric milk were earlier satiated and finished feeding faster.

Conclusions: Our longitudinal data show decreasing milk protein and energy content from age 1 to 3 months, while fat and carbohydrate tended to decrease. Macronutrient composition, particularly fat content, differed considerably between mothers. Milk fat and energy content at 3 months associated with gain in FM% from age 1 to 6 months, indicating that higher fat and energy content associate with higher gain in FM% during the critical window for adiposity programming. As infants receiving higher caloric breastfeeding were earlier satiated, this self-regulatory mechanism might prevent intake of excessive macronutrients.

Online trial registry: NTR, NL7833.

1. Introduction

Breastfeeding is considered to be the gold standard infant feeding, because it can result in health benefits for mother and child [1]. Breastfeeding lowers the risk of adiposity during childhood [2–6]. Breastfeeding is also a protective factor against several infections [1], asthma development [7], eczema and allergic rhinitis [8] by supporting the development of the immune system and microbiota [9]. For mothers, breastfeeding lowers the risk of breast cancer [1].

Human milk is a dynamic fluid, with changes in composition from early to late lactation [10]. It is likely that human milk composition affects infant growth [11]. Human milk is composed of macronutrients, micronutrients and bioactive factors [10]. Different techniques exist for analyzing human milk macronutrient composition [12]. Nowadays, infrared human milk analyzers (HMA), a fast

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Abbreviations

ADP	Air-displacement plethysmography
BEBQ	Baby eating behavior questionnaires
BF	Breastfeeding
BMI	Body mass index
CVD	Cardiovascular diseases
DXA	Dual energy x-ray absorptiometry
FFM	Fat-free mass
FFMI	Fat-free mass index
FFM%	Fat-free mass percentage
FM	Fat mass
FMI	Fat mass index
FM%	Fat mass percentage
HMA	Human milk analyzers
IQR	Interquartile range
SDS	Standard deviation score

method to estimate macronutrient composition, are being used in clinical settings [13], mainly to determine adequate fortification of donor human milk for feeding preterm infants [14]. In research, some studies have used HMA to assess macronutrient human milk composition in term-born infants [11,15–17].

The first 1000 days period, from conception until age 2 years, is important for body and brain development, and obesity prevention [18]. Studies investigating human milk macronutrients in association with longitudinal body composition (fat mass percentage (FM) and fat-free mass (FFM)), and appetite during this period are very scarce in healthy term-born infants [11]. One study stated that human milk protein inversely associated with the rate of body fat gain until age 4 months in 41 infants [19]. Another study presented the effect of only carbohydrates on body composition measured by skinfolds and bioelectrical impedance measurements in 20 infants until age 12 months and concluded that human milk carbohydrate concentrations associated with decreased FM% and FMI [20].

Within this 1000 days period, the first 6 postnatal months are considered a critical window for adiposity programming [21–24]. Accelerated weight gain during that period associated with more adiposity and a less favorable health profile at 21 years [21]. We previously found that not only weight gain, but specifically accelerated gain in FM% in the first 6 months of life associated with higher FM%, already at age 2 years [22]. We now present data on human milk macronutrients and their associations with longitudinally measured body composition, abdominal FM and appetite during this critical window for adiposity programming and at age 2 years in healthy term-born infants.

The primary objective of this study was to investigate longitudinal human milk macronutrient composition and the associations with body composition and abdominal fat mass in infants during the first 6 months of life and at age 2 years. We hypothesized that human milk with higher caloric value would result in infants having more body fat mass and visceral fat mass during early life compared to those receiving low caloric human milk. The secondary objective was to investigate the associations between human milk macronutrient composition and infant appetite.

2. Subjects and methods

2.1. Study settings and subjects

The study population consisted of healthy, term-born infants, participating in the Sophia Pluto Study, a birth cohort study in the Rotterdam area (The Netherlands). Between January 2013 and March 2020, infants were recruited to obtain detailed data on body composition and growth during early life. The Sophia Pluto Study obtained approval by the Medical Ethics Committee of Erasmus University Medical Center and parents gave written informed consent.

All participants fulfilled the following inclusion criteria: term born (≥37 weeks of gestation), age < 28 days, uncomplicated neonatal period without signs of severe asphyxia (defined as an Apgar score < 3 after 5 min), sepsis or long-term complication of respiratory ventilation. For this study, infants who were exclusively breastfed during the first 3 months of life, with breastmilk samples of their mothers at age 1 and 3 months, were included. Infants were excluded if they had known congenital or postnatal diseases, confirmed intrauterine infection, maternal use of corticosteroids during pregnancy or a significant maternal medical condition that could interfere with the study results.

2.2. Data collection and measurements

Outpatient clinic visits were scheduled at age 1, 3, 6, 9, 12, 18 and 24 months (Table 1). Data on pregnancy and birth were obtained from medical records and measurements were performed by trained staff. Ethnicity groups were classified as White/Caucasian [1], Black/African [2], Asian [3], Latin-American [4] or other [5].

2.3. Anthropometrics

Weight was measured with an electronic infant scale to the nearest 5 g (SECA 717, Hamburg, Germany). Length was measured twice by the two-person technique with an infantometer to the nearest 0.1 cm (SECA 416). Head circumference was measured twice as the widest frontal-occipital circumference with a measuring tape to the nearest 0.1 cm (SECA 201). Weight-for-length, weight-for-age and height-for-age standard deviation scores (SDS) were calculated using Growth Analyser (https://growthanalyser.org/; Talma, 2010).

2.4. Body composition measurements

Up to and including age 6 months, body composition was assessed by air-displacement plethysmography (ADP by PEA POD, COSMED, Italy) as described in detail elsewhere [25]. The PEA POD was calibrated daily, according to standard protocol [26].

From 6 months onwards, a Dual Energy X-ray Absorptiometry (DXA) scan was performed at every visit in all infants, with the use of a vacuum cushion (465 75100, B.u.W Schmidt GmbH, Germany) to reduce movement artifacts [27]. All DXA scans were performed with the same device (DXA, Lunar Prodigy, GE Healthcare, UK) and software (enCORE software version 14.1). We previously reported that FM% was measured in triplicate at the transition point of 6 months in 278 infants; by PEA POD and DXA with versus without vacuum cushion. Median FM% was 24.1 by ADP and 25.0 by DXA with vacuum cushion, with a median difference of 0.9% between both measurements and no potential bias (Bland–Altman analysis: $p = 0.32$) [27]. DXA without vacuum cushion did show potential bias, most likely due to movement artifacts and was thus inaccurate [27].

Fat mass index (FMI) was determined by dividing fat mass (kg) by height squared (m^2).

2.5. Abdominal fat mass measurements

Abdominal subcutaneous and visceral fat thickness (cm) were measured by ultrasound at every visit starting from age 3 months,

Table 1
Clinical characteristics of exclusively breastfed infants.

N [Male]		Birth	1 month	3 months	6 months	9 months	12 months	18 months	24 months
		133 [66]	133 [66]	133 [66]	133 [66]	133 [66]	133 [66]	133 [66]	133 [66]
Weight (kg)	M	3.51 [0.46]	4.52 [0.58]	6.29 [0.68]	7.85 [0.82]	8.96 [0.94]	9.79 [1.09]	11.22 [1.22]	12.50 [1.43]
	F	3.41 [0.50]	4.20 [0.53]	5.75 [0.65]	7.21 [0.73]	8.35 [0.80]	9.20 [0.87]	10.71 [0.98]	12.01 [1.19]
Length (cm)	M	51.4* [2.32]	55.3 [2.03]	62.3 [1.98]	68.4 [2.48]	72.6 [2.38]	76.4 [2.72]	82.8 [3.07]	88.5 [3.78]
	F	50.3* [2.23]	54.3 [2.13]	60.6 [2.30]	66.8 [2.35]	70.9 [2.62]	74.8 [2.60]	81.5 [2.76]	87.7 [3.20]
Head circumference (cm)	M	NA	37.6 [1.30]	40.7 [1.34]	43.5 [1.43]	45.3 [1.41]	46.4 [1.44]	48.0 [1.41]	48.7 [1.72]
	F	NA	36.5 [1.04]	39.5 [1.12]	42.1 [1.11]	44.0 [1.30]	45.0 [1.22]	46.6 [1.25]	47.6 [1.29]
FM (%)	M	NA	16.3 [4.20]	23.0 [4.50]	24.5 [4.28]	22.1 [4.84]	20.6 [4.02]	17.9 [4.54]	17.2 [3.91]
	F	NA	17.3 [3.89]	22.9 [4.76]	25.3 [5.06]	23.5 [5.14]	21.0 [4.76]	19.5 [5.06]	18.8 [4.34]
FMI (kg/m^2)	M	NA	2.44 [0.76]	3.76 [0.97]	4.13 [0.93]	3.78 [1.02]	3.46 [0.85]	2.96 [0.91]	2.76 [0.78]
	F	NA	2.48 [0.67]	3.60 [0.90]	4.13 [0.99]	3.93 [1.00]	3.47 [0.95]	3.17 [0.95]	2.95 [0.81]
Subcutaneous FM (cm)	M	NA	NA	0.43 [0.12]	0.45 [0.12]	0.40 [0.10]	0.35 [0.09]	0.31 [0.09]	0.33 [0.09]
	F	NA	NA	0.39 [0.12]	0.44 [0.13]	0.38 [0.09]	0.33 [0.09]	0.33 [0.10]	0.34 [0.10]
Visceral FM (cm)	M	NA	NA	2.34 [0.65]	2.18 [0.56]	2.22 [0.56]	2.40 [0.58]	2.40 [0.70]	2.23 [0.53]
	F	NA	NA	2.36 [0.62]	2.11 [0.61]	2.36 [0.69]	2.44 [0.52]	2.30 [0.54]	2.16 [0.55]

Data expressed as pooled means [pooled standard deviation of the means] for male (M) and female (F). *Available for 37 boys and 44 girls. NA; not applicable.

because earlier measurements are unreliable [25,28]. Unsuccessful ultrasound measurements of visceral fat mass, without visualization of the lumbar vertebra, were excluded from analyses.

2.6. Breastmilk samples

Mothers were instructed to collect hind milk samples, thus after their infants were breastfed (BF), at infant's age of 1 and 3 months. Samples were frozen at −18 °C at home until study visits at the hospital and thereafter at −80 °C until analysis. Breastmilk concentrations of fat, crude and true protein, carbohydrate and energy were analyzed using a Human Milk Analyzer (HMA, MIRIS, Uppsala, Sweden). Before analysis, samples were warmed to 40 °C and homogenization was obtained by an ultrasonic processor (MIRIS, Uppsala, Sweden). The HMA was cleaned and calibrated according to manufacturer's protocol and samples with protein values <0.5 g/100 ml were classified as 'bad samples'. We used the same MIRIS HMA device for all analyses, which were performed by the same investigator (KF).

Human milk composition was measured in triplo. The intra-assay mean [95% confidence interval] coefficients of variance were 1.1% [1.0–1.2] for fat, 2.9% [2.5–3.2%] for crude protein, 2.9% [2.5–3.3] for true protein, 1.7% [1.1–2.3] for carbohydrate and 1.1% [0.9–1.2] for energy. The samples were divided in groups based on time of collection to investigate circadian variations: during night/early morning (00:00–09:00) and morning (09:00–12:00). For the comparison of fasting versus non-fasting human milk samples, we defined samples as fasting samples if mothers collected the milk sample in the morning before their breakfast. For comparison of macronutrient content in breastmilk versus formula feeding, we calculated median macronutrients per 100 ml of 6 common formula feeding brands available in the Netherlands.

2.7. Baby Eating Behavior Questionnaires (BEBQ)

At age 1 and 3 months, mothers were asked to fill out the Baby Eating Behavior Questionnaire (BEBQ) to assess infant appetite [29]. Each item was answered using a five-point Likert frequency scale (1 = never, 2 = rarely, 3 = sometimes, 4 = often and 5 = always). To investigate whether exclusively breastfed infants receiving high caloric human milk felt satiated faster and finished feeding earlier, we used items of "satiety responsiveness" (SR, e.g. "my baby gets full up easily"), slowness in drinking tempo called "slowness in eating" (SE, e.g. "my baby finishes feeding quickly", "my baby takes more than 30 min to finish feeding" and "my baby sucks more and more slowly during the course of a feed") and "food responsiveness" (FR, e.g. "my baby frequently wants more milk than I provide", "even when my baby has just eaten well, he/she is happy to feed again if offered" and "my baby is always demanding a feed").

2.8. Statistical analysis

Clinical characteristics are expressed as median (IQR). Differences in clinical characteristics were determined by independent Student t-test or Mann–Whitney U-test for non-parametric parameters. Differences between multiple groups were determined by one-way ANOVA or Kruskal–Wallis one-way ANOVA on ranks for non-parametric parameters. Differences between related samples at two time points were determined by Wilcoxon matched-pair signed-rank in infants with 2 milk samples (n = 121) and categorical variables were determined by chi-squared test. Correlations were determined by Pearson's correlation coefficient or Spearman's correlation coefficient for nonparametric variables.

Missing data on growth and body composition, mainly because of infants had not yet reached age 2 years or showed resistance at measurements, were imputed using a multiple imputation approach in SPSS to generate 20 imputed datasets. Although small differences in some effect estimates were observed between analyses with imputed missing data and complete cases only, the main conclusions of the results were similar [22]. Linear regression analyses were performed to investigate the associations between human milk macronutrients and infant body composition, with adjustments for sex, parity, gestational age and (postnatal) age. For determining FM% SDS, we could only use infants with measured FM % and SDS scores for FM% were calculated based on reference data of our large, total cohort [27]. SPSS statistical package version 25 (SPSS Inc. Chicago, Illinois) was used. *P*-values <0.05 were considered statistically significant at the two-sided level.

3. Results

The study population consisted of 133 infants receiving exclusive breastfeeding during the first 3 months, of whom 49.6% was male and 63.2% Caucasian. Median gestational age at birth was 40.1 [39.3–40.7] weeks. Clinical characteristics of the subjects by sex are presented in Table 1.

3.1. Human milk macronutrient composition in exclusively breastfed infants at age 1 and 3 months

Median (IQR) concentrations of human hind milk macronutrients at 1 and 3 months are presented in Table 2. Median crude and true protein decreased from 1 to 3 months from 1.3 to 1.0 g/100 ml ($p < 0.001$) and 1.0 to 0.8 g/100 ml ($p < 0.001$), respectively. Energy decreased from 1 to 3 months from 81.6 to 74.6 kcal/100 ml ($p = 0.016$). Median fat and carbohydrate tended to decrease over time ($p = 0.066$ and 0.081, respectively). Of all macronutrients, milk fat showed the largest differences in concentration between mothers.

3.2. Human milk macronutrient composition compared to formula macronutrient composition

For comparing macronutrients in breastmilk versus formula, we calculated median (g/100 ml) macronutrient levels in the 6 most common formula feeding brands available in our country; 3.5 g fat, 7.4 g carbohydrate and 1.4 g protein and 66 kcal energy per 100 ml formula feeding. In contrast, macronutrients in human hind milk samples showed a large difference compared to macronutrients in formula feeding (Fig. 1). Of the 133 hind milk samples received by exclusively BF infants, 68.4% had higher fat, 78.2% higher energy, 92.5% higher carbohydrates and 31.6% higher protein per 100 ml compared to the average macronutrient levels in formula feeding at age 1 month. At age 3 months, 55.6% of hind milk samples received by exclusively BF infants had higher fat, 66% higher energy, 92.5% higher carbohydrates and 3.8% higher protein per 100 ml compared to the average macronutrient levels in formula feeding.

3.3. Associations of human milk macronutrient composition and body composition during early life in exclusively breastfed infants

Table 3 shows the associations of milk macronutrient composition with infant body composition, adjusted for sex, gestational age, parity and age at visit. Human milk macronutrients at 1 month were not associated with FM% at 1, 3 and 6 months. Human milk fat (g/100 ml) and energy (kcal/100 ml) at 3 months were associated with FM% at 6 months (beta 0.387 (0.006–0.767) and beta 0.040 (0.000–0.081), respectively, both $p \leq 0.049$), but not with FM% at 3 months.

Human milk fat (g/100 ml) and energy (kcal/100 ml) at 3 months were also associated (beta 0.088 (0.005–0.171), $p = 0.039$ and beta 0.009 (0.000–0.018), $p = 0.045$, respectively) with the change in FM% SDS from 1 to 6 months, the critical window for adiposity programming.

Human milk fat (g/100 ml) and energy (kcal/100 ml) at 1 month were associated with subcutaneous FM (cm) at 3 months (beta 0.013 (0.002–0.025) and beta 0.001 (0.000–0.003), respectively, both $p \leq 0.027$). Crude and true protein (g/100 ml) at 3 months were inversely associated with visceral FM (cm) at 6 months (beta −0.271 (−0.539 to 0.004) and beta −0.335 (−0.670 to 0.001), respectively, both $p \leq 0.049$). As ultrasound measurements were performed from age 3 months onwards, only the change in abdominal fat mass from age 3 to 6 months was investigated. Human milk macronutrients at 1 and 3 months were not associated with the change in subcutaneous and visceral FM (cm) from 3 to 6 months.

Human milk macronutrients at both 1 and 3 months were not associated with FM% and subcutaneous FM (cm) at age 2 years. Only milk carbohydrate (g/100 ml) content at 3 months tended to associate with visceral FM (cm) at age 2 years (beta 0.290 (−0.014 to 0.595), $p = 0.06$).

3.4. Human milk macronutrient composition and infant appetite

Infant's appetite at 1 and 3 months was assessed by using the items of the Baby Eating Behavior Questionnaire (BEBQ), regarding satiety and slowness in drinking, to investigate if exclusively breastfed infants receiving higher caloric human milk would satiate faster (Table 4).

At age 1 month, milk fat and energy content correlated positively with “my baby gets full up easily”, and inversely with “even when my baby had just eaten well, he/she is happy to feed again if offered”, indicating that if milk fat concentration is higher, infants gets full up more easily and are less eager to feed again if offered. Furthermore, milk fat correlated inversely with “my baby is always demanding a feed” and “my baby sucks more and more slowly during the course of a feed”, also indicating that a higher fat content is associated with more satiety and less frequently demanding a feed. At age 3 months, milk fat and energy content correlated with “my baby finishes feeding quickly” and “my baby gets full up easily”, indicating that also at age 3 months higher fat and energy content are associated with faster satiety and infants finishing feeding earlier.

3.5. Variables with potential influence on human milk macronutrient composition in infants with exclusive breastfeeding at 1 and 3 months

In addition, we investigated variables with potential influence on human milk macronutrient composition.

3.5.1. Sex

At age 1 month, only milk fat and energy content were higher in samples received by boys compared to girls (both $p < 0.05$). At 3 months, all macronutrients were similar between boys and girls (all $p \geq 0.38$) (Table 2).

3.5.2. Fasting versus non-fasting samples

At age 1 month, there were no differences in macronutrient composition between fasting and non-fasting milk samples. At 3 months, only carbohydrate concentrations were higher in non-

Table 2
Macronutrient composition of human milk received by exclusively breastfed boys and girls at the age of 1 and 3 months.

	Total study group		*p*-value*	Male	Female	*p*-value	Male	Female	*p*-value
	1 month	3 months		1 month			3 months		
Energy (kcal/100 ml)	81.6 [71.4–94.4]	74.6 [62.6–90.3]	**0.016**	84.5 [72.6–99.6]	79.1 [67.7–90.7]	**0.018**	73.7 [64.4–90.3]	76.7 [61.8–92.0]	0.92
Fat (g/100 ml)	4.4 [3.3–5.8]	3.9 [2.6–5.6]	0.066	4.7 [3.6–6.3]	4.1 [3.3–5.5]	**0.048**	3.7 [2.6–5.6]	4.0 [2.6–5.6]	0.92
Carbohydrate (g/100 ml)	8.7 [8.5–8.9]	8.7 [8.5–8.8]	0.081	8.8 [8.6–8.9]	8.7 [8.5–8.9]	0.24	8.7 [8.4–8.8]	8.6 [8.5–8.8]	0.39
Crude protein (g/100 ml)	1.3 [1.1–1.5]	1.0 [0.9–1.2]	**<0.001**	1.3 [1.1–1.5]	1.3 [1.1–1.5]	0.59	1.0 [0.9–1.2]	1.0 [0.9–1.2]	0.59
True protein (g/100 ml)	1.0 [0.9–1.2]	0.8 [0.8–0.9]	**<0.001**	1.1 [0.9–1.2]	1.0 [0.9–1.2]	0.61	0.8 [0.7–0.9]	0.8 [0.7–1.0]	0.59

Data expressed as median [IQR]. *Comparison of samples from mothers who collected 2 breastmilk samples at age 1 and 3 months.
Bold value signifies p < 0.05.

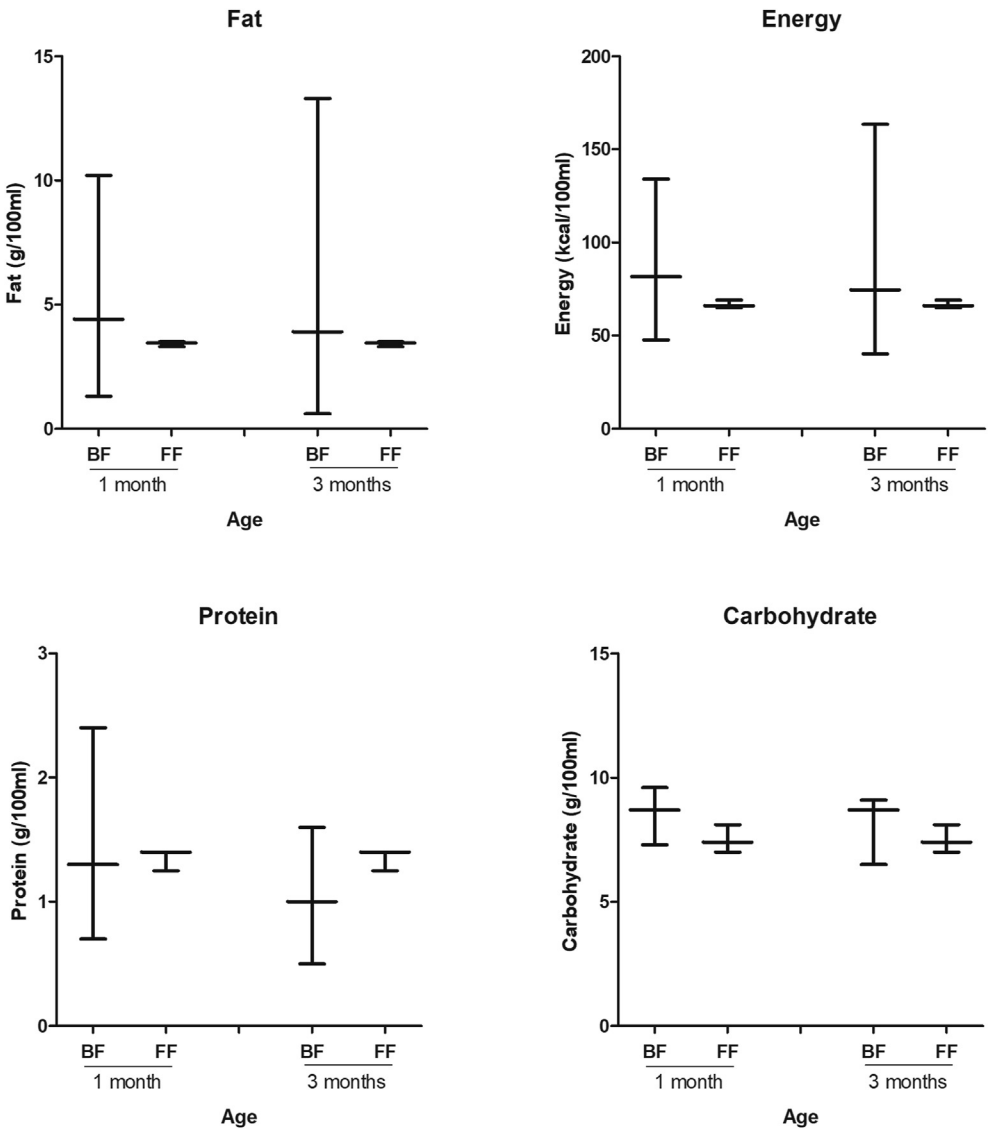

Fig. 1. Macronutrient composition in human hind milk samples versus formula feeding. Data are expressed as median (total range: min–max). Abbreviations: BF; breastfeeding, FF; formula feeding.

Table 3
Univariate linear regression analyses for human milk macronutrient composition and (Δ) FM% during the first 6 months and at age 2 years in infants with exclusive breastfeeding.

	FM% 1 month		FM% 3 months		FM% 6 months		FM% 2 years		ΔFM% SDS 1–6 months		ΔFM% SDS 1 month–2 years	
	β	*p*-value	β	*p*-value	β	*p*-value	β	*p*-value	β	*p*-value	β	*p*-value
Macronutrients												
At 1 month												
Energy (kcal/100 ml)	0.014	0.52	0.020	0.40	0.012	0.66	−0.001	0.74	−0.004	0.47	−0.013	0.17
Fat (g/100 ml)	0.154	0.44	0.206	0.36	0.105	0.68	−0.004	0.79	−0.046	0.42	−0.121	0.18
Carbohydrate (g/100 ml)	−0.385	0.68	−0.054	0.96	1.033	0.39	−0.015	0.85	0.177	0.52	−0.036	0.94
Crude protein (g/100 ml)	−2.139	0.18	−1.565	0.39	−1.702	0.39	−0.102	0.39	0.024	0.96	−0.233	0.75
True protein (g/100 ml)	−2.716	0.18	−1.996	0.38	−2.158	0.38	−0.129	0.38	0.026	0.96	−0.287	0.76
At 3 months												
Energy (kcal/100 ml)			0.045	0.09	**0.040**	**0.049**	0.001	0.31	**0.009**	**0.045**	−0.002	0.77
Fat (g/100 ml)			0.298	0.08	**0.387**	**0.047**	0.014	0.30	**0.088**	**0.039**	−0.020	0.80
Carbohydrate (g/100 ml)			−0.171	0.86	0.444	0.68	−0.014	0.84	−0.094	0.71	−0.087	0.84
Crude protein (g/100 ml)			0.502	0.83	−0.443	0.86	0.040	0.80	0.426	0.45	−0.410	0.69
True protein (g/100 ml)			0.625	0.83	−0.597	0.85	0.048	0.81	0.523	0.46	−0.499	0.69

Adjusted for sex, gestational age, parity and age at visit (Δ age at visits was used for ΔFM% SDS). β represents unstandardized regression coefficients. Associations for FM% 2 years are presented based on log-transformed outcomes.
Bold value signifies $p < 0.05$.

Table 4
Baby Eating Behavior Questionnaire items in relation to human milk macronutrient composition in exclusively breastfed infants.

	Energy		Fat		Carbohydrate		Crude protein		True protein	
	R	*p*-value	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value
At age 1 month										
My baby frequently wants more milk than I provide	0.025	0.79	0.033	0.72	−0.018	0.85	0.126	0.171	0.125	0.17
Even when my baby has just eaten well, he/she is happy to feed again if offered	**−0.238**	**0.009**	**−0.236**	**0.009**	0.092	0.31	−0.024	0.80	−0.026	0.78
My baby is always demanding a feed	−0.172	0.06	**−0.195**	**0.034**	−0.021	0.82	0.022	0.81	0.021	0.82
My baby gets full up easily	**0.241**	**0.008**	**0.202**	**0.026**	−0.148	0.10	0.145	0.11	0.143	0.12
My baby finishes feeding quickly	0.136	0.14	0.081	0.38	0.075	0.41	0.165	0.07	0.165	0.07
My baby takes more than 30 min to finish feeding	−0.077	0.41	−0.059	0.52	−0.165	0.07	−0.126	0.17	−0.128	0.17
My baby sucks more and more slowly during the course of a feed	−0.172	0.06	**−0.184**	**0.044**	0.098	0.29	−0.153	0.10	−0.152	0.10
At age 3 months										
My baby frequently wants more milk than I provide	0.111	0.23	0.100	0.28	0.143	0.12	0.070	0.45	0.069	0.46
Even when my baby has just eaten well, he/she is happy to feed again if offered	0.039	0.67	0.040	0.67	0.105	0.26	−0.039	0.68	−0.040	0.67
My baby is always demanding a feed	0.079	0.39	0.077	0.40	0.110	0.23	−0.014	0.88	−0.013	0.88
My baby gets full up easily	**0.271**	**0.003**	**0.272**	**0.002**	0.001	0.99	0.139	0.13	0.138	0.13
My baby finishes feeding quickly	**0.215**	**0.019**	**0.219**	**0.016**	−0.058	0.53	0.162	0.08	0.162	0.08
My baby takes more than 30 min to finish feeding	−0.114	0.21	−0.121	0.18	0.100	0.27	−0.031	0.74	−0.028	0.76
My baby sucks more and more slowly during the course of a feed	−0.104	0.25	−0.116	0.21	0.015	0.87	−0.012	0.90	−0.012	0.89

Data are presented as correlations with *R*; correlation coefficient.
Bold value signifies p < 0.05.

fasting samples compared to fasting samples (8.9 vs 8.7 g/100 ml, $p = 0.002$).

3.5.3. Circadian variation

At age 1 and 3 months, no differences were found between macronutrients in milk samples collected during night/early morning or during late morning (all $p \geq 0.23$).

3.5.4. Delivery mode, birthweight and ethnicity

Milk macronutrient composition at both time points were not different between infants born via normal vaginal delivery or caesarean section (all $p \geq 0.19$). No correlations were found between birthweight SDS and macronutrient composition (all $p \geq 0.24$) and macronutrients at both time points were not different across ethnicity groups (all $p \geq 0.26$).

3.5.5. Parity

At age 3 months, milk fat content was higher in human milk samples collected from mothers of second children ($n = 44$) compared to first ($n = 72$) or third ($n = 12$) children (4.5 vs 4.0 and 2.7, respectively, $p = 0.048$). No differences were found for other macronutrients nor for human milk samples collected at the age of 1 month.

3.5.6. Maternal characteristics

No correlations were found between pre-pregnancy BMI or maternal weight gain during pregnancy and macronutrient composition.

4. Discussion

In 133 healthy, term-born, exclusively breastfed infants, we showed that human milk protein and energy content decreased significantly from age 1 to 3 months, while fat and carbohydrate content tended to decrease. Human milk macronutrient composition showed remarkable differences between mothers, particularly in fat levels. Human milk fat and energy content at 3 months were associated with FM% at 6 months and the gain in FM% SDS from 1 to 6 months, the critical window for adiposity programming. Infants receiving higher caloric human milk were satiated earlier and finished feeding faster.

Human milk protein and energy decreased from age 1 to 3 months, which is in line with literature [15–17,30,31], while fat and carbohydrate content tended to decrease. Differences in macronutrient levels in breastmilk samples were considerable and more than half of the breastfed infants would receive more fat, energy and carbohydrates until age 3 months compared to formula fed infants when they would drink the same amount of milk. Protein content decreased from 1 to 3 months in human milk, while formula feeding maintains the same concentration of proteins resulting in a higher protein per 100 ml intake in formula fed infants at age 3 months.

Our study is the first to investigate human milk macronutrient composition in relation to longitudinally measured body composition and abdominal FM in early life in a large group of term-born infants. Fat and energy content at 3 months were associated with FM% at 6 months and the change in FM% SDS from 1 to 6 months. Another study showed that human hind milk %fat at age 4–8 weeks, without assessment of intake, was inversely related to gain in weight and adiposity, which was assessed by skinfolds at age 3 and 12 months [32]. The seemingly contradictory results could be explained by the physiological changes in body composition during the first year of life as we previously showed that FM% increases until 6 months and decreases thereafter [27]. We now present data in a large group of exclusively breastfed infants with longitudinally measured FM% and show that higher human milk fat and energy content at age 3 months associates with a higher FM% at age 6 months and a higher gain in FM% during the critical window for adiposity programming.

Our findings are in line with those of our PROGRAM-study, where subjects with versus without a higher gain in weight in early life had significantly higher FM% and unfavorable metabolic and cardiovascular risk profiles at age 21 years [21,33,34]. Also our previous study in infants showed that a higher gain in FM% from 1 to 6 months associated with a higher FM% at age 2 years [22]. In current study, human milk fat and energy did associate with higher FM% at age 6 months and a higher gain in FM% from age 1 to 6 months, but not with a higher FM% at 2 years. A possible explanation for the latter might be that current study comprised

of one third of the previously investigated infants as we only included exclusively breastfed infants with collected human milk samples.

Increased abdominal visceral FM has been specifically associated with an unfavorable metabolic health profile during childhood and thereafter [35,36]. The duration of exclusive breastfeeding has been associated with subcutaneous rather than visceral fat mass at age 3 and 6 months [37]. We now show that specifically human milk fat and energy concentrations at 1 month associate with subcutaneous FM at 3 months, but not with visceral FM. Only milk carbohydrate content at 3 months tended to associate with visceral FM at 2 years. Since all associations are not strong, more research in a large group of infants is needed to investigate associations between human milk macronutrients and abdominal fat mass development during infancy and childhood.

To investigate whether exclusively breastfed infants receiving higher caloric human milk are feeling satiated faster and finish feeding earlier, we used BEBQ items of 'satiety', 'food responsiveness' and 'slowness in drinking' [29]. The correlations of human milk fat and energy at 1 and 3 months with 'my baby gets full up easily' indicate that infants receiving higher fat and energy satiated faster. This was confirmed by the correlation at 3 months between human milk fat and energy with 'my baby finishes feeding quickly'. In breastfed infants, without use of expressed milk, it is difficult to determine the exact amount of human milk intake, and thus the total intake of macronutrients, which is a limitation. It could be a self-regulatory mechanism that higher caloric human milk leads to infants feeling satiated faster and longer while finishing feeding more quickly, thus preventing excessive intake of milk which in turn protects from adiposity programming. This finding could explain why some infants drink for a longer time and others finish earlier. It is our experience that this is reassuring for mothers, care takers, professionals and researchers. For future research, it is important to investigate these correlations in fore milk samples as well and to investigate body composition and metabolic health beyond age 2 years.

In addition, we investigated potential influencing factors of human milk macronutrients. Human milk samples of mothers with boys contained more fat and energy compared to girls at age 1 month, but all macronutrients were similar at 3 months. No differences were found for macronutrient composition between samples collected during night/early morning or during late morning. Others also reported similar concentrations of macronutrients throughout the day [38], but the difference between samples collected at night/early morning and during later in the morning had not been investigated before.

Macronutrient composition of human milk was not different between delivery mode. This is in contrast to two contradicting studies showing higher protein levels either after vaginal delivery [39] or Caesarian section [15]. Both studies, however, investigated human milk samples after birth or until 1 month only.

Regarding parity, human milk samples received by second children contained more fat compared to samples received by first and third children, which is partly in line with a small study describing higher lipid levels with increasing parity [31]. That study did, however, not use a Human Milk Analyzer, but weighed fat after using a modification of the gravimetric method which could affect comparison between studies.

Pre-pregnancy BMI and maternal weight gain during pregnancy were not correlated with macronutrient composition, which was in contrast to studies showing that human milk samples of overweight mothers had higher fat and protein and lower carbohydrate levels [40], and that maternal BMI was related to human milk fat and energy content [41].

In conclusion, longitudinal data on human milk macronutrient composition show that protein and energy content decrease from age 1 to 3 months, while fat and carbohydrate content tended to decrease. Human milk macronutrient composition shows remarkable differences between mothers, particularly in fat levels. Human milk fat and energy content at 3 months associate with FM% at 6 months and with the gain in FM% from age 1 to 6 months, the critical window for adiposity programming. Exclusively breastfed infants receiving higher caloric human milk satiate earlier and finish feeding faster. This self-regulatory mechanism might protect them from receiving excessive amounts of fat and energy.

Authors' contributions

AHK designed research project; KF, IB, LB and AHK conducted research; KF and AHK analyzed data; KF, GK and AHK wrote the paper; Critical revision of the manuscript for important intellectual content: GK, IB, LB, BvdH, MAB. KF and AHK had primary responsibility for final content. All authors read and approved the submitted version.

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Data sharing

Data described in the manuscript, code book, and analytic code will be made available upon request.

Conflict of interest

The Sophia Pluto Study is an investigator-initiated study; AHK received an unrestricted research grant from Danone Nutricia Research. BvdH and MAB are employees of Danone Nutricia Research. All authors were involved in writing the manuscript and had final approval of the submitted version.

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