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Macronutrient composition in human milk from mothers of preterm and term neonates is highly variable during the lactation period

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ABSTRACT

Objective: To investigate the macronutrient content of human milk during the first 28 days of lactation of mothers who delivered preterm infants, and to compare preterm to term milk.
Methods: A prospective and longitudinal study of mothers at various stages of lactation was conducted in the Amiens-Picardie University Hospital (France). Fat, true protein, carbohydrate and energy contents were estimated in human milk collected from each participant.
Results: Macronutrients in human milk were: fat (g/100 mL), 3.36±1.01; true protein (g/100 mL), 1.34±0.61; carbohydrate (g/100 mL), 7.23±0.68; energy (kcal/100 mL), 72.97±9.21 for extremely preterm human milk; fat (g/100 mL), 3.47±1.14; true protein (g/100 mL) 1.32±0.63; carbohydrate g/100 mL), 7.28±1.10; energy (kcal/100 mL), 76.18±12.84 for very preterm human milk; fat (g/100 mL), 3.48±0.87; true protein (g/100 mL), 1.26±0.46;

Abbreviations: HM, human milk; HMB, human milk bank; nICU, neonatal intensive care unit; nMU, neonatal medicine unit; Wk, week; WG, week gestational; BW, birth weight; GA, gestational age; Yrs, years; CV, coefficient of variation; SD, standard deviation; APUH, Amiens-Picardie University Hospital; BM, breast milk; OMM, own mother's milk; LCPFA, long-chain polyunsaturated fatty acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; HMO, human milk oligosaccharide.

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carbohydrate (g/100 mL), 7.36±0.47; energy (kcal/100 mL), 76.47±8.21 for moderate preterm human milk; fat (g/mL), 3.48±1.57; true protein (g/100 mL), 1.23±1.03; carbohydrate (g/100 mL), 7.36±0.63; energy (kcal/100 mL), 76.56±13.57 for term human milk.

Conclusion: Marked variations in macronutrient contents were observed between preterm and term human milk. The macronutrient score of extremely and moderate preterm human milk versus term human milk was significant, p=0.0001.

1. Introduction

During the first year of life, infants have special nutritional requirements to maintain a healthy body and support rapid growth and development [1]. Human milk (MH) is a complex fluid, essential for child and recommended not only for infants born at term, but also for infants born preterm. Infants and babies derive the bulk of their energy from glucose and therefore from the transformation of the lactose contained in HM. The long-chain polyunsaturated fatty acids (LCPUFA), docosahexaenoic acid (DHA,*n*-3) and arachidonic acid (ARA,*n*-6) are always present in HM [2]. This HM also contains proteins and especially the essential amino acids that child cannot make. During this first period of life, HM is the best source of nutrition that must supply the infant with appropriate amounts of energy and nutrients. HM contains also enzymes, hormones, growth factors, and host defense agents. The latter are a combination of various specific and nonspecific factors such as oligosaccharides, lactoferrin, vitamin A,C and B complex, binding proteins, lysozyme, antibodies (secretory IgA and low concentrations of IgG and IgM), and immune proteins soluble CD14 receptor, transforming growth factor-β-2 (TGF-β-2),α-defensin 5 (HD 5),β-defensins (HBD1 and HBD2),interleukins (IL-6,IL-10,IL-13),interferon-Y,TNF-α and lysozyme [3–6].

Preterm infants require substantially more protein than term infants to achieve growth comparable to normal intrauterine rates. Extremely preterm infants are particularly vulnerable [7] with poor postnatal growth for weeks or months resulting in lifelong neurodevelopmental consequence [8,9]. HM is optimal for growth, immunity and protection against infection of both preterm and full-term infants [10]. However, despite inter and intra-individual variations in nutrient content, HM provides sufficient nutritional intake for newborn growth HM [11].

Macronutrient content of HM samples is influenced by many factors including gestational age [12], lactation stage [12,13] mother's body mass index (BMI) [14], parity number [14] diurnal variations [15], maternal diet, and the technique used to pump milk [16,17]. HM composition change within the feeding, with fat content increasing and protein content decreases [18–20] this same periods.

To make the target fortification strategy feasible in the Intensive care unit (nICU) and human milk bank (HMB), a simple and rapid method to measure macronutrient content at beside is required. The milk analysis device available in our HMB since 2017 is the MIRIS Human Milk Analyzer (MHMA) (Uppsala, Sweden).

The optimal nutrition of preterm infants still needs to be investigated. During the last few years, advances in the preterm neonates have stimulated research on the best dietetic program to improve survival and to reduce handicap incidence. Little is known about the composition of milk from mothers giving birth prematurely. The aim of this study was to quantify different macronutrients (the fat, true protein, carbohydrate and energy content) in HM during the lactation period by using the autoanalyzer MHMA method and to investigate possible association between macronutrients and certain maternal and infant characteristics during the first 28 days after birth.

2. Materials and methods

2.1. Study design and participants

An observational prospective and longitudinal study was conducted from January 1, 2017 to June 30, 2017 in nICU, neonatal medicine unit (nMU), and to milk bank of Amiens-Picardie University Hospital (APUH), France.

A group of 88 breastfeeding mothers at various stages of lactation was included in this study of whom 72 (81.8%) had preterm deliveries [extremely preterm (n = 22), range 24–27 weeks gestational (WG); Very preterm (n = 25), range 28–38 WG, and moderate preterm (n = 25), range 32–36 WG [21]]. The remaining, 16 (19.2%) had term deliveries, 37–41 WG, served as the comparison group (Table 1).

Eighty-eight breastfeeding mothers and their newborns were enrolled in the study. Of these lactating mothers, 65 were healthy, and 23 had chronic illness like [chronic smoking (n = 8),

Table 1
Characteristics and medical history of women with primary cesarean delivery.

Mother's age (years) at delivery	Total (N = 38)	Primiparous women:N = 21 (55.3%)	Multiparous women; N = 17 (44.7%)
20–24	8 (21.0%)	5 (23.9%)	3 (17.6%)
25–29	15 (39.5%)	9 (42.9%)	6 (35.3%)
30–34	11 (28.9%)	4 (19.0%)	7 (41.3%)
35 or older	4 (10.6%)	3 (14.2%)	1 (5.8%)
Gestational age (weeks)			
Less than 28	9 (23.7%)	5 (23.8%)	4 (23.5%)
28–32	18 (47.4%)	10 (47.6%)	8 (47.0%)
33–36	8 (21.0%)	3 (14.3%)	5 (29.5%)
37–41	3 (7.9%)	3 (14.3%)	–
Comorbidities		N = 10 (55.5%)	N = 8 (44.5%)
Tobacco addiction	9 (50.0%)	6 (60.0%)	3 (37.5%)
Hypertension	5 (27.8%)	1 (10.0%)	4 (50.0%)
Diabetes mellitus			
Gestational diabetes	4 (22.2%)	3 (30.0%)	1 (12.5%)
Indications for primary cesarean delivery			
Number	N = 91	N = 49 (53.8%)	42 (46.2%)
Intrauterine growth retardation	18 (19.8%)	9 (18.4%)	9 (21.4%)
Nonreassuring fetal heart rate tracing	13 (14.5%)	9 (18.4%)	4 (9.5%)
Preeclampsia or HELPP syndrome	11 (12.2%)	7 (14.3%)	4 (9.5%)
Preterm rupture of membranes (12 h)	11 (12.2%)	6 (12.3%)	5 (11.9%)
Fetal malpresentation	10 (10.9%)	6 (12.3%)	–
Breech	6	5	1
Transverse	4	1	3
Metrorrhagia	6 (6.6%)	3 (6.3%)	4 (9.5%)
Multiple gestation	6 (6.6%)	–	6 (14.2%)
Retroplacental hematoma	3 (3.3%)	2 (4.0%)	1 (2.4%)
Umbilical cord abnormalities	3 (3.3%)	2 (4.0%)	1 (2.4%)
Double nuchal cord	2	2	–
Cord prolapse	1	–	–
Eclampsia	2 (2.2%)	1 (2.0%)	1 (2.4%)
Hydramnios	2 (2.0%)	1 (2.0%)	1 (2.4%)
Oligoamnios	2 (2.0%)	1 (2.0%)	1 (2.4%)
Chorioamnionitis	1 (1.0%)	1 (2.0%)	–
Failure of non-commitment of the fetal presentation	1 (1.0%)	1 (2.0%)	–
Failure of the fetal presentation	1 (1.0%)	–	1 (2.4%)
Twin-to-twin transfusion syndrome	1 (1.0%)	–	1 (2.4%)

42.9% of primiparous women were between the ages of 25 and 29 yrs versus 35.3% of multiparous women, while 41.3% of multiparous women were the ages between 30 and 34 yrs versus 19% of primiparous women.There was no significant difference ($p > 0.05$) between the gestational age of primiparous women and that of multiparous women.Multiparous women were more likely to have hypertension (50%), while primiparous women were more likely to present tobacco addiction (60%) and diabetes mellitus (30%).

hypertension (=5), gestational diabetes (=5), chronic smoking + gestational diabetes + hypertension (n = 2), type 1 diabetes (n = 1), type 2 diabetes (=1), and chronic smoking + gestational diabetes (=1)].

The characteristics of women who had a primary cesarean delivery and medical histories differed by parity (Table 1). Of these breastfeeding mothers, 45 (51.2%) were primiparous and 43 (48.8%) were multiparous including 30 single multiparas and 13 grand multiparas. Among the 45 primiparous women, 21 (46.6%) had a primary cesarean delivery and 24 (53.4%) had a vaginal delivery. Among the 43 multiparous women, 17 (39.5%) had primary cesarean delivery, 4 (9.3%) had repeat cesarean delivery, and 22 (51.2%) had a vaginal delivery. The overall primary cesarean rate in the study was 43.3%.

The most common indications for primary cesarean delivery included in order of frequency: intrauterine growth retardation (19.8%), non reassuring fetal heart rate tracing (14.5%), preeclampsia or help syndrome (12.2%), preterm rupture of membranes (>12 h) (12.2%), fetal malpresentation (10.9%) (Table 1).

2.2. Human milk bank, collection and storage of raw milk

The donated milk was analyzed at the APUH' s HM bank (HMB). The donations were made either directly for donor's own hospitalized preterm newborn or for other children as a free-of-charge, anonymous donation.

Upon delivery of the raw milk to the HMB samples were registered, screened, stored, thawed, rescreened and lastly frozen. At the time of sample registration, each donor filled a questionnaire to check for the absence of formal contraindications to donation: tobacco use, alcohol consumption, blood transfusion or a positive serologic test for HIV (Human Immunodeficiency Virus), HTLV (Human T-Lymphotropic Virus), HBV (Hepatitis B Virus) and HCV (Hepatitis C Virus). If these contraindications are absent, the sample is processed.

Milk samples were collected according to recommended protocol [22]. Before taking the milk samples, mothers perform a thorough washing of the hands and forearms with soap and water, then the breasts and nipples. The hands are dried with paper towels (collection at home). In the hospital, the hands dried with paper are decontaminated with a hydro-alcoholic solution and this before each expression of milk. To minimize any effect of diurnal variation, sample collection was done in the morning hours between 8:00 AM and 11:00AM. Milk was expressed from both breasts using either manual expression or an electric pump into a sterile polycarbonate (about 10–15 min for each breast). Extracted milk was poured into a labeled sterile container. All samples were stored frozen at −20 °C until processed. Any breast pain, fever, medication used and crevices were reported to staff.

One average, a total volume of 60–200 mL was collected from each mother each day (Days 7–20, 21–25, and 26–28 and stored at −20 °C for 48 h). Thirty raw milk samples were also collected from 16 healthy lactating full-term mothers on days 26–28 after delivery and survey as the control group. Samples for analysis were taken from the total pooled daily milk. A total of 256 milk samples analyzed, covered a range of infant's ages and lactation periods. All sampling expressed HM from a 24-h pool reduces nutrient variation [23].

2.3. MIRIS Human Milk Analyzer

The fat, true protein, carbohydrate and energy content was measured in milk from mothers delivering preterm or term neonates by using MHMA Version 2.87 according to manufacturer's recommendations (Uppsala, Sweden). The MHMA is an analytical instrument which is calibrated against standard methods and uses mid-infrared transmission spectroscopy. This method was carried out to direct determination of the nutritional composition of HM on the basis of its spectral content [24–28]. The unprocessed calibration was used for sample analysis according to the manufacturer's recommendations. A daily calibration check and cleaning steps every 10 analyses were performed using the calibration solution. Both solutions were supplied by manufacturer. The energy content in HM samples was calculated from the individual fat, true protein, and carbohydrate values using the equation: energy= (9.25 kcal/gxg fat) + (4.40 kcal/gxg protein) + (3.95 kcal/gxg lactose). The percentage values of MIRIS were converted into g/100 mL for better reliability.

Briefly, 10 mL of milk samples were aliquoted into 15 mL centrifuge tubes and stored at −20 °C in the field for further analysis. The frozen samples were thawed in a refrigerator for a maximum of 48 h and heated in water bath until 40 °C during 20 min. In this water bath there is a thermometer to determine when the samples reached 40 °C. The sample was then homogenized with an ultrasonic vibrator MIRIS SONICATOR™ (1.5 s/mL of sample) before analysis. The milk sample is analyzed immediately otherwise it is stored in the water bath for a maximum of 15–20 min longer. Before the analysis, the homogenized sample is thoroughly mixed by delicate inversions 10 times. Three mL of samples are taken with a syringe and were immediately injected into the MHMA™ analysis cell leaving about 0.5 mL in the syringe on the inlet, and start the analysis by pressing the << start>> key of the device.

Within the infrared spectrum, fat, true protein, and carbohydrate have characteristic absorption wavelength. When the intensity of the light transmitted at precisely these wavelengths are measured and the concentration of the compounds is determined.

2.4. Statistical methods

The results of this study were expressed as mean ± standard deviation (SD), median and range of target parameters were calculated for each mother's age at childbirth and gestational age (GA). Correlation between target parameters were described using Spearman's correlation coefficient. Fisher's exact test was used to compare the sex and the mean difference between the preterm and the term results. Wilcoxon-Mann Whitney was used to study the influence of parity on milk macronutrients. The preterm milks were correlated against the term milks. *p*-values were considered statistically significant in the case of $p < 0.05$. Variability within each epoch of milk collection was evaluated by calculating the Coefficient of Variation (CV). The CV was calculated using following formula: CV=Standard Deviation/Mean.

3. Results

3.1. Subjects

The maternal and newborn subjects were distributed into four groups on the basis of GA (Table 2): group I, 22 cases [24–27 weeks (mothers delivered extremely preterms)]; group II, 25 cases [28–30 weeks (mothers delivered very preterms)]; group III, 25 cases [32–36 weeks (mothers delivered moderate preterms)], and group IV, 16 cases [37–41 weeks (mothers delivered at term)]. The mean maternal age in each group was 29–40 years (yrs), mean, 29.40 ± 6.08 for group I; 29.76 ± 6.37 for group II; 29.12 ± 4.85 for group III, and 31.4 ± 4.39 for group IV.

Eighty-eight newborns admitted in care units of APUH are consisted of 48 males and 40 females for a sex ratio M/F of 1.2. Of these children, 72 (81.8%) belonged to preterm group of whom 38 (52.7%)

Table 2
General characteristics of breastfeeding mothers and their newborn infants.

Subject group (N = 88)	Distribution of values	Mother's age at childbirth (years)	Gestational age (GA-weeks)
Extremely Preterm	Mean ± SD	29.40 ± 6.08	25.95 ± 2.08
(I) N = 22 (25%)	Median	28	25
	Range	22–44	24–30
Very Preterm (II)	Mean ± SD	29.76 ± 6.37	29.6 ± 1.32
N = 25 (28.4%)	Median	30	29
	Range	19–45	27–32
Moderate Preterm	Mean ± SD	29.12 ± 4.85	33.64 ± 1.35
(III) N = 25	Median	30	33
(28.4%)	Range	19–39	32–37
Term infant (IV)	Mean ± SD	31.4 ± 4.39	38.8 ± 1.25
N = 16 (18.2%)	Median	33	39
	Range	21–38	37–41

Milk samples were collected from a total of 88 mothers including 72 (81.8%) women who delivered at ≤ 37 weeks of gestation. Mothers who delivered prematurely had a mean age±SD of 29 ± 5.7 years.

males and 34 (47.3%) females and 16 (18.2%) belonged to term group including 10 (62.5%) males and 6 (37.5%) females. There was no significant difference in the sex distribution between preterm group and term group ($p = 0.5$).

Fourty-six (52.3%) of these children were born by vaginal delivery and 42 (47.7%) by cesarean delivery ($p > 0.05$). Twenty-two (25%) of these newborns were extremely preterms, of whom, 18 (81.8%) were admitted for prematurity and respiratory distress, and 4 (18.2%) for management prematurity; one (4.6%) child is returned home, 7 (31.8%) had dead in the unit and the remaining 14 (63.6%) had been transferred to other units. Twenty-five (28.4%) were very preterms, 8 (32%) and 17 (68%) of them were admitted for prematurity and respiratory distress respectively, and then, six (24%) are returned to home and 19 (76%) had been transferred to other units. Twenty-five (28.4%) were moderate preterms, of these, 5 (20%) were admitted for management of prematurity, 5 (20%) for other reasons and 15 (60%) for prematurity and respiratory distress; 1 (4%) died in unit, 6 (24%) are returned to home and 18 (75%) had been transferred to other units. Sixteen children (18.2%) were term babies hospitalized for various reasons, 6 (37.5%) and 10 (62.5%) of them are returned to home or transferred to other units respectively. The results on the length of stay of children in the units are: 644 days (mean: 29.27 ± 17.80) for extremely preterm group; 401 days (mean: 16.04 ± 12.37) for very preterm group; 261 days (mean: 10.4 ± 5.60) for moderate preterm group, and 87 days (mean: 5.43 ± 5.46) for term infant group.

3.2. Human milk composition

Over the first 28 days of lactation, the mean ± SD, macronutrient composition per 100 mL were as follow: extremely preterm milk, 3.36 ± 1.01 g for fat; 1.34 ± 0.61 g for protein; 7.23 ± 0.68 g for carbohydrate, 72.97 ± 9.21 kcal for energy content; very preterm milk, 3.47 ± 1.14 g for fat; 1.32 ± 0.63 g for protein; 7.28 ± 1.10 g for carbohydrate; 76.18 ± 12.84 kcal, 69.48 kcal for energy content; moderate preterm milk, 3.48 ± 0.87 g for fat; 1.26 ± 0.46 g for protein; 7.36 ± 0.47 g for carbohydrate; 76.47 ± 8.21 kcal for energy content. Finally, for term milk, 3.48 ± 1.57 g, for fat; 1.23 ± 1.03 g for protein; 7.36 ± 0.63 g for carbohydrate, 76.56 ± 13.57 kcal for energy content (Table 3).

Table 3 shows macronutrients and energy content of the HM samples of mothers with preterm and term deliveries on the specified four days during first 28 days of lactation. The macronutrient composition changed with increasing postpartum age. In the preterm group, it was observed that, over the first 4 weeks, there was a significant decline in the protein content ($p < 0.001$). On the other hand, fat ($p = 0.001$),carbohydrate ($p < 0.001$) as well as energy ($p = 0.032$) contents increased with postpartum age ($p = 0.001$). These results were identical to those observed by Hsu et al. [29]. Comparisons of the preterm and term HM for macronutrient and energy contents on days between 26 and 28 were carried out. For term infants, the changes were in the same direction that the preterm infants. The differences in macronutrient composition between the preterm and term HM at days 26–28 were not significant ($p > 0.05$). A marked variability was observed in macronutrient composition of preterm HM and term HM (Table 3).

The Pearson's product-moment correlation method was used for comparing macronutrient mean values between extremely preterm HM *vs* term HM: Qobs, 80.460, P:0.9998, 95% IC [0.9922; 1], $p = 0.0001$, and *vs* moderate preterm HM: Qobs, 98.190, P:0.9999, 95% IC [0.9948; 1], $p = 0.0001$ (the differences are significant). In contrast, for the other combinations, the differences are not significant ($p > 0.05$).

Macronutrients (fats, protein, and carbohydrate) a relatively stable during storage and heat processing, although they can broken down by heat treatment and freeze–thaw cycles may not have the same bioactivity after undergoing these treatments. Given the influence of these factors on macronutrients it was necessary to carry out a correlation study between the different macronutrient within the same group and if they are or not significantly different. Spearman's rank correlation coefficients (rs) were computed to compare the macronutrient scores derived from preterm and term HM using MIRIS method (Table 4). In extremely preterm HM, the correlation between protein and fat was lower positive ($r = 0.0112$), between protein and energy was also low positive ($r = 0.1143$), and between fat and energy was strong positive ($r = 0.8288$). The correlation between carbohydrate and energy was lower negative ($r = -0.0734$), between fat and carbohydrate was low negative ($r = -0.1475$), and then between protein and carbohydrate was moderately negative ($r = -0.4949$). The relation between

Table 3
Means ± standard deviation and sample size of macronutrients and energy content of human milk at various periods of lactation.

Macronutrients	Extremely Preterm human milk (group I), N = 22 (<21 days)			Very Preterm human milk (group II), N = 25 (21–25 days)			Moderate Preterm human Milk (group III),N = 25 (26–28 days)			Term Infant human milk (group IV) N = 16 as control (26–28 days)		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Mean ± SD	Median	Range
Fat (g/100 mL)	3.36 ± 1.01	3.9	1.52–8.57	3.47 ± 1.14	3.49	0.79–8.29	3.48 ± 0.87	3.38	1.61–4.8	3.48 ± 1.57	2.9	1.77–10.04
True protein (g/100 mL)	1.34 ± 0.61	1.15	0.46–4.99	1.32 ± 0.63	1.24	0.1–5.01	1.26 ± 0.46	1.21	0.49–3.06	1.23 ± 1.03	1.31	0.37–6.00
Carbohydrates (g/100 mL)	7.23 ± 0.68	7.27	2.28–9.04	7.28 ± 1.10	7.46	2.28–8.89	7.36 ± 0.47	7.44	5.30–8.48	7.36 ± 0.63	7.37	4.70–8.14
Energy (kcal/100 mL)	72.97 ± 9.21	70.88	52.45–114.11	76.18 ± 12.84	69.48	18.22–112.1	76.47 ± 8.21	68.05	49.59–82.77	76.56 ± 13.57	64.31	51.87–123.7
	[a]CV			[a]CV			[a]CV			[a]CV		
Fat (g/100 mL)	30%			32%			25%			45%		
True protein (g/100 mL)	45%			47%			36%			83%		
Carbohydrates (g/100 mL)	9%			15%			6%			8%		
Energy (kcal/100 mL)	12%			16%			10%			17%		

Protein content of preterm human milk decreased from day < 21 to 28 days.Fat and energy contents followed the same trend, and gradually increase from days <21 to 28 days.The carbohydrate content also increased from 7.23 g/100 mL in the first <21 days to 7.36 g/100 mL by the 28th day.

[a] CV:Coefficient of Variation. The high CV indicates that the group was more variable and less stable or less uniform. A low CV indicates that the group is less variable and more stable or more uniform.

Table 4
Spearman rank correlation (r) on paired macronutrients in the same group.

Group		Protein/Fat	Protein/Carbohydrate	Protein/Energy	Fat/Carbohydrate	Fat/Energy	Carbohydrate/ Energy
Extremely Preterm Human milk	*Qobs*	108,501.763	164,044.089	97,193.785	125,921.261	14,787.698	117,790.981
	rs	0.0112	−0.4949	0.1143	−0.1475	0.8288	−0.0734
	p-value	0.91	1.10	0.29	0.17	3.81	0.49
Very Preterm Human milk	*Qobs*	63,369.993	119,515.130	56,111.088	70,083.109	2895.785	68,701.157
	rs	0.167	−0.571	0.2624	0.0788	0.9619	0.0969
	p-value	0.14	5.87	[a]0.02	0.49	5.30	0.40
Moderate Preterm Human milk	*Qobs*	48,807.791	67,386.525	41,274.449	31,836.390	5188.856	29,799.915
	rs	-0.1174	−0.5427	0.0551	0.2711	0.8812	0.3178
	p-value	0.35	3.60	0.66	[a]0.03	7.63	[a]0.01
Term Human milk	*Qobs*	4377.490	5548.556	3105.549	3428.907	423.673	4048
	rs	−0.198	−0.5185	0.1501	0.0616	0.8841	−0.0996
	p-value	0.31	[a]0.004	0.44	0.75	4.47	0.61

There was a significant correlation between protein and lactose (p = 0.004) in term human milk, between protein and energy (p = 0.02) in very preterm human milk, between fat and lactose (p = 0.03) in moderate preterm human milk and between lactose and energy in moderate preterm human milk.
[a] $p < 0.05$ is statistically significant.

variables was negative because as one variable increases, the other variable decreases. For all correlations between these variables, $p > 0.05$.

In the very preterm HM, correlations between fat and carbohydrate, and between carbohydrate and energy, were lower positive ($r = 0.0788$, and $r = 0.0969$ respectively), between protein and fat ($r = 0.167$), between protein and energy ($r = 0.2624$) were low positive, and between fat and energy, was strong positive ($r = 0.9615$). The correlation between protein and carbohydrate was moderate negative ($r = -0.571$). The p-value for r between protein and energy was significant ($p = 0.02$), while for remaining variables the rs were not significant ($p > 0.05$).

In the moderate preterm mother's milk, the correlation between protein and energy was lower positive ($r = 0.0551$), between fat and carbohydrate, and between carbohydrate and energy were low positive (rs 0.2711 and 0.3178 respectively), and between fat and energy was strong positive ($r = 0.8812$). The p-values for rs between fat and carbohydrate ($p = 0.03$) and between carbohydrate and energy ($p = 0.01$) were significant. The correlation between protein and fat was low negative ($r = -0.1174$), and between protein and carbohydrate was moderate negative ($r = -0.5427$). The rs of remaining variables had $p > 0.05$.

In the term HM, the correlation between fat and carbohydrate was lower positive ($r = 0.0616$), between protein and energy was low positive ($r = 0.1501$), and between fat and energy was strong positive ($r = 0.8841$), $p > 0.05$. Finally, the correlation between carbohydrate and energy was lower negative ($r = -0.0996$), between protein and fat was low negative ($r = -0.198$), and between protein and carbohydrate was moderate negative ($r = -0.5185$). p-value for r between protein and carbohydrate is significant ($p = 0.004$), and for remaining variables, $p > 0.05$.

3.3. Association between parity and HM composition

The women were allocated into three groups: primiparous (para 1), single multipara (para 2 or 3 times), and grand multipara (para 4 or more times). This study investigated the influence of lactating parity on fat, true protein, carbohydrate and energy contents of milk. We observed that primiparous women have a milk with a higher concentration of fat and carbohydrate. In contrast the concentration of proteins is not affected by parity. The grand multiparity is associated with a decrease in the fat, carbohydrate and energy contents (Table 5). The concentration of fat (rs:−0.1161; rs:−0.2312) and energy content (rs:−0.1277; rs:−0.2214), were negatively correlated in primiparous and single primipara respectively. The carbohydrate content is also negatively correlated in primiparous (rs:−0.2554) and in grand multipara (rs:−0.2936). There was a statistically significant decrease in fat

Table 5
Relationship between parity and human milk composition for the different groups of parity.

Macronutrients	Primiparus[1]			Single multipara[2]			Grand multipara[3]		
	mean ± SD	Wilcoxon Mann Whitney	Spearman's correlation	mean ± SD	Wilcoxon Mann Whitney	Spearman's correlation	mean ± SD	Wilcoxon Mann Whitney	Spearman's correlation
Fat (g/100 mL)	3.36 ± 1.46	p = 7.301	rs:−0.1161	3.24 ± 1.09	p = 0.0007	rs:−0.2312	3.15 ± 1.07	p = 0.001	rs: 0.2209
True protein (g/100 mL)	1.47 ± 0.76	p = 0.0008	rs: 0.0697	1.66 ± 0.97	p = 0.0001	rs: 0.1252	1.47 ± 0.41	p = 1.45	rs: 0.569
Carbohydrate (g/100 mL)	7.00 ± 1.08	p = 2.536	rs:−0.2554	6.96 ± 1.35	P = 6.296	rs: 0.0433	6.91 ± 1.00	p = 0.004	rs:−0.2936
Energy (Kcal/100 mL)	67.45 ± 13.79	p = 2.543	rs:−0.1277	67.27 ± 10.02	P = 6.318	rs:−0.2214	65.35 ± 16.01	p = 1.45	rs: 0.2555[1]

We did not observed any statistically significant correlation between parity and milk macronutrient composition except in grand multipara where there is significant correlation with true protein (rs:0.569;p=0.042).
Primiparas have a milk with a high average concentration of fat, carbohydrate and energy (3.36±1.46 g/100 mL; 7.00±1.08 g/100 mL,and 67.45±13 Kcal/100 mL respectively).This concentration is statistically significant in single and grand multipara for fat (p=0.0007 and 0.0001 respectively); in primiparous and single multipara for true protein (p=0.0008 and 0.0001 respectively) and in grand multipara for carbohydrate (p=0.004).
rs: Spearman's correlation of coefficient.
[1] Primiparous: a woman who has given birth once.
[2] Single multipara: a woman who has given birth two or three times.
[3] Grand multipara: a woman who has given birth four or more times.

content compared with parity, 3.36–3.15 g/mL ($p = 0.0007$ and $p = 0.001$). A statistically significant decrease in protein from primiparous to single multipara ($p = 0.0008$ and $p = 0.0001$) and carbohydrate in grand multipara ($p = 0.004$) contents was also observed (Table 5).

4. Discussion

For this work, we used the MHMA to quantitatively measure the fat, true protein, carbohydrate and energy content in the HM. This study documents the longitudinal changes in the composition of HM during the first four postpartum weeks in a group of 88 mothers delivered preterm and term infants. The preterm HM is rich in protein content initially and with increasing in postpartum age, and then decline and an increase in the amount of fat, carbohydrate and energy. These trends occur in mothers with preterm as well as term deliveries. In the present study, we showed that the macronutrient and energy composition of infant's own mother's milk (OMM) and banked donor HM used for nutrition in preterm infants in the nICU are highly variable, leading to high rate of protein and energy deficits. As shown in Table 3 there are marked variations in milk composition between and within mothers and across the course of lactation. These variations are more pronounced in the protein and fat contents. Such results have been reported elsewhere [30,31]. Bauer et al. [15] found in OMM between 28 and 32 weeks, the protein content could be as high as 2.3–1.9 g/dL, whereas the fat and the energy content account for 4.4 g/dL and 77 kcal/dL respectively. These trends are according to this study. Of the other hand, de Halleux et al. [32] shown that protein, fat, and energy contents ranged from 0.8 to 2.4 g/dL for protein, from 1.8 to 6.6 g/dL for fat, and from 47 to 85 kcal/dL for energy. These results differ from ours because of these authors [32] shown that the protein content progressively increases whereas the fat and energy content depicts the same trends that our results. These variations could be several explanations, incomplete milk expression and manipulations of HM during storage, transport, and processing. Other factors such as woman-to-woman, day-to-night, day-to-day, feeding-to-feeding and the various maternal dietary factors potentially may affect HM composition [16].

Protein is key nutrient for growth and brain development of preterm infants [9]. Other Researchers have shown that the protein content of HM is related to premature or term birth and is not associated with the birth weight of the infant or lactation volume of the mother [33]. In this study, there is a large CV in protein content (Table 3). To overcome the variability of the protein content in each meal, it has been recommended that HM given to premature infants is pooled over 24 h [33]. In this study, the preterm HM contains protein values between 0.1 and 5.01 g/100 mL. These protein values are higher than those of Anderson et al. [34] (1.8–2.4 g/100 mL) and than those of Bauer et al. [15] (2.7–3 g/100 mL). This difference is due probably by the technique and method used, and the protein type evaluated [35]. Anderson et al. [34] have suggested that differences noted between the preterm group and the term group may be related to 24-h milk volume.

The fat content of HM is rich in LCPUFA, such as ARA and DHA that are derived from linoleic acid (*n*-6) and α-linolenic acid (*n*-3) (ALA) [36–38]. These LCPUFA are major constituents of neuronal cell membranes. They increase the fluidity of these membranes and thus influence their functional characteristics. The incorporation of these fatty acids into the neonatal nervous tissue depends on the amount of LCPUFA available in HM, but also of their respective precursors. All are dependent on the amount present in the maternal diet during breastfeeding as during pregnancy. Both *n*-3 and *n*-6 fatty acids are found in significant amounts in the brain and visual tissues [39]. DHA is highly concentrated in the retina and the brain in human and is essential for normal visual and brain function. It is a major component of the phospholipid structure of the cell membrane wall in the nervous system. LCPUFA also play an important role in inflammatory and immune response, through the production of eicosapentaenoic acid (EPA) derived from *n*-3 fatty acids are thought to be anti-inflammatory [39,40]. Infants have a limited ability to synthesis LCPUFA themselves [41]. However, infants undergo a rapid period of neurodevelopment in utero and during the first two years of life. It is therefore critical that LCPUFA are available to the foetus via the maternal diet during pregnancy and following birth to the infant through breastfeeding or supplemented infant formula [42]. The fat content of HM,particularly omega-3 fatty acids can be influenced by maternal nutrition [43]. A lactating mother with high omega-3 LCPUFA consumption directly influences the omega-3 LCPUFA content in HM [43]. In the present study we did not collect data on maternal diet, but we cannot exclude the possibility that this might have influenced our results. The fat to achieve appropriate growth, the energy intake must match growth. In our study, the CV of fat content is more variable (Table 3), and impressively confirms that fat shows the highest variability after the protein content. This result is similarly to this reported by Fusch et al. [26] and Shiwani et al. [44]. Vinod et al. [45] have reported that the fat content in HM increased during the first 7 days after birth, but also to be high initially and then decrease within the first week after delivery. Zachariassen et al. [46] found fat content to be high among mothers who gave birth to the most preterm born infant, but also among mothers who gave birth to term infants. These authors found that the fat content varies between samples during the entire study period. Kent et al. [16] showed that, this may be due to sampling technique and diurnal variation in fat content. They also found a low fat content at night and a high fat content during the day and evening in HM.

Analysis of the carbohydrate component and the calculated caloric density of HM using MHMA included the presence of both lactose and oligosaccharide [35,47,48]. Carbohydrate measured followed the same trend, which was to increase gradually over weeks 1–4 and remain relatively stable from then on. It has been shown that a more fat-dominated composition of non-protein calories favors protein oxidation, an unwanted metabolic pathway for a growing organism. In contrast, higher carbohydrate intakes facilitate protein accretion and thus grow. This study shows the CV of carbohydrate and energy contents are less variable and more stable in HM and are in line with literature data [26]. HM oligosaccharides (HMOs) are not digested and absorbed in the small intestine but are readily fermented in the colon [49]. As HMOs are not digestible in the small intestine, and thus do not contribute to caloric density. They become the major source of carbone and energy for the colon flora. In fact, HMO containing N-acetylglucosamine (the bifidus factor) are necessary from the growth of *Bifidobacterium bifidum* in the large bowel. These oligosaccharides form precursors in the biosynthesis of muramic acid, a component of the bacterial cell wall. Short-chain fatty acids such as acetic, propionic and butyric acids produced by fermentation are absorbed across the large-bowel wall, providing nutrition for the colonocyte and a source of energy to body. As much as 70% of the energy in the carbohydrate can be "salvaged" by this mechanism [50]. Thus, most of the energy in HMOs may eventually become available to the infant. Carbohydrate content of HM will also influence the growth and weight gain of newborn [51]. Lactose is the predominant disaccharide carbohydrate in HM (approximately 70–83% of total carbohydrates). It produces 40% of calories of HM. HM also contains free glucose and galactose, as well as numerous oligosaccharides [31,52].

This study was planned to minimize the impact of variation on breastfeeding. These variations illustrate the complexity of the HM production response, of collecting, evaluating and feeding HM to preterm infants. All these variations have been described previously [53]. This stress that our understanding of the physiological responsible for the macronutrient composition in HM is still limited. Despite increasing evidence that a lactogenic hormone complex is required for the initiation of lactation at parturition, the origin of higher protein content and variations of macronutrient levels content in preterm HM is not completely understood [54,55]. It seems likely that the discrepancies are due to differences in hormonal balance and metabolic regulation associated with shorter gestational period and certain aspects of immaturity of mammary gland [54–56]. Research in the hormonal regulation of lactation in prematurity may be suggested that the mammary gland of the mother delivering prematurely is functionally developed and undergoes regular maturational processes, which are responsible for changes in milk composition [54–57]. The analysis of this study showed for the overall variables, correlations that were in acceptable range for all groups.

In this study, we did not analyze the relationship between GA and BW and macronutrients taken individually, because this was not the goal of our study. Such studies have already been done elsewhere [29].

The development of nICU has resulted in the survival of an increasingly larger proportion of preterm infants. In this study, of 72 preterm neonates hospitalized at nICU during the study period, 64 (88.8%) have survived, while only 8 (11.2%) have dead. In the term babies group, no deaths were reported in the maternity ward, but many questions remain about the optimal method of feeding the preterm babies. It has been suggested that HM may provide some protection against infection and necrotizing enterocolitis [58,59]. The other possibility is that the nutritional needs of the preterm infant are matched by the composition of HM produced by mothers delivering prematurely. These data have led to studies designed to evaluate the nutritional composition and adequacy of HM produced by mothers delivering to ≥37 weeks of gestation [34,45,60–62]. To minimize variations that may affect milk composition in this study, each participant received professional support by lactation consultants providing instruction in sampling milk during the entire investigation including home visits after discharge.

5. Study limitations

One of the limitations of this study was that the MHMA we used could only measure macronutrient components. It was not capable of measuring the immunological and biochemical properties of HM, which may also change after storage, processing and associated with other factors. Another limitations is that studies focusing on the macronutrient composition of HM from mothers of extremely preterm neonates, particularly under 28 weeks of gestation, are limited due to hormonal and physiological factors that cause insufficient milk output.

6. Conclusions

The milk analyzed in this study was collected by a standard procedure designed to minimize inter-individual variations. This shows that macronutrient composition of HM is highly variable and its content is important when feeding preterm infants fortified HM during hospitalization, but agreements on protocols for HM sampling and analysis are definitely needed in future research on HM to optimize nutrition of extremely preterm infants. The use of autoanalyzers such as MIRIS appears to be more suitable for routine use in neonatology units or HMB. It improves nutritional management in vulnerable premature infants and facilitates more precise supplementation of HM than currently possible.

Ethics approval and consent to participate

The study protocol and the informed consent form were reviewed and approved by the local Person Protection Committee, Ile de France 3,n° ID-RCB; 2017-AO3540-53, opinion favorable, Ref.DS/LG/2018-039, July 21,2018. Furthermore, the study was performed in accordance with the Declaration of Helsinki. The participants of this study were all breastfeeding mothers having given their written, independent, informed consent.

Consent for publication

Not applicable.

Availability of data and material

Please contact author for data requests.

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Authors contributions

All authors listed on the title page participated in meetings and follow-up discussions that culminated in preparation of this manuscript. A. Léké and M. Biendo were involved in the concept, design, acquisition of data, analysis and interpretation of data, and drafted the manuscript. S. Grognet was involved in mother's milk sample collection and milk processing. M Deforceville, S. Goudjil, C. Chazal, G. Kongolo were involved in drafting of the manuscript.B. Elion Dzon assisted in the preparation of the materials necessary for this study. All authors have read and approved the final version to be submitted.

Conflict of interest

None.

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