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Original article

Temporal changes of major protein concentrations in preterm and term human milk. A prospective cohort study

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SUMMARY

Background: Proteins are major contributors to the beneficial effects of human milk (HM) on preterm infant health and development. Alpha-lactalbumin, lactoferrin, serum albumin and caseins represent approximately 85% of the total HM protein. The temporal changes of these proteins in preterm (PT) HM and its comparison with term (T) HM is poorly characterized.

Aims: To quantify and compare the temporal changes of the major proteins in PT HM and T HM.

Methods: HM was collected for 4 months postpartum at 12 time points for PT HM (gestational age 28 0/7 −32 6/7 weeks; 280 samples) and for 2 months postpartum at 8 time points for T HM (gestational age 37 0/7−41 6/7 weeks; 220 samples). Proteins were measured with a micro-fluidic LabChip system.

Results: Casein, alpha-lactalbumin and lactoferrin decreased with advancing stages of lactation in PT and T HM, whereas serum albumin remained stable. Only marginal differences between PT and T HM were observed for alpha-lactalbumin during postpartum weeks 3−5 and for serum albumin at the first week. However, a comparison of HM provided to preterm and term infants at the same postmenstrual ages revealed that alpha-lactalbumin contents were significantly lower in PT HM than in T HM during the 39 −48 postmenstrual weeks.

Conclusions: This study provides comprehensive information of the longitudinal changes of major proteins in PT and T HM, and suggests limited availability of alpha-lactalbumin, a nutritionally important protein, in breastfed PT infants after reaching the term corrected age. This information may be important to optimize HM protein fortification, although its biological relevance needs to be confirmed by intervention studies.

Clinical trial registry: ClinicalTrials.gov (NCT02052245), https://clinicaltrials.gov/ct2/show/NCT02052245.

1. Introduction

Human milk (HM) feeding, especially with mother's own milk (MOM), reduces the risk of mortality and morbidities associated with premature birth and has a positive impact on preterm infant neurodevelopment [1,2]. Consequently, MOM is the recommended nutritional support for preterm infants, during their stay at the neonatal intensive care unit (NICU) and after hospital discharge [3–5]. When MOM is not available, donor milk – often HM offered by mothers having given birth to term infants [6] – is the second preferred option [3].

HM proteins appear to be major contributors to the beneficial effects of HM on preterm infant health, growth and development. Proteins provide nitrogen and amino acids required for growth. They can also act as biologically active molecules able to assist to nutrient digestion and absorption in the preterm immature intestine, to confer protection against pathogens, and to modulate the immune maturation and immune response [7,8]. Yet, among other nutrients, it is recommended to supplement MOM and donor HM with additional protein to prevent postnatal growth failure during

Abbreviations list: PT HM, preterm human milk; T HM, term human milk; NICU, neonatal intensive care unit; CV, coefficient of variation.

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hospital stay [4] and, occasionally, after hospital discharge [5]. Furthermore, fortification with specific, biologically active proteins such as lactoferrin may be beneficial even in preterm infants fed freshly expressed MOM, as suggested by some intervention trials [9–11]. Accurate, quantitative data on the concentration and variability of specific proteins in preterm (PT) and term (T) HM is needed i) to optimize the amino acid profile of the protein used to fortify MOM and donor HM and ii) to identify potential needs and doses of supplements containing biologically active proteins.

The HM proteome is complex, with up to 2500 different protein species identified [12]. Of them, alpha-lactalbumin, lactoferrin, serum albumin and caseins are amongst the most abundant species, accounting for approximately 85% of the total protein content of HM [13]. These proteins are not only the most important source of nitrogen and amino acids in HM, but may also contribute to a range of its functional benefits [7].

Concentration of total protein can undergo large variations along lactation and is reported to be higher in PT HM than in T HM [14]. Other factors like mode of delivery [15] or even infant sex [16] are also proposed to affect total protein content in HM. However, studies quantifying the major proteins in HM are scarce. Most studies report levels of only one of these proteins at the time – mostly lactoferrin – and use a wide range of analytical methods, which precludes a consistent compilation of their results [20]. Longitudinal, simultaneous quantification of the major milk proteins is not yet available, possibly because the methods used in the past required a substantial amount of HM, which was therefore unavailable for infant feeding. In addition, traditional methods required long analysis times. We have recently developed a new method that allows high-throughput with concurrent analysis of alpha-lactalbumin, lactoferrin, serum albumin and total caseins in microliter volumes of HM [17].

The objectives of this study were twofold: 1) to simultaneously quantify and compare the temporal changes of the major proteins (lactoferrin, alpha-lactalbumin, serum albumin and caseins) in HM from mothers of preterm and full-term infants; and 2) to investigate other potential sources of variability such as mode of delivery, infant sex and multiple delivery.

2. Subjects and methods

2.1. Subjects

This research was part of a prospective cohort study aiming at characterizing the PT and T HM nutritional composition. It was conducted at the NICU and at the maternity ward of the University Hospital in Lausanne (CHUV), Switzerland, between October 2013 and July 2014.

The study included women older than 18 years of age, given birth to preterm (gestational age 28 0/7–32 6/7 weeks) or to full-term (gestational age 37 0/7–41 6/7 weeks) infants and intending to breast-fed for a minimum of 4 months. Gestational age was calculated as the time elapsed since the first day of the last menstrual period, whenever this time differed in less than 7 days from that estimated by early (less than 16 weeks) ultrasonography. When the difference between both methods was 7 days or more, the ultrasonography estimate was chosen. The study excluded the mothers diagnosed with type I or II diabetes, having consumed alcohol or illicit drugs during pregnancy and/or with insufficient skills to follow the study procedures.

After informed consent, subjects were enrolled before postpartum day 3 by the two referent pediatricians (CJF, LB). Subjects were followed during 16 weeks postpartum for the preterm group, and 8 weeks for the term group, or until lactation discontinuation (whatever came first), by a dedicated research nurse. This nurse, who was qualified as lactation consultant, closely interacted with the subjects all along the study by telephone interviews and home visits to encourage breastfeeding, answer to mother questions and collect the HM samples.

2.2. Data collection

Upon subject enrollment, neonatal demographic and delivery data were collected from the medical electronic charts. Neonatal data included single or multiple gestation, mode of delivery, sex, weight, length, head circumference and gestational age at birth. Birth weight was monitored with an electronic scale accurate to the nearest 5 g, crown-heel length with a height gauge and head circumference with a tape. Maternal weight at delivery was measured with an electronic scale, whereas maternal age, height and weight before pregnancy was self-reported.

2.3. Human milk sampling and processing

After delivery, PT HM samples were collected on 12 instances, every 7 days ± 1 day during the first 8 weeks, then every 14 days ± 1 day during the following 8 weeks. For T HM, a total of 8 samples were collected every 7 days ± 1 day for the first 8 lactation weeks (Supplemental Fig. 1).

Samples were collected from the first HM expression in the morning, between 6 and 12 am. Full HM expression from a single breast was performed either at home or at the hospital with the help of an electric, double, breast pump (Symphony®, Medela, 6340 Baar Switzerland). The collected HM was homogenized and a 10 mL sample (1–3 mL for the first two sampling time points in the PT group) was held for biochemical characterization. The rest of the HM was used for infant feeding. Mothers transferred the HM samples to 15 mL polypropylene tubes (Falcon ™, Fisher Scientific, Switzerland), previously labeled with subject number and collection information. For collection at home, subjects stored the samples at −18 °C in the home freezer during 1 week maximum before transfer to the hospital. At the hospital, samples where temporarily kept at −80 °C before shipment to the Nestlé Research Centre (Lausanne, Switzerland). To avoid multiple thawing/freezing cycles, HM samples were thawed once for splitting into 15 aliquots then stored at −80 °C until analyses of major proteins and other HM components (results reported in independent publications).

2.4. Protein quantification

The four major HM proteins alpha-lactalbumin, lactoferrin, serum albumin and total caseins were quantified as described previously [17]. Briefly, all samples were analyzed in triplicates, using a volume of 25 μL of HM, on a LabChip GX-II microfluidic device (Perkin Elmer, Waltham, MA, USA). HM samples were diluted 5-fold with water (Merck Lichrosolv quality) followed by denaturation and derivatization according to the LabChip protocol. Separation and detection was performed on a HT Protein Express protein chip according to the instrument manual. Individual calibration curves were generated using pure human milk proteins (alpha-lactalbumin, lactoferrin, and serum albumin from Sigma, St. Louis, MO, USA) and bovine caseins (alpha-, beta- and kappa-casein from Sigma; human caseins not available). The individual casein proteins could not be fully resolved on the LabChip system. Because of that, all casein peaks were integrated as one peak and thus one value for total casein concentration in HM was obtained (sum of α-, β- and κ-casein). System performance was monitored by applying a quality control sample (pooled HM from Lee Biosolutions Inc., Maryland Heights, MO, USA) every 20th sample in the analytical series.

Total protein content in HM was measured using the colorimetric bicinchoninic acid (BCA) method according to the protocol provided with the BCA assay kit (ThermoFisher Scientific).

2.5. Statistics

The paucity of quantitative data on the major protein content in preterm HM precluded a proper power calculation in this exploratory study. Study size was initially set at n = 20 subjects per group (preterm and term infant mothers) according to the estimated recruitment feasibility at the study center within one year period.

The temporal changes of protein contents were compared in PT and T HM at equivalent infant 1) postpartum ages and 2) postmenstrual ages. For both comparisons, mixed linear models were used to estimate the differences between preterm and term infants. The models used age (either postpartum or postmenstrual), term/preterm status, and interaction between age and term/preterm status, multiple/single delivery, delivery mode and gender as fixed effects. Within subject variability was accounted by declaring the subject ID as a random effect. Contrast estimates of the model were calculated by comparing PT and T HM groups at each time point. No imputation method was applied for missing data (both in between visits and loss to follow up) as the method used does not require a complete data set. A conventional 2-sided 5% error rate was used without adjusting for multiplicity.

Similar methods were used to analyze the effects of gender, delivery mode and multiple/single delivery and their interaction with age.

Statistical analyses were done with SAS 9.3 and R 3.2.1.

2.6. Ethics and study registration

The study followed the Declaration of Helsinki's guidelines. The study protocol with all procedures involving human subjects were approved by the local Ethical Committee (Commission cantonale d'éthique de la recherché sur l'être humain du Canton de Vaud, Switzerland; Protocol 69/13, clinical study 11.39.NRC) in April 9, 2013. All the subjects participating to the study signed an inform consent before the enrollment.

The study was registered at ClinicalTrials.gov (NCT02052245).

3. Results

3.1. Subject characteristics

In all, this cohort study included 27 mothers having delivered 33 preterm infants and 34 mothers having delivered 34 term infants. Figure 1 displays the study flow diagram. Two out of 27 (7.4%) preterm infant mothers and 6 out of 34 (17.6%) term infant mothers were lost for follow up. No serious adverse events were reported along the study period. In total, 500 HM samples, 280 from preterm and 220 from full-term infant mothers, were available for protein analyses.

Table 1 reports mother and infant demographic and baseline anthropometric data. Maternal baseline characteristics were comparable among groups. Maternal health status, anthropometric and socioeconomic data at recruitment and the absence of serious adverse event reporting along the study indicate that all the subjects were healthy. Cesarean delivery was more frequent in the preterm cohort. As expected, preterm and full term infants significantly differed in all baseline parameters except for gender distribution. Multiple deliveries (twins) were frequent (36%) in the preterm group, but absent in the term cohort.

3.2. Concentration of the major proteins in PT HM and T HM

This study quantified the four major proteins in HM samples from preterm and full-term infant mothers, from postpartum week 1 up to week 16 and week 8, respectively. The study analyzed first the impact of preterm delivery on protein changes along lactation weeks 1–8, as well as the influence of mode of delivery, single or twin delivery, and infant gender. In a secondary analysis, we further compared the protein concentration in the milk received by preterm and term infants at equivalent postmenstrual ages (39–48 weeks).

Lactoferrin was the most abundant of the whey proteins analyzed in the early samples, with values reaching 5.1 ± 1.2 g/L in PT HM and 5.7 ± 2.1 g/L in T HM during the first postpartum week. Lactoferrin content sharply decreased during the first month of lactation, then remained fairly constant, without significant differences between PT HM and T HM at any time point (Fig. 2A, Supplemental Table 1). When compared at equivalent infant postmenstrual age, lactoferrin concentration was higher in T HM than in PT HM at postmenstrual weeks 39–40, and lower at week 45 (Fig. 2B, Supplemental Table 2).

Alpha-lactalbumin was the most abundant analyzed whey protein in the transitional and mature HM samples. Its concentration decreased along lactation in both T (3.9 ± 0.8 to 2.8 ± 0.5 g/L) and PT (from 3.8 ± 0.7 to 2.2 ± 0.6 g/L) HM. T HM displayed significantly higher values than PT HM during the postpartum weeks 3–5 only (Fig. 3A, Supplemental Table 1). When compared at equivalent infant postmenstrual age, alpha-lactalbumin content was consistently (30%–65%) higher in T HM than in PT HM all along the study period (Fig. 3B, Supplemental Table 2).

Serum albumin was the least abundant protein assessed. Its concentration was essentially constant all along the study period and similar in both PT and T HM, except for the first postpartum week, in which T HM displayed significantly higher values (Fig. 4A, Supplemental Table 1). The comparison by infant postmenstrual age revealed only occasional differences between both cohorts (Fig. 4B, Supplemental Table 2).

Collectively, total caseins constituted the most abundant protein analyzed in the study samples and their temporal changes marginally differed in PT and T HM. Specifically, total casein contents continuously decreased along lactation in PT HM, from 8.0 ± 2.8 to 4.9 ± 1.7 g/L. In T HM, casein concentration initially increased (week 1–2) from 7.0 ± 3.6 to 8.0 ± 2.4 g/L then decreased to 5.5 ± 1.9 mg/mL at postpartum week 8. No significant differences were observed in PT and T HM at any postpartum time (Fig. 5A, Supplemental Table 1). The comparison by infant postmenstrual age revealed significantly higher levels in T than in PT HM during weeks 41–43 (Fig. 5B, Supplemental Table 2).

The magnitude of the inter-individual variability differed between the studied proteins. It was lowest for alpha-lactalbumin, with coefficient of variation (CV) values ranging between 14% and 31%. In contrast, lactoferrin and serum albumin showed high variability and reached CV values higher than 150% at some postpartum times (Supplemental Table 1). Mode of delivery, twin vs single delivery and infant gender did not significantly affect the concentration of any of the studied proteins in either group (data not shown).

Macronutrient composition of all HM samples from this study was analyzed independently to this work and will be reported separately (manuscript in preparation). However, available data on the total protein content measured with the BCA assay allowed for comparison with the summed-up individual protein amounts from the LabChip analysis of all HM samples. A graphical representation of the correlation result is shown in Supplemental Fig. 2. Although only the major HM proteins have been quantified in this study, representing around 80% of the total HM content, a good

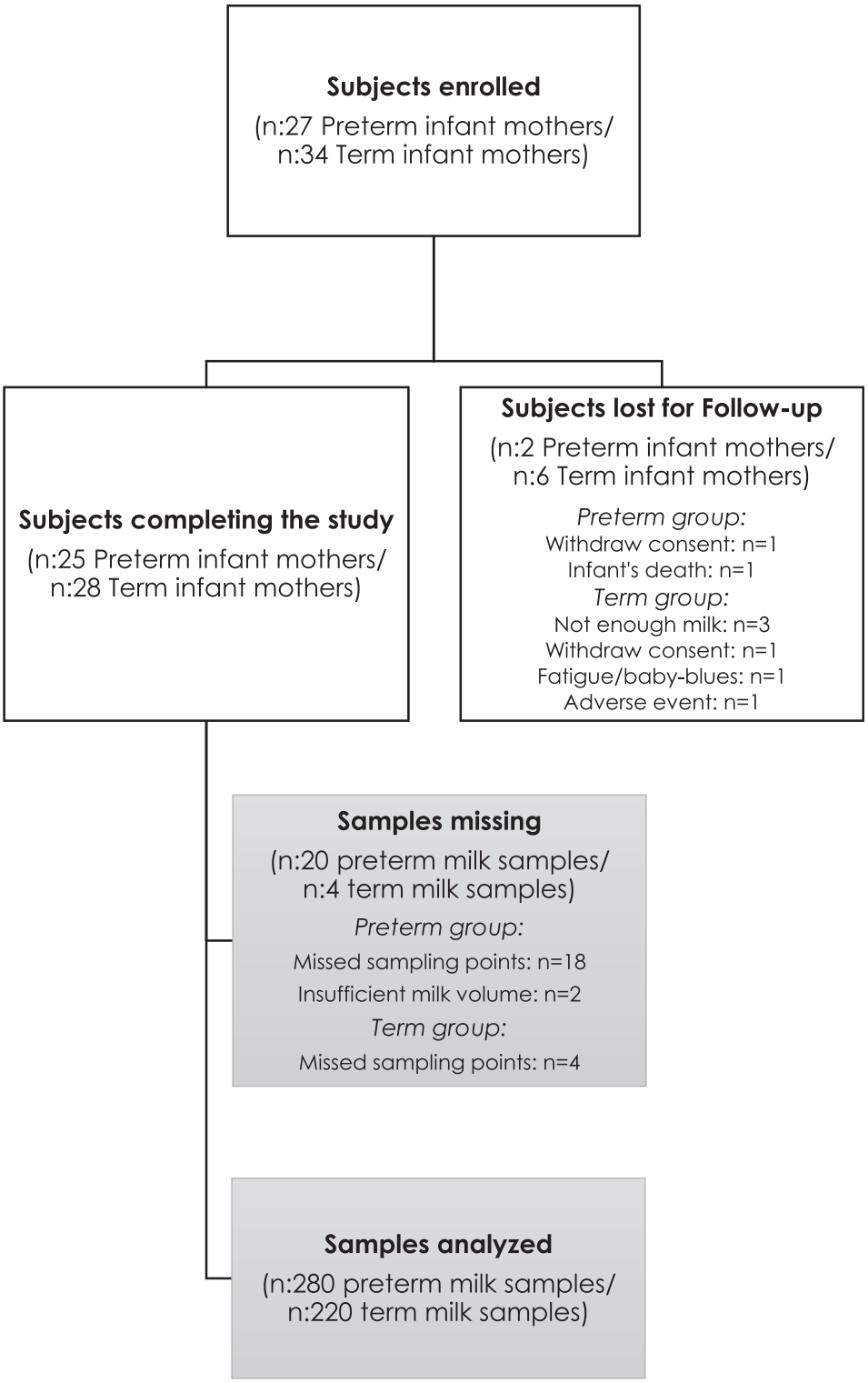

Fig. 1. Study flow chart.

correlation (Pearson r = 0.758; $R^2 = 0.574$) was observed between the two analytical approaches.

4. Discussion

This cohort study characterized the concentrations of the three most abundant whey proteins and of the total caseins in PT and T HM, from the first week post-delivery until week 16 and week 8, respectively. The absolute contents of the four proteins were quantified simultaneously in a single analytical run for each of 500 HM samples. The temporal changes of protein contents were compared in PT HM and T HM at equivalent infant postpartum and postmenstrual ages. Both PT HM and T HM displayed close protein contents and comparable changes all along the studied postpartum

Table 1
Mother and infant characteristics.

Study population	Preterm	Term	*P*-value[a]
Mother	$n = 27$	$n = 34$	
Age (years), mean ± SD	32.4 ± 5.6	31.2 ± 4.2	0.3173
Height (cm), mean ± SD	165.2 ± 7.1	166.8 ± 6.6	0.3601
Weight before pregnancy (kg), mean ± SD	62.1 ± 9.5	64.3 ± 12.0	0.4479
Weight at birth (kg), mean ± SD	70.3 ± 10.6	74.5 ± 11.3	0.1426
BMI before pregnancy (kg/m^2), mean ± SD	22.8 ± 3.4	23.2 ± 4.9	0.6990
BMI at delivery (kg/m^2), mean ± SD	25.8 ± 3.7	26.9 ± 4.7	0.3141
Caesarean delivery, *N* (%)	63.0	23.5	0.0019
Infant	$n = 33$	$n = 34$	
Gestational age at birth (weeks), mean ± SD	30.8 ± 1.4	39.5 ± 1.0	<0.0001
Males, *N* (%)	54.5	52.9	0.8952
Twins, *N* (%)	36.4	0.0	0.0001
Birth weight (g), mean ± SD	1421 ± 373	3278 ± 354	<0.0001
Birth height (cm), mean ± SD	40.4 ± 3.2	49.4 ± 1.7	<0.0001
Birth head circumference (cm), mean ± SD	27.8 ± 2.1	34.4 ± 1.5	<0.0001

[a] *t*-test and Fisher test of proportions were used for the comparison of continuous and discrete variables, respectively.

period. However, when compared at equivalent infant postmenstrual ages, contents of alpha-lactalbumin and, to a lesser extent, total caseins and lactoferrin in PT HM were lower than those in T HM.

Total caseins and alpha-lactalbumin were the most abundant protein species in the majority of our study samples and their content generally decreased from early to late lactation, as already described [17–19]. To our knowledge, only one publication has reported the absolute concentration of these two proteins in PT HM, and only in early PT HM [20]. This former study found significantly lower casein and alpha-lactalbumin contents in PT HM than in T HM in the first postpartum week. We also found marginally (around 10%) but significantly lower alpha-lactalbumin content in PT HM during early lactation. These results are consistent with recent data showing lower alpha-lactalbumin gene expression in HM cells isolated from PT HM than from T HM [21] and may reflect transient immaturity of the mammary gland shortly after premature delivery.

Alpha-lactalbumin and caseins are key to the nutrition of the breast-fed infant. Besides being the most abundant proteins in HM, they encompass a remarkably high proportion of indispensable amino acids [22]. Moreover, alpha-lactalbumin and caseins appear to be more readily digested in the preterm infant gastrointestinal tract than other major HM proteins, such as lactoferrin [23]. Beyond their role as amino acid sources, these proteins contain bioactive peptides (e.g. with mineral chelating or anti-microbial activity) that may transiently exert their activity (e.g. increased mineral absorption or prevention of infection) upon digestion in the infant intestine [7].

Lactoferrin was the most abundant whey protein in both milks during the first few weeks of lactation. Then, its content sharply decreased and remained essentially constant in mature HM, which is in good agreement with previous literature [24]. We did not find significant differences in lactoferrin content between PT HM and T HM at any of the studied postpartum times. Although early publications reported higher content of lactoferrin in PT than in T HM [20,25,26], a recent systematic review [24] and two subsequent studies [27,28] failed to find any difference, in line with our results. The nutritional significance of lactoferrin as source of amino acids is unclear [29]. However, this protein is proposed to have anti-infective and anti-inflammatory activities able to reduce morbidity (sepsis, necrotizing enterocolitis) and mortality in the preterm infant, as shown in lactoferrin intervention trials in preterm infants during the NICU stay [9–11]. An unpowered subgroup analysis in one of these intervention studies [9] showed that the benefits of lactoferrin supplementation extended to infants receiving fresh MOM, suggesting that the requirements of this at risk population may be higher than the doses provided in HM.

Serum albumin was the least abundant protein in our study samples, remained essentially constant along the studied postpartum period and was similar in T and PT HM. Comparison with literature data [30] showed marginally lower values in the present study, which may be related to the differences in the analytical methodologies. In this line, the range of serum albumin concentrations observed in the current study were similar to that found in a previous cross-sectional study in T HM from a very different origin (Chinese mothers) which used the same analytical approach [17]. The role of this protein in HM is largely underexplored [30]. It likely participates to the provision of amino acids, as it can be digested, at least partially, by the infant [31,32]. The amino acid sequence of serum albumin in HM is identical to that of the circulating serum albumin. Because of that, it has long believed to be transferred to the mammary gland from maternal blood [30]. However, data from other mammalian species suggest that part of the milk serum albumin may be synthesized in the mammary gland [33]. The large inter-individual variability observed in our study samples suggests a lack of tight regulation in the synthesis and/or blood-to-mammary gland transfer of this protein.

In summary, this study show several differences in protein profiles between PT HM and T HM. The most important factor driving variability in the concentration of the three major HM proteins – alpha-lactalbumin, lactoferrin and total caseins – was the time elapsed after delivery without major impact of the other factors evaluated, including gestational age at delivery. These results suggest that, independently on whether the preterm infant receives PT HM or T HM, protein fortification shall primarily account for the temporal changes in the levels of these proteins occurring along lactation.

To evaluate a possible need of optimizing the protein quality of HM fortification after discharge, this study further compared the major protein contents in PT HM and T HM at 39–48 infant postmenstrual weeks (age at which most preterm infants have been discharged from the hospital). This secondary analysis was prompted by the assumption that preterm infants may have, at minimum, the same protein requirements for growth and development than their term pairs of equivalent postmenstrual age. We based this assumption on the fact that the growth standards of infants born at term are used as reference for optimal growth of

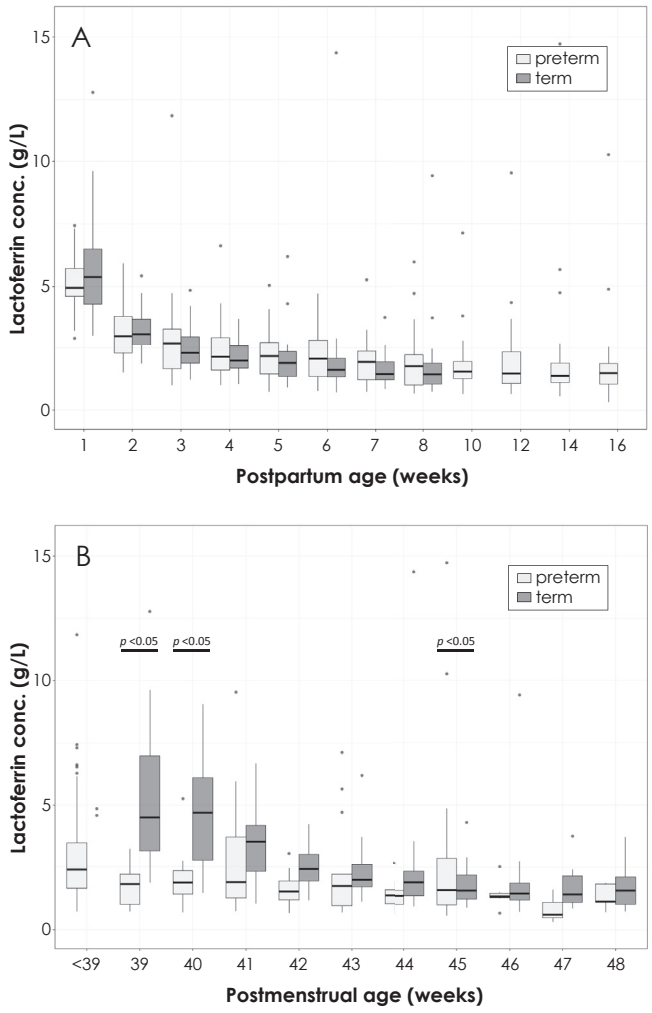

Fig. 2. Comparison of lactoferrin content in preterm (PT) and term (T) human milk (HM) over lactation (A) and at equivalent infant postmenstrual age (B). Box plots represent medians with 25th and 75th percentile, min–max range and outliers. Mixed linear models were used to estimate the differences. The models used infant age (either postpartum or postmenstrual), term/preterm delivery, delivery mode, single/multiple delivery and gender as fixed effects. Within subject variability was taken into consideration by declaring the subject ID as a random effect. Contrast estimates of the model were calculated by comparing the two groups (PT and T HM) in each time point (either postpartum or postmenstrual age). No adjustment for multiplicity was applied and a conventional 2-sided 5% error rate was applied. *P* values of the differences between PT and T HM lower than 0.05 are reported.

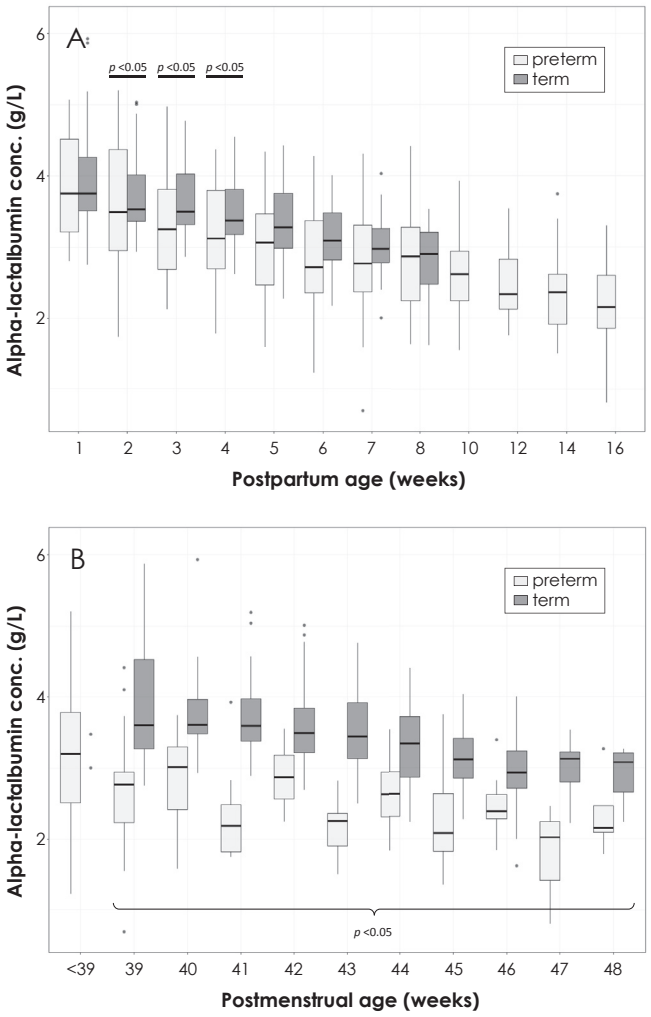

Fig. 3. Comparison of alpha-lactalbumin content in preterm (PT) and term (T) human milk (HM) over lactation (A) and at equivalent infant postmenstrual age (B). Box plots represent medians with 25th and 75th percentile, min–max range and outliers. Mixed linear models were used to estimate the differences. The models used infant age (either postpartum or postmenstrual), term/preterm delivery, delivery mode, single/multiple delivery and gender as fixed effects. Within subject variability was taken into consideration by declaring the subject ID as a random effect. Contrast estimates of the model were calculated by comparing the two groups (PT and T HM) in each time point (either postpartum or postmenstrual age). No adjustment for multiplicity was applied and a conventional 2-sided 5% error rate was applied. *P* values of the differences between PT and T HM lower than 0.05 are reported.

preterm infants after they reach the term corrected age [34]. Such comparison revealed a substantially lower alpha-lactalbumin concentration in the HM given to preterm infants of 39–48 postmenstrual weeks than in HM received by their term pairs. Lower levels of total caseins and lactoferrin in PT HM samples were also found but were only statistically significant during short periods of time. This gap in nutritionally and functionally important proteins between PT and T HM may contribute to the increased risk of growth failure [35] and disease [36] experienced by the preterm infant in the weeks that follow discharge from the hospital. Whether preterm infants may benefit from a specific protein supplementation during this period warrant further investigation.

Main limitations of this study that may curb the generalization of the results are the limited sample size and the mono-centric design in one single hospital in Switzerland. However, the study results were generally close to the data reported in the studies available, including those obtained in very different areas of the globe [17], suggesting that the content and temporal progression of the major proteins in HM is a well-conserved evolutionary trait.

Another limitation in this research relates to the fact that HM samples were collected from the first breast expression in the morning, which, as shown for other nutrients [37], may not reflect the major protein composition in HM along the 24 h. Nevertheless, total protein content does not appear to change along the day in preterm BM [38] and although marginal circadian variations in specific amino acids have been reported [39], it is unlikely that these changes reflect large circadian variations in the major protein content.

Finally, this study only quantified 4 major HM proteins out of around 2500 reported in HM [12]. In addition, it fails to provide data on the levels of the individual casein species. The reason was

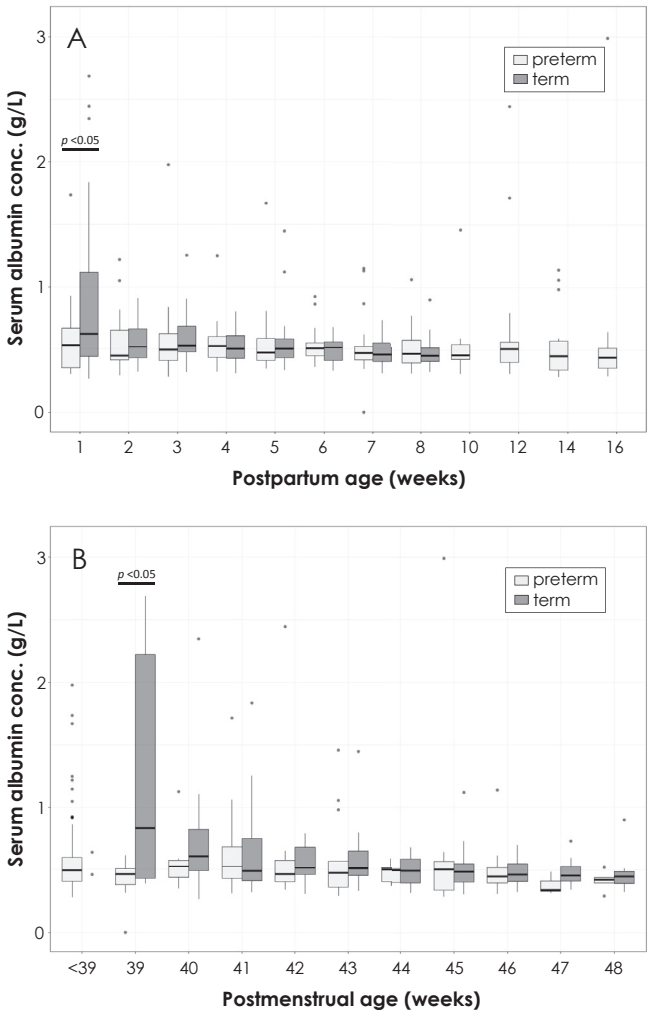

Fig. 4. Comparison of serum albumin content in preterm (PT) and term (T) human milk (HM) over lactation (A) and at equivalent infant postmenstrual age (B). Box plots represent medians with 25th and 75th percentile, min–max range and outliers. Mixed linear models were used to estimate the differences. The models used infant age (either postpartum or postmenstrual), term/preterm delivery, delivery mode, single/multiple delivery and gender as fixed effects. Within subject variability was taken into consideration by declaring the subject ID as a random effect. Contrast estimates of the model were calculated by comparing the two groups (PT and T HM) in each time point (either postpartum or postmenstrual age). No adjustment for multiplicity was applied and a conventional 2-sided 5% error rate was applied. *P* values of the differences between PT and T HM lower than 0.05 are reported.

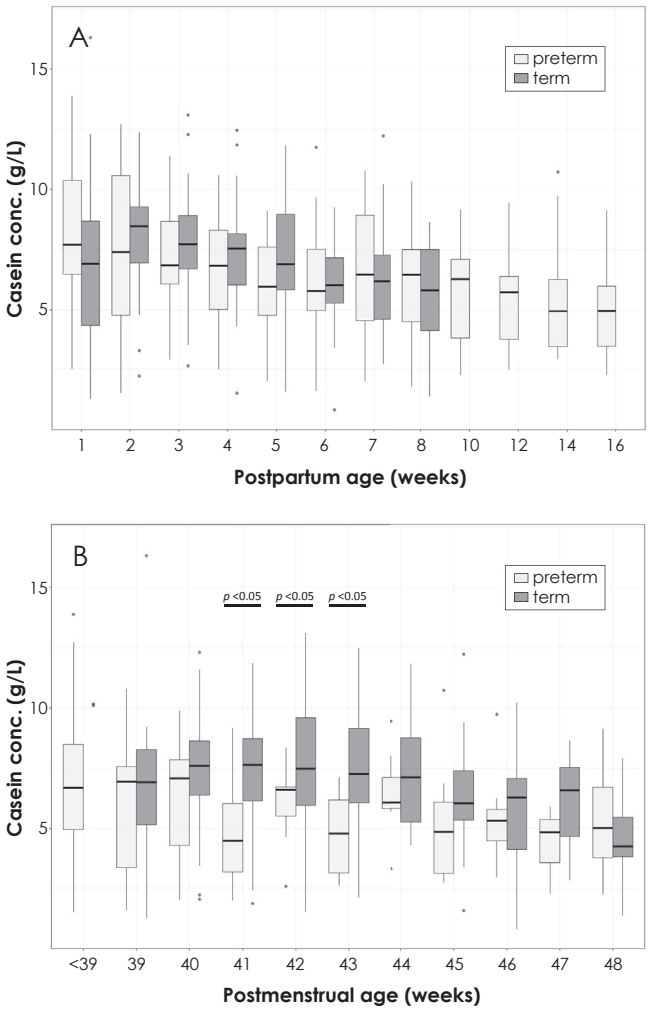

Fig. 5. Comparison of casein content in preterm (PT) and term (T) human milk (HM) over lactation (A) and at equivalent infant postmenstrual age (B). Box plots represent medians with 25th and 75th percentile, min–max range and outliers. Mixed linear models were used to estimate the differences. The models used infant age (either postpartum or postmenstrual), term/preterm delivery, delivery mode, single/multiple delivery and gender as fixed effects. Within subject variability was taken into consideration by declaring the subject ID as a random effect. Contrast estimates of the model were calculated by comparing the two groups (PT and T HM) in each time point (either postpartum or postmenstrual age). No adjustment for multiplicity was applied and a conventional 2-sided 5% error rate was applied. *P* values of the differences between PT and T HM lower than 0.05 are reported.

linked to the compromise between high-throughput but precise quantitative analysis of specific HM proteins versus in-depth but only qualitative profiling of hundreds of proteins using a typical proteomics approach. The studied proteins were selected not only because of their relative abundance in HM but also because of their relevance to the preterm infant nutrition and protection. However, many other proteins in HM are believed to have important biological functions [7], and the development of new quantitative methodologies permitting their accurate characterization in small volumes and large numbers of HM samples is a priority in future human milk research.

Important to note is the high rate of lactation success at 4 months in the study preterm population. This can be due to selection bias, to Hawthorne effect, but also to the fact that mothers were closely (weekly) supported by the dedicated lactation nurse during the entire study period. This close interaction allowed excellent compliance with the HM collection procedures and limited dropout rates, despite the burden of the protocol. This observation confirms the feasibility of successful and durable breastfeeding in this vulnerable population when lactation is adequately supported [40].

In conclusion, this study thoroughly characterized the temporal changes of the major HM proteins, poorly described in previous studies, in particular in preterm HM [30]. It confirmed previous observations that the content of the most abundant proteins rapidly and strongly decreases along lactation, without substantial differences between PT and T HM. Our comparison of the protein content in HM available to preterm and term infants of equivalent postmenstrual age suggests that the provision of nutritionally and functionally important proteins may fall below the requirements for growth and protection after the preterm infant reaches the term corrected age. Whether these observations shall be accounted for in the HM fortification practice warrants further investigation.

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This study was funded by Nestlé Research, Lausanne (Nestec Ltd.). Nestlé Research sponsored the study, participated to the protocol elaboration, and provided the infrastructure and the material and human resources to analyze the HM samples and protein data. Employees of Nestlé Research further participated to the interpretation of the results and to the manuscript writing.

Author's contributions

CGR contributed to the interpretation of the results and drafted the manuscript.

CADC performed the statistical analysis, participated to manuscript writing and approved the final version.

JFT, CJFF contributed to the study design and to the development of overall research plan, conducted the research, reviewed the manuscript and approved the final version.

LB, RJ and SKT conducted the research, reviewed the manuscript and approved the final version.

MA designed and conducted the research, contributed to the interpretation of the results and to manuscript writing and approved the final version.

Conflicts of interest

CLGR, CADC, RJ, SKT and MA are all employees of Nestec Ltd. LB, JFT, and CFF declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.07.016.

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