Early Human Development 91 (2015) 7–11

Contents lists available at ScienceDirect

Early Human Development

journal homepage: www.elsevier.com/locate/earlhumdev

Effect of gestation length on the levels of five innate defence proteins in human milk

Marita Broadhurst [a], Keryn Beddis [a], Janet Black [b], Harold Henderson [a], Arun Nair [b], Thomas Wheeler [a,*]

[a] AgResearch, Ruakura Research Centre, Hamilton, New Zealand
[b] Neonatal Unit, Waikato Hospital, Private Bag 3200, Hamilton, New Zealand

ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form 3 November 2014
Accepted 6 November 2014
Available online xxxx

Keywords:
Lactoferrin
Immunoglobulin A
Immunoglobulin G
Complement C3
Secretory component protein

ABSTRACT

Background: Human milk contains a range of host defence proteins that appear to contribute to health and wellbeing, but their variability in abundance among individuals has not been very well characterised. Milk from mothers of premature infants has altered composition, but the effect of gestation length on the host-defence properties of milk is not known. A study was therefore undertaken to determine the variability and effect of gestation length on the abundance of five host-defence proteins in milk; lactoferrin, secretory IgA, IgG, secretory component, and complement C3.

Methods: Milk was obtained from 30 mothers at their second and fifth week of lactation. These were from three groups of ten mothers having had very premature (V; 28–32 weeks gestation), premature (P; 33–36 weeks) or full term deliveries (T; 37–41 weeks). The concentration of each of the five proteins was measured in each milk sample by either ELISA or quantitative western blotting.

Results: The concentration of IgG, and complement C3 ranged 22- and 17-fold respectively between mothers, while lactoferrin, secretory IgA, and secretory component ranged 7-, 9-, and 4-fold, respectively. The V group had significantly lower concentrations of four of the five proteins, the exception being IgG. Levels of these four proteins also decreased between weeks 2 and 5 of lactation in the P and T groups. Significant correlation was found between the concentrations of the host defence proteins within individual mothers, indicating some degree of co-ordinate regulation.

Conclusions: Mothers vary widely in the levels of host defence proteins in milk. Very short gestation length results in decreased abundance of host-defence proteins in milk. This may have functional implications for very premature infants.

1. Introduction

Milk plays a critical role in providing nutrition to the newborn mammal as well as protecting the neonate against infection. This latter functionality of milk is delivered in part through a range of antimicrobial, pathogen-recognition and other host defence proteins which form part of the complement of minor proteins in milk [1–4]. The abundance of some of these proteins varies considerably between individuals. Some milk proteins are up-regulated as part of the inflammatory response that occurs during infection of the mammary gland [5–7]. Some host defence proteins are also known to be altered in abundance with the stage of lactation [8–11]. This variability in the protein composition of milk, at least in part, may reflect the changing need of the offspring during its growth and development.

Premature babies have specific nutritional requirements [12] and infection is of particular concern. The composition of the milk from preterm mothers is known to be altered compared with full-term mothers [13,14]. However it is not clear whether this is a response to addressing the premature infant's needs, whether it is due to immaturity of mammary development, or for other reasons. Several studies have addressed whether a shorter than normal gestation length affects the host defence proteins in milk. Immunoglobulin A has been reported to be higher in milk from short gestation mothers compared with normal length gestation [15–18]. In contrast, other studies have reported lower IgA levels in milk from mothers of very pre-term babies [19,20]. These studies revealed a high degree of variability in the concentration of IgA between the individual donors. The levels of another host defence protein, lactoferrin, has also been reported to be either lower [18,19] or higher [21] in milk from mothers of premature babies. Thus there is a lack of clarity as to what extent the concentration of host defence proteins vary among individuals, and if the composition of these host defence proteins is altered in pre-term mothers' milk compared with full term mothers.

In a study of 30 mothers (10 in each of three gestation length groups) we wished to determine the extent of within-mother and between-mother variability in the abundance of a range of host defence

* Corresponding author at: AgResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, 3240 New Zealand.
E-mail address: tom.wheeler@agresearch.co.nz (T. Wheeler).

http://dx.doi.org/10.1016/j.earlhumdev.2014.11.006
0378-3782/

proteins in milk during established lactation, and the extent to which the levels of these proteins were altered by gestation length. The levels of five host defence proteins were measured; lactoferrin, secretory IgA, IgG, secretory component, and complement C3. Large variations were found for each of the proteins, with some differences between the gestation-length groups. These data will provide a basis for developing approaches to provide better natural defence against infection for premature infants.

2. Materials and methods

2.1. Collection of milk samples

Between 3 and 5 ml of breast milk was collected either manually or using a breast pump from a total of 30 mothers on two separate occasions; two weeks and five weeks after giving birth. Ten of the mothers had delivered between 28 and 32 weeks of gestation (very premature, V), another ten had delivered between 33 and 36 weeks of gestation (premature, P), while the remaining ten had delivered between 37 and 41 weeks of gestation (normal term, T). The mothers were recruited to the study at the Waikato Hospital Neonatal Unit, Hamilton, New Zealand over a seven month period based on willingness to participate, the absence of any health issue in the mother–infant dyad at time of each collection, and no signs of mastitis since giving birth. A formal informed consent was obtained from each mother. The recruitment procedure was approved by the Northern Y Regional Ethics Committee, based in Hamilton, New Zealand. All the mothers underwent a spontaneous parturition, with 12 of them having a caesarean section delivery. All were non-smokers, and there were no significant differences among the groups in age of the mother, parity or the sex of the babies. However, the P group was more diverse in age of the mother compared to the other groups, and there were 3, 2 and 1 multiple births in the V, P and T groups, respectively.

The milk samples were collected at home and chilled to approximately 6 °C within 10 min of collection and transported on ice to the laboratory within 24 h. The protease inhibitor phenylmethanesulfonyl fluoride (PMSF) was added to a concentration of 1 mM. The milk was centrifuged at 800 *g* for 10 min at 4 °C. The fat layer was removed and aliquots of the skim milk were transferred into fresh tubes and frozen at −20 °C. These were used for all the subsequent analysis. The integrity of the milk proteins was confirmed by electrophoretic analysis.

2.2. Analysis of milk samples

Total protein concentration of each skimmed milk sample was determined by Bradford assays using commercially supplied reagents (Bio-Rad, Hercules, CA). The concentrations of lactoferrin, secretory IgA, and IgG in skimmed milk were measured by ELISA using commercially supplied kits (Bethyl Laboratories, Montgomery, TX). The concentration of the complement factor C3 was also measured by ELISA using a commercially supplied kit (Genway Biotech, San Diego, CA). Each sample was diluted 1/3000 and 1/5000 for lactoferrin, secretory IgA, and IgG assays, and 1/80,000 and 1/160,000 for the C3 assay. Each dilution was measured in duplicate alongside a set of standards and the results averaged. No matrix effects were observed for the sample analyses at the dilutions used.

The concentration of secretory component in the milk samples was measured using quantitative western blotting using a commercially available polyclonal anti-human secretory component antibody raised in sheep (Sigma, St Louis, MO). Five serial dilutions of purified secretory IgA (Sigma) were used as a standard and analysed on the same gel as the milk samples to be quantified. The quantity of secretory component was calculated based on its known stoichiometry in secretory IgA. Samples and standards were resolved by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose, blocked with 4% (w/v) nonfat milk powder and probed with anti-secretory component antibody at 53 ng/ml. After washing, the blot was probed with anti-sheep IgG conjugated to horseradish peroxidase (Sigma). After three washes the blot was incubated for 2 min in a freshly prepared mixture of 1.25 mM luminol (Sigma), 67 μM p-coumaric acid (Sigma) and 0.01% (w/v) hydrogen peroxide in 0.1 M Tris.HCl, pH 8.8. The luminol and p-coumaric acid were added from stocks of 500 mM luminol and 168 mM p-coumaric acid in DMSO. The hydrogen peroxide was added from a 30% (v/v) stock solution (Scharlau, Sentiment, Spain). The chemiluminescent signal was captured using a CCD camera-based detector (Chemidoc, Bio-Rad) and processed to produce quantities of secretory component in the samples and standards using the Quantity One software package (Bio-Rad). Between two and eight replicate analyses of each sample were performed, at a range of dilutions.

2.3. Statistical analysis

The data were subjected to ANOVA using GenStat. Statistical analysis of the total protein concentration, and the logarithm of individual protein concentrations were analysed with a linear mixed model analysis in GenStat with gestation group, week of lactation and their interaction as fixed effects, and individual mother and individual mother-within-week as random effects. Within-assay variability was assessed for individual protein concentrations, with coefficients of variation attained between 10 and 15%. Pearson's correlation analysis was performed using the GenStat software package.

3. Results

The abundance of total protein, as well as that of five host-defence proteins (lactoferrin, secretory IgA, IgG, complement C3 and secretory component) was determined in milk samples collected from each of 30 mothers, falling into one of three groups; 10 having had a normal term gestation (T) of 37 to 40 weeks, 10 having had a premature delivery (P) between 33 and 37 weeks, and 10 having had a very premature delivery (V) of between 26 and 33 weeks. For all but one of the pre-term mothers, samples were obtained at both week 2 and week 5 of lactation. The concentration of total protein obtained from one of the term mother's week 5 sample was very much higher than the others (Fig. 1). This sample also had very high levels of the individual proteins — between 2 fold and 40 fold higher than the average for the remaining samples. This outlier was most likely due to an inflammatory response as a result of a subclinical infection of the mammary gland at the time of collection. The values obtained from this sample were therefore removed from subsequent statistical analyses and figures. A large and significant degree of variation among the mothers was observed for each of the proteins.

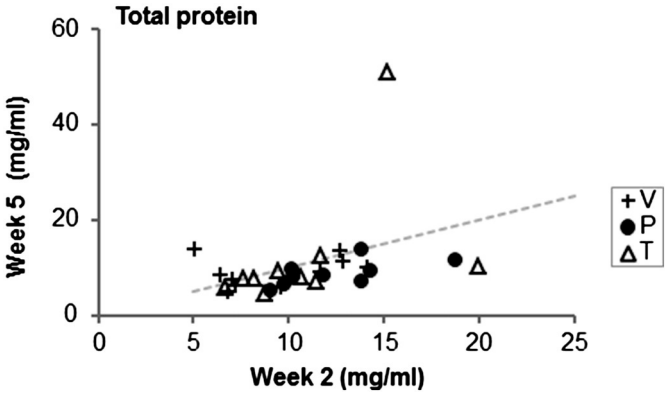

Fig. 1. Total protein concentration of milk from mothers at weeks 2 and 5 of lactation. The protein concentrations (mg/ml) obtained from milk samples from each mother at week 5 was plotted against the protein concentration of the milk sample obtained at week 2 from the same mother, for each of the 10 mothers in each of the groups (very premature (V), premature (P), and full term (T)). The dashed line represents equal concentration for weeks 2 and 5.

The coefficients of variation are two- to five-fold greater than that of the technical variation, with a 3- to 17-fold range in concentration, depending on the protein measured. The magnitude of the variation indicates that it is of biological origin. The means, standard errors, coefficients of variation, and ranges obtained for total protein concentration as well as for each of the individual proteins are summarised in Table 1.

The variability in the abundance of the proteins could be due to environmental or physiological circumstances at the time of milk collection, or could be the result of inherent physiological differences between individual mothers. In order to begin to address this question, the total protein abundance, as well as that of the individual proteins, was compared between the milk samples taken at weeks 2 and 5 after birth for each of the mothers. A decrease in abundance between weeks 2 and 5 was observed for many of the mothers (Fig. 2). Statistical analysis showed these differences were significant ($p < 0.05$) for total protein, lactoferrin, secretory IgA, complement C3 and secretory component in either the premature delivery or term delivery groups. The abundance of IgG appeared to be unchanged between weeks 2 and 5 (Fig. 2). One possible explanation for the large variation is that it is due to the milk being diluted to a variable amount among individual samples, either *in vivo* or during sample preparation. To address this, the abundance of each of the five proteins relative to total protein concentration of the sample was determined, and the variability was analysed as above. In this case the CV% values obtained were 40, 59, 62, 59, and 55 for lactoferrin, secretory IgA, IgG, secretory component and C3, respectively. These values are 2- to 4-fold greater than the technical variation, and there is a 5 to 21-fold range in the relative concentrations of each protein among individual samples. These CV% values and ranges are similar to those obtained using absolute concentrations, indicating that dilution of the milk is not a significant contributor to the biological variation.

A correlation analysis of the abundance was performed on all possible pairs of the five proteins, using log-transformed data, in order to determine if their concentrations are linked to each other within an individual mother. Analyses on the samples from week 2 of lactation resulted in a significant correlation ($p < 0.05$) between lactoferrin and IgG ($r = 0.46$), lactoferrin and C3 ($r = 0.52$), secretory IgA and IgG ($r = 0.43$), IgA and C3 ($r = 0.67$), and IgG and C3 ($r = 0.66$). A similar analysis using the concentrations at week 5 of lactation resulted in a significant correlation (r ranged from 0.37 to 0.86) for all pairs. The threshold correlation coefficient for significance with a 95% confidence level ($p = 0.05$) was 0.36.

The above analyses suggest that both the time after delivery and the mother's gestation length influence the abundance of the host defence proteins in her milk. Therefore, the data were also examined to determine if for each collection time, the differences among the groups were statistically significant (Fig. 3). This analysis showed that statistically significant decreases occurred between the V group and the other groups for four of the five proteins, particularly between the very premature and premature groups at week 5 ($p < 0.05$ for all the proteins except IgG).

4. Discussion

This study has shown that there is a large variability in the concentration of at least five host defence proteins in mature milk among a sample size of 30 mothers. There is a 4- to 17-fold range in concentration, depending on the protein. Similar variation is apparent when the abundance relative to total protein abundance is considered, indicating that the variability is not due to a dilution effect. These levels of variation are well above the technical variability produced by the assays (10–15% CV), so these differences are biological in origin and may be significant for the host defence function of the milk. A recent study found that total protein concentration of skimmed milk varied among 25 mothers with a CV of 28% [22], similar variation to that obtained in this study. Further, these authors have also shown variation in milk composition over time within mothers [23,24]. The comparison between milk from weeks 2 and 5 of lactation in the present study showed that there is less biological variation between the two milk samples within a mother over time compared with among the mothers, indicating that inherent differences among the population accounts for at least some of the variability, rather than day-to-day physiological status as described in the previous studies. Thus, different mothers appear to produce milk with distinct inherent host-defence functionalities. The consequence of this for the baby requires further investigation.

Comparison among the different gestation length groups showed only slight differences compared with the variation among mothers in general. Nevertheless, the milk from the V group was distinct from the P and T groups in that the host-defence proteins did not decrease in concentration between weeks 2 and 5. This may reflect either a response to the particular needs of a very premature infant or the result of incomplete development of the mammary gland during the shortened pregnancy. In either case, it appears that the host defence composition of milk can be influenced by the physiological context.

The concentrations of the five host defence proteins were not all identically regulated. Despite a 22-fold range of concentrations among all the samples, the concentration of IgG appeared to be relatively little altered between the groups or between weeks 2 and 5 of lactation. In contrast, the concentration of the other four proteins was altered, either between at least two of the groups or between weeks 2 and 5. Correlation analysis revealed some linkage of the concentration between pairs of host defence proteins within an individual sample, suggesting some degree of co-ordinated regulation of expression of the proteins. This is consistent with the concept that the overall host defence functionality of milk is subject to some degree of centralised control within each mother, and that the setting of this control varies among mothers.

The results presented here help to resolve uncertainties resulting from contrasting earlier reports on the concentration of proteins in mature milk. The present study produced a mean total protein concentration of 9.8 mg/ml and showed no significant effect of gestation length on total protein concentration. This is comparable with one study which reported a concentration of 12–14 mg/ml [21] with no significant change with gestation length, but in contrast to another study that

Table 1
Concentrations of total protein and five specific proteins in milk from three gestation length groups at two times in lactation*.

		Total protein (mg/ml)	Lactoferrin (mg/ml)	sIgA (mg/ml)	IgG (μg/ml)	SC (μg/ml)	C3 (μg/ml)
All samples	Mean	9.84	2.69	0.52	20.4	63.0	157
	SE	(0.44)	(0.15)	(0.04)	(2.06)	(2.67)	(96.0)
	CV%	34.0	42.0	58.0	76.0	32.0	61.0
	range	4.73–19.9	0.88–6.23	0.06–1.87	4.56–103	32.0–128	28.3–476
Week 2	V	9.37 (1.03)	3.34 (0.27)	0.54 (0.09)	23.4 (6.71)	77.5 (7.10)	165 (29.2)
	P	12.4 (0.92)	3.48 (0.37)	0.52 (0.09)	16.5 (1.84)	62.1 (6.65)	163 (16.1)
	T	11.0 (1.26)	2.61 (0.37)	0.68 (0.14)	20.0 (2.57)	56.2 (4.79)	177 (37.0)
Week 5	V	9.01 (0.97)	2.92 (0.33)	0.59 (0.09)	28.2 (8.83)	72.3 (7.13)	213 (43.0)
	P	8.86 (0.78)	1.98 (0.25)	0.34 (0.06)	14.6 (1.15)	54.4 (5.07)	93.5 (9.37)
	T	8.15 (0.78)	1.65 (0.18)	0.45 (0.06)	19.1 (2.80)	53.7 (4.13)	119 (22.8)

(*) Data is presented as arithmetic means, with their standard errors in parentheses. The % coefficient of variation and range of concentrations over all the samples is also presented. V; very premature delivery, P; premature delivery, T; full term delivery.

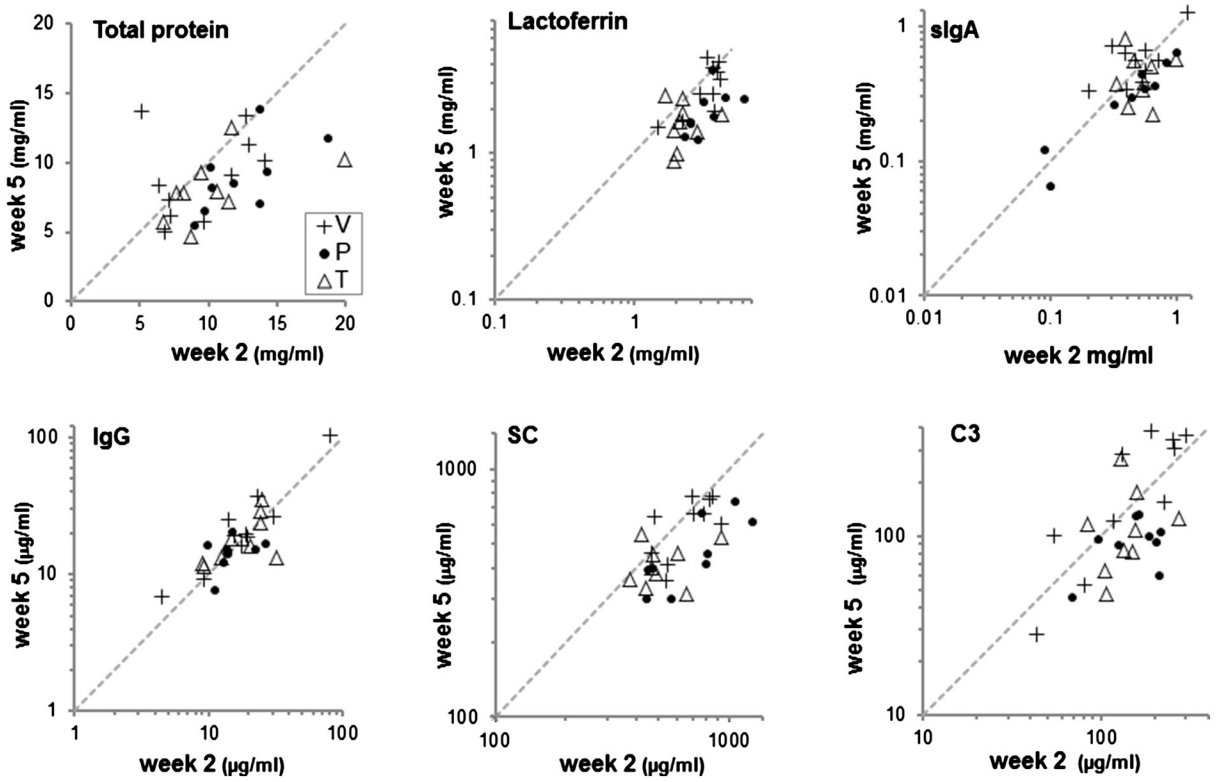

Fig. 2. Change in concentration of individual proteins between weeks 2 and 5 of lactation. The concentration of total protein as well as that of lactoferrin, secretory IgA, IgG, secretory component and complement C3 obtained from each mother at week 5 was plotted against that from the same mother at week 2. The gestation group of the mother (very premature (V), premature (P) and full term (T)) is indicated by crosses, filled circles and open triangles, respectively. The diagonal dashed line represents equal concentrations for weeks 2 and 5.

reported a 15% higher overall protein concentration in milk from short gestation length mothers compared with normal term delivery [25]. Given the large inter-individual variation (34% CV in this study) it would seem that an effect of this magnitude would be of marginal significance. Lactoferrin in mature milk has been reported to be present at 1 mg/ml [26], 0.5 mg/ml [18], or 3.3 mg/ml [21]. The present study

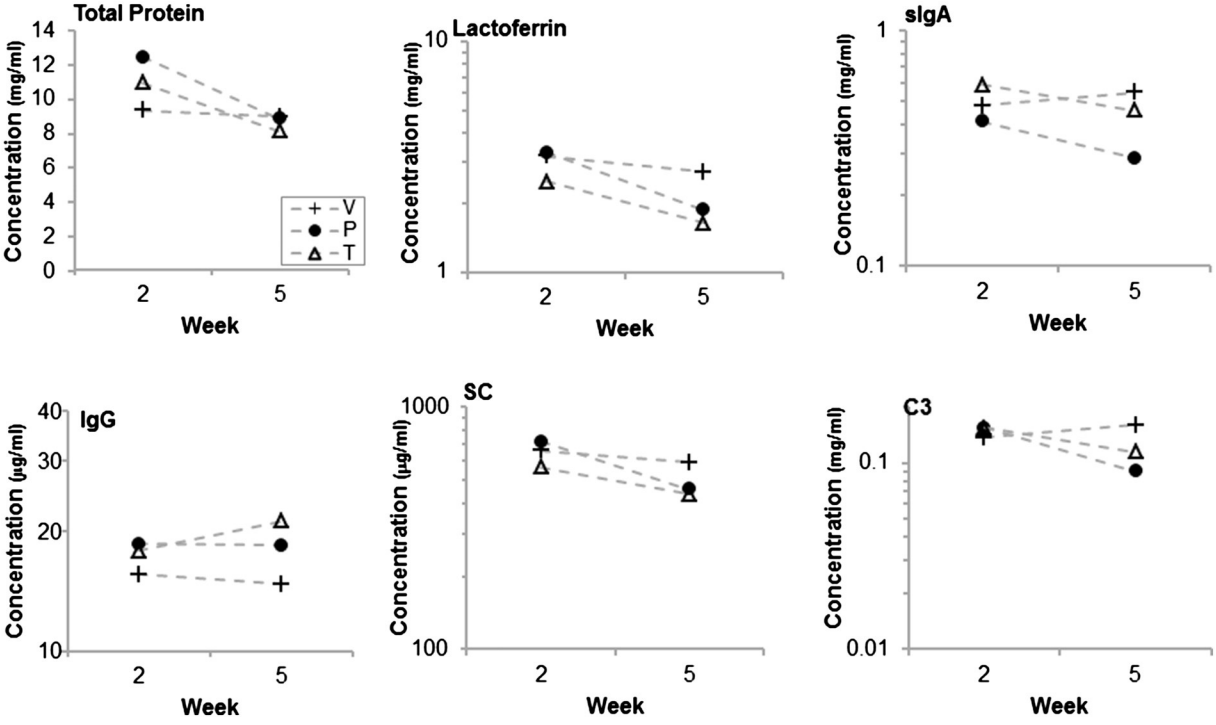

Fig. 3. Change in mean concentration of gestation groups for individual proteins between weeks 2 and 5 of lactation. The group means for the concentration of total protein, lactoferrin, secretory IgA, IgG, secretory component, and complement C3 are shown for all the milk samples taken at week 2 as well as those taken at week 5 of lactation. The means for the very premature (V), premature (P) and full term (T) group are indicated by crosses, filled circles and open triangles, respectively.

produced an estimate of 2.7 mg/ml, with a CV% of 42 and concentrations ranging from 0.9 to 6.2 mg/ml. Therefore the differences among the earlier studies are likely to be due to the large inter-individual variability. Similarly, the concentration and variability of secretory IgA found in this study (mean = 0.52 mg/ml, range 0.06–1.87) accounts for the differences reported in previous studies [15,18]. The concentration of IgG determined here (0.02 mg/ml) is similar to at least one previous study which reported 0.05 mg/ml in full term colostrum [27]. The average concentration of complement C3 in mature milk obtained in this study (0.16 mg/ml) is similar to two previous studies reporting values of 0.17 and 0.12 mg/ml [28], but higher than two others, which reported concentrations of 0.03 mg/ml [29] and 0.016 [30]. To our knowledge, the concentration of secretory component has not previously been reported for human milk.

The large degree of biological variation observed in this and previous studies could have many possible sources. Inherent biases in the sample population could potentially affect the results. As would be expected, the T group has a significantly increased ($p = 0.042$) proportion of normal vaginal deliveries (9 out of 10) compared with the other groups (9 out of 20). However, there is no known mechanism to account for how this would lead to altered abundance of the five proteins measured in this study. The parity was similar between the groups. Other possible sources of variation which were not assessed include the presence of subclinical disease, diet, lifestyle choices, ethnicity, and the age of the mother.

It has been reported that the total concentration of protein in colostrum and milk is increased in mothers with short gestation [16,27]. In this study of mature milk, while the protein concentrations varied substantially between individual samples, no such increase was observed. One reason for this may be the stage of lactation, as the composition of milk changes markedly in the transition from colostrum to mature milk.

Taken together, the findings from this study suggest that the host defence function of milk varies widely among the population of nursing mothers. However, it appears that the levels of the five host defence proteins examined here are not significantly different between short gestation-length mothers and the other groups, except that their levels remain elevated later in lactation in the V group compared with the other groups. The abundance of the host defence proteins in the mother's milk could conceivably influence susceptibility of the infant to infection. Whether the high variability and effect of gestation length reported here are significant for the infant's wellbeing awaits further investigation.

Conflict of interest statement

All authors declare they have no conflict of interest.

Acknowledgements

The contributions of Kerst Stelwagen, Alison Hodgkinson, Rex Humphrey and Elisabeth Carpenter to discussions on developing the study design are acknowledged. Preparation of the ethics application was done with the help of Ms Bronwyn Clothier. The study was made possible through grants from the Waikato Medical Research Foundation and the New Zealand Ministry of Business, Innovation and Employment (contract number C10X0707). The generosity of the mothers who participated in the study is acknowledged.

References

[1] Hosea Blewett HJ, Cicalo MC, Holland CD, Field CJ. The immunological components of human milk. In: Taylor S, editor. Advances in Food and Nutrition Research. Academic Press; 2008. p. 45–80.
[2] Hettinga K, van Valenberg H, de Vries S, Boeren S, van Hooijdonk T, van Arendonk J, et al. The host defense proteome of human and bovine milk. PLoS One 2011;6(4):e19433.
[3] Picariello G, Ferranti P, Mamone G, Klouckova I, Mechref Y, Novotny MV, et al. Gel-free shotgun proteomic analysis of human milk. J Chromatogr A 2012;1227:219–33.
[4] Wheeler TT, Smolenski GA, Harris DP, Gupta SK, Haigh BJ, Broadhurst MK, et al. Host-defence-related proteins in cows' milk. Animal 2012;6(3):415–22.
[5] Ramadan MA, Salah MM, Eid SZ. The effect of breast infection on the composition of human milk. J Reprod Med 1972;9(2):84–7.
[6] Fetherston CM, Lai CT, Hartmann PE. Relationships between symptoms and changes in breast physiology during lactation mastitis. Breastfeed Med 2006;1(3):136–45.
[7] Boehmer JL. Proteomic analyses of host and pathogen responses during bovine mastitis. J Mammary Gland Biol Neoplasia 2011;16(4):323–38.
[8] Trégoat VS, Cuillière ML, Molé CM, Béné MC, Faure GC, Montagne PM. Dynamics of innate and cognitive immune components in human milk during lactation. J Food Compos Anal 2003;16(1):57–66.
[9] Reinhardt TA, Lippolis JD. Developmental changes in the milk fat globule membrane proteome during the transition from colostrum to milk. J Dairy Sci 2008;91(6):2307–18.
[10] Liao Y, Alvarado R, Phinney B, Lonnerdal B. Proteomic characterization of human milk whey proteins during a twelve-month lactation period. J Proteome Res 2011;10(4):1746–54.
[11] Gao X, McMahon RJ, Woo JG, Davidson BS, Morrow AL, Zhang Q. Temporal changes in milk proteomes reveal developing milk functions. J Proteome Res 2012;11(7):3897–907.
[12] Senterre T, Rigo J. Optimizing early nutritional support based on recent recommendations in VLBW infants and postnatal growth restriction. J Pediatr Gastroenterol Nutr 2011;53(5):536–42.
[13] Atkinson SA. Effects of gestational age at delivery on human milk components. In: Jensen RG, editor. Handbook of Milk Composition. San Diego: Academic Press; 1995. p. 222–37.
[14] Molinari CE, Casadio YS, Hartmann BT, Livk A, Bringans S, Arthur PG, et al. Proteome mapping of human skim milk proteins in term and preterm milk. J Proteome Res 2012;11(3):1696–714.
[15] Ronayne de Ferrer PEA. Immunoglobulin A level in human milk frommothers delivering preterm. Am J Clin Nutr 1984;40:465–7.
[16] Araujo ED, Goncalves AK, Cornetta Mda C, Cunha H, Cardoso ML, Morais SS, et al. Evaluation of the secretory immunoglobulin A levels in the colostrum and milk of mothers of term and pre-term newborns. Braz J Infect Dis 2005;9(5):357–62.
[17] Ballabio C, Bertino E, Coscia A, Fabris C, Fuggetta D, Molfino S, et al. Immunoglobulin-A profile in breast milk from mothers delivering full term and preterm infants. Int J Immunopathol Pharmacol 2007;20(1):119–28.
[18] Mehta R, Petrova A. Biologically active breast milk proteins in association with very preterm delivery and stage of lactation. J Perinatol 2011;31(1):58–62.
[19] Pamblanco M, Ten A, Comin J. Proteins in preterm and term milk from mothers delivering appropriate or small-for-gestational age infants. Early Hum Dev 1986;14(3–4):267–72.
[20] Castellote C, Casillas R, Ramirez-Santana C, Perez-Cano FJ, Castell M, Moretones MG, et al. Premature delivery influences the immunological composition of colostrum and transitional and mature human milk. J Nutr 2011;141(6):1181–7.
[21] Ronayne de Ferrer PEA. Lactoferrin levels in term and preterm milk. J Am Coll Nutr 2000;19(3):370–3.
[22] Khan S, Casadio YS, Lai CT, Prime DK, Hepworth AR, Trengove NJ, et al. Investigation of short-term variations in casein and whey proteins in breast milk of term mothers. J Pediatr Gastroenterol Nutr 2012;55(2):136–41.
[23] Khan S, Hepworth AR, Prime DK, Lai CT, Trengove NJ, Hartmann PE. Variation in fat, lactose, and protein composition in breast milk over 24 hours: associations with infant feeding patterns. J Hum Lact 2013;29(1):81–9.
[24] Khan S, Prime DK, Hepworth AR, Lai CT, Trengove NJ, Hartmann PE. Investigation of short-term variations in term breast milk composition during repeated breast expression sessions. J Hum Lact 2013;29(2):196–204.
[25] Anderson GH. The effect of prematurity on milk composition and its physiological basis. Fed Proc 1984;43(9):2438–42.
[26] Houghton M, Santoso H, Soetjiningsih Gracey M. Lactoferrin concentrations in the breast milk of Indonesian mothers. Paediatr Indones 1985;25(9–10):163–6.
[27] Koenig A, de Albuquerque Diniz EM, Barbosa SF, Vaz FA. Immunologic factors in human milk: the effects of gestational age and pasteurization. J Hum Lact 2005;21(4):439–43.
[28] Ogundele M. Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. Immunol Cell Biol 2001;79(1):1–10.
[29] Tregoat V, Montagne P, Cuilliere ML, Bene MC, Faure G. C3/C4 concentration ratio reverses between colostrum and mature milk in human lactation. J Clin Immunol 1999;19(5):300–4.
[30] Prentice A, Prentice AM, Cole TJ, Paul AA, Whitehead RG. Breast-milk antimicrobial factors of rural Gambian mothers. I. Influence of stage of lactation and maternal plane of nutrition. Acta Paediatr Scand 1984;73(6):796–802.