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ORIGINAL ARTICLE

Combining hand techniques with electric pumping increases the caloric content of milk in mothers of preterm infants

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Objective: We previously reported that preterm mothers' milk production can exceed levels of term mothers by using early hand expression and hands-on pumping (HOP) with the highest production (955 ml per day) in frequent users of hand expression. In this study, we compared milk composition between mothers stratified by early hand expression frequency.

Study Design: A total of 67 mothers of infants <31 weeks gestation were instructed on hand expression and HOP. Subjects submitted expression records and 1-ml samples from each pumping session over 24 h once weekly for 8 weeks.

Result: 78% (52/67) of mothers completed the study. But for Week 1, no compositional differences (despite production differences) were noted between the three groups. Protein and lactose tracked reported norms, but fat and energy of mature milk (Weeks 2–8) exceeded norms, 62.5 g l^{-1} per fat and 892.7 cal l^{-1} (26.4 cal oz^{-1}), respectively.

Conclusion: Mothers combining manual techniques with pumping express high levels of fat-rich, calorie-dense milk, unrelated to production differences.
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Keywords: hand expression; breastmilk; breastfeeding; lactation; preterm infants; milk composition

Introduction

Although human milk is the preferred nutrition for premature infants, the major limitations have been inadequate supply[1,2] and suboptimal nutrients for the infant to achieve optimal growth.[3] More effective milk expression techniques may address both issues by removing a larger fraction of nutrient-dense 'hindmilk'.

We previously reported that mothers of preterm infants can attain and sustain high milk production levels by combining the use of electrical pumps with two manual techniques, hand expression and hands-on pumping (HOP) (Figure 1).[4] Mothers who initiated expression by using frequent hand expression (>5 × per day × 3 days) produced the highest subsequent volumes. The influence of hand expression in the first 3 days was independent of pumping frequency and affected production up to Week 8. We concluded that early hand expression may have removed viscous colostrum more effectively than pump suction alone, and thereby influenced the percentage of alveoli (milk secretory units) recruited for subsequent milk production.

Once milk volume increased, all mothers received instruction on the second manual technique, HOP, at 20.6 ± 9.6 days postpartum. This technique combines electric pumping with breast compression, massage, stripping and, if needed, hand expression. The use of HOP did not increase pumping time and was associated with longer uninterrupted sleeping time.[4]

This was the first report of mothers of preterm infants with a steady increase in production over 8 weeks, which surpassed reference levels for mothers of term infants. Recently, a pump with a newer suction pattern was shown to improve volume over earlier pumps, and yet subjects did not achieve production levels on a par with those we reported for mothers using frequent early hand expression. This pump was designed to mimic the suction pattern of the breastfeeding baby, but there is no feature to match the infant's oral compression and milking action.[5] We speculate that combining electric pumping with manual techniques (which include breast compression and a milking action) results in more effective breast emptying, thereby increasing milk production. If correct, one would expect compositional changes to include a higher percentage of hindmilk fat, possibly improving the nutritional adequacy of human milk for the preterm infant.

Milk fat, but not other macronutrients increases significantly in relation to breast emptying.[6] Special attributes of human milk fat for preterm infants have included the benefits of polyunsaturated fatty acids on neuronal development,[7] the superiority of absorption of human milk fat versus cow milk formula fat,[8] the improved caloric value of high fat feeding, and increased availability of fat-soluble vitamins.[9] Concerns about the nutritional inadequacies of human milk for the preterm infant have led to routine multi-nutrient fortification of human milk both pre- and

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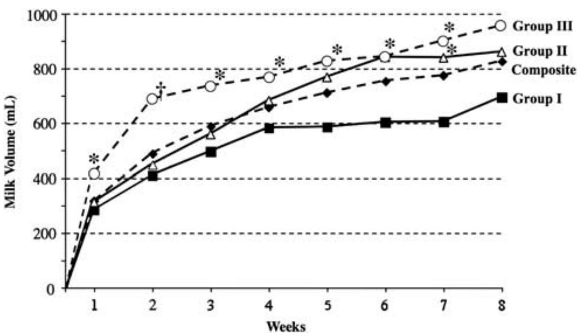

Figure 1 Mean daily volumes (MDV) of expressed milk over the course of the 8-week study of three groups as defined by frequency of hand expression during the first three postpartum days. Group I (<2 times per day, $n = 15$), Group II (2 to 5 times per day, $n = 18$) and Group III (>5 times per day, $n = 16$) volumes are shown along with the output data of the Composite Group. Statistical comparisons using analysis of variance were performed only between Groups I, II and III. $P<0.05$ *vs Group I, vs Groups I and II. (Morton J, Hall JY, Wong RJ, Thairu L, Benitz WE and Rhine WD. Combining hand techniques with electric pumping increases milk production in mothers of preterm infants. *J Perinatol* 2009; **29:** 757–764, reprinted with permission).

post-discharge, with limited regard for compositional variation.[3] If expression techniques consistently and significantly affect the nutritional value, a more selective approach to fortification may be appropriate.

Currently, reports on the composition of milk of mothers of preterm infants and donor milk are primarily based on samples expressed using passive pump suction.[9–11] A possible influence of breast massage on milk composition was proposed by Foda *et al.*,[12] who reported total solids, lipids, casein and gross energy increase in milk hand expressed after using a Japanese method of breast massage.

The composition of milk expressed by combining pump suction with manual techniques has not previously been reported. Our objective was to investigate the constituents of milk in mothers using two techniques, which influence production (early hand expression of colostrum and HOP of mature milk). Furthermore, we compare milk composition in high production mothers (those initiating expression using hand expression $>5\times$ day) to that in lower production mothers (those initiating expression using hand expression $<2\times$ day, yet pumped with the same frequency).

Methods

Study population

From 2004 to 2006, a convenience sample of eligible mothers of preterm infants (<1500 g infants, <31 weeks gestation) were invited to participate. Exclusion criteria included a moribund infant, breast surgery, substance abuse, severe maternal illness and known plans to transfer the infant to another facility. Written informed consent was obtained from all participants. The study was approved by the Stanford University Institutional Review Board and the Human Research Ethics Committee, The University of Western Australia.

Study design

Mothers were provided Symphony pumps (Medela, McHenry, IL, USA) for either the duration of the study or the hospitalization of the infant, which ever was longer. Maternal perinatal information and histories were obtained through chart review, questionnaires and interviews. Mothers recorded their own milk expression information, including date, time, duration and volume removed from each breast for 8 weeks.

Manual techniques (hand expression and HOP) combined with electric pumping

Participants were instructed to begin pumping within 6 h following delivery. Before the onset of copious milk production, they were instructed to ‘double pump’ (pump both breasts simultaneously) eight times per day for 15 min, and to hand-express colostrum as frequently as possible in the first three postpartum days. Once milk volume increased, mothers were advised to pump eight times per day until they could express only drops. As the capability of mothers varied, partners and family members were invited to learn the techniques to help with hand expression.

Once discharged, mothers returned for monitored pumping sessions where they were instructed on ‘HOP’. In contrast to relying only on pump suction for milk removal, this technique combined both electric pumping with manual maneuvers, which included breast massage, compression, stripping and, if needed, hand expression. Instructions for hand expression and ‘HOP’ can be viewed at http://newborns.stanford.edu/Breastfeeding/HandExpression.html and http://newborns.stanford.edu/Breastfeeding/MaxProduction.html.

Sample collection

Mothers were asked to donate milk samples once they felt doing so would not deprive their infants. They were given pre-labeled 1-ml cryogenic vials (Econo-lab, Chambly, QC, Canada) and asked to contribute right and left breastmilk samples after completing expression by swirling the bottles of pooled milk collected from each breasts and pouring mixed milk into the vials. Subjects labeled the vials with time and date. They were asked to provide right and left breastmilk samples from one expression on postpartum days 1 to 14 and similarly, right and left breastmilk samples after every pumping for a 24-h period once a week, beginning week 2. They were instructed to freeze the samples immediately, transport them in cold storage and give them to staff for placement in a freezer. They were logged in, shipped in frozen storage to Perth, Australia for analyses at The University of Western Australia.

Table 1 Composition values (mean ± s.d. in $g\,l^{-1}$ and $cal\,l^{-1}$) of milk expressed using hand techniques and electric pumping

	WEEK 1 (n)	*WEEK 2* (n)	*WEEK 3* (n)	*WEEK 4* (n)	*WEEK 5* (n)	*WEEK 6* (n)	*WEEK 7* (n)	*WEEK 8* (n)
Lactose ($g\,l^{-1}$)	60.7 ± 9.3 (48)	67.1 ± 6.8 (43)	68.6 ± 7.0 (51)	70.6 ± 7.8 (61)	71.7 ± 6.9 (54)	73.5 ± 7.6 (50)	73.6 ± 7.2 (46)	75.3 ± 7.8 (45)
Lactose ($cal\,l^{-1}$)	235.0 ± 36.1 (48)	259.5 ± 26.5 (43)	265.5 ± 27.1 (51)	273.1 ± 30.1 (61)	277.5 ± 26.5 (54)	284.3 ± 29.5 (50)	294.5 ± 28.9 (46)	291.6 ± 30.4 (45)
Protein ($g\,l^{-1}$)	20.1 ± 3.5 (48)	17.8 ± 2.9 (50)	17.2 ± 2.7 (50)	15.7 ± 2.3 (58)	14.7 ± 2.3 (53)	14.5 ± 2.3 (49)	13.8 ± 2.2 (46)	12.7 ± 3.1 (45)
Protein ($cal\,l^{-1}$)	85.9 ± 15.0 (48)	76.1 ± 12.2 (43)	73.5 ± 11.7 (50)	67.1 ± 10.0 (58)	62.9 ± 9.9 (53)	61.9 ± 9.8 (49)	58.8 ± 9.3 (46)	54.1 ± 13.1 (45)
Fat ($g\,l^{-1}$)	51.8 ± 20.3 (48)	60.8 ± 23.5 (42)	63.1 ± 16.0 (50)	65.2 ± 16.6 (63)	62.8 ± 18.8 (52)	64.3 ± 17.2 (51)	63.2 ± 21.8 (45)	57.9 ± 19.7 (45)
Fat ($cal\,l^{-1}$)	454.9 ± 178.8 (48)	538.4 ± 206.9 (42)	555.0 ± 140.7 (50)	573.1 ± 146.3 (63)	551.9 ± 165.1 (52)	565.2 ± 150.9 (51)	555.4 ± 191.4 (45)	509.2 ± 173.6 (45)
Total $cal\,l^{-1}$	775.8	874.0	894.0	913.3	892.3	911.4	908.7	854.9
Total $cal\,oz^{-1}$	19.4	24.7	26.4	27.0	26.4	27.0	26.9	26.6

Fat, lactose and protein concentrations represent the averages of samples from the last day of that week for all study mothers.

Sample analyses

Esterified fat assay. Esterified fat assay was determined by a method described by Atwood and Hartmann.[13] Triolein was used as a standard and the recovery of a known amount of fat added to milk samples was 98.4 ± 5.5% ($n=15$). The detection limit of this assay was $0.27 \pm 0.03\,g\,l^{-1}$ ($n=15$) and the interassay coefficient of variation (CV) was 7.3% ($n=15$).

Lactose. Lactose was determined by an enzymatic method described by Arthur and Hartmann.[14] The recovery of a known amount of lactose added to defatted milk samples was 99.5 ± 2.3% ($n=10$). The detection limit of this assay was $11.3 \pm 0.3\,g\,l^{-1}$ ($n=10$) and the interassay CV was 5.3% ($n=10$).

Proteins. The protein assay was performed on defatted milk samples using the Bio-Rad Protein Assay (Hercules, CA, USA) according to manufacturer's instructions. Kjeldahl's method was used to determine the protein concentration of a defatted human milk sample. The defatted milk sample was then diluted and used as a protein standard for the Bio-Rad Protein Assay. The recovery of a known amount of protein added to defatted milk samples was 100.5 ± 3.8% ($n=7$). The detection limit of this assay was 0.14 ± 0.02 g/l ($n=10$) and the interassay CV was 6.6% ($n=10$).

Energy. For mature milk, energy values based on bomb calorimetry agree with calculations based on conversion formulas, summing the contribution to total energy from each of the major components of milk.[15,16] Energy conversion calculations are based on the specific Atwater conversion factors: proteins $\times 4.27\,kcal\,g^{-1}$; fats $\times 8.79\,kcal\,g^{-1}$; lactose $\times 3.87\,kcal\,g^{-1}$.[13]

Statistical analyses. Laboratory investigators were masked as to breakdown of subjects in the hand expression groups. Statistical analyses were performed using SAS v9.2 Software (SAS Institute, Cary, NC, USA). The factors affecting changes in milk production for an individual mother were analyzed using analysis of variance and with Student's paired *t*-tests. Comparisons of milk production volumes for any given timeframe were done using unpaired *t*-tests. The level of significance was set at $P \leqslant 0.05$. All data are expressed as mean ± s.d.

Results

Demographics

A total of 71 mothers were recruited. Three refused and one was ineligible because of breast implants. As described previously, of the 67 enrolled mothers, 52 remained in the study for the entire 8 weeks.[4] No significant demographic differences among the three groups were found.

Hand expression

In all 49 reported their frequency of use of hand expression in days 1 to 3. Based on the frequency of hand expression, these participants were stratified into three self-selected groups: Group I (no/low, <2 times per day, $n=15$); Group II (medium, 2 to 5 times per day, $n=18$); and Group III (high, >5 times per day, $n=16$). Up until Week 8, significant production differences were reported between the three groups, with Group III maintaining the highest levels. By Week 8, mean production in all Groups was 658 ± 267, 859 ± 430, and 955 ± 667 ml per day, respectively, as we have previously reported.[4] No statistical differences in mean pumping frequency in Groups I, II and III over the first 3 days postpartum or over days 1 to 14 were found.

Sample collection and analyses

Overall, 5684 samples were analyzed. Table 1 and Figure 2 presents the average values for fat, lactose and protein concentrations for study mothers from Week 1 to 8. Fat, lactose and protein concentrations represent the averages of samples from the last day of that week for each study mother. The fat content increased from $51.8\,g\,l^{-1}$ at Week 1 to $63.1 \pm 16.0\,g\,l^{-1}$ at Week 3 and then remained relatively constant, with a mean fat value for mature milk (Weeks 2–8) of $62.5\,g\,l^{-1}$ per fat. The concentration of lactose also increased from Week 1 (60.7 ± 9.3) through Week 8 ($75.3 \pm 7.8\,g\,l^{-1}$); whereas, the concentration of protein decreased from Week 1 ($20.1 \pm 3.5\,g\,l^{-1}$) to Week 8 ($12.7 \pm 3.1\,g\,l^{-1}$).

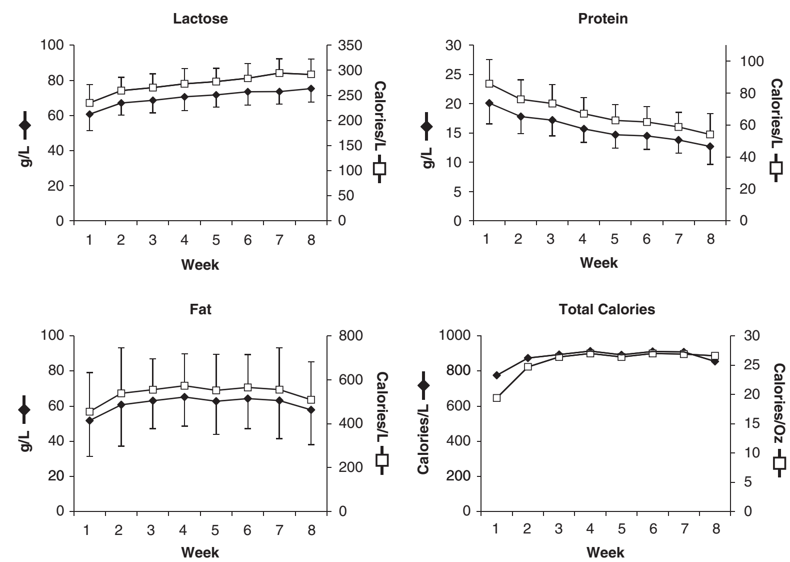

Figure 2 Compositional ($g\,l^{-1}$) and caloric ($cal\,l^{-1}$) values of milk expressed using hand techniques and electric pumping over the 8-week study period. Fat, lactose and protein concentrations represent the averages of samples from the last day of that week for each study mother (see also Table 1).

$cal\,l^{-1}$ are calculated by summing the contribution to metabolizable energy from each of the major components of milk, that is, fats, lactose and total proteins. Primarily weighted by the fat contribution, energy values of mature milk (Weeks 2–8) averaged 892.7 $cal\,l^{-1}$ or 26.4 $cal\,oz^{-1}$.

We evaluated the values of fat, protein, lactose and energy in breastmilk over an 8-week study period from the three groups of mothers categorized by frequency of early hand expression: Group I (<2 times per day), Group II (2 to 5 times per day), and Group III (>5 times per day). Despite production differences between groups up to Week 8,[4] there are no significant compositional or energy differences between groups after Week 1 ($P = 0.05$).

Discussion

Many of the immediate and long-term benefits of breastfeeding for both the infant and mother depend on exclusivity and duration,[17] with the strongest determinant being the volume of milk produced by the pump-dependent mother.[18] By combining manual techniques with electric pumping, sufficient production can be achieved by preterm mothers who have been reported to be 2.8 times more likely to have insufficient production compared with term mothers.[1,2]

Despite significant differences in subsequent milk production between subjects who used early hand expression with varying frequency, there were no significant differences in the composition of their milk after Week 1, demonstrating that subsequent volume, but not milk composition, was impacted by the early use of hand expression. Our findings on the changes in the concentration of lactose and protein in milk up to Week 8 of lactation are consistent with those reported for preterm mothers by other investigators.[10]

The most striking observation is the high fat content of milk (>60 $g\,l^{-1}$) expressed using the combination of electric pump and manual techniques. Although a wide range of values in fat concentration has been reported in both preterm and term milk, the typical range is between 25–45 $g\,l^{-1}$.[19,20] Reports of high fat content of milk have been associated with late lactation and a decline in milk production. An increase of 50% in fat content from early to late lactation has been observe with crematocrit values rising as high as 28%.[21] Before electric pumps were in common use, Dewey *et al*[15] reported high fat content in milk of mothers who used either hand expression and/or a manual pump. Mean values of 51.6 ± 27.3 and $62.2 \pm 28.7\,g\,l^{-1}$ were found in mothers with infants 7–11 months and 12–20 months of age, respectively. These investigators suggested that the duration of lactation influences the fat content of milk.

We hypothesize that both high production levels and fat content are related to effective removal of viscous milk, (colostrum early on and the 'hindmilk' fraction of mature milk) enhanced by combining hand techniques with electric pumping. The triple combination of external breast compression, pump suction and the milk ejection reflex possibly removes a greater fraction of milk from individual alveoli than pump suction alone. Breast compression may increase intramammary and intraductile pressure, thereby improving the removal of more viscous, fat-rich milk, especially from the periphery of the breasts. Yet a near doubling of fat content as compared with reported norms was unexpected and calls for examination of collection, sampling, transportation and analyses.

During the collection process, variables that affect fat concentration are the interval between pumping sessions and the percent of available milk removal. With a long versus brief interval between expressions, more foremilk (fat-poor milk) may be collected. By collecting milk from each expression over a 24-h period once a week for each subject, differences related to expression schedules should be insignificant. For each subject, the 24-h mean from week to week varied minimally, suggesting consistency in her collection technique.

Sampling unmixed milk could lead to errors. Therefore, subjects were specifically cautioned not to provide samples from the tail end of the collection or from unmixed milk. If these instructions were inconsistently followed by some subjects, one would expect larger standard deviations than those reported in studies of comparable size, which is not the case.

If transportation processes exposed samples to evaporation, one would expect the lactose and protein content to be higher than reported norms. Instead, lactose is similar to norms ($70\,g\,l^{-1}$),[10,22] and the protein is somewhat lower, ($13-18\,g\,l^{-1}$).[9,10]

The investigators performing the analyses of samples in this report followed the same technique used in their previously published reports on milk composition. In addition, they have recently compared the esterified fat analysis with infrared spectophotometric analysis and found good agreement between the two methods.[23] Furthermore, noting that fat content rises with breast emptying, these investigators have proposed formulas for calculating breast emptying based on pre- and post-fat content.[24] Although they have reported values above 60 g/l for breastfeeding mothers of term infants, the mean was $41.1 \pm 7.8\,g\,l^{-1}$ (range: 22.3 to 61.6).[20] These values are similar to those reported from other laboratories.[25]

The acceptability of HOP to study mothers was uniformly positive, as >90% of mothers saw their production increase from pre- to post-instruction by an average of 48% (583 ± 383 to 863 ± 506; $P<0.0003$). The high caloric value of milk could not be appreciated but may be a motivational factor for advising future mothers to use the technique. As demonstrated, this benefit is similar in high and low producers.

Study limitations

Data from this observational study are provocative. Sampling 24-h pooled and mixed collections may offer advantages in accuracy, but poses practicality issues. Until larger randomized controlled studies confirm these results, caution must be taken not to assume all milk collected using these techniques has the identical fat/caloric value.

Conclusion

The two most clinically challenging concerns regarding the use of breastmilk for preterm infants are insufficient availability and suboptimal infant growth. When two manual techniques are combined with electric pumping, (hand expression of colostrum and HOP once milk volume increases) both milk production and the fat content may be improved. The fat-rich, calorie-dense milk expressed using these techniques is not related to production differences between mothers.

We propose that a simple, no-cost, risk-free intervention, combining electric pumping with two manual techniques has a beneficial influence on milk production, as well as composition, by removing fat-rich hindmilk more effectively than pump suction alone.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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References

1 Furman L, Minich N, Hack M. Correlates of lactation in mothers of very low birth weight infants. *Pediatrics* 2002; **109**: e57.

2 Hill PD, Aldag JC, Chatterton RT, Zinaman M. Comparison of milk output between mothers of preterm and term infants: the first 6 weeks after birth. *J Hum Lact* 2005; **21**: 22–30.

3 Kuschel CA, Harding JE. Multicomponent fortified human milk for promoting growth in preterm infants. *Cochrane Database Syst Rev* 2004; CD000343.

4 Morton J, Hall JY, Wong RJ, Thairu L, Benitz WE, Rhine WD. Combining hand techniques with electric pumping increases milk production in mothers of preterm infants. *J Perinatol* 2009; **29**: 757–764.

5 Meier PP, Engstrom JL, Janes JE, Jegier BJ, Loera F. Breast pump suction patterns that mimic the human infant during breastfeeding: Greater milk output in less time spent pumping for breast pump-dependent mothers with premature infants. *J Perinatol* 2011 (in press), doi:10.10138/jp.201164.

6 Saarela T, Kokkonen J, Koivisto M. Macronutrient and energy contents of human milk fractions during the first six months of lactation. *Acta Paediatr* 2005; **94**: 1176–1181.

7 Innis SM. Polyunsaturated fatty acids in human milk: An essential role in infant development. *Adv Exp Med Biol* 2004; **554**: 27–43.

8 Schanler RJ, Garza C, Nichols BL. Fortified mothers' milk for very low birth weight infants: Results of growth and nutrient balance studies. *J Pediatr* 1985; **107**: 437–445.

9 Bishara R, Dunn MS, Merko SE, Darling P. Nutrient composition of hindmilk produced by mothers of very low birth weight infants born at less than 28 weeks' gestation. *J Hum Lact* 2008; **24**: 159–167.

10 Faerk J, Skafte L, Petersen S, Peitersen B, Michaelsen KF. Macronutrients in milk from mothers delivering preterm. *Adv Exp Med Biol* 2001; **501**: 409–413.

11 Wojcik KY, Rechtman DJ, Lee ML, Montoya A, Medo ET. Macronutrient analysis of a nationwide sample of donor breast milk. *J Am Diet Assoc* 2009; **109**: 137–140.

12 Foda MI, Kawashima T, Nakamura S, Kobayashi M, Oku T. Composition of milk obtained from unmassaged versus massaged breasts of lactating mothers. *J Pediatr Gastroenterol Nutr* 2004; **38**: 484–487.

13 Atwood CS, Hartmann PE. Collection of fore and hind milk from the sow and the changes in milk composition during suckling. *J Dairy Res* 1992; **59**: 287–298.

14 Arthur PG, Smith M, Hartmann PE. Milk lactose, citrate, and glucose as markers of lactogenesis in normal and diabetic women. *J Pediatr Gastroenterol Nutr* 1989; **9**: 488–496.

15 Dewey KG, Finley DA, Lonnerdal B. Breast milk volume and composition during late lactation (7–20 months). *J Pediatr Gastroenterol Nutr* 1984; **3**: 713–720.

16 Nommsen LA, Lovelady CA, Heinig MJ, Lonnerdal B, Dewey KG. Determinants of energy, protein, lipid, and lactose concentrations in human milk during the first 12 mo of lactation: The DARLING Study. *Am J Clin Nutr* 1991; **53**: 457–465.

17 Gartner LM, Morton J, Lawrence RA, Naylor AJ, O'Hare D, Schanler RJ *et al.* Breastfeeding and the use of human milk. *Pediatrics* 2005; **115**: 496–506.

18 Hill PD, Aldag JC, Zinaman M, Chatterton RT. Predictors of preterm infant feeding methods and perceived insufficient milk supply at week 12 postpartum. *J Hum Lact* 2007; **23**: 32–38.

19 Gross SJ, Geller J, Tomarelli RM. Composition of breast milk from mothers of preterm infants. *Pediatrics* 1981; **68**: 490–493.

20 Kent JC, Mitoulas LR, Cregan MD, Ramsay DT, Doherty DA, Hartmann PE. Volume and frequency of breastfeedings and fat content of breast milk throughout the day. *Pediatrics* 2006; **117**: e387–e395.

21 Mandel D, Lubetzky R, Dollberg S, Barak S, Mimouni FB. Fat and energy contents of expressed human breast milk in prolonged lactation. *Pediatrics* 2005; **116**: e432–e435.

22 Paul VK, Singh M, Srivastava LM, Arora NK, Deorari AK. Macronutrient and energy content of breast milk of mothers delivering prematurely. *Indian J Pediatr* 1997; **64**: 379–382.

23 Casadio YS. Evaluation of a mid-infrared analyzer for the determination of the macronutrient composition of human milk. *J Hum Lact* 2010; **26**: 376–383.

24 Daly SE, Di Rosso A, Owens RA, Hartmann PE. Degree of breast emptying explains changes in the fat content, but not fatty acid composition, of human milk. *Exp Physiol* 1993; **78**: 741–755.

25 Michaelsen KF, Larsen PS, Thomsen BL, Samuelson G. The Copenhagen Cohort Study on Infant Nutrition and Growth: Breast-milk intake, human milk macronutrient content, and influencing factors. *Am J Clin Nutr* 1994; **59**: 600–611.