HOMOLOGOUS FORTIFICATION OF HUMAN MILK FOR THE PRETERM VERY LOW BIRTH WEIGHT INFANT IN DEVELOPING COUNTRIES

by

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Abstract

Human milk is considered as the gold standard for infant nutrition. However, preterm, term, and banked human milk is not nutritionally adequate for the high nutrient requirements of the small premature infant. To meet the nutritional needs of the preterm infant, the nutrient content of human milk needs to be increased. Several studies have been conducted using human milk components as supplements (homologous fortification), and these studies have shown that the rate of weight gain of the premature infants fed fortified human milk approximated intrauterine growth rate. However, the expense associated with this technology limits its practical use, especially in developing countries where finances allocated to the care of the preterm infants are limited and often do not allow for the purchase of such fortifiers.

One of the objectives of this study was to assess the feasibility of a simple and inexpensive technique of human milk concentration described by Martinez (1989). Another objective was the preparation of a homologous human milk fortifier using the concentrated human milk. The final objective was to assess the potential nutritional adequacy of the prepared fortified human milk by comparing its nutrient content with the nutrient requirements of the preterm very low birth weight infant.

Sixteen donor milk (dHM) samples were concentrated (evaporation and subsequent lactose removal) under vacuum using a rotary evaporator. The concentrated human milk (cHM) obtained had a significantly higher nutrient content (p<0.001) compared to that of dHM. Protein, fat, and lactose content of cHM increased 2.94±0.67, 2.35±0.79, and 2.36±0.33 times, respectively compared to dHM. Calcium, phosphorus, and sodium content of cHM increased 3.69±0.69, 3.67±0.84, and 2.09±0.47 times, respectively compared to dHM.
The cHM obtained was then used to fortify dHM resulting in the production of a fortified human milk (fHM). Three different types of fHM ("low", "medium", and "high") were prepared by mixing dHM and cHM at different proportions. The protein content of the fHM preparations was significantly higher than that of dHM (p<0.05). Fat content of the fHM preparations was not significantly different from dHM, with the exception of "high" fHM (p<0.05). Calcium and phosphorus content of the fHM preparations was significantly higher than that of dHM (p<0.001). Only the "medium" and the "high" fHM had a significantly higher sodium content (p<0.001). The osmolality of the fHM increased markedly and was significantly higher in the fHM preparations compared to dHM (p<0.001). The energy content of the fHM preparations was not significantly different from dHM, except for the "high" fHM (p<0.05). Overall, the nutrient content of the fHM preparations was higher than that of dHM.

In conclusion, the concentration and homologous fortification of human milk using a simple and inexpensive technique was achieved. However, the increment of the nutrients determined in the cHM showed a high degree of variability. In spite of the increased concentration of nutrients in the fHM, protein, calcium and phosphorus levels may still not meet the requirement of the preterm very low birth weight infant.
Table of Contents

Abstract ii
Table of Contents iv
List of Tables viii
List of Figures ix
Acknowledgement x
1 Introduction 1
   1.1 Nutritional management of the preterm very low birth weight baby 1
   1.2 The situation in developing countries 2
   1.3 Study objectives 3
   1.4 Null hypotheses 3
2 Literature Review 4
   2.1 Preterm, low birth weight and very low birth weight infants 4
      2.1.1 Definition 4
      2.1.2 Epidemiology 5
      2.1.3 Nutritional management of very low birth weight babies 6
   2.2 Nutritional requirements of the preterm and VLBW infant 7
      2.2.1 Growth and body composition of the human fetus 7
      2.2.2 Estimated nutrient and energy requirements of the preterm VLBW infant 8
      2.2.3 Energy requirements 8
      2.2.4 Protein requirements 9
      2.2.6 Fat and essential fatty acids (EFA) requirements 11
      2.2.7 Carbohydrate requirements 12
      2.2.8 Calcium and phosphorus requirements 12
      2.2.9 Sodium requirements 13
   2.3 Breastfeeding and human milk 14
      2.3.1 Non-nutritional benefits of human milk 15
         2.3.1.1 Anti-infective and anti-allergic properties of human milk 15
         2.3.1.2 Human milk protective factors 16
            2.3.1.2.1 Antigen-specific system 17
            2.3.1.2.2 Non-specific protective mechanism 18
            2.3.1.2.3 Anti-inflammatory agents in human milk 19
         2.3.1.3 Human milk interaction with the gastrointestinal tract 20
            2.3.1.3.1 Gastrointestinal hormones 20
3.5 Data analysis

4 Results

4.1 Characteristics of human milk samples
4.2 Characteristics of the concentrated human milk (cHM)
4.3 Comparison of the nutrient content of donor milk (dHM) with that of concentrated human milk (cHM)
4.4 Concentration process
4.5 Comparison between expected and observed nutrient increment in the concentrated human milk
4.6 Fortified human milk
   4.6.1 Comparison of the nutrient composition of the fortified human milk (fHM) with that of the donor milk (dHM)
   4.6.2 Adequacy of the fortified human milk for the nutritional management of the preterm VLBW infant

5 Discussion

5.1 Analysis of donor human milk
   5.1.1 Protein
   5.1.2 Fat
   5.1.3 Lactose
   5.1.4 Energy and osmolality
   5.1.5 Minerals
5.2 Milk concentration process
   5.2.1 Nutrient losses
   5.2.2 Osmolality
   5.2.3 Protein modifications
   5.2.4 Lipid oxidation products and risk of oxidative damage
5.3 Nutritional adequacy of the fortified human milk (fHM) for the preterm VLBW infant
   5.3.1 Protein, fat and energy
      5.3.1.1 Prevention of hypoproteinemia
      5.3.1.2 Growth of the VLBW infant
      5.3.1.3 Protein to energy ratio and weight gain composition
   5.3.2 Lactose and osmolality
   5.3.3 Calcium, phosphorus and sodium
      5.3.3.1 Calcium absorption and retention
      5.3.3.2 Calcium and fat interaction
List of Tables

| Table 1 | Characteristics of donor human milk samples used in this study. | 60 |
| Table 2 | Macronutrient and mineral content of donor human milk (dHM) used in this study. | 61 |
| Table 3 | Comparison of the composition of donor human milk used in this study with values found in the literature. | 62 |
| Table 4 | Macronutrient and mineral composition of donor and concentrated human milk, and comparison between the observed and the expected concentration factor. | 68 |
| Table 5 | Volumes of human milk samples before evaporation (donor human milk), after evaporation (evaporated human milk) and after crystallization and removal of lactose (concentrated human milk). | 75 |
| Table 6 | Macronutrient and mineral composition of donor and fortified human milk | 78 |
| Table 7 | Comparison of the amount of nutrients present in 150 or 180 ml of fortified human milk with the nutrient requirements of a preterm VLBW infant per kg body weight per day. | 83 |
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Outline of the protocol followed for the preparation of the concentrated human milk.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Rotary evaporator (Tecnal TE 120) used for the evaporation of human milk.</td>
<td>43</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Outline of the protocol followed for the fortification of human milk.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Illustration of the procedure used to quantify the lactose content of human milk.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Outline of the procedure used to simultaneously quantify the amount of protein and fat content of human milk.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Illustration of the procedure used to quantify the phosphorus content of human milk.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Variability in the fat content of donor human milk samples used in this study.</td>
<td>63</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Variation in the calcium content of donor human milk depending on the postpartum age of the human milk sample.</td>
<td>66</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Comparison of the mean protein, fat and lactose content of donor human milk with concentrated human milk.</td>
<td>69</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Mean fat content of donor and concentrated human milk.</td>
<td>70</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Comparison of the calcium, phosphorus and sodium content of donor human milk with concentrated human milk.</td>
<td>71</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Protein, fat and lactose content of donor human milk, &quot;low&quot;, &quot;medium&quot; and &quot;high&quot; fortified human milk.</td>
<td>79</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Calcium, phosphorus and sodium content of donor human milk, &quot;low&quot;, &quot;medium&quot; and &quot;high&quot; fortified human milk.</td>
<td>80</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Osmolality and energy content of donor human milk, &quot;low&quot;, &quot;medium&quot; and &quot;high&quot; fortified human milk.</td>
<td>81</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Comparison of the amount of nutrients presents in 180 ml of &quot;high&quot; fortified human milk (fHM) with the nutrient requirements of a preterm VLBW infant per kg body weight per day.</td>
<td>84</td>
</tr>
</tbody>
</table>
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1 Introduction

1.1 Nutritional management of the preterm very low birth weight baby

With increased knowledge in perinatal care and better technology in intensive care units, the survival of premature infants with low birth weight (LBW) and very low birth weight (VLBW) has increased markedly. Neonatologists and pediatricians are now facing new challenges, one being the nutrition of the preterm VLBW infant.

The nutritional management of the small premature infant in the special care unit, where the "external fetus" is at high risk of overt infection and perinatal problems, is not an easy task. The very rapid weight gain and increased nutritional requirements occurring in the normal fetus between 26 and 36 weeks of gestation combined with the limited nutritional stores of the fetus at this stage of life place the preterm VLBW baby at higher risk for nutritional deficiencies.

Due to its protective agents and nutritional qualities, human milk is recognized to be the best food for the nutrition of the full-term newborn, and therefore, breastfeeding is recommended by the majority of pediatricians. Human milk not only provides high quality nutrients in adequate amounts, but it also contains protective and trophic factors that are not present in commercial formulas. Moreover, it helps create a natural bond between mother and child, which is of primary importance for the overall health of the infant.

Yet, it is becoming increasingly recognized that milk produced by mothers giving birth to both term and preterm infants, as well as pooled banked human milk, is not perfectly adequate for the special needs of the preterm VLBW infant.

In industrialized countries, where financial resources are available, several alternatives are used in order to overcome the "deficiencies" of preterm milk for preterm VLBW infants. These alternatives are based on the volume of milk available (Gross et al., 1983). When the lactating mother produces milk in adequate quantity, preference is given to human milk fortifiers (liquid or
powder). On the contrary, when milk output is low, special preterm formulas are used in quantities as high as 50% of the infant’s daily ingested volume (Schanler et al., 1985a).

Some studies have shown the benefit (in terms of growth) of giving human milk fortified with a commercial fortifier to the small premature infant. However, the protein composition of these fortifiers, made from cow’s milk, is not quite adequate for the premature infant and may induce several metabolic disorders such as metabolic acidosis, hyperammonemia and aminoaciduria (Räihä, 1994). Therefore, the best way to give small premature babies all the protection and the nutrients needed, would be to provide them with their own mother's milk fortified with human milk constituents (Lucas et al., 1980; Itabashi et al., 1992). Several studies have demonstrated the benefits of this approach (Rönholm et al., 1986; Moro et al., 1991; Boehm et al., 1993); however, the techniques used to separate and extract the human milk constituents are highly complicated, time consuming and costly.

1.2 The situation in developing countries

In developing countries, finances allocated to the care of the preterm VLBW infant are limited and the nutritional management of preterm babies is different than in industrialized countries. Because fortifiers and special formulas are the privilege of a few, preterm babies in the special care unit of the Hospital das Clinicas da Faculdade de Medicina de Ribeirão Preto (HCFMRP), Brazil, are fed human milk. Whenever possible, the mother's milk is preferred. However, if the mother's milk is not available or if it is present in insufficient amounts (which is so in the majority of the cases), babies are fed mature pasteurized banked human milk, especially during the first 2 weeks of life (Mattar, 1994). This practice may have short and long-term adverse consequences on the health and development of the preterm VLBW infant. Consequently, it is important to i) implement policies and practices that facilitate the establishment and maintenance of lactation and ii) discover simple and inexpensive techniques that will allow the separation of human milk constituents and thereby permit the autologous/homologous fortification of human milk.
Dr. Francisco E. Martinez, pediatrician at the HCFMRP, has adapted a concentration technique used by the dairy industry (Hunziker, 1949), which allows the preparation of a human milk concentrate (cHM). The cHM could then be used for the fortification of mother's milk. This technique of human milk concentration and fortification is simple and affordable, and it is potentially applicable, at some extra cost to all milk banks in developing countries.

1.3 Study objectives

This study was designed 1) to assess the feasibility and practicality of the human milk concentration technique described by Martinez (1989) for use in a practical setting. 2) to investigate the possibility of using the prepared human milk concentrate as a human milk fortifier (homologous fortification), and 3) to assess the nutritional adequacy of the prepared homologous fortified human milk for the preterm VLBW infant.

1.4 Null hypotheses

1) there will be no difference in the composition of the concentrated human milk (cHM) compared to that of donor human milk (dHM).

2) there will be no difference between the expected and observed nutrient increment of the concentrated human milk (cHM).

3) there will be no difference in the nutritional composition of the fortified human milk (fHM) compared to that of donor human milk (dHM).

4) there will be no difference between the amount of nutrient supplied by a given volume of fHM with the estimated nutrient requirements of the preterm VLBW infant.
2 Literature Review

2.1 Preterm, low birth weight and very low birth weight infants

2.1.1 Definition

Preterm (gestational age < 37 weeks) and low birth weight infant (birth weight < 2500 g) are often referred to as one group or category and these terms are often used interchangeably. However, there are some fundamental differences separating these two groups of newborns. Differences between low birth weight and preterm infants have been recognized, and in 1961 these two groups were separated and classified by the World Health Organization (WHO).

It is now widely recognized that both gestational age and fetal accretion rate are determinants for birth weight (Raju, 1986). Fetal growth may be normal, leading to appropriate-for-gestational age (AGA), or abnormal, leading to small-for-gestational age (SGA)\(^1\) (Arnold \textit{et al.}, 1991). Therefore, LBW infants may be premature or full-term SGA babies (O'Leary, 1989). In 1979 the percentage of SGA among LBW infants represented 24% in Finland, 57% in England and Whales, 45% in Sweden and the United States, 34% in Kenya, 56% in Tanzania, 83% in Guatemala and 38% in Cuba. In India, as high as 75% of the LBW were SGA (WHO, 1980). In Brazil, Gonçalves (1992) determined that 49.5% of LBW born between 1978 and 1979 in the area of Ribeirão Preto, São Paulo, were preterm. A recent study in Nigeria, showed that 61% of the LBW infants were premature (Wright, 1990).

The term very low birth weight (VLBW) was introduced in order to differentiate babies born with a LBW (birth weight<2500 g) with those with birth weight<1500 g. Extremely small (VLBW) or immature (preterm) babies represent two distinct groups. Still, VLBW is often used synonymously with prematurity. In this thesis, the terms preterm and very low birth weight will be

\(^{1}\) The term small-for-gestational age was introduced by the American Academy of Pediatrics in 1967
used interchangeably due to the widespread acceptance that VLBW infants are premature babies in the majority of cases.

2.1.2 Epidemiology

According to the World Health Organization 1979 statistics, out of 122 million live infants, nearly 21 million were LBW babies, representing about 17% (or 1 in 6) of all the births in that year (WHO, 1980). In 1983, LBW in the US represented about 50 live birth per 1000 births and VLBW represented about 10 live birth per 1000 births. Non-whites were at greater risk of giving birth to VLBW infants (Raju, 1986). In developing countries, the incidence of LBW babies is higher than in western countries (WHO, 1980). In Asia for example, the incidence of LBW in 1979 represented 20% of all live births, whereas it represented 15% in Africa, 11% in Latin America, 8% in Europe and 7% in North America. More striking is the fact that among the 21 million LBW infants born in 1979, 90% were born in developing countries (WHO, 1980).

Low birth weight is recognized to be an important determinant of the newborn's chance for survival, healthy growth and development. The neonatal mortality ratio (NMR = number of deaths before 28 days/number of live births x 1000) increases exponentially with a decrease in birth weight and gestational age for both white and black population in the US (Raju, 1986). Neonatal survival of VLBW infants is also determined by perinatal care and nutritional management. If inadequate, the newborn may be at risk for short and/or long-term sequel. Challenges faced by neonatologists include respiratory abnormalities, hypothermia, sepsis, necrotizing enterocolitis, and intraventricular hemorrhage which may lead to death (Nishida, 1993). Once the critical perinatal period is over, LBW and VLBW babies are at risk for long-term sequel such as central nervous system disorders, hearing and visual defects and mental retardation. Nishida (1993) found that between 1984 and 1990, 17% of VLBW Japanese infants born in one of Tokyo's hospitals showed signs of major neurological sequel when evaluated at 1 to 8 years of age.


2.1.3 Nutritional management of very low birth weight babies

Improvements in technology, knowledge of the pathophysiology of the neonate, and advances in perinatal care have led to a progressive and significant improvement in the survival rate of preterm infants, including those of lowest gestational age and VLBW (Raju, 1986; Martinez, 1987; O'Leary, 1989). With the introduction of intensive care units in the 1970's, the mortality rate of VLBW infants declined significantly from over 90% to below 50% (Nishida, 1993). Survival rate of VLBW infants is now estimated to be over 80% (Nishida, 1993). Still, new challenges face neonatologists and pediatricians regarding the care of the high-risk VLBW infant. One of these challenges is nutritional management.

Nutritional management of the high risk VLBW infant is difficult because of the

  i) limited knowledge of the nutrient requirements of the VLBW infant.
  ii) the narrow margin between nutritional deficiency and overnutrition.
  iii) lack of self regulation of food intake.
  iv) lower functional ability of the gastrointestinal tract.
  v) common presence of life-threatening illnesses.
  vi) the vulnerability of the process of maturation and growth to nutritional insults.

Low nutritional body stores, rapid growth and high nutrient requirements, render VLBW babies highly vulnerable to nutritional insufficiencies. Inadequacy of postnatal nutrition may lead to several nutritional imbalances and thus influence their long-term developmental outcome, leading to impaired growth and development (Lucas, 1992, 1994 ; Decsi, 1994).
2.2 Nutritional requirements of the preterm and VLBW infant

2.2.1 Growth and body composition of the human fetus

The normal human fetus experiences very rapid growth between 26 and 36 weeks of gestation (Adamkin, 1986). The fetus grows at a rate of 1 to 2 percent per day between 28-40 weeks (Ziegler et al., 1976) and therefore triples in body weight within three months. During the last trimester of intrauterine life infant body composition undergoes major changes. There is a rapid increase in cell number, size and differentiation, leading to functionally organized tissues and systems (Adamkin, 1986). Brain size, for example, doubles in two months in a 28-week infant (the infant's brain accounts for 15% of body weight at 28 weeks compared to only 2% of an adult's weight). Moreover, important changes in body water content and in nutrient accretion occurs during the last trimester of their in utero life (McQuaid Cox et al., 1993; Ziegler et al., 1976). The body water content decreases from nearly 90% of body weight at 16-20 weeks of gestation to 79% at 24-28 weeks and finally to 62.5% at 36-40 weeks (Ziegler et al., 1976; Baumgart, 1990). Protein accretion increases from 10.8 g/d to 13.9 g/d and fat accretion from 7.8 g/d to 19.8 g/d. Overall nutrient accretion increases from 16.8 g/d to 27.1 g/d with a peak of 30.7 g/d at 32-36 weeks (Ziegler et al., 1976).

At birth, the infant's survival is dependent primarily on fat and glycogen stores (Adamkin, 1986). However, since fat accretion occurs mainly during the last 6 weeks of gestation, the preterm infant may have very limited fat and glycogen stores. It is estimated that the small premature infant weighting less than 1000g has a total fat content of only 1% of body weight compared to 16% in the full term infant weighing 3500g (O'Leary, 1989). Glycogen and fat stores are rapidly depleted at birth and lack of adequate nutrition may lead to severe protein-energy malnutrition (Adamkin, 1986), which may impair future short- and long-term outcomes for the newborn. Lack of proper nutrition may decrease the immuocompetence of the newborn, therefore increasing the risk of infection, (Chandra, 1981) and weakening the respiratory or cardiac
muscles. Early nutrition is also critical to brain development. During the prenatal and early infancy periods, neurons continue to be formed and cerebellar control develops. Brain growth and function may therefore be irreversibly impaired by an inadequate nutrient intake (Lucas, 1990, 1994; O'Leary, 1989).

2.2.2 Estimated nutrient and energy requirements of the preterm VLBW infant

The small premature infant, which may be comparable to an ex utero fetus, has higher nutrient and caloric requirements than the full term baby. Preterm infants are in a very rapid state of growth and lack sufficient stores of fat and glycogen therefore being at high risk for nutritional deficiencies (Farrel et al., 1988; Neu et al., 1990). The normal clinical practice for the nutritional management of the VLBW infant consists of trying to achieve the same in utero growth rates as a normal fetus. This practice is still questionable. Nevertheless, several authors and the American Academy of Pediatrics (AAP) have the opinion that "achieving postnatal growth that approximates the in utero growth of a normal fetus at the same postconceptional age appears to be the most logical approach at present" (AAP, 1985).

2.2.3 Energy requirements

Small premature infants experience increased energy expenditure and high energy losses due to a high surface area/body mass ratio (Jéquier, 1993). Therefore the energy requirements are higher. The Committee on Nutrition of the American Academy of Pediatrics (1985) recommends an intake of approximately 120 kcal per kilogram of body weight per day in order to achieve extra-uterine weight gain similar to intrauterine growth rates. This estimate may, in certain cases, be too conservative, and higher intakes (130-150 kcal/kg/d) may be needed. Physiological stress (fever, short bowel syndrome, chronic lung disease), environmental stress (thermal environment),
medication, birth weight, postnatal age, type and method of feeding increase the energy needs for
these infants (O'Leary, 1989).

2.2.4 Protein requirements

The preterm VLBW infant has higher protein requirements compared to the full-term infant. Schulze et al. (1987) for example, fed preterm infants 2.25 g protein per kilogram per day and 113 kcal per kilogram per day. These authors were unable to show an appropriate weight gain, indicating the inadequacy of this diet in terms of growth rate. The daily protein accretion in the fetus is estimated to be 1.7 g/kg at 28 weeks of gestation and 2.0 g/kg at 36 weeks of gestation. According to Ziegler et al. (1976) the human fetal accumulation of nitrogen between 24 and 36 weeks of gestation is 320 to 350 mg per kilogram per day. Taking into account dermal and urine losses and considering 87% absorption, an average of 3.5 to 4 g per kilogram per day of protein is advisable (AAP, 1985; Ziegler et al., 1993). Based on the reference fetus (between 28 and 32 weeks of gestational age), Fomon et al. (1977) estimates that the daily protein requirement of the small premature infant is around 3.7 g/kg (or 2.5 g/100 kcal).

High protein intake may exceed the metabolic capacity of immature babies and may stress the sick infant, resulting in fever, lethargy, poor feeding, metabolic acidosis, hyperammonemia, azotemia, hyperproteinemia, high levels of amino acids (tyrosine, phenylalanine) and aminoaciduria (Neu et al., 1990; Steichen et al., 1987). Yet, it has been shown by Hagelberg et al. (1982) that levels of protein intake equal to 3.0-3.5 g/kg/d at energy levels averaging 110 kcal/kg/d were well tolerated and were effective in enhancing and reaching a growth rate similar to the intrauterine growth rate in infants fed human milk enriched with human milk protein.

Kashyap et al. (1988) showed that in preterm LBW babies (32 weeks gestational age) protein intake as high as 3.8 g/kg/d was incompletely utilized when the energy intake was about 120 kcal/kg/d. These investigations emphasized the importance of the protein-to-energy ratio on protein utilization showing that a protein intake of 3.9 g/kg/d, was better utilized when the energy
intake was 142 kcal/kg/d than 3.8 g/kg/d at 120 kcal/kg/d. Although there was no evidence of metabolic acidosis in the infants receiving 3.8 and 3.9 g protein/kg/d, several plasma amino acids were found to be elevated. As well, although the higher protein and energy intakes resulted in better growth and nitrogen retention rates compared to lower protein intake, an increase in fat accretion was also observed (Schulze et al., 1987). Whether this is a desirable outcome is still questionable. Kashyap et al. (1988) suggest a protein and energy intake of 2.8 g/kg/d and 119 kcal/kg/d respectively. This recommendation is based on their observations that such an intake was effective in achieving adequate rates of weight gain and nitrogen retention without producing apparent metabolic disturbances.

It has been shown that protein and energy influence the composition of weight gain (Schulze et al., 1987; Kashyap et al., 1988). Thus, it is estimated that the ratio of protein and fat deposition should be close to 1. This also seems to be achieved with a protein intake of 3 to 3.5 g/kg/d and an energy intake of 100 to 120 kcal/kg/d (Salle, 1993). Such high protein and energy requirements cannot be met by the usual daily volume of preterm human milk taken by the infant (approximately 150 ml/kg/day). An excessive volume intake is thus necessary to meet these requirements, which could however be harmful for the premature infant (i.e. increasing the risk of opening the ductus arteriosus, increasing the risk of congestive heart failure and necrotizing enterocolitis) (Guerrini, 1994). Moreover, many of these infants suffer from chronic respiratory disorders such that fluid restriction may be necessary (Garza et al., 1984). Maintenance of growth rate similar to the intrauterine rate of growth is therefore necessary to increase the protein and energy intake without increasing the fluid volume ingested.

**Amino acid considerations**

Preterm infants have incomplete metabolic development and therefore exhibit several biochemical immaturities. Several amino acid pathways are incomplete hence some amino acids like cysteine, and taurine may be essential and must be supplied by the diet (Neu et al., 1990).
Amino acid catabolism is also incomplete. Phenylalanine and tyrosine are not readily metabolized (Rassin cited by Rönnholm et al., 1982) and a high protein intake may result in hyperaminoacidemia, metabolic acidosis, and hyperammonemia (Räihä, 1983). Therefore "because of immaturities in both the gastro-intestinal tract and the liver, the amino acid composition of the protein given to preterm infants may be at least as important as the quantity" (Neu et al., 1990).

### 2.2.6 Fat and essential fatty acids (EFA) requirements

Fetus fat stores increase during the last two months of gestation. Fat and fatty acids are essential for cellular structure and integrity, central nervous system development (e.g. brain and retinal development), liposoluble vitamin transport, energy storage, body insulation, and hormone synthesis. Preterm infants have very limited reserves of fat and essential fatty acids (EFA). Due to very low fat stores and high fat and caloric requirements, they are at a higher risk for essential fatty acid deficiency, if these are not provided in the early diet (Farrel et al., 1988; Decsi et al. 1994). Farrel et al. (1988) showed that in 63 premature infants (<37 weeks of gestation; <2000g), 67% had low plasma linoleic acid levels (plasma linoleic acid< 26% of total plasma fatty acids) and 44% had abnormally high triene-tetraene ratio (> 0.2) by day 7. Among infants who did not receive lipid-containing feeds (enterally or parenterally) by day 7 (n=25), a high proportion displayed abnormal plasma fatty acid profile. In contrast, infants fed lipid containing feeds by day 2 (breast milk or formula, n=20), did not show any abnormal fatty acid status. These authors suggest that early intakes of linoleic acid (approximately 1.19 g/kg/d or 10% of total caloric intake) are required to prevent or reverse EFA deficiency. These estimates are higher than the suggested intrauterine accretion rate of 400 mg/kg/d (Clandinin et al. 1980). However, due to high energy requirements, a certain proportion may be utilized for energy production purposes, justifying the higher estimate of 1.19 g/kg/d (Farrel et al., 1988).
2.2.7 Carbohydrate requirements

At 34 weeks of gestation, activity of alpha-glycosidases (sucrase, isomaltase and maltase) is about 70% of that found in the full-term infant. However, lactase (beta-glycosidase) activity is only 30% of that of the term infant (Auricchio et al., 1965; Lebenthal et al., 1986). From in vitro studies using small intestinal homogenate, Auricchio et al. (1965) estimated that a preterm newborn (between 7 and 8 months gestational age) should be able to hydrolyze about 6.4 g of lactose/24h (range 2.8-8.3). Newborns of gestational age between 8-9 months should be able to digest approximately 23.4 g of lactose/24 h (range 13.2-33.4), and full-term babies should hydrolyze as much as 62 g of lactose/24 h (range 57-67).

Maximum specific activity of the beta glycosidase does not occur until the end of normal gestation, therefore the premature infant exhibits low activity levels of lactase at birth. The reduced activity of the small intestinal mucosal lactase may lead to lactose malabsorption and intolerance (fermentative diarrhea secondary to incomplete lactose digestion and absorption). Nevertheless, lactose malabsorption does not seem to affect overall carbohydrate absorption (McQuaid Cox et al., 1993). Hydrolysis by colonic bacteria into absorbable two- and three carbon fragments may decrease carbohydrate losses into the feces (Neu et al., 1990; Lifschitz et al., 1995). Also, after premature birth the level of lactase activity rises rapidly, thus lactose intolerance does not seem to be a common problem beyond the first few days of life (Auricchio et al., 1965; O'Leary, 1989).

2.2.8 Calcium and phosphorus requirements

Calcium accumulation increases markedly from 24 weeks of gestation and peaks between 32 and 34 weeks of gestation, with an accretion rate of approximately 120 mg per kilogram per day (Tsang, 1985; Neu et al., 1990). Other authors cited by Steichen et al. (1980) estimate that intrauterine calcium accretion is as high as 150 mg/kg/d by the 36th to 38th week of gestation. Phosphorus accretion during the last trimester of pregnancy approximates 65-75 mg/kg/d (Neu et
It has been shown by several authors that preterm LBW newborn show signs of phosphate deficiency at birth and therefore are at risk for limited bone synthesis (Natal-Pujol et al., 1993). The fetal accretion rate for calcium and phosphorus in a VLBW infant weighing 1000g and gaining weight at a rate of 20 g/d has been estimated to be 150 mg and 90 mg, respectively (Heine, 1992). Taking into account the limited intestinal absorption of calcium (only 35-50% of dietary calcium is thought to be absorbed), Steichen et al. (1980) estimated that an ingestion of 210 to 250 mg per kilogram per day of calcium and 110 to 125 mg per kilogram per day of phosphorus is needed to achieve adequate bone mineralization.

Human milk is considered to be the optimal source of calcium and phosphorus for the feeding of normal term infants due to its calcium/phosphorus ratio and low phosphorus concentration. Yet, in the case of preterm babies, human milk is not the optimal source of these minerals as its content does not meet the infant requirements when administered at normal volumes (about 150 ml/kg/d). Osteopenia of prematurity has been reported in premature infants fed breastmilk (Steichen et al., 1980).

Preterm infant needs to retain around 80 to 100 mg of calcium /kg/d whereas term babies need only 20 mg/kg/d (Ziegler et al., 1993). With these high requirements for calcium and phosphorus (190 mg/kg/d and 120 mg/kg/d, respectively) (Ziegler et al., 1993), it has been estimated that a preterm VLBW infant should receive between 400-600 ml of human milk (calcium=24-34 mg/100 ml; phosphorus=11-16 mg/100 ml) per day (Heine, 1992). This is not feasible since it increases the risk for opening the ductus arteriosus, and the risk of congestive heart failure.

### 2.2.9 Sodium requirements

Drastic changes in sodium content of the fetal body occurs during the last trimester of pregnancy. Sodium content falls from about 94 mmol/kg at 25 weeks of gestation to about 74 mmol/kg at term. The daily rate of sodium accretion is estimated to be between 0.85 to 1.1
mmol/kg (Wharton, 1987). Fomon et al. (1977) estimate that an infant gaining weight at a rate of 20 g/d retains 1.48 mEq of sodium/d. However, due to immaturity of the renal tubular function and differences in environment and feeding (in utero vs. ex utero), sodium requirements are much higher in preterm infants. This immaturity in tubular function leads to higher amounts of sodium being excreted (approximately 1 mEq/kg/d; Fomon et al., 1977). Sodium deficiency may lead to hyponatremia (plasma sodium concentration<130 mmol(mEq)/l), increasing the risk for water intoxication (shift of water into the cells due to hypotonicity of the extra cellular fluid). Gross (1983) showed that hyponatremia (serum sodium concentration <133 mmol(mEq)/l) developed during the first six weeks of life in 50% of the infants fed pooled human milk, in 20% of the infants receiving a formula, and in 15% of infants receiving preterm breast milk.

Fomon et al. (1977) estimate that a preterm infant absorbing 87% of the ingested sodium would need to ingest at least 2.8 mEq/kg/d. According to several reports (cited by Wharton et al., 1987), it is estimated that at least 2.9 mmol(mEq)/kg/d of sodium are necessary to promote growth and to maintain an appropriate plasma sodium concentration around 134 mmol/l. Al-Dahhan et al. (1984) showed that intakes of 4-5 mEq/kg/d reduced the incidence of hyponatremia in preterm LBW infants without causing any visible side effects. Therefore, the AAP: Committee on Nutrition (1985) recommend an intake of 2.5-3.5 mEq/kg/d (in formulas) for low birth weight babies and an intake varying between 4 and 8 mEq/kg/d for VLBW infants.

2.3 Breastfeeding and human milk

Human milk is considered by most health professionals as the gold standard for infant nutrition. The American Dietetic Association (ADA, 1993), states that breastfeeding should be advocated "because of the nutritional and immunologic benefits of human milk for the infant, the physiological, social, and hygienic benefits of the breastfeeding process for the mother and infant, and the economic benefits to the family and health care". Aside from the recognized psychological advantages of breastfeeding (i.e. bonding between the mother and the baby), human milk provides
the baby with other numerous factors that are advantageous compared to other forms of nutrition such as formula feeding.

2.3.1 Non-nutritional benefits of human milk

It is now well recognized that, in addition to being a source of nutrients, human milk also contains non-nutritive components such as anti-infective factors, hormones, enzymes and growth factors, which may be of great importance for the health and development of the VLBW infant. Human milk provides passive immunologic protection and active immunostimulation and prevents the VLBW infants from antigenic and toxic loads.

2.3.1.1 Anti-infective and anti-allergic properties of human milk

A multitude of epidemiological studies have been carried out and the results have been concurrent in showing the benefits of human milk over infant formulas (Brown cited by Jelliffe and Jelliffe, 1988). The results of these studies may have been confounded by several factors such as unreliable water supply, poor sanitation, inadequate facilities for cleaning bottles and nipples, and literacy. However, several studies controlling for these confounders have clearly shown the benefit of feeding breastmilk (Chandra, 1979; Narayanan, 1981; Lucas, 1990; Cunningham et al., 1991). Breastfed babies have been shown to have a significantly lower incidence of common infections of the gastrointestinal and respiratory systems than non-breastfed babies (Chandra, 1979; Cunningham et al., 1991). Human milk has been shown to protect small premature babies against diarrhea and neonatal necrotizing enterocolitis (NEC) (Lucas et al., 1990), sepsis and death (Narayanan, 1981), respiratory infection and otitis (Chandra, 1979). Moreover, retinopathy of prematurity (ROP) may also be prevented by human milk feeding (Johnson et al., 1985). Interestingly, feeding breast milk seems to decrease the incidence of non-gastrointestinal infections (including pneumonia, bacteremia and meningitis) in newborns (Cunningham et al., 1991). Lower
morbidity due to infection and allergies was demonstrated in both developing and industrialized countries. This was shown in exclusively breastfed infants and formula fed infants, living in a rural community in India (n=70) and in an urban community in Canada (n=60) (Chandra, 1979). In India, breastfeeding was significantly associated with a reduction in the incidence of respiratory infections, otitis, diarrhea, dehydration and pneumonia. In Canada, incidence of otitis and respiratory disease was significantly lower in the exclusively breastfed babies. In a prospective study performed in India with 62 high-risk infants, Narayanan et al. (1981) showed that in the human milk-fed group no major infections were recorded whereas in the formula-fed group, the occurrence of infection was significantly higher. In a case control study, Victora et al. (1987) found that among Brazilian children (mean age 4.3 months), infants who did not receive any breast milk had a 14.2 times higher risk of death from diarrhea and a 3.6 times higher risk of death from respiratory infections than exclusively breastfed infants. Lucas et al. (1990) showed that human milk feeding offers protection against NEC. Fifty one cases of NEC were recorded in 926 infants (birth weight <1850 g). The incidence of NEC was 1.2% in the human milk fed group versus 7.2% in the preterm formula fed group, showing a significant protective effect of human milk compared to preterm formula. Lucas (1990) estimates that 500 cases of NEC, as well as 150 abdominal surgeries and 100 deaths per year in Britain are attributable to use of artificial feeding products.

2.3.1.2 Human milk protective factors

Protection from a variety of injurious agents like microorganisms, parasites, malignant cells, allergens, toxins and free radicals (superoxide, oxygen radicals, hydrogen peroxide and lipid hydroperoxides) depends upon many complex interacting mechanisms. These include antigen-specific immune responses, non-specific barriers to infection and anti-inflammatory agents (Chandra, 1981; Goldman et al., 1986). Human milk contains a wide variety of protective agents. These include a large number of white cells (such as T and B lymphocytes, neutrophils,
macrophages, polymorphonuclear leukocytes) and several protective substances such as secretory immunoglobulin A (sIgA), IgG, IgM, IgE, IgD, lysozyme, lactoferrin, lactoperoxidase, interferon, Bifidus factor, enzymes, antienzymes, antioxidants, glycoconjugates, and fatty acids (Joneja, 1992; Arnold et al., 1993). These biochemical agents protect the newborn infant from infection.

2.3.1.2.1 Antigen-specific system

Secretory antibodies against viruses, enterobacteria and enterotoxins are present in human milk. One very important component present in high quantities in human milk and especially colostrum is secretory IgA (sIgA; antibodies secreted by the mammary gland of the mother) which may act against common enteric pathogens and it is thought to play a local protective role in the infant's intestine. Secretory IgA interferes with the attachment of microorganisms to mucosal surfaces and neutralizes toxins and virulence factors from microbial pathogens (Goldman et al., 1986).

It has been shown that due to the existence of an enteromammary circulation, the antibody content of breastmilk reflects the immunologic experience of the mother's gastrointestinal tract. Secretory antibodies against enteric pathogens common to the mother-infant environment or against some food substances ingested by the mother are produced in the mammary gland by lymphocytes that migrate from the Peyer's patches in the intestinal mucosa to the mammary gland. Therefore, pathogen-specific or food-specific secretory IgA antibodies are found in breast milk (Goldman et al., 1986; Cunningham et al., 1991). Moreover, sIgA antibodies directed against certain food antigens may reduce the allergic reactions by preventing the macromolecular absorption of the exogenous food antigen or by competing with the antibodies produced by the infant to those antigens. The antigen-antibody complex formed may then prevent the inflammatory response by preventing the activation of the complement system by IgM and IgG (Goldman et al., 1986).
Leukocytes (the most abundant of which are neutrophils and macrophages) are also found in human milk. However, it is not clear whether they play a substantial role in the protection of the newborn (Goldman et al., 1986; Joneja, 1992).

2.3.1.2.2 Non-specific protective mechanism

Non-specific mechanisms of defense include the skin and mucous membranes, lactoferrin, interferon, lysozyme, complement system and phagocytes (Chandra, 1981). The complement components and polymorphonuclear leukocytes and macrophages act in conjunction with specific immunity mechanisms (antibody response and cell-mediated immunity) (Chandra, 1981).

Lactoferrin is one of the most important whey proteins in human milk. It is a glycoprotein that chelates free iron present in human milk and transports it to the intestinal mucosa. By doing so, it lowers the amount of free iron thus inhibiting the growth of iron-dependent enteric microorganisms (Joneja, 1992).

The antimicrobial agent lysozyme is a protein which lyses susceptible bacteria by hydrolyzing beta-1,4 linkages in cell walls (Joneja, 1992). Like lactoferrin and sIgA, lysozyme resists digestion by digestive enzymes of the gastrointestinal tract and therefore remains active throughout the digestive tract (Davidson et al., 1987; Joneja, 1992).

Human milk also contains several oligosaccharides that are analogs to the receptors of the gastrointestinal epithelium and of the retropharyngeal epithelium. It is thought that microorganisms bind to the oligosaccharide fraction of human milk and are thus rendered unable to bind to the intestinal mucosa (Goldman et al., 1986; Joneja, 1992; Coppa et al., 1993). Human milk also exerts non-immunoglobulin inhibitory activity against E. Coli heat-labile enterotoxin (LT), cholera toxin, rotavirus and respiratory syncytial virus (Kolstø Otnaess et al., 1984; Joneja, 1992). Taurine, which is also present in human milk, appears to be involved in the stabilization of certain cellular membranes like retinal membranes thereby protecting against retinal damages (Gaull et al., 1984; Johnson et al., 1985). Taurine concentration in the retina increases from birth until about
two months postpartum. Taurine seems to regulate membrane excitability, promotes homeostasis and protects membranes against oxidant damage. Retinal abnormalities may thus result from taurine deficiency (Johnson et al., 1985). Results of a follow up study of partially breastfed infants and non-breastfed babies (n=385; birth weight <2000 g) between 1979 and 1981, suggested that breast milk has a protective effect against retinopathy of prematurity (ROP). It was suggested that this protective effect could be due to improved taurine nutrition (Johnson et al., 1985).

2.3.1.2.3 Anti-inflammatory agents in human milk

Human milk contains certain anti-inflammatory substances which appear to reduce or modify host responses to microbial and food antigens, potentially decreasing the incidence of several complications (Goldman et al., 1986). In newborn siblings of children with atopic disease, exclusively breastfed babies developed allergies at a significantly lower level (eczema: p<0.001; recurrent wheezing: p<0.01) than the formula fed matched group (Chandra, 1979). Moreover, in a prospective randomized trial, Lucas et al. (1990) found that at 18 months of age, infants fed preterm formula had 1.5 times the amount of wheezing, greater than two times the incidence of eczema, and three times the incidence of food and drug reactions when compared with breastmilk-fed infants. According to Goldman et al. (1986), lactoferrin, secretory IgA and lysozyme exhibit anti-inflammatory properties. Lactoferrin seems to inhibit the complement system. As well, secretory immunoglobulin A not only prevents the attachment of microorganisms to mucosal surfaces but also seems to inhibit the chemotaxis of neutrophils. Neutrophils, principal actors of the inflammation process, are also influenced by lysozyme. Lysozyme is thought to reduce chemotaxis and to limit the production of toxic oxygen compounds by neutrophils during phagocytosis.

Human milk contains several free oxygen radical scavengers that protect the intestinal mucosa from oxidative injuries. These antioxidants include alpha tocopherol, cysteine, and
ascorbic acid. Moreover, catalase and glutathione peroxidase are also present in human milk and they may play a role in the protective function of human milk (Goldman et al., 1986).

Several other potential protective factors such as protease inhibitors, histaminase, arylsulfatase and lymphocyte inhibitors have been identified in human milk and may be implicated in the reduction of the inflammatory responses (Goldman et al., 1986).

2.3.1.3 Human milk interaction with the gastrointestinal tract

Adaptation to the external environment and to extrauterine nutrition are of primary importance for the survival of the newborn. Enteral feeding of human milk, even at volumes as low as 12 ml/kg of body weight, has been shown to have a direct effect on the maturation and development of the gastrointestinal tract (Lucas et al., 1986; Lebenthal et al., 1988; Slagle et al., 1988; Currao et al., 1988). Lucas et al. (1990) showed that infants receiving human milk had better feeding tolerance than infants receiving artificial formulas. These infants vomited less, and less time was required for breastmilk-fed babies to establish full enteral feeding (20 days versus 45 and 48 days for the formula fed groups).

2.3.1.3.1 Gastrointestinal hormones

It is suggested that gastrointestinal hormones may play a key role in the development and maturation of the gastrointestinal tract after birth (Lucas et al., 1986). Increased plasma concentration of gut hormones including enteroglucagon, gastrin, gastric inhibitory peptide (GIP), motilin and neurotensin have been identified in enterally fed infants. Lucas et al. (1986) studied 104 preterm infants and showed that daily intakes of human milk at volumes as low as 12 ml/kg body weight were sufficient to induce a surge in the plasma concentration of the different gut hormones. Moreover, they showed that with an intake of 50 ml/kg/d, the increase in regulatory
peptides was maximal. Therefore, enteral feeding of human milk seems to be a potent stimulus to the development and maturation of the gastrointestinal tract (Lucas et al., 1986).

2.3.1.3.2 Growth modulators in human milk

Growth modulators, including small molecules such as the amino acid taurine and its structural lipid analogs ethanolamine and phosphoethanolamine, and small hormone-like proteins such as epidermal growth factor (EGF) and nerve growth factor (NGF), have been found in human milk (Gaull et al., 1984). In vitro studies have confirmed the influence of certain growth modulators on cell proliferation. Gaull et al. (1984) have shown that taurine was effective in the proliferation of human lymphoblastoid cells. Possible physiological roles of EGF and NGF have been shown. EGF seems to act on the immature gastrointestinal tract promoting growth and differentiation of epithelial cells and other tissues, such as the liver (Moran, 1983; Read, 1984). The development of the gastrointestinal mucosa induced by EGF may strengthen the mucosal barrier and therefore limit the penetration of antigens into the mucosa (Goldman et al., 1986). Moreover, since EGF inhibits the release of gastric acid, it might also act as a pH regulator (Gaull et al., 1984).

2.3.1.4 Human milk and subsequent neurodevelopment

According to Lucas et al. (1992), an interesting but controversial issue, is that human milk feeding seems to cause long-term differences in neurological development. Infants fed their own mother's milk, tested at 71/2-8 years of age, had an IQ advantage of 10 points compared to children who were not fed their own mother's milk. These results tend to support the hypothesis that breastmilk promotes neurodevelopment in newborns. Therefore, it seems that human milk is one of the determinants of the long-term neurodevelopment of the infant.
2.3.2 Nutritional benefits of breastmilk

From a nutritional point of view, human milk is a complete food for the full-term infant, providing all the nutrients needed to sustain the infant for the first six months of life. Heine (1992) stated that "human milk feeding as compared with formula feeding has the advantage of more effective utilization of proteins, fat, minerals and trace elements". The presence of 60-70 enzymes in human milk, such as the fat-splitting lipase (bile salt stimulated lipase, BSSL), and certain binding factors, enhance the bioavailability and the absorption of the different nutrients in human milk thus preventing deficiencies.

2.3.2.1 Protein quality

Human milk proteins, which are primarily whey proteins (whey:casein ratio equal to 60:40) are of high biological value for the preterm infant. They are better tolerated, digested and absorbed than casein proteins, and have been shown to induce less metabolic acidosis than casein-predominant proteins (O'Leary, 1989). The amino acid composition of whey proteins seems to be more appropriate than casein (found in higher proportions in cow's milk proteins) for the preterm infant because it contains high amounts of cysteine and taurine and relatively low amounts of methionine, phenylalanine and tyrosine (Räihä, 1985; Adamkin, 1986). Cystathionase (enzyme leading to the conversion of methionine to cysteine) activity is reduced in preterm infants (Wharton et al., 1994), therefore, cysteine is considered to be an essential amino acid. Consequently, human milk proteins are better suited for the premature infant than cow's milk proteins due to the relatively higher amount of cysteine.
2.3.2.2 Essential Fatty Acids (EFA)

Multiplication of neuronal cells, dendritic arborization and myelinization peaking during the last trimester of gestation and after birth, require large amounts of lipids, especially EFA (Decsi et al., 1994). The lipid composition of cellular membranes is influenced by the lipid composition of the diet (Ballabriga, 1994), therefore, EFA like linoleic acid (C18:2n-6), alpha-linolenic (C18:3n-3) and pre-formed arachidonic acid (C20:4n-6) should be given to the premature infant.

Lipid-rich neuronal tissues including the brain and retina are especially vulnerable to EFA deficiency (Decsi et al., 1994). It has been shown that the linoleic acid (C18:3n-6) content of the brain sharply increases after birth, and arachidonic acid (C20:4n-6) brain accretion increases as soon as the 32\textsuperscript{nd} week of gestation (Heim, 1983). It is estimated that approximately 400 mg of omega-6 and 50 mg of omega-3 EFA /kg of body weight are deposited per day (Clandinin et al. 1980).

Arachidonic acid (C20:4n-6), docosatetraenoic acid (C22:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) are major constituents of the central nervous system and accumulate rapidly during the 3\textsuperscript{rd} trimester of gestation and the first 18 months after birth (Chirouze, 1993). Docosahexaenoic acid (DHA) is particularly important for the development of the cerebral cortex and the retina because the preterm infant may have limited capacity to synthesize this from alpha-linolenic acid (Uauy, 1990; Lucas, 1993). Farrel et al. (1988) showed that because of their limited stores of linoleic acid and adipose tissue and due to their high energy requirements, preterm VLBW infants are particularly susceptible to EFA deficiencies if not provided by their early diet. Lipid supply during the early postnatal period is thus very important, and insufficiencies or imbalances in lipid intakes, especially in EFA, may modify and impair the growth and development of the nervous system, leading in some cases to reduced visual acuity (Decsi et al., 1994). In this regard, human breastmilk is the optimal food because it contains sufficient amounts of long chain poly-unsaturated fatty acids (PUFA) omega-3 and omega-6 derivatives to allow...
adequate intake and meet the requirements for neural tissue and allow normal neurodevelopment (Heim, 1983; Lucas, 1992, 1993).

2.3.2.3 Fat digestion

Normal fat digestion is catalyzed by different lipases, for example gastric lipase which initiates the hydrolysis of triglycerides (TG) in the stomach, and pancreatic colipase-dependent lipase which continues the digestion in the upper small intestine. In newborn and especially in VLBW infants, digestion and absorption of TG are impaired due to low pancreatic lipase and low synthesis of bile salts (Bläckberg et al., 1993). The amount of bile salts synthesized by the small preterm newborn is insufficient to solubilize all the long-chain lipids and therefore fat digestion and absorption depend on a compensatory mechanism such as the action of extrapancreatic lipases, lingual lipase and bile salt stimulated lipase (BSSL) (Hamosh, 1983). The presence of BSSL in breast milk is thus essential for fat digestion, absorption and hence for the growth of the preterm infant. Bile salt stimulated lipase has been shown to improve fat absorption in preterm babies fed fresh human milk as compared with those fed a commercial formula or heated breastmilk (Hamosh, 1978, 1980, 1981, 1983). Bile salt stimulated lipase, which is present only in a limited number of species (humans, gorillas, cats, and dogs) increases the rate of lipolysis. This yields free fatty acids (FFA) and free glycerol which are then absorbed by the enterocytes of the intestinal mucosa (Bläckberg et al., 1993).

2.3.2.4 Fat utilization/ metabolism

Newborns have an immature carnitine synthetic pathway and thus impaired beta-oxidation of long-chain fatty acids. This impaired metabolic mechanism may therefore affect the utilization of fat by the premature infant. Human milk contains very high concentrations of carnitine (Neu et al., 1990), providing adequate amounts to the preterm infant.
2.4 Preterm human milk

Feeding human milk to premature infants has been shown to be beneficial for their growth, development and survival. Human milk is highly digestible, and the presence of trophic and antiinfectious factors seems to protect the infant from a wide variety of infections. Nevertheless, nutritional deficiencies have been shown in VLBW infants fed preterm human milk (Rowe et al., 1979; Atkinson et al., 1983; Cooper et al., 1985) and the suitability of human milk for the premature infant remains controversial. Still, even though clinicians' opinions vary greatly, there seems to be a growing advocacy for the use of human milk from the infant's own mother (Lawrence, 1994).

The composition of preterm human milk (milk obtained from mothers delivering prematurely) has not been as well studied as term milk. Some reports indicate that preterm human milk has a similar composition to term human milk (Sann et al., 1981; Anderson et al., 1983), whereas other authors have shown that it has a different composition from mature human milk expressed from mother a giving birth to baby at term. Preterm milk has higher concentration of protein, sodium, chloride and potassium than term milk (Gross et al., 1980; Anderson et al., 1981; Butte et al., 1984; Dawodu et al., 1990; Lucas, 1993). It has therefore been suggested that preterm milk meets the nutrient needs of premature infants more adequately than pooled term milk (Gross et al., 1980; Anderson et al., 1981; Atkinson et al., 1981; Gross, 1983; Dawodu et al., 1990). Preterm infants fed their mother's milk have been shown to experience a more rapid increase in weight, length and head circumference compared to preterm infants fed full-term human milk (Atkinson et al., 1981; Gross, 1983). Infants fed preterm human milk had more adequate growth rate and protein accretion compared to the intrauterine estimates (Chessex et al., 1983; Hendrickse et al., 1984). Yet, as lactation progresses protein and mineral content of preterm human milk decline and problems such as hypoproteinemia, hyponatremia and metabolic bone disease are common in VLBW infants fed preterm human milk (Fomon et al., 1977; Atkinson et al., 1983; Rönnholm et al. 1982; Gross, 1983; Rowe et al., 1979; Steichen et al., 1980). Hypophosphatemic
rickets and poor bone mineralization, leading to osteopenia or rickets of prematurity, have been reported in VLBW infants who have been fed solely with their own mothers' preterm human milk (Rowe et al., 1979, 1984, 1987; Atkinson et al., 1983).

2.4.1 Limitations of preterm human milk

2.4.1.1 Protein composition of preterm human milk

According to the AAP (1985), a milk intake of at least 180-200 ml/kg/d is necessary to meet the fetal growth requirement of nitrogen. This high volume load may lead to increased risk of opening the ductus arteriosus and congestive heart failure. The level of protein in preterm milk is approximately 21% higher than that found in milk of mothers delivering term infants (Garza et al. 1984). However, the higher protein concentration seems to be due to large amounts of indigestible proteins (Lindblad et al., 1984; Beijers et al., 1992). Higher amounts of poorly-absorbable antigenic factors such as sIgA, lactoferrin and lysozyme are present in preterm human milk (Lindblad et al., 1984; Davidson et al., 1987 Räihä, 1988; Heine, 1992; Joneja, 1992), emphasizing the need for protein fortification of preterm human milk for the VLBW infants.

2.4.1.2 Calcium and phosphorus concentrations in preterm human milk

Butte et al. (1984) found lower concentrations of calcium and phosphorus in preterm milk compared to term milk, whereas Gross et al. (1980) found similar concentrations in both types of human milk. Garza et al. (1984) showed that the mean calcium and phosphorus concentrations of preterm human milk were consistently lower than those found in full-term breast milk. Salle et al. (1988) found slightly different results. Nevertheless, these authors reported that even if the calcium and phosphorus content of preterm human milk was the same as in mature milk (i.e. 27-32 mg of calcium/100 ml and 14-15 mg of phosphorus/100 ml), the increased requirement for calcium
needed to match the intrauterine accretion rate (120-130 mg/kg/day) could not be met by preterm milk. This result is consistent with the findings of Gross et al. (1980, 1983) who showed that the estimated requirements for calcium and phosphorus were not met by preterm human milk.

Rowe et al. (1979) and Gross (1983) emphasized the need for appropriate supplementation of preterm milk with calcium and phosphorus to meet the mineral requirements of the rapidly growing premature infant. Moreover, Raupp et al. (1990) point out that in some cases, prolonged fortification of mother's milk with minerals may be necessary.

Lockitch (1993) indicated that the calcium and phosphorus content of both term and preterm human milk is insufficient to supply the needs of the preterm infant without added supplementation, emphasizing the need for adequate fortification of human milk for preterm and VLBW infants.

2.5 Banked human milk

2.5.1 Banked human milk for preterm and very low birth weight infants

It has been routine practice to feed preterm infants with pooled banked human milk from mothers who had delivered at term. This practice was dictated in part by socio-economical reasons (mothers were not available to provide the milk for their babies) and in part by the inability for the preterm infant to suckle from the breast. This feeding alternative gave the infant the immunological and nutritive constituents of human milk as opposed to formula feeding. However, due to its low protein, energy and mineral content, banked human milk is not considered to be adequate in supporting the high requirements of the rapidly growing LBW infant (Senterre et al., 1984, Rönholm et al., 1986; Marques, 1990). Based on theoretical calculations by Fomon et al. (1977), full-term human milk seems to be insufficient in supporting tissue accretion at the intrauterine rate. Several investigators have demonstrated that banked human milk obtained from mothers of term infants is unable to promote intrauterine growth and nitrogen retention rates (Atkinson et al., 1981;
Gross, 1983; Tyson et al., 1984). It was reported that in infants fed pooled human milk, weight gain was lower than that of their formula fed counterparts (Gross, 1983; Senterre et al., 1984; Marques, 1990). Several other authors have questioned the practice of giving pooled human milk to preterm infants, showing that preterm babies fed pooled human milk at volumes approximating 180 ml/kg/d did not achieve optimal weight gain, confirming the inadequacy of pooled human milk, especially in terms of protein retention, for optimal infant growth (Putet, 1983; Räähä, 1983). Contradictory results were reported by Järvenpää et al. (1983) who demonstrated adequate weight gain in preterm infants fed pooled human milk at volumes of 185 and 200 ml/kg/d. However, 45% of the milk provided to the babies was preterm human milk, therefore, increased amount of protein and other nutrients might have been responsible for this high growth rate.

One potential problem in these studies which may have confounded the results, may be the loss of nutrients during tube feeding (Martinez et al., 1987). Retention of fat and delivery of low calorie-dense milk is a problem with the continuous method of gavage feeding using a syringe infusion pump (Narayanan et al., 1984a). Human milk fat globules seem to separate and adhere to the gavage tube and syringe during tube feeding (Greer et al., 1984; Stocks et al., 1985), potentially creating a desequilibrium in the protein-to-energy balance intake of the infant (Martinez et al., 1987). Moreover, losses of fat soluble constituents like fat soluble vitamins, iron, copper, zinc, calcium, and magnesium (Fransson et al., 1984) may occur during tube feeding, thus interfering with infant growth and development. One way of reducing fat losses during tube feeding is to homogenize human milk using ultrasound treatment. Martinez et al. (1987) were able to show a significant reduction in fat losses during a laboratory simulated tube feeding experiment. These results were confirmed by Dhar (1989) and Rayol (1993). Dhar (1989) also studied the effect of ultrasonication on certain components of banked human milk (IgA, long-chain polyunsaturated fatty acids, free fatty acids, peroxide levels). She showed that ultrasonication resulted only in slight losses of IgA and LC-PUFA, and that these losses were still minimal compared to losses which occurred during tube infusion of untreated milk. Rayol et al. (1993), fed banked human milk homogenized by ultrasonic treatment to premature infants. They found that
infants fed ultrasonically homogenized human milk had better weight and length gain and triceps skinfold thickness than the infants fed non-homogenized banked human milk. More studies are needed in order to assess the changes in the quality of human milk after ultrasonic treatment (Hamosh, 1988).

Examination of the chemical composition of banked human milk shows that it is inadequate for meeting the needs of the VLBW infant and that fortification is needed (Garza et al., 1986). Some authors have shown the superiority of preterm human milk, by demonstrating that infants fed preterm human milk for the first 2 weeks of life grew more rapidly than infants fed banked human milk (Atkinson et al., 1981). Therefore, if banked human milk is to be used for the nutritional management of the preterm VLBW infant, it requires fortification.

2.5.2 Safety of banked human milk

Several cases of viral and bacterial contamination by infected untreated banked human milk have been reported in human milk-fed infants. Salmonella, Streptococcus, hepatitis B surface antigen, cytomegalovirus and HIV have been shown to be present in the breast milk of infected women (Ryder et al., 1977; Kenny et al., 1977; Nutrition Committee, Canadian Pediatric Society, 1985; Van De Perre et al., 1993). With the increased risk of transmission of pathogens and viruses (HIV, cytomegalovirus, adult T-cell leukemia virus) to the newborn through pooled human milk, the practice of giving untreated banked pooled human milk has been replaced by the use of pasteurized human milk. Heat treatment of human milk destroys HIV while retaining most of its immunologic and nutritional properties (Arnold et al., 1993).
2.5.3 Handling and storage of human milk

Processing and storage of human milk have been associated with loss of certain nutritional and antimicrobial factors (Freier et al., 1984; Barrois-Larouze et al., 1984; Lyster et al., 1984; Garza et al., 1986; Lavine et al., 1987).

2.5.3.1 Nutritional changes during pasteurization

Although found effective in destroying the majority of bacterial contaminants, pasteurization of human milk has significant adverse effects on the protective immunological constituents of human milk (Liebhaber et al., 1977; Wills et al., 1982; Garza et al., 1986), and on the absorption of fat by the newborn (Williamson et al., 1978). Holder pasteurization (30 minutes at 62.5°C) has been shown to inactivate the different lipases present in the milk (BSSL and LPL) (Williamson et al., 1978) and to reduce the amount of IgA lysozyme and lactoferrin (Barrois-Larouze et al., 1984; Garza et al., 1986; Arnold et al., 1993). Moreover, the cellular components of breast milk are completely destroyed during heat treatment (Freier et al., 1984).

Björkstén et al. (1980) recommend that whenever possible, raw breastmilk should be used, with the baby being fed it's own mother's milk. However, when recipient mother's milk is not available, the use of pasteurized banked human milk is preferable and considered a safe alternative, compared to the costly formulas (Arnold et al. 1993). Narayanan et al. (1981; 1984b) have shown that even after pasteurization, human milk was still protective if used as the sole source of alimentation. It thus seems wise in countries where the risk of infection is very high, as is the case in most of the developing countries, that the practice of pasteurization be continued.
2.5.3.2 Lipid oxidation products

Lipid peroxidation is based on pro-oxidative mechanisms/agents and on the presence of antioxidative mechanisms/agents. The presence of macrophages in human milk may increase the production of reactive oxygen radicals that are prone to initiate a chain reaction with PUFA, leading to lipid peroxides. Moreover, the presence of human milk lipases results in the release of FFA (Lavine et al., 1989). Length of storage and increased temperature of storage (-70°C to 25°C) have been shown to lead to an increased release of FFA like free linoleic acid (C18:2n-6) and arachidonic acid (C20:4n-6) which are susceptible to peroxidation (Lavine et al., 1987). Van Zoeren-Grobben et al. (1993) have demonstrated a significant increase in free linoleic acid hydroperoxide (C18:2OOH) in unpasteurized human milk stored at 4°C for four days due to increased peroxidation. Because pasteurization destroys almost completely the BSSL (Björkstén et al., 1980), the increase in FFA is unlikely to occur in pasteurized human milk stored at -20°C. Since the antioxidant vitamin C is reduced during pasteurization and during storage at 4°C, and as the amount of vitamin C and riboflavin (cofactor of glutathione reductase) is decreased due to exposure to light (Hamosh et al., 1984), the formation of oxygen radicals might be increased in pasteurized human milk.

2.6 Milk fortifiers for the preterm and VLBW infant

The nutritional goal for VLBW babies as outlined by the Committee on Nutrition of the American Academy of Pediatrics (AAP, 1985) is to "support the intrauterine rate of weight gain and nitrogen retention without producing metabolic disturbances". Even though preterm human milk has been shown to lead to a more rapid rate of growth in weight, length, and head circumference, as well as to a shorter time to regain birth weight than does milk from the mothers of term infants (Gross, 1983), term, preterm and banked human milk cannot be considered fully satisfactory for the dietary management of the VLBW infants (Garza et al., 1986; Rönnholm et al.,
Inadequacy in calcium and phosphorus is well documented and normal growth can only be achieved if VLBW babies are fed large quantities of preterm milk (Järvenpää et al., 1983, Chessex et al., 1983). Nutrient supplementation of human milk for VLBW infants should be advocated in order to increase the nutrient content of term, preterm or pooled human milk and to meet the needs of the rapidly growing preterm baby (Hagelberg et al., 1982; Senterre et al., 1984; Rönnholm et al., 1986; Kashyap et al., 1990). According to Lucas (1993b) "if breast milk is to be used as the major food, it needs fortification".

2.6.1 Commercial milk fortifier or human milk fortifier

In order to maintain the biological advantages of maternal milk, to ensure an adequate intake of the different minerals and macro nutrients, and to meet the estimated daily requirements and growth rates of human milk-fed premature infants, various fortifiers (lyophilized fractions of human milk) and nutritional additives (protein-mineral supplements) have been mixed into human milk (fresh or banked donor milk).

2.6.1.1 Protein and fat

There is currently no agreement on the type of supplement, protein or fat, which should be used as a human milk fortifier for the special needs of the VLBW infant. Schanler et al. (1985a) found adequate growth and macronutrient balance in VLBW infants fed their mothers' milk fortified with additional skim and cream components of human milk. Singhania et al. (1989) used a medium-chain triglyceride (MCT) and sugar formula and found that the feeding of fortified fresh preterm mother's milk to LBW infants at the average rates of 178 ml/kg/d was sufficient to achieve postnatal gains of weight and height similar to intrauterine rates. Rönnholm et al. (1986) found that protein supplementation of human milk with an extra 0.9 g/100 ml improved the growth of the VLBW infant, whereas MCT alone did not significantly affect the growth rate compared with that
of the infants fed their own mother's milk. Ehrenkranz et al. (1989) supplemented human milk with a powdered protein-mineral supplement and found that the rates of weight gain by the premature babies were similar to fetuses during the third trimester of pregnancy. The same results were achieved by Itabashi et al. (1992) using human milk protein, and by Raschko et al. (1989) who used a commercial liquid human milk fortifier. Moro et al. (1991) used a human milk and a cow's milk protein fortifier and found that at intakes of 3.3-3.6 g/kg/d, LBW infants gained weight at a higher rate than the estimated in-utero accretion rate. However, it should be noted that in this study all infants received calcium and phosphorus supplementation at levels of 90 mg/kg/d of calcium and 68 mg/kg/d of phosphorus. Boehm et al. (1993a,b) fed human milk fortified with either bovine milk or human milk protein to VLBW babies. The total protein and energy intakes were estimated to be 3-3.5 g/kg/d and 120 kcal/kg/d respectively. They found similar rates of weight gain as well as nitrogen retention and fat absorption. Moreover, no apparent signs of metabolic imbalances using either the bovine or human milk protein were found.

Although commercial fortifiers and formulas are designed for the preterm infant, they are not considered to be the perfect nourishment for VLBW babies (Jensen et al., 1992). Preterm infant formulas contain several components in excess of the infants' needs and thus constitute a metabolic load that may lead to several disturbances such as metabolic acidosis, hyperammonemia, hyperproteinemia, high levels of amino acids and aminoaciduria. Commercially available fortifiers of human milk are based on cow's milk which can lead to the development of allergy to proteins due to very early contact with heterologous proteins. Moreover, necrotizing enterocolitis (NEC) has been shown to predominate in formula-fed infants (Narayanan et al., 1981; Richardson et al., 1982) and disturbances in intestinal motility are some of the most common problems with oral formula feeding of extremely VLBW infants. Another concern, for the physiological and biochemical development of the infant, is the quality of the protein administered and therefore its impact on protein synthesis. This can be evaluated by monitoring the plasma amino acid levels and other metabolic indicators of amino acid catabolism. Plasma amino acid levels of VLBW infants fed human milk or infant formulas may be compared to values of umbilical cord blood obtained at
the delivery of full term infants, fetal cord blood obtained between 19 and 29 weeks of gestation, or plasma amino acid levels of infants fed pooled banked human milk. Moro et al. (1991) stated that the serum amino acid profile of infants depends on the quality of milk protein given. Infants fed whey or casein predominant formulas have a different serum amino acid profile than those fed exclusively human milk proteins. Amino acids abundant in bovine whey proteins, such as threonine, valine, histidine, alanine, glycine, and serine, show high concentrations in the plasma of infants fed whey-predominant formulas (Räihä et al., 1986), whereas infants fed casein-predominant formulas will show high plasma concentrations of tyrosine and phenylalanine. Priolisi et al. (1992) found that plasma concentrations of threonine, valine, methionine, and phenylalanine were significantly higher in formula fed infants than those fed human milk protein-fortified human milk. Thus the amount of plasma essential amino acids and plasma amino acid concentrations may be higher in the formula fed infants, thereby increasing the risk of metabolic acidosis, hyperammonemia, and aminoaciduria. Räihä (1994) emphasizes the fact that protein composition of formulas must be modified (i.e. more similar to the composition found in human milk) to avoid metabolic imbalances. Moreover, Kunz et al. (1990) showed that throughout lactation, there was an increase in the casein content of preterm and full term human milk, emphasizing the inadequacy of the fixed protein proportions found in commercial infant formulas. Thus, it seems that there is a need for tailor-made human milk fortifiers in order to ensure optimal supply (quantity and quality) of protein and to achieve a plasma amino acid pattern similar to that found in human milk fed infants.

2.6.1.2 Calcium and phosphorus

The low phosphorus content of breast milk may lead to a decrease in the serum level of phosphorus, thus predisposing the VLBW infant to an impairment in bone mineralization. This could be due to high levels of 1,25-(OH)2 D triggered by the low serum levels in phosphorus, and a subsequent increase in serum alkaline phosphatase (Pettifor et al., 1989).
Schanler et al. (1992) reported that preterm infant fed mineral-fortified breast milk (Ca: 80 mg/dl, P: 40 mg/dl) from week 2 through week 8 after birth and human milk for at least 2 months after hospital discharge had a lower mid-radius bone mineral content (BMC) compared with infants fed mineral-fortified breast milk during the first weeks of life but formula after hospital discharge. This difference was still detectable at 1 year. Itabashi et al. (1992) showed that calcium and phosphorus deficiencies occurred even in VLBW infant fed fortified preterm human milk, strengthening the fact that calcium and phosphorus supplementation along with protein is necessary for the improvement of preterm human milk composition. These results show that an early nutritional management with an adequate mineral fortification is critical for the bone mineral development of the premature infant. Adequate fortification may thus decrease the risk of poor bone mineralization among preterm infants thus reducing the risk of rickets, bone deformity, fractures and poor thoracic mineralization. Schanler et al. (1992) suggest that bone mineralization "catch-up", observed at 2 years of age in VLBW infants fed human milk after hospital discharge, may occur earlier if feeding mineral-fortified human milk is continued during the post-hospitalization period.

2.7 "Lactoengineering"^2

It is believed by several authors that because the growth of VLBW infants is restricted by nutrient deficiencies of human milk (preterm, term and pooled banked human milk), enrichment of human milk given to preterm babies would lead to increased growth. Guerrini (1994) compared the growth of 7 VLBW infants fed their own mother's milk fortified with a commercial fortifier and the growth of 14 infants fed a high-caloric-density formula. He found that the nutritional requirements of the VLBW infants were satisfied when fed the fortified human milk. It was concluded that, whenever possible, feeding the VLBW baby with human milk, preferably their

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^2 Technological alteration of human milk composition
own mother's milk, fortified with human milk products, is preferable to formula feeding. As suggested by Lucas et al. (1980), the most suitable way to give the VLBW babies all the needed energy, nutrients, minerals, vitamins and immunoproteins, without compromising the superiority of preterm human milk and without introducing foreign proteins, is to feed them with their own mother's milk supplemented with a tailored and well balanced human milk fortifier. This modification in human milk composition should be performed without disturbing the immunological and enzymatic properties of fresh human milk and without introducing foreign proteins.

Autologous or homologous fortification can be accomplished by two different approaches. First, one can simply concentrate freshly expressed human milk and thereby increase the nutrient composition of the milk. The problem in doing so is that the concentration process may lead to the reduction of immunologic properties of human milk and an excessive increase in osmolality. The second method is to produce human milk constituents (protein, fat and minerals) from the baby's mother (autologous fortification) or from pooled human milk (homologous fortification) and then mix them with the mother's freshly expressed breast milk. This process would then lead to an improved nutrient composition without introduction of non-human products and conserving at the same time the anti-infective properties of human milk. As stated by Lucas (1993), early nutrition may have a strong influence on long-term health and neurodevelopment, therefore emphasis should be made on the quality, not only the quantity of nutrients provided to the VLBW infant.

The dairy industry has developed several methods to make milk powder from fresh cow's milk and some of these processes have been adapted and used to prepare milk components from human milk (Rönnholm et al., 1982; Hagelberg et al., 1982; Hylmö et al., 1984; Garza et al., 1984; Williams, 1984; Lindblad et al., 1984, Goldblum et al., 1985; Schanler et al., 1985a; Polberger et al., 1987; Itabashi et al., 1992). These processes involve separation of the cream, ultrafiltration (reverse osmosis) of the skim fraction in order to reduce lactose and mineral content, and lyophilization of both cream and skim fractions. Even if useful and well designed, these processes are time consuming, expensive (Senterre at al., 1984; Boehm et al., 1993a) and require
large amounts of milk. Thus, they are not very suitable for routine clinical use and may represent extra costs in the management of milk banks in developing countries. Yet, the different procedures utilized can be adapted on a smaller scale in order to produce adequate quantities of human milk components. These components (proteins, fat and minerals) could then be incorporated and mixed with fresh mother's milk in order to feed the VLBW infants.

A simple and inexpensive way of producing human milk components (proteins, fat and minerals) is to concentrate the human milk by evaporating the milk water. One of the problems encountered using this procedure is that the resultant human milk concentrate has a high lactose content, thereby leading to increased osmolar load which potentially could be harmful for the VLBW infant (Billeaud et al., 1982; Koo et al., 1991). In order to concentrate human milk and to remove the excess lactose, Martinez (1989) adapted a method described by Hunziker (1949), which was used to precipitate lactose from evaporated cow's milk. The preliminary results show that it is an easy and low-cost procedure, which uses simple techniques and commonly available equipment, and could thus be a promising way of removing lactose from human milk. However, the possible removal of other nutrients, especially minerals, during precipitation and removal of lactose has not yet been studied. The possible risk of nutrient losses due to the separation of lactose precipitate from the supernatant is also of concern. Nevertheless, after resolving some methodological problems, this process seems to be an appropriate way for the preparation of an homologous fortified human milk for preterm VLBW infants, especially in developing countries.
3 Materials and Methods

This study was divided into two phases.

**Phase I** consisted of concentrating donor milk thereby increasing the protein, fat, lactose, calcium, phosphorus and sodium content of the milk per volume.

**Phase II** consisted of fortification of donor milk with the prepared concentrated human milk.

3.1 Donor human milk

Milk samples were obtained from the milk bank of the "Hospital das Clinicas, Faculdade de Medicina, Ribeirão Preto" (HCFMRP), University of São Paulo, São Paulo, Brazil. The Hospital das Clinicas da Faculdade de Medicina de Ribeirão Preto runs a milk bank which collects and stores human milk, with donor milk being preferentially given to preterm babies (Mattar, 1994). Human milk is donated by volunteer mothers who hand express their milk at home after feeding their own babies. Nurses operating the milk bank provide instructions to the mothers on how to express and store their breast milk. The milk is collected in a sterile glass receptacle (supplied by the milk bank) and then stored at home in a freezer compartment. The same receptacle is used to gather the expressed breastmilk until collected by the milk bank nurse (3 times/week). Therefore, each bottle collected represents milk expressed over approximately a 72 hour period. Once collected, the milk is transported to the milk bank in insulated containers and then pasteurized at 62.5°C for 30 minutes and stored in a freezer at -20°C. Records of the volumes of milk and time of collection were not available for the milk samples used during this study.

Mothers who donate their milk are routinely tested for HIV, hepatitis and syphilis. Donor milk samples used in this study were either freshly hand expressed and stored (-20°C) or
Materials and Methods

Pasteurized and stored. The length of storage at -20°C of the donor milk samples used in this study was not available, therefore, studies on the effect of storage on human milk composition could not be performed.

According to Dos Santos (1994) who concentrated human milk samples using the same technique utilized in this study, milk samples aged less than 2 months postpartum are not suitable for the concentration process. Their high protein and antiinfective content increases milk viscosity thereby interfering with lactose crystallization (Hunziker, 1949). Therefore, all milk samples used in this study were donated by mothers having given birth more than two months before the collection of the milk sample (> 2 months postpartum).

The choice to use mature milk samples (between 2 and 6 months) was dictated by several other factors. First, colostrum and transitional milk which are produced in smaller quantities are of primary importance to the baby (antiinfective properties, presence of growth factors, etc.) and therefore should be exclusively reserved for the early nutrition of the preterm VLBW infant. Second, mature milk is likely to be the most available form of milk in all milk banks in developing and developed countries. Therefore it was important in this study, to use the most commonly available form of milk in order to mimic practical settings. Third, due to the low numbers of volunteers, the low milk volumes donated and the high milk demand from the intensive care unit of the HCFMRP, the only available milk for this study was mature milk.
3.2 Phase I: Concentration process

The concentration process used in this study was adapted for human milk by Martinez (1989) from a method used for cow's milk which was described by Hunziker (1949). The original method was used to evaporate cow's milk and to precipitate its lactose in order to extract it. Each milk sample was concentrated separately.

The concentration process illustrated in Figure 1 consisted of:

I. evaporation of the human milk  
II. lactose crystallization and extraction

Approximately 100 ml of donor milk samples (fresh or pasteurized) stored at -20°C were thawed at room temperature. Once at room temperature, an aliquot of each of the milk samples was taken to analyze its composition (protein, fat, lactose, calcium, phosphorus, sodium) prior to the concentration process. Each donor milk sample was then concentrated.

The concentration factor (i.e. initial volume divided by the volume of the evaporated milk) was set at 4 (i.e. 100 ml to 25 ml) in order to obtain a supersaturated solution of lactose that would allow further lactose crystallization (Martinez, 1989; Dos Santos, 1994). Based on the precipitation curves described for cow's milk by Hunziker (1949), Martinez (1989) estimated the magnitude of the concentration factor for human milk. Assuming that at 4°C the maximum lactose concentration would be between 16% and 18%, Martinez (1989) estimated that in order to reduce the lactose content of the evaporated human milk by 50%, it would have been necessary to obtain a solution containing 32% lactose. A preliminary study by Dos Santos (1994) showed that a concentration factor of 2 (i.e. 100 ml to 50 ml) did not cause lactose crystallization, possibly due to the fact that the solution obtained was not sufficiently saturated, whereas a concentration factor of 4 (i.e. 100 ml to 25 ml) was effective in promoting further lactose crystallization.
3.2.1 Evaporation of human milk

The main problem of evaporating human milk is the temperature used during the process. High temperatures lead to substantial alterations of human milk components, therefore it was important to minimize these alterations using a low temperature. According to Barrois-Larouze (1984), human milk heated at a temperature lower than 58°C for 30 minutes conserves over 99% of its IgA and lactotransferrin proteins, and over 90% of its iron-binding capacity and its lysozyme activity. Consequently, the temperature was empirically set at 40±1°C for the evaporation process (Martinez, 1989; Dos Santos, 1994).

One hundred milliliters of donor milk were placed into the 500 ml flask of the rotary evaporator (Rotavapor TE120 Tecnal; Figure 2) and subsequently immersed in a 40±1°C water bath (Figure 1). A vacuum pump was connected to the rotary evaporator in order to create the negative pressure necessary to enable milk to evaporate at 40±1°C. After a few minutes at 40±1°C and under negative pressure, milk started bubbling, demonstrating that the vacuum pump generated sufficient vacuum, between -20 and -40 mmHg (Dos Santos, 1994), which was sufficient to induce evaporation of water from the milk. During this study two different vacuum pumps were used, so that the time necessary to evaporate 75% of the water (volume of the evaporated human milk = 25 ml) was not constant and depended on the pump and the negative pressure generated. Theoretically, with a vacuum pump operating around -30 mmHg, the time necessary to reduce the volume to 1/4 of the initial volume, should be about 15-20 min. (Martinez 1994, personal communication).
Materials and Methods

Figure 1: Outline of the protocol followed for the preparation of the concentrated human milk.

Each donor milk sample (=100ml) was concentrated separately.
Figure 2: Rotary evaporator (Tecnal TE 120) used for the evaporation of human milk
3.2.2 Lactose crystallization

Lactose in solution is present in two forms, alpha and beta lactose. It remains as a stable solution in milk until its concentration reaches the precipitation point where it starts to form lactose crystals. Crystallization rate depends on the degree of supersaturation, the surface area available for deposition, and the diffusion rate to the crystal surface, which in turn is based on viscosity, agitation, temperature of the solution and the rate of mutarotation of the alpha to beta form (Webb et al., 1974). Lactose crystallization can be summarized by the following reactions:

\[
\text{beta-lactose} \leftrightarrow \text{alpha-lactose} \leftrightarrow \text{alpha-lactose hydrate crystals}
\]

Due to the crystallization of the alpha lactose, the beta form is converted into the alpha lactose form in order to maintain the normal alpha-beta ratio present in the native non saturated solution. This mutation is responsible for the slow rate of crystallization found in milk (Hunziker, 1949).

The evaporated human milk obtained during the previous phase (section 3.2.1 and Figure 1) leads to a highly supersaturated solution of lactose (i.e. very low water to lactose ratio), thus inducing crystal formation. According to Dos Santos (1994) the mean lactose concentration in the evaporated milk is approximately 28-30 g/100 ml. Thus, there is more lactose present than can be maintained in solution. Hence, according to Hunziker (1949), as the temperature drops and the supersaturation increases, there is a spontaneous inevitable tendency for the lactose to crystallize. Newly formed lactose crystals catalyze crystallization, and as supersaturation increases with a decrease in temperature, crystallization becomes more efficient. Lactose crystallization continues until increased viscosity and concentration of colloidal substance interfere with the crystallization process (Hunziker, 1949).

In this study, evaporated human milk (≈25 ml), was obtained from donor milk (≈100 ml) using the previously described procedure (section 3.2.1). It was then transferred from the evaporator flask into an acid-washed glass tube (>25 ml glass tube). During this transfer, a
significant amount of milk fat was lost; the milk fat globules adhering to the glass surface of the evaporator flask.

The tubes containing the evaporated human milk, were sealed and set aside at -20°C to allow lactose crystallization (Figure 1). Dos Santos (1994) showed that the formation of lactose crystals was effective if the evaporated solution was kept at -20°C for at least 12 hours with a maximum crystallization occurring after 24 hours. Longer cooling periods did not seem to affect the amount of lactose crystallized (Dos Santos, 1994).

After a minimum of 24h, the tubes containing the evaporated human milk were thawed at room temperature and centrifuged at 2000 rpm for 20 minutes in a refrigerated centrifuge at approximately 4°C. After centrifugation, the formation of the lactose crystals was clearly visible. A large white pellet was formed at the bottom of the tube. The supernatant (i.e. concentrated human milk) was then removed using a Pasteur pipette and transferred into another acid-washed glass tube and stored at -20°C for further analysis (Figure 1). In order to avoid the removal of lactose crystals during the transfer of the concentrated human milk (i.e. the supernatant), small volumes of concentrated human milk (situated at the interface between the liquid and the solid phase) were left in the tubes, therefore leading to increased nutrient losses.

Volumes of the evaporated human milk and the concentrated human milk were measured using graduated glass tubes. The pellet, which theoretically contains only lactose crystals (Martinez, 1994), was solubilized using 10 ml of deionized water and later analyzed for protein, fat, calcium, and phosphorus content in order to quantify the losses of those nutrients during the evaporation and crystallization phases.
3.3 Phase II: Fortification of donor milk

The autologous/homologous fortification of a donor milk using a concentrated human milk was designed to increase the nutritive value of the donor milk given routinely to the very low birth weight (VLBW) babies in certain intensive care units. As stated previously (section 2.6), preterm, term and banked human milk do not contain enough macro and micronutrients to allow for adequate rate of growth and development of the premature VLBW infant. Therefore, if donor milk is to be used for the nutrition of the preterm VLBW infant, it has to be fortified (Lucas, 1993b). The approach followed in this study was to fortify donor milk with human milk constituents, in order to 1) eliminate the risk of introducing heterologous proteins (which would increase the risk of allergies), 2) prevent abnormal plasma amino acid profiles, 3) conserve the normal high bioavailability found in human milk for proteins, fat and minerals, and 4) lower the risk of postnatal complications often found when using commercial formulas.

The procedure followed to fortify donor milk is illustrated in Figure 3. Donor and concentrated human milk (fresh or pasteurized) stored at -20°C was thawed at room temperature, mixed thoroughly (using a vortex) and then a predefined proportion of concentrated human milk (cHM) was added to donor milk (dHM). One of the objectives of this study was to estimate the adequate proportion of the concentrated human milk (cHM) needed to be mixed with donor milk (dHM) in order to meet the estimated requirements of the VLBW infant. Therefore, three different types of fortified human milk (fHM) were prepared using different proportions of concentrated milk added to the donor milk. As shown in Figure 3, aliquots of the cHM were taken and then added to different amounts of dHM in order to obtain three different types of fortified human milk. The following proportions of donor and concentrated human milk were used:

- \( \frac{2}{3} \) donor milk + \( \frac{1}{3} \) concentrated human milk = "high" fHM
- \( \frac{3}{4} \) donor milk + \( \frac{1}{4} \) concentrated human milk = "medium" fHM
- \( \frac{4}{5} \) donor milk + \( \frac{1}{5} \) concentrated human milk = "low" fHM.
Materials and Methods

Donor human milk (pasteurized or unpasteurized)

Concentrated human milk

2 ml donor human milk + 1 ml concentrated human milk (2/3:1/3)
3 ml donor human milk + 1 ml concentrated human milk (3/4:1/4)
2 ml donor human milk + 0.5 ml concentrated human milk (4/5:1/5)

"high" fortified human milk
"medium" fortified human milk
"low" fortified human milk

Figure 3: Outline of the protocol followed for the fortification of human milk.

Banked human milk (dHM) is fortified at different proportions using the concentrated human milk (cHM) previously obtained from dHM.
The resulting fortified human milk (fHM), if found nutritionally adequate, could then be used for the nutrition of the very low birth weight (VLBW) premature infant.
Materials and Methods

The different concentrated and donor milk samples used for the fortification process were randomly chosen from the previously analyzed samples (i.e. milk samples from the 16 donors were randomly fortified with aliquots of the concentrated human milk). Fortification of donor milk using randomly chosen concentrated human milk was selected because it reproduced the practical situation of homologous fortification (i.e. fortify mother's milk with concentrated human milk obtained from one or several different mothers).

The fortified human milk (fHM) obtained was then vigorously shaken and stored at -20°C for further macronutrient and mineral analysis. In order to assess the nutritional adequacy for the preterm VLBW infant of the different fHM obtained ("high", "medium" and "low" fHM), protein, fat, lactose, osmolality, calcium, phosphorus and sodium contents of the fHM were measured. As well, the caloric content of the fHM was estimated.
3.4 Assay procedures

In this study, the techniques used to quantify the macronutrient and mineral content of human milk were chosen from those available at the HCFMRP. These techniques are simple, affordable and are representative of those available in developing countries.

Lactose content was determined using the colorimetric method of Barnett and Tawab (1957). Protein and fat content were assessed simultaneously using the Nakai and Chi Le method (1970). Total calcium content was assessed by atomic absorption spectrophotometry, and total phosphorus content was determined from dry ashes (600°C, 24h) using Richterich's modified method (Martinez 1994, personal communication). All assays were done in duplicate. All glass ware was acid-washed.

3.4.1 Determination of lactose content of human milk

The determination of lactose was conducted according to the colorimetric method of Barnett and Tawab (1957) (Figure 4). Milk aliquots were diluted in deionized water; 0.05 ml of donor milk or 0.04 ml of concentrated human milk in 100 ml deionized water. Two milliliters of diluted milk solution were assayed for lactose content; 0.15 ml of phenol (80% vol.) was added and the solution was shaken using a vortex mixer. Then, 5 ml of sulfuric acid were added. The solution was thoroughly shaken and set aside for 15 min. and the absorption of the solution was read using a spectrophotometer (Spectronic 20D, Baush&Lomb) at 420nm. The spectrophotometer was set at zero using a blank prepared from 2 ml of water instead of the diluted milk (Figure 4).
Materials and Methods

Figure 4: Illustration of the procedure used to quantify the lactose content of human milk.

(*) 0.05 ml of donor milk (dHM), 0.05 ml of fortified human milk (fHM) or 0.04 ml of concentrated human milk (cHM) were used for the assay.
Due to the high temperature produced by the addition of the 5 ml sulfuric acid, Pyrex tubes were used. Because of the instability of the reagents, a new standard curve was made for each measurement using several dilution of a 10 mg pure lactose/100 ml solution.

Phenol-sulfuric acid reagent appears to be specific for carbohydrates, and the recovery obtained in Barnett and Tawab' (1957) study (95% to 102%) indicate that this method although rapid, it is accurate when measuring lactose concentrations ranging from zero to 100μg /2 ml of diluted solution.

### 3.4.2 Spectrophotometric determination of protein and fat in human milk

Nakai and Chi Le (1970) developed a simple method for the determination of fat and protein using an ordinary spectrophotometer (Figure 5). The method consists of adding 5 ml of 97% acetic acid to 0.05 ml of milk in order to dissociate both protein and fat. The solution is vigorously shaken and the protein content is determined from the absorbance at 280 nm measured (spectrophotometer Zeiss PM6K) against a blank made of 0.05 ml of deionized water and 5 ml of 97% acetic acid.

The subsequent addition of 2.5 ml of urea-imidazole solution (20% w/v of urea, 0.2% w/v of imidazole) enhances the formation of fat globules resulting in the development of turbidity. The solution is mixed thoroughly and allowed to stand for 30 min. Then the fat content is calculated from the absorbance at 400 nm measured (spectrophotometer Spectronic 20D, Baush&Lomb) against a blank (Figure 5).

This method was validated by comparison with different standardized methods. Nakai and Chi Le (1970) determined that the correlation coefficient for the protein content measured with this method and the micro-Kjeldahl method was 0.991 (n=38). Although not strictly linear when compared with the Babcock method for fat (Nakai and Chi Le, 1970), the fat content determined using this method was highly correlated, r=0.977 (n=25) when compared with the Roese-Gottlieb method (Hundrieser et al., 1984).
Materials and Methods

5 ml of 97% acetic acid

0.05 ml of human milk + 2.5 ml urea-imidazole solution, homogenization

Absorption at 280 nm (=> protein content)

Absorption at 400 nm (=> fat content)

Figure 5: Illustration of the procedure used to simultaneously quantify the protein and fat content of human milk.
Materials and Methods

Providing a calibration curve with standardized solutions, the standard error of estimations for protein and fat should not be higher than 0.117% and 0.123% respectively (Nakai and Chi Le, 1970). A standard curve was obtained using several dilution of a commercial formula Nanon® (Nestlé) of known protein and fat concentrations. Analysis of the reconstituted formula for fat content using the Van de Kramer method (1948) showed a variation of ±10% from the estimated amount of fat derived from the manufacturer’s information. However, the goal of this study was not to assess the exact amount of protein and fat but to determine the relative changes in these macronutrients between the donor and concentrated samples. Accounting for the fact that the method was used simultaneously for the donor and concentrated milk samples, it can be assumed that the relative change in fat and protein content is consistent and correct for all milk samples.

3.4.3 Phosphorus determination

Phosphorus was quantified based on the colorimetric method described by Richterich (1965) Modifications of the original method were made and these are described in Appendix VIII. Milk samples (500μl) were previously dried for 24h at 100°C and subsequently ashed at 600°C for 24h. Ashes were then dissolved using 200μl of HCl and diluted to 5 ml or 10 ml (donor milk or concentrated human milk, respectively). Milk dilution were treated according to the modified Richterich’s method (Figure 6). One milliliter of borate-pyrosulfite was added to 0.1 ml of diluted milk ashes. Then 0.25 ml of molybdic acid and 0.25 ml of hydroquinone ascorbate were added to the milk solution. The solution was vigorously shaken using a vortex mixer and, after 15 minutes, 3 ml of carbonate-sulfite were added. The solution was again mixed thoroughly and after 5 minutes the phosphorus content was determined by measuring the absorption of the solution at 578 nm (Spectronic 20D, Baush&Lomb). A standard curve was previously made and for each set of measurements, several points of this curve were repeated to control the accuracy and reliability of the measurements.
Materials and Methods

0.05 ml of human milk

0.02 ml HCl (1N)

100°C, 24 h

600°C, 24 h

diluted to 5 or 10 ml *

milk ashes solution

0.1 ml of diluted milk ashes
+ 1 ml of borate pyrosulfite
+ 0.25 ml of molybdic acid
+ 0.25 ml of hydroquinone ascorbate

3 ml of carbonate-sulfite

homogenization, wait for 15 minutes

homogenization, wait for 15 minutes

Absorption at 578 nm

Figure 6: Illustration of the procedure used to quantify the phosphorus content of human milk.

(*) Donor human milk (dHM) and fortified human milk (fHM) ashes were diluted to 5 ml whereas concentrated human milk (cHM) ashes were diluted to 10 ml.
3.4.4 Determination of calcium in milk samples

Calcium content was analyzed using an atomic absorption spectrophotometer (Perkin Elmer 380). Quality control was assessed using Acutrol® solution (Sigma®) (9.3 mg of calcium/100 ml at 422.7 nm). The accuracy and reliability of the spectrophotometer was estimated at ± 10%. In order to eliminate the possible interference of phosphorus, a 5 mg% lanthanum solution (58.64g La2O3 with 50 ml H2O plus 250 ml concentrated HCl, made up to 1 liter) was used.

Milk samples were diluted with deionized water: 50 times for the donor milk samples and 100 times for the concentrated milk sample (donor milk: 0.1 ml + 0.1 ml Lanthanum 5 mg% made up to 5 ml; concentrated human milk: 0.1 ml + 0.2 ml Lanthanum 5 mg% made up to 10 ml). Diluted milk samples were then placed into the atomic absorption spectrophotometer and the amount of calcium was read automatically by the apparatus. Calcium content was expressed in mg/100 ml.

3.4.6 Determination of sodium in human milk

An atomic absorption spectrophotometer (Micronal flame photometer B361 coupled with the automatic dilutor B335) was used to determine the amount of sodium present in the human milk. Milk samples were diluted using the automatic dilutor (Micronal B335) and were then ignited in the flame of the spectrophotometer. The amount of sodium present in the milk was automatically calculated and values were expressed in mEq/l.
3.4.5 Measurement of osmolality

Osmolality was assessed using an automatic osmometer (KNAUER Halbmikro-Osmometer). Using the freezing point depression method, the osmolality was determined and expressed as mOsm/kg of water. Salt standards of 100, 200 and 500 mOsm/kg of water were used to verify the accuracy and reliability of the apparatus. A volume of 0.2 ml of milk was used to measure the osmolality.

3.4.7 Estimation of the energy content

The following formula was used to calculate the total caloric content of the human milk samples; [protein] g/l x 4.27 kcal/g + [fat] g/l x 8.87 kcal/g + [lactose] g/l x 3.87 kcal/g (Gross et al., 1980). The energy content was expressed in kcal/l.
3.5 Data analysis

Data were analyzed statistically using the SYSTAT® software. Level of significance was set at p<0.05. Results of the biochemical analyses performed on the human milk prior to and after processing (donor and concentrated human milk), were compared using the paired Student's t-test. Correlation between postpartum age and the macronutrient and mineral content of donor milk was computed. Pearson correlation coefficient between concentrated and donor milk was calculated for each macro and micronutrient assayed. The degree of correlation between macronutrients and minerals of the concentrated human milk was also calculated. The expected nutrient increment ("concentration factor") of the concentrated human milk was estimated (based on the reduction in volume of the human milk sample after evaporation) and compared with the observed increment (estimated by dividing the nutrient content of the concentrated human milk by the nutrient content of donor milk) using the paired Student's t-test. The nutrient content of the different proportions of fortified human milk ("high", "medium" and "low" fHM) obtained during this study was compared with that of donor milk (dHM). The degree of statistical difference between donor milk and the fHM was assessed by analysis of variance (ANOVA) and post hoc pairwise comparison test (Tukey's Honestly Significant Difference). Since the number of samples being compared was unequal, the Tukey-Kramer adjustment was performed. Finally, the estimated daily intakes of each macronutrient and mineral assayed were calculated and were compared with the estimated requirements for the preterm VLBW infant.
Results

Overall, sixteen donor milk samples (100-150 ml; postpartum age between 2-6 months) were collected, analyzed for their nutrient content, osmolality and energy density, and were then concentrated according to Martinez' technique (1989). We selected single donors because it reflected the practical situation for autologous fortification (i.e. each mother providing their own milk for their baby).

4.1 Characteristics of human milk samples

Human milk samples (donor milk) were provided by fully lactating mothers at various stages of lactation. The date of milk collection was recorded and the postpartum time at which the milk was expressed was calculated, for each sample, subtracting the date of birth from the date of collection. This is referred to as postpartum age and is expressed in months. The postpartum age of each milk sample is presented in Table 1. It varied between 2 and 6 months with an average of 3.7±1.2 months. As described in Table 1, out of 16 human milk samples, 12 were stored unpasteurized and 4 were pasteurized (62.5°C for 30 min.) before storage. All milk samples were stored at -20°C for at least one week. The length of storage of the pasteurized and unpasteurized human milk samples was not recorded, therefore the effect of storage on the macronutrient content of human milk could not be determined. The macronutrient and mineral composition of each of the donor milk (dHM) samples used in this study is displayed in Table 2. The mean macronutrient and mineral content of dHM, as determined in this study, and the mean values found in the literature are presented in Table 3.

**Protein:** As can be seen in Table 2, the protein content of the 16 donor milk (dHM) samples showed little variation. It averaged 0.89 ± 0.05 g/100ml with a range of 0.83-1.00 g/100ml. The protein content of dHM was negatively correlated with postpartum age (r=-0.48, p=0.055): such
that, milk protein content tended to decrease as the stage of lactation increased. Protein content was correlated with calcium and sodium content ($r=0.53$, $p<0.05$ and $r=0.63$, $p<0.01$ respectively). No correlation was found between protein and phosphorus content ($r=0.10$).

**Fat:** As shown in Figure 7, there was a very wide range of variation in the fat content of donor milk (dHM). The average fat content was $3.55 \pm 2.27$ g/100ml with a range of $0.51$-8.00 g/100ml (Table 2). Analysis of variance showed that fat content was not significantly correlated with postpartum age ($r=0.02$) nor was it with the other macronutrients and minerals assayed. Only a small but not significant correlation was found between fat and lactose content of donor milk ($r=-0.467$).

**Lactose:** Lactose content was quite constant among the 16 donor milk samples. Mean lactose content was $9.94 \pm 0.74$ g/100ml ranging between $8.56$ and $11.16$ g/100ml. No correlation was found between lactose and any of the nutrients assayed. According to Coppa et al. (1993), lactose content significantly increases with postpartum age. Surprisingly in this study, lactose content was not found to be correlated with postpartum age.

**Energy:** The mean energy content of the 16 milk samples was $73.82 \pm 18.94$ kcal/100ml. As presented in Table 2, sample #11 had the highest energy density with 107.75 kcal/100ml. The lowest energy content was found in sample #13 with only 51.98 kcal/100ml. Energy density was strongly correlated with fat content ($r=0.99$, $p<0.001$).

**Osmolality:** Mean osmolality of donor milk was $274.06 \pm 11.29$ mOsm/kg of water with a range of 245-300 mOsm/kg of water (Table 2). Both lactose and phosphorus contents were positively correlated with osmolality ($r=0.575$ and $r=0.57$, respectively; $p<0.05$ for both). No significant correlation was found between osmolality and the other macronutrients and minerals assayed.
Table 1: Characteristics of donor human milk samples used in this study

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Donor human milk sample *</th>
<th>Postpartum age ¥ (months of lactation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pasteurized</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Unpasteurized</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Unpasteurized</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Unpasteurized</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Unpasteurized</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>Unpasteurized</td>
<td>3.5</td>
</tr>
<tr>
<td>8</td>
<td>Pasteurized</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>Pasteurized</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>Unpasteurized</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>Unpasteurized</td>
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<tr>
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<tr>
<td>13</td>
<td>Unpasteurized</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>Pasteurized</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>Unpasteurized</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>Unpasteurized</td>
<td>2</td>
</tr>
</tbody>
</table>

Mean ± SD ≈ 3.7 ± 1.2

* Milk samples were either pasteurized and stored in the freezer at -20°C or stored unpasteurized at -20°C, prior to their utilization for the concentration process.

¥ Postpartum collection was calculated subtracting the date of delivery from the date of milk collection.
Table 2: Macronutrient and mineral content of donor human milk (dHM) used in this study

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Protein g/100 ml</th>
<th>Fat g/100 ml</th>
<th>Lactose g/100 ml</th>
<th>Energy kcal/100 ml</th>
<th>Osmolality mOsm/kg (H2O)</th>
<th>Calcium mg/100 ml</th>
<th>Phosphorus mg/100 ml</th>
<th>Sodium mEq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.91</td>
<td>1.04</td>
<td>10.55</td>
<td>53.93</td>
<td>275</td>
<td>19.40</td>
<td>12.33</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>0.85</td>
<td>7.23</td>
<td>8.97</td>
<td>102.42</td>
<td>285</td>
<td>22.15</td>
<td>11.56</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>0.87</td>
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<td>10.42</td>
<td>63.77</td>
<td>270</td>
<td>25.06</td>
<td>10.41</td>
<td>15</td>
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<td>4</td>
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<td>9.20</td>
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<td>25.99</td>
<td>11.07</td>
<td>14</td>
</tr>
<tr>
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<td>1.00</td>
<td>4.70</td>
<td>10.28</td>
<td>85.80</td>
<td>275</td>
<td>26.99</td>
<td>9.27</td>
<td>23</td>
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<td>6</td>
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<td>4.14</td>
<td>9.94</td>
<td>79.08</td>
<td>280</td>
<td>20.64</td>
<td>10.01</td>
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<td>2.72</td>
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<td>67.30</td>
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<td>8.00</td>
<td>8.56</td>
<td>107.75</td>
<td>245</td>
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<tr>
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<td>2.14</td>
<td>10.48</td>
<td>63.08</td>
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<td>10.22</td>
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<td>9.39</td>
<td>51.98</td>
<td>280</td>
<td>27.85</td>
<td>12.53</td>
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<td>7.33</td>
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<tr>
<td>15</td>
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<td>2.79</td>
<td>10.52</td>
<td>68.96</td>
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<td>84.95</td>
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<td>11.08</td>
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<td>Mean</td>
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<td>9.94</td>
<td>73.82</td>
<td>274.06</td>
<td>24.33</td>
<td>11.50</td>
<td>17.71</td>
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<td>± SD</td>
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<td>2.27</td>
<td>0.74</td>
<td>18.94</td>
<td>11.29</td>
<td>3.56</td>
<td>2.46</td>
<td>4.61</td>
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Table 3: Comparison of the composition of donor human milk used in this study with values found in the literature

<table>
<thead>
<tr>
<th>Studies</th>
<th>Protein (g/100ml)</th>
<th>Fat (g/100ml)</th>
<th>Lactose (g/100ml)</th>
<th>Energy (kcal/100ml)</th>
<th>Calcium (mg/100ml)</th>
<th>Phosphorus (mg/100ml)</th>
<th>Sodium (mEq/l)</th>
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</thead>
<tbody>
<tr>
<td>Lauber et al. 1979</td>
<td>0.96±0.11</td>
<td>3.07±0.65</td>
<td>6.74±1.04</td>
<td>61±7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gross et al. 1980</td>
<td>1.42±0.05</td>
<td>4.01±0.30</td>
<td>7.26±0.17</td>
<td>69.7±2.9</td>
<td>24.9±0.18</td>
<td>15.8±0.13</td>
<td>8.5±1.8</td>
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<tr>
<td>Gross et al. 1983</td>
<td>1.01±0.03</td>
<td>3.97±0.37</td>
<td>7.06±0.20</td>
<td>66.8±1.0</td>
<td>26.8±2.1</td>
<td>12.1±0.8</td>
<td>6.65±0.91</td>
</tr>
<tr>
<td>Dewey et al. 1983</td>
<td>1.25-1.32</td>
<td>4.30-4.62</td>
<td>7.13-7.75</td>
<td>73.6-78.7</td>
<td>24.8-27</td>
<td>-</td>
<td>5.8-8.0</td>
</tr>
<tr>
<td>Butte et al. 1984</td>
<td>0.9±0.16</td>
<td>4.24±1.57</td>
<td>-</td>
<td>66±12.8</td>
<td>26±0.26</td>
<td>13.6±0.27</td>
<td>5.65±1.7</td>
</tr>
<tr>
<td>Michaelsen et al. 1990</td>
<td>0.63-1.43</td>
<td>1.84-8.9</td>
<td>6.42-7.65</td>
<td>50-115</td>
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<td>-</td>
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<tr>
<td>Allen et al. 1991</td>
<td>-</td>
<td>-</td>
<td>6.53±0.13</td>
<td>-</td>
<td>25.2±0.8</td>
<td>-</td>
<td>6±0.4</td>
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<td>Nommsen et al. 1991</td>
<td>1.14±0.15</td>
<td>3.77±0.96</td>
<td>7.44±0.19</td>
<td>70.7±9.2</td>
<td>-</td>
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<tr>
<td>Western countries</td>
<td>0.9-1.6</td>
<td>3.5-5.2</td>
<td>5.3-7.6</td>
<td>65-75</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Developing countries</td>
<td>Lauber et al. 1995</td>
<td>0.85-1.78</td>
<td>2.3-4.1</td>
<td>6.1-7.9</td>
<td>53-68</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Present study 1995</td>
<td>0.89±0.05</td>
<td>3.55±2.27</td>
<td>9.94±0.74</td>
<td>73.82±18.94</td>
<td>24.33±3.56</td>
<td>11.50±2.46</td>
<td>17.71±2.46</td>
</tr>
</tbody>
</table>
Figure 7: Variability in the fat content of donor human milk used in this study.
The figure displays the fat content of the 16 samples of donor human milk. Fat content in donor milk averaged 3.55±2.27 g/100 ml with a range of 0.51-8.00 g/100 ml.
**Results**

**Calcium:** Calcium content of donor milk averaged 24.33 ± 3.56 mg/100ml with a range of 18.70 to 29.66 mg/100ml (Table 2). Calcium was negatively correlated with postpartum age (r=-0.848, p<0.001). As can be seen in Figure 8, the older the postpartum age, the lower the calcium content of donor milk. Although, Butte et al. (1984) report no longitudinal changes in calcium content of milk produced by mothers delivering term infants, this result is consistent with a previous report indicating a decrease in calcium content with postpartum age (Karra et al., 1988). Milk casein is known to form a complex with calcium (90% of skim-milk calcium content is found as calcium caseinate in cow's milk; Webb et al., 1974). It is not surprising that calcium content of the donor milk samples used in this study was correlated with protein content (r=0.536, p<0.05).

**Phosphorus:** Phosphorus content of the 16 donor milk samples was estimated to be 11.05 ± 2.46 mg/100ml with a range of 7.33-17.74 mg/100ml. A small but not significant correlation was found between phosphorus and lactose content (r=0.425). Also, phosphorus content exhibited a negative but not significant correlation with fat content (r=-0.333). Casein forms a complex with calcium and phosphorus (Webb et al., 1974). Still, in this study, phosphorus was not correlated with protein content (r=0.109). According to Kunz et al. (1990), as lactation advances, the ratio between phosphorylated beta-casein and glycosylated k-casein decreases, indicating that less beta-casein and therefore potentially less phosphorus is present, possibly explaining the fact that no correlation was found between protein and phosphorus content of donor milk. Phosphorus which forms complexes with calcium in human milk (colloidal calcium phosphate; Webb et al., 1974) was unexpectedly not correlated with calcium content (r=0.273).

**Sodium:** Mean sodium content of expressed human milk samples was estimated to be 20.53 ± 12.13 mEq/l ranging from 11 to 60 mEq/l. The very high sodium content found in sample #14 (60 mEq/l) was probably due to contamination during storage or handling. Moreover sodium content of milk sample #11 could not be determined because its high fat content interfered with the measurement. All but these two milk samples were taken into account for the calculation of the mean sodium content of dHM. After elimination of sample #11 and #14, the average sodium
Results

content was 17.71 ± 4.61 mEq/l with a range of 11-28 mEq/l (Table 2). Sodium content was negatively correlated with postpartum age ($r=-0.55; n=14; p<0.05$) and more strongly correlated with calcium and protein ($r=0.672$ and $r=0.636$, respectively; $n=14; p<0.01$ for both).
Figure 8: Variation in the calcium content of donor human milk depending on the postpartum age of the human milk sample. The Pearson correlation coefficient between calcium and postpartum age was $r = -0.848$ ($p < 0.001$).
4.2 Characteristics of the concentrated human milk (cHM)

The mean nutrient contents of both donor (dHM) and concentrated (cHM) human milk are presented in Table 4. The macronutrient and mineral contents of the concentrated human milk (cHM) compared to those of donor milk (dHM) are presented in a graphic form in Figures 9, 10 and 11.

**Protein:** The protein content of the cHM averaged 2.62 ± 0.63 g/100ml ranging from 1.57-3.63 g/100ml.

**Fat:** cHM fat content averaged 6.97 ± 2.41 g/100ml with a range of 1.16-9.09 g/100ml. Like dHM, fat content of the cHM exhibited a wide range of variations. As expected, the fat content of cHM was positively correlated with the fat content of dHM (r=0.766, p<0.001); that is to say, the higher the fat in dHM, the higher the fat in cHM.

**Lactose:** The lactose content of cHM averaged 23.43 ± 3.30 g/100ml with a range of 14.44-29.86 g/100ml. No significant correlation was found between lactose content before (dHM) and after concentration (cHM). This result was not surprising because lactose crystals were removed from the concentrated milk prior to the determination of the lactose content.

**Osmolality:** After concentration and removal of excess lactose, the osmolality of cHM averaged 917.5 ± 122.7 mOsm/kg of water, ranging from 760-1250 mOsm/kg of water. Surprisingly osmolality of the cHM was not correlated with lactose (r=-0.076), calcium (r=0.479), fat (r=0.406) or protein content of cHM (r=0.378).
Table 4: Macronutrient and mineral composition of donor and concentrated human milk, and comparison between the observed and the expected concentration factor

<table>
<thead>
<tr>
<th>Component</th>
<th>Donor human milk (dHM)</th>
<th>Concentrated human milk (cHM)</th>
<th>Observed concentration factor (cHM/dHM)</th>
<th>Expected concentration factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (a) ±SD</td>
<td>Mean (b) ±SD</td>
<td>Mean (c) ±SD</td>
<td>Mean (d) ±SD</td>
</tr>
<tr>
<td>Protein</td>
<td>g/100 ml</td>
<td>0.89 ± 0.05</td>
<td>2.62 ± 0.63</td>
<td>2.94 ± 0.67</td>
</tr>
<tr>
<td>Fat</td>
<td>g/100 ml</td>
<td>3.55 ± 2.27</td>
<td>6.97 ± 2.41</td>
<td>2.35 ± 0.79</td>
</tr>
<tr>
<td>Lactose</td>
<td>g/100 ml</td>
<td>9.94 ± 0.74</td>
<td>23.43 ± 3.30</td>
<td>2.36 ± 0.33</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/100 ml</td>
<td>24.33 ± 3.56</td>
<td>90.70 ± 24.63</td>
<td>3.69 ± 0.69</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg/100 ml</td>
<td>11.50 ± 2.46</td>
<td>41.18 ± 8.76</td>
<td>3.67 ± 0.84</td>
</tr>
<tr>
<td>Sodium</td>
<td>mEq/l</td>
<td>17.71 ± 4.61</td>
<td>36.50 ± 9.10</td>
<td>2.09 ± 0.47</td>
</tr>
<tr>
<td>Energy</td>
<td>kcal/100 ml</td>
<td>73.82 ± 18.94</td>
<td>163.65 ± 22.19</td>
<td>2.32 ± 0.50</td>
</tr>
<tr>
<td>Osmolality</td>
<td>mOsm/kg (H2O)</td>
<td>274.06 ± 11.29</td>
<td>917.50 ± 122.77</td>
<td>3.35 ± 0.41</td>
</tr>
</tbody>
</table>

a) Mean values calculated from raw data shown in Table 2
b) Mean values calculated from raw data shown in Appendix I
c) The concentration factor was calculated by dividing the nutrient content of the cHM by the corresponding value of dHM. The detailed calculations are presented in Appendix V.
d) The expected concentration factor was calculated as described in Table 5.

†: significant difference between cHM and dHM (p<0.001)
‡: significant difference between the observed and the expected concentration factor (p<0.001)
¥: significant difference between the observed and the expected concentration factor (p<0.005)
fl: no significant different between the observed and the expected concentration factor (p=0.05)
Figure 9: Comparison of the mean protein, fat and lactose content of donor milk with concentrated human milk.

Mean concentration is shown on the top of each bar. It is expressed in g/100 ml.

* statistical difference between donor and concentrated human milk (p<0.001).
Donor human milk was subdivided into two categories, high fat (>4 g/100 ml, n=7)) and low fat (<4 g/100 ml, n=9).

Mean concentration values (g/100 ml) are displayed on the top of each bar.

* The fat increment in the concentrated human milk was significantly higher for the low fat group when compared with that in the high fat group (= 2.9 vs. = 1.6 times) (p<0.001).
Figure 11: Comparison of the calcium, phosphorus and sodium content of donor milk with concentrated human milk.

Mean concentration values are shown on the top right of each bar. Values are expressed in mg/100 ml for calcium and phosphorus concentration and in mEq/l for sodium content.

* The concentrations of all the micro nutrients were significantly higher in the concentrated compared to donor milk (p<0.001)
Results

Calcium: The calcium content of the cHM averaged 90.70 ± 24.63 mg/100ml with a range of 48.77-129.52 mg/100ml. Calcium content of cHM was significantly correlated with protein content of cHM (r=0.578, p<0.05). It was also significantly correlated with calcium content of dHM (r=0.785, p<0.001), indicating that calcium was efficiently concentrated and that minor calcium losses occurred during the concentration process.

Phosphorus: The mean phosphorus content of the concentrated human milk samples was 41.18 ± 8.76 mg/100ml ranging from 21.59-55.82 mg/100ml. No correlation was found between initial (dHM) and final (cHM) phosphorus content. Phosphorus content of cHM was correlated with calcium (r=0.518, p<0.05) and protein (r=0.488, p<0.05).

Sodium: Mean sodium content of cHM was 38.88±21.62 mEq/l ranging from 4-107 mEq/l. If we excluded sample #14 that may have been contaminated (107 mEq/l) and sample #11 (4 mEq/l, measurement error), we found that the sodium content of the 14 cHM samples averaged 36.5 ±9.10 mEq/l with a range of 15-48 mEq/l. Sodium content of cHM was significantly correlated with calcium and lactose (r=0.671 and r=0.620, respectively; p<0.01 and p<0.05, respectively).

4.3 Comparison of the nutrient content of donor milk (dHM) with that of concentrated human milk (cHM)

After analysis of the macronutrient and mineral content of both dHM and cHM, the nutrient increment (or "concentration factor") for each milk sample was estimated (Table 4; Appendix V and VI). The comparison of the mean nutrient content of dHM with that of cHM is presented in Table 4, and in Figures 9, 10 and 11. Student's paired t-test was performed for all macronutrients and minerals assayed. Results confirmed the significant increase in the nutrient content of cHM compared to that of dHM (p<0.001).

Macronutrients: The mean protein, fat and lactose content were significantly higher in the cHM compared to dHM (p<0.001).
Results

Minerals: Figure 11 illustrates the difference in mineral content found between dHM and cHM. There was a marked increase in calcium, phosphorus and sodium content of human milk after the concentration process. Statistical analysis performed for all the minerals assayed showed a significant difference ($p<0.001$) between the mineral content of dHM and of cHM. Sodium concentration was computed using 14 samples, samples #11 and #14 being eliminated from the calculation due to their erroneous values.

Osmolality and energy content of the concentrated human milk (cHM) were significantly higher than that of dHM ($p<0.001$).

4.4 Concentration process

The 16 samples of donor milk were concentrated by evaporation with subsequent removal of lactose crystals as described above in section 3.2. It should be noted that this process involved several glass ware (bottle, evaporator flask, graduated cylinders, tubes, Pasteur pipets) and that fat globules tend to adhere to glass surfaces (Goldblum et al., 1981). Thus, at every step during the manipulation, small quantities of milk fat globules and liposoluble vitamins were lost in the different glass containers leading to a substantial loss of fat. Moreover, it was noted that the size of the lactose pellet formed was highly variable, depending on the amount of lactose and other nutrients present in the supersaturated lactose solution (i.e. evaporated human milk).

Expected "concentration factor": Milk volumes were measured before and after the evaporation of the milk water in order to assess the degree of concentration ("concentration factor") that should be expected in the concentrated human milk. Volumes of each milk sample before, after evaporation, and after crystallization and removal of lactose are shown in Table 5. The expected "concentration factor" was calculated dividing the milk volume before evaporation by the volume obtained after evaporation. The average expected "concentration factor" was $4.11 \pm 0.77$ (Table 5);
such that, the concentrated human milk (cHM) is expected to have a protein, fat, calcium, phosphorus and sodium content 4.11±0.77 times higher than that of donor milk (dHM).

4.5 Comparison between expected and observed nutrient increment in the concentrated human milk

Based on the milk volume reduction that occurred during the concentration process, the expected increment (or "concentration factor") in protein, fat, calcium, phosphorus and sodium was estimated to be 4.11±0.77 (Table 5). To estimate the actual nutrient increment in the cHM, mean nutrient content of cHM was divided by the corresponding mean nutrient content of dHM. The expected and observed increments (or "concentration factors") for each nutrient are presented in Table 4 (and Appendix V and VI).
Table 5: Volumes of human milk samples before evaporation (donor human milk), after evaporation (evaporated human milk) and after crystallization and removal of lactose (concentrated human milk)

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Volume before evaporation (ml)</th>
<th>Volume after evaporation (ml)</th>
<th>Volume after concentration (ml)</th>
<th>Expected concentration factor *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>40.00</td>
<td>37.70</td>
<td>2.50</td>
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<tr>
<td>2</td>
<td>100</td>
<td>24.30</td>
<td>13.00</td>
<td>4.12</td>
</tr>
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<td>3</td>
<td>100</td>
<td>21.00</td>
<td>6.50</td>
<td>4.76</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>25.00</td>
<td>13.70</td>
<td>4.00</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>26.00</td>
<td>16.50</td>
<td>3.85</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>25.00</td>
<td>16.80</td>
<td>4.00</td>
</tr>
<tr>
<td>7</td>
<td>75</td>
<td>20.00</td>
<td>9.30</td>
<td>3.75</td>
</tr>
<tr>
<td>8</td>
<td>110</td>
<td>28.00</td>
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</tr>
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<td>9</td>
<td>100</td>
<td>25.00</td>
<td>14.00</td>
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<td>100</td>
<td>16.00</td>
<td>4.80</td>
<td>6.25</td>
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<td>100</td>
<td>25.00</td>
<td>22.30</td>
<td>4.00</td>
</tr>
<tr>
<td>15</td>
<td>88</td>
<td>22.00</td>
<td>11.10</td>
<td>4.00</td>
</tr>
<tr>
<td>16</td>
<td>75</td>
<td>18.50</td>
<td>10.60</td>
<td>4.05</td>
</tr>
<tr>
<td>Mean</td>
<td>95.50</td>
<td>23.96</td>
<td>15.16</td>
<td>4.11</td>
</tr>
<tr>
<td>± SD</td>
<td>10.23</td>
<td>5.38</td>
<td>8.07</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* The evaporation of the milk water produced a more concentrated milk. The concentration factor is calculated by dividing the volume prior to evaporation by the volume after evaporation. It indicates how many times the human milk nutrients are expected to be concentrated (i.e. the nutrient content of the concentrated human milk is expected to be equal to 4.11±0.77 times the nutrient content of donor human milk).
The observed "concentration factors" differed significantly from the expected value of 4.11±0.77 times (Table 4). The observed protein increment in the cHM was 2.94±0.67 and was significantly different from the expected value (p<0.001). The average fat increment was 2.35±0.79 times and was significantly different from the expected value (p<0.001). Separation of dHM samples into 2 subgroups, "high fat" (> 4 g/100ml, n=7) and "low fat" (< 4 g/100ml, n=9) subgroups, and comparison between the nutrient content of dHM with that of cHM indicated that there was a significant difference in the fat increment in the cHM depending on the initial fat content of dHM (p<0.001); the "high fat" subgroup having a fat increment of 1.61±0.37 times and the "low fat" subgroup having an increment of 2.92±0.47 times (Figure 10). Calcium increment averaged 3.69±0.69 times, and even though it was close to the value of 4.11±0.77, Student's paired t-test confirmed that it was significantly different from the expected value (p<0.01). Phosphorus increment was 3.67±0.84 times and was not significantly different from the expected increment (p=0.052). Sodium increment averaged 2.09±0.47 times and was significantly different from the expected increment of 4.11±0.77 times (p<0.001). As expected, lactose and osmolality had a lower increment due to lactose crystallization and extraction during the concentration process. Lactose content increased only by a factor of 2.36±0.33. The lactose crystals' removal should have led to a significant decrease in osmolality. Surprisingly, the osmolality increment was quite high, averaging 3.35±0.41 times the initial osmolality.

**Nutrients losses during milk concentration**

During the milk concentration process, substantial losses of protein, fat, and calcium occurred. Apart from the visible fat losses that took place during the transfer of the evaporated human milk from the evaporator flask to the test tube (Figure 1), analysis of the lactose pellets (after lactose crystallization and removal of the supernatant) showed presence of protein, fat, and calcium, leading to the confirmation that substantial nutrient losses occurred during this phase of the concentration process. The results are presented in Appendix VII. Macronutrient and mineral losses during the concentration process were quite variable. Protein losses were significant and
averaged 28.05%±17.95 of the initial content of dHM. Fat and calcium losses averaged 12.04%±8.26 and 19.37%±5.38, respectively. There was a wide range of variations in the percentage of nutrients lost, especially in the case of protein and fat. It probably depended on the viscosity and fat content of the milk, and on the residual milk left in the glassware.

4.6 Fortified human milk

4.6.1 Comparison of the nutrient composition of the fortified human milk (fHM) with that of the donor milk (dHM)

Concentrated human milk was mixed with donor milk as described in section 3.3. Different proportions of fortified human milk were prepared ("high", "medium" and "low" fHM) in order to determine the one ("high", "medium" or "low" fHM) which would fulfill the nutrient requirements of the preterm VLBW infant. Table 6 summarizes the mean macronutrient and mineral content, as well as the osmolality and the energy content of the different fortified human milks ("high", "medium" and "low" fHM). The detailed data are presented in Appendix II, III, and IV. The macronutrient and mineral content of all the fHM ("high", "medium" and "low" fHM) were significantly different from that of dHM. Figures 12, 13, and 14 illustrate the differences in the nutrient composition existing among the different fHM and dHM. The protein content was higher in the fHM compared to the dHM. The difference in the protein content between dHM and the "low" fHM was very small. Donor human milk had an average protein content of 0.89±0.05 g/100ml while the "low" fHM had a mean protein content of 1.03±0.03 g/100ml. Analysis of variance (ANOVA) and Tukey's HSD test demonstrated that the difference in protein content between donor and "low" fHM was significant (p<0.05). As well, there was a significant difference between "medium" fHM and dHM and between "high" fHM and dHM (p<0.001).
Table 6: Macronutrient and mineral composition of donor and fortified human milk (a)

<table>
<thead>
<tr>
<th>Component</th>
<th>Donor human milk (dHM)</th>
<th>&quot;Low&quot; fortified human milk</th>
<th>&quot;Medium&quot; fortified human milk</th>
<th>&quot;High&quot; fortified human milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>Protein</td>
<td>g/100 ml</td>
<td>0.89 ± 0.05</td>
<td>1.03 ± 0.04 ¥</td>
<td>1.19 ± 0.18 ✲</td>
</tr>
<tr>
<td>Fat</td>
<td>g/100 ml</td>
<td>3.55 ± 2.27</td>
<td>3.95 ± 1.44</td>
<td>4.99 ± 2.00</td>
</tr>
<tr>
<td>Lactose</td>
<td>g/100 ml</td>
<td>9.94 ± 0.74</td>
<td>10.70 ± 0.83</td>
<td>12.34 ± 1.45 ✲</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/100 ml</td>
<td>24.33 ± 3.56</td>
<td>36.78 ± 3.60 ¥</td>
<td>41.39 ± 5.40 ✲</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg/100 ml</td>
<td>11.50 ± 2.46</td>
<td>19.90 ± 2.39 ✲</td>
<td>20.05 ± 2.16 ✲</td>
</tr>
<tr>
<td>Sodium</td>
<td>mEq/1</td>
<td>17.71 ± 4.61</td>
<td>26.20 ± 9.14 ✲</td>
<td>37.10 ± 10.67 ✲</td>
</tr>
<tr>
<td>Energy</td>
<td>kcal/100 ml</td>
<td>73.82 ± 18.94</td>
<td>80.85 ± 13.49</td>
<td>97.11 ± 19.14</td>
</tr>
<tr>
<td>Osmolality</td>
<td>mOsm/kg (H2O)</td>
<td>274.06 ± 11.29</td>
<td>397.50 ± 20.72 ✲</td>
<td>430.50 ± 25.87 ✲</td>
</tr>
</tbody>
</table>

Data come from Table 2 and Appendix II, III, and IV.
a) "Low" fortified human milk (4/5 donor + 1/5 concentrated human milk), "Medium" fortified human milk (3/4 donor + 1/4 concentrated human milk), "High" fortified human milk (2/3 donor + 1/3 concentrated human milk).

¥ : significant difference between fortified human milk and donor human milk (p<0.05)
 トラック : significant difference between fortified human milk and donor human milk (p<0.001)
Figure 12: Protein, fat and lactose content of donor milk, "low", "medium" and "high" fortified human milk.

Mean values are expressed in g/100 ml and are displayed on the top of each bar.

* significantly higher than donor milk (p<0.05)
** significantly higher than donor milk (p<0.001)
Results

Calcium, phosphorus and sodium content of donor milk, "low", "medium" and "high" fortified human milk (fHM).

Mean concentration is displayed on the top of each bar. Calcium and phosphorus are expressed in mg/100 ml, whereas sodium concentration is expressed in mEq/l.

Calcium and phosphorus content of all three fHM is significantly higher compared to that of donor milk (p<0.001).

Due to the high variability in the sodium content of donor milk, only the "medium" and "high" fHM had a significantly higher sodium content compared to donor milk (p<0.001).

Figure 13: Calcium, phosphorus and sodium content of donor milk, "low", "medium" and "high" fortified human milk (fHM).
Figure 14: Osmolality and energy content of donor milk, "low", "medium" and "high" fortified human milk (fHM).

Mean osmolality (mOsm/kg of water) and energy content (kcal/100ml) of each milk group is displayed on the top right of each bar.

* Due to the high fat variability of the different fHM, only the "high" fHM had an energy content that was significantly different compared to that of donor milk (p<0.05).

** fHM had a significantly higher osmolality than donor milk (p<0.001)
Because the range of variations in the fat content of donor and concentrated human milk was large, the fortified human milk displayed the same pattern. Analysis of variance and subsequent Tukey's test confirmed that the difference in fat content between the "low" fHM and dHM, and between "medium" fHM and dHM was not significant. The only significant difference was seen comparing "high" fHM with dHM (3.55±2.26 g/100ml vs. 6.92±0.81 g/100ml; p<0.05).

Lactose content of the "low" fHM was not statistically different from that of dHM. The "medium" and "high" fHM had a lactose content significantly higher than that of dHM (p<0.001). Calcium and phosphorus content of fHM was significantly higher compared to dHM (p<0.001). Sodium content was not significantly different in the "low" fHM compared to dHM. Sodium content of the "medium" and "high" fHM was significantly higher than that of dHM (p<0.001). Osmolality was significantly higher in all fHM compared to dHM (p<0.001).

The average energy content of the different fHM was higher than that found in dHM. Due to the wide range of fat variation found in the milk samples (dHM, cHM and fHM), only the "high" fHM had a significantly higher energy content than dHM (p<0.05).

4.6.2 Adequacy of the fortified human milk for the nutritional management of the preterm VLBW infant

In order to assess the adequacy of the fHM for the needs of the preterm VLBW infant, we estimated the nutrient content of a certain volume of the different fHM ("low", "medium" and "high" fHM). Because volumes received by the preterm infant may vary widely, we calculated the nutrient content for both volumes usually used (150 or 180 ml). The estimated nutrient content of fHM are listed in Table 7. As can be seen from these estimates, protein, calcium and phosphorus content of the fHM at both volumes are below the estimated requirements of the preterm VLBW infant. Figure 15 illustrates the estimated nutrient content of 180 ml of "high" fHM. Comparison between the assumed nutrient intakes (at 180 ml/kg/d) and the estimated requirements confirmed that protein and mineral fortification of fHM is still needed in order to fulfill the high nutrient requirements of the preterm VLBW infant.
Table 7: Comparison of the amount of nutrients present in 150 or 180 ml of fortified human milk with the nutrient requirements of a preterm VLBW infant per kg body weight per day.

<table>
<thead>
<tr>
<th>Component</th>
<th>&quot;Low&quot; fHM (at 150 ml/kg/d)</th>
<th>&quot;Low&quot; fHM (at 180 ml/kg/d)</th>
<th>&quot;Medium&quot; fHM (at 150 ml/kg/d)</th>
<th>&quot;Medium&quot; fHM (at 180 ml/kg/d)</th>
<th>&quot;High&quot; fHM (at 150 ml/kg/d)</th>
<th>&quot;High&quot; fHM (at 180 ml/kg/d)</th>
<th>Requirements (AAP, 1985)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/kg/d)</td>
<td>1.54 ±0.05</td>
<td>1.85 ±0.06</td>
<td>1.78 ±0.28</td>
<td>2.14 ±0.33</td>
<td>2.17 ±0.26</td>
<td>2.60 ±0.31</td>
<td>3.5-4</td>
</tr>
<tr>
<td>Fat (g/kg/d)</td>
<td>5.93 ±2.16</td>
<td>7.11 ±2.59</td>
<td>7.49 ±3.00</td>
<td>8.99 ±3.60</td>
<td>10.39 ±1.22</td>
<td>12.47 ±1.47</td>
<td>-</td>
</tr>
<tr>
<td>Lactose (g/kg/d)</td>
<td>16.05 ±1.25</td>
<td>19.26 ±1.50</td>
<td>18.51 ±2.17</td>
<td>22.21 ±2.62</td>
<td>21.45 ±1.18</td>
<td>25.74 ±1.41</td>
<td>-</td>
</tr>
<tr>
<td>Calcium (mg/kg/d)</td>
<td>55.18 ±5.40</td>
<td>66.21 ±6.48</td>
<td>62.09 ±8.10</td>
<td>74.51 ±9.73</td>
<td>80.16 ±9.42</td>
<td>96.19 ±11.31</td>
<td>200-250</td>
</tr>
<tr>
<td>Phosphorus (mg/kg/d)</td>
<td>29.84 ±3.59</td>
<td>35.81 ±4.31</td>
<td>30.07 ±3.24</td>
<td>36.09 ±3.89</td>
<td>40.26 ±9.26</td>
<td>48.31 ±11.11</td>
<td>110-125</td>
</tr>
<tr>
<td>Sodium (mEq/kg/d)</td>
<td>3.93 ±1.37</td>
<td>4.71 ±1.64</td>
<td>5.56 ±1.60</td>
<td>6.67 ±1.92</td>
<td>5.91 ±0.73</td>
<td>7.09 ±0.88</td>
<td>4.0-8.0</td>
</tr>
<tr>
<td>Energy (kcal/kg/d)</td>
<td>121.28 ±20.24</td>
<td>145.54 ±24.29</td>
<td>145.67 ±28.71</td>
<td>174.80 ±34.45</td>
<td>184.41 ±11.41</td>
<td>221.29 ±13.69</td>
<td>120</td>
</tr>
</tbody>
</table>
Figure 15: Comparison of the amount of nutrients presents in 180 ml of "high" fortified human milk (fHM) with the nutrient requirements of a preterm VLBW infant per kg body weight per day.
5 Discussion

Feeding human milk to preterm VLBW infants has the advantage of 1) providing species-specific anti-infectious factors, 2) minimizing metabolic stress, and 3) avoiding exposure to foreign proteins like cow's milk proteins. However, it has been shown that neither term nor preterm human milk is fully adequate for the higher nutritional requirements of the preterm VLBW infant (Gross, 1983; Senterre et al., 1984). Therefore, in order to provide the preterm VLBW infant with an adequate amount of macro and micronutrients, as well as the other inherent factors contained in human milk, human milk needs to be fortified (Lucas et al., 1980). The fortification of human milk has been carried out using different techniques and products. The approach of this study was to fortify human milk with human milk's native components (protein, fat, calcium, phosphorus). This approach has been used by several investigators; however, the technique used to obtain the human milk constituents is technically and financially not feasible, particularly in developing countries. Therefore, a simpler technique to obtain a human milk fortifier was investigated in this study.

5.1 Analysis of donor human milk

The objective of this study was to assess the feasibility and the reliability of the concentration and fortification technique as described by Martinez (1989). Human milk constituents were prepared from pasteurized or unpasteurized donor human milk (Table 1). The composition of donor milk used was assessed prior to and after the processing. Since the main purpose of this study was not to investigate the quality and quantity of donor human milk expressed by Brazilian mothers, factors such as time of collection, type of expression (manual or mechanical) and maternal nutritional status were not taken into consideration.
In order to assess the validity of our biochemical methods, the composition of 16 samples of donor human milk were compared with the composition of term milk reported in the literature (Lauber et al., 1979; Gross et al., 1980; Dewey et al., 1983; Gross et al., 1984; Butte et al., 1984; Michaelsen et al., 1990; Nommsen et al., 1991; Allen et al., 1991). Table 3 illustrates the donor milk composition as measured during this study and its comparison with the literature values. Milk composition obtained from Brazilian mothers who gave birth to term babies was found to exhibit a wide range of fat content (Figure 7), but was still similar to the composition of milk reported in the literature and within the normal range found in both developing and developed countries. As can be seen from Table 3, with the exception of lactose and sodium content, the composition of donor human milk is within the published range, with regards to protein, fat, calcium and phosphorus.

There are considerable variations in the composition of human milk among women. The composition of milk depends on the timing of milk collection. Also, within feed (i.e. fore- and hind-milk) and within day variation, has been demonstrated for milk yield, energy, mineral, protein and fat levels (Hytten, 1954a,b; Harzer et al., 1983; Neville et al., 1984; Garza et al., 1986; Karra et al., 1988). As described previously, donor milk used in this study is a "3-day pool" of manually expressed milk, hence milk samples may have been collected at different times and thus exhibited high variability. They did not represent a standard 24-h collection period unlike most of the studies cited in the literature.

5.1.1 Protein

In the present study, the protein content of donor milk was found to be lower than that found in most developed countries. The protein content of donor human milk was negatively correlated with the stage of lactation. A similar pattern was found by Gross et al. (1980); however, in their study, only the first 28 days of lactation were considered. Butte et al. (1984) monitoring the longitudinal changes in the protein content of human milk, found a significant decrease of milk protein associated with postpartum time. As has been noted, protein content of human milk is
negatively correlated with milk volume (Michaelsen et al., 1990), thus mothers producing high volumes could have a low milk protein content. In this study, there was a lack of information concerning the volume of milk expressed in 24-hours. Therefore, critical comparison of the protein content of human milk from Brazilian mothers with that of mothers from other countries could not be made.

5.1.2 Fat

Fat in human milk supplies the major source of energy for the newborn. Approximately 50% of the baby's daily caloric intake comes from fat. As shown in Figure 7, fat content of human milk expressed from Brazilian mothers was highly variable. High fat variability was also reported by Lauber et al. (1979), Prentice et al. (1980b) and Lavine et al. (1987).

Among the principal factors influencing total lipid content of human milk are 1) collection methods (hand expression, electric pump, drip milk), 2) type of milk collected (i.e. hind- or fore-milk), 3) stage of lactation, 4) time of collection, 5) breast drained (right or left), 6) gestational age and 7) diet (Jensen, 1989; Jensen et al., 1995).

There is lack of agreement on the dietary effect on the milk fat content. Fat concentration in milk from poorly nourished women may vary depending on dietary protein intakes or energy derived from proteins (Nommsen et al., 1991). However, as pointed out by Lönnerdal (1986) and confirmed by Mattar (1994), the total fat content of human milk does not seem to be affected by dietary practices. Changes in the diet have been shown to induce only changes in the fatty acid (FA) composition of human milk (Jelliffe and Jelliffe, 1978; Koletzko et al., 1992).

Mattar (1994) showed that manually expressed milk had significantly higher fat levels compared with milk expressed by a manual pump. Also, Neville et al. (1984) showed a significant increase in milk fat from fore-milk to hind-milk (2.3% to 4.6%). Therefore, samples with a very low amount of fat may come from mothers who first expressed their milk (fore milk) and later fed their babies.
Moreover, the amount of milk fat also depends on maternal fat stores (Prentice et al., 1981b; Brown et al., 1986; Michaelsen et al., 1990). Fat content of human milk seems to be correlated with the mother's BMI\(^1\) (Michaelsen et al., 1990), therefore women with higher BMI will have milk with higher fat content than mothers of low BMI. Mattar (1994) showed that mothers donating their milk to the human milk bank in Ribeirão Preto were adequately nourished. However, the nutritional status of the mothers donating their milk for this study was not assessed, and critical evaluation on the relation between milk fat content and the aforementioned variables could not be made.

5.1.3 Lactose

In this study, the mean lactose concentration of donor human milk was 9.94±0.74 g/100 ml. The normal range found in the literature is between 5.3 and 7.9 g/100 ml (Table 3; Lauber et al., 1979; Finley et al., 1985; Viverge et al., 1986; Allen et al., 1991; Coppa et al., 1993). The discrepancy regarding lactose content of human milk found between this study and other published studies could be due to the colorimetric method of lactose determination used in this study (Barnett and Tawab, 1956), which differs from the methods used by the other researchers. It is not known to what extent the method used in this study also measured monosaccharides and complex polysaccharides; however, since the monosaccharide content of human milk represents less than 1 % of the total carbohydrate content (Coppa et al. 1993), it is unlikely that it would affect the final result.

Milk lactose content has been shown to increase throughout lactation (Gross et al., 1980; Viverge et al., 1986; Dawodu et al., 1990; Allen et al., 1991). The longitudinal increase in lactose content is paralleled by a decrease in oligosaccharides (Coppa et al., 1993), hence the high lactose content is not due to an increase in monosaccharides and oligosaccharides. Yet, Allen et al. (1991) do not

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1 Body Mass Index: weight expressed in kg/(height expressed in meters)

2
showed that lactose content of term human milk at 180 days postpartum was still lower than the value found in this study.

Undernourished mothers have been shown to express milk with high lactose content (Prentice et al., 1983). The nutritional status of the mother donating their milk from this study was not assessed. Mattar (1994) studying the nutritional status of the volunteers that donate their milk to the milk bank of the Hospital das Clinicas da Faculdade de Medicina de Ribeirão Preto, found that the nutritional status of 68.4% of the volunteers was adequate. Only 2.6% were underweight, and human milk composition was not correlated with maternal diet. Thus, one can assume that the high milk lactose values found in this study, using the colorimetric technique (Barnett and Tawab, 1956), do not reflect the actual lactose content of the human milk.

### 5.1.4 Energy and osmolality

The energy content of human milk expressed from Brazilian mothers as determined by this study was higher than that found in most developing countries but still within the range cited in the literature (Table 3). Because the energy content of human milk was calculated using the protein, fat and lactose concentrations and not directly measured, and because lactose content has probably been overestimated, it is clear that it does not reflect the actual energy density and it can be postulated that the actual energy content of donor human milk was probably lower than reported in this study.

The osmolality of donor human milk was within the range seen in the literature. We found an average osmolality content of 274.06±11.29 mOsm/kg H₂O which is approximately equal to the values found by Fomon et al. (1977) and Keenan et al. (1982).
5.1.5 Minerals

Calcium and phosphorus content of donor human milk used during this study was similar to that found by Gross (1983) and by Butte et al. (1984) in term human milk and was within the normal range of 24-28 mg/100 ml (Table 3).

In the present study calcium content of human milk declined from 29.6 mg/100 ml at 2 months postpartum to 18.7 mg/100 ml at 6 months postpartum (Figure 8). The longitudinal decline in calcium content with months of lactation was also found by Vaughan et al. (1979), Finley et al. (1985) and Allen et al. (1991). Vaughan et al. (1979) found that at 1-3 months of lactation, the calcium content of human milk was approximately 25.7 mg/100 ml and fell to 23.6 mg/100 ml between 4-6 months of lactation. The fall in calcium concentration of human milk found in this study corresponded to a decrease of 36% and was higher than the previously reported decrease of 9% (Karra et al., 1986). Karra et al. (1986) reported that this trend was due in part to a decrease in the frequency of feedings per day. According to Rea et al. (1990), 70% of Brazilian mothers stop breastfeeding before the second month postpartum and only 30% continue breastfeeding their babies over six months postpartum. A possible explanation for this might be that the volunteer mothers were weaning their baby while donating their milk and therefore had a higher decrease in milk calcium content.

Sodium content of human milk determined in this study was extremely high compared to values found in the literature. Gross (1983) and Allen et al. (1991) estimated the sodium content of mature human milk at 6.5-6.65 mEq/l, whereas we found a sodium content of 17.71 mEq/l.

The high sodium content of human milk can be explained by the fact that the milk samples used during this study were not pooled milk samples but came from individual mothers. Episodes of mastitis or local inflammation may be responsible for the increased sodium content of human milk (Neville et al., 1984; Jensen, 1989). A high risk of infection due to poor housing and sanitation conditions among inhabitants of Ribeirão Preto has been reported (Desai et al., 1980).
However, it seems unlikely that all 16 mothers had an inflammation of the mammary gland at the time they expressed the milk for this study. Nevertheless, since no clinical examination was performed on the volunteers, this hypothesis cannot be ruled out.

High sodium content in human milk might also be due to decreased milk expression and production occurring during weaning (Neville et al., 1991). As stated previously (section 5.1.5.1) it is possible that mothers were weaning their babies while donating their milk, which may in part explain the high milk sodium content.

5.2 Milk concentration process

5.2.1 Nutrient losses

Some losses of nutrients occurred during the milk concentration process. Therefore, the level of several nutrients in the concentrated human milk (cHM) was lower than expected. High amounts of fat globules were lost in the evaporator flask and in all the glassware used (tubes, pipettes, etc.) because fat globules and fatty acids tend to adhere to glass and plastic surfaces (Narayanan et al., 1984; Greer et al., 1984; Stocks et al., 1985; Mehta et al., 1991). Considerable quantities of fat globules were also lost during the separation of the supernatant from the precipitated lactose. This step might have been the most crucial for milk fat, protein and mineral losses. Analysis of the lactose pellet as shown in Appendix VII clearly demonstrates that high amounts of fat, protein, and calcium were lost during the crystallization process. Nonetheless, calcium and phosphorus losses were minimal compared to fat and protein losses. The low predictability/reliability of the concentration process is therefore a major limiting factor for its routine clinical use. The high standard deviations obtained when computing the observed macronutrient and mineral increment clearly demonstrate that the concentration process needs further improvement and standardization.
A way to decrease fat losses may be to ultrasonicate human milk prior to the concentration process in order to reduce the size of milk fat globules. It has been shown by Martinez et al. (1987) and Rayol et al. (1993) that milk fat losses that occur during tube feeding were reduced using ultrasonicated human milk. However, a pilot study conducted prior to this study (same experimental conditions) showed that ultrasonicated human milk did not allow crystallization of lactose. Moreover, due to the activation of milk lipases, ultrasonication of human milk may lead to increased FFA and may be detrimental to the infant (Hamosh, 1988). Since ultrasonication of milk samples have been shown to interfere with lactose crystallization, and because of potential negative effects induced by the ultrasonic treatment (Hamosh, 1988), one practical way to reduce milk fat losses would be to use large volumes of banked human milk. It has been shown by Michaelsen et al. (1990) that pooling human milk resulted in lower fat variability. Also, as shown in this study, fat losses are significantly lower when low-fat milk samples are concentrated (Figure 10). Consequently high volumes of low-fat milk could be used and concentrated in order to obtain a more uniform concentrated human milk.

5.2.2 Osmolality

Surprisingly, the osmolality of concentrated human milk (cHM) increased sharply and was not correlated with the increase in lactose and other macro nutrients and minerals. A couple of reasons can be proposed to explain this increase in osmolality. First, the native milk fat globule in human milk is resistant to the action of the different lipases as long as its structure remains intact (Berkow et al., 1984). Once damaged, the triglycerides are available to the milk lipases (lipoprotein lipase and Bile-Salt-Stimulated-Lipase) and lipolysis occurs. Freezing, heating and thawing may induce those damages (Wardell et al., 1981; Berkow et al., 1984). Hamosh et al. (1984) showed the effect of storage (after freezing and thawing) on the FFA content of human milk. According to these authors (Hamosh et al., 1984), the outer membrane of the milk fat globule is not affected by two thawing and freezing cycles whereas the core triglycerides are
hydrolyzed leading to increased amounts of FFA. Also, the storage at -20°C led to increased amounts of FFA compared to storage at -70°C (Berkow et al., 1984). This rise in lipolysis does not seem to be due to an increase in activity of the two human milk lipases, but to an increase in the activity of the bile salt-stimulated esterase which has lost the bile salt dependency (Hamosh et al., 1983, 1984). Increased amounts of FFA and/or phosphorus from phospholipids may be the cause of a rise in osmolality. Yet, Björkstén et al. (1980) reported that human milk lipase activity was completely eliminated after pasteurization at 62.5°C. Thus, the increase in osmolality may not be due to increase in FFA and phosphorus. Second, pasteurization of bovine milk does not affect lactose hydrolysis (Björkstén et al., 1980), therefore release of glucose and galactose from the lactose molecule does not seem to be the cause of rise in osmolality. Denaturation of human milk protein that occurred during heat treatment might have released ionized calcium and phosphorus, thereby increasing the osmolality. However, osmolality was not correlated with either calcium or phosphorus, thereby, ruling out this hypothesis.

Formation of novel peptides may also be implicated in the rise in osmolality. Whether this is the cause of the elevated osmolality needs further research.

5.2.3 Protein modifications

Biological activity of certain protective factors (like sIgA and lactoferrin), and of certain enzymes (like BSSL) may be lost during the concentration process due to heat treatment. Heat treatment is known to denature proteins. However, according to Lyster et al. (1984) sIgA does not seem to be affected by temperatures below 45°C and some proteins like the BSSL seem to be fairly stable at these temperatures. Moreover, Hylmø et al. (1984), who heated human milk at 45 to 50°C found that the activities of alpha1-antitrypsin, amylase and BSSL were conserved exhibiting only a small loss (15% of the normal activity). Therefore, at 40±1°C, the temperature used during the concentration process, protein denaturation does not seem to be of major concern and significant changes in the protein quality are unlikely to have occurred. Still, denaturation and
formation of novel peptides should not be excluded and more studies are needed in order to characterize and quantify the possible changes in milk composition after the concentration process.

5.2.4 Lipid oxidation products and risk of oxidative damage

Human milk contains considerable amount of long-chain polyunsaturated fatty acids (PUFA) that are essential for the normal development of the preterm infant (Decsi et al., 1994). PUFA may be susceptible to oxidation in the presence of air, heat, light, and pro-oxidants. During the entire concentration process, human milk has been exposed to air, heat and light. Moreover, due to the use of a rotary evaporator (Figure 2), the surface area of human milk in contact with air was increased due to recycling action. In addition, milk was heated at 40±1°C in the presence of light, thus, potentially increasing the formation of lipid peroxides.

Preterm VLBW infants are more prone to suffer from disorders (e.g. respiratory distress, hemodynamic failure, proven sepsis, aspiration syndromes and perinatal asphyxia) believed to enhance free-radical formation. Along with endogenously formed oxygen radicals, the exogenous lipid peroxides provided by the diet may increase the total reactive oxygen radical load. There are potential side effects of preformed exogenous lipid peroxides, especially for the immature VLBW infant that has poorly developed antioxidant defense systems (Decsi et al., 1994; Van Zoeren-Grobben et al., 1993). Therefore, increased oxidative load (endogenous and exogenous) may increase the risk of necrotizing enterocolitis, bronchopulmonary dysplasia, lesions in the retina and brain, and hemolysis (Sosenko et al., 1988; Lunec, 1990; Van Zoeren-Grobben et al., 1993). Because the fatty acid composition of the red blood cell membrane is primarily determined by the fatty acid composition of the diet (Ballabriga, 1994), infants fed large amounts of polyunsaturated fatty acids have increased erythrocyte susceptibility to oxidative destruction (O'Leary, 1989) and the risk of hemolytic anemia may be high, especially if antioxidant (vitamin E, ascorbic acid, etc.) levels are low (Van Zoeren-Grobben et al., 1993). Therefore, amounts of lipid peroxides formed during the milk concentration process should be thoroughly assessed.
5.3 Nutritional adequacy of the fortified human milk (fHM) for the preterm VLBW infant

The following discussion focuses only on the theoretical/estimated nutritional adequacy of fHM. Only feeding studies could determine the in vivo nutritional adequacy of the fHM prepared in this study. The final aim of this study was to prepare a fortified human milk (fHM) that would be nutritionally and immunologically adequate for the preterm VLBW infant. The fHM was intended to provide the preterm newborn with human milk proteins, bypassing the need of the addition of foreign proteins that have been shown to produce abnormal plasma amino acid profiles in the babies fed bovine protein-based formulas (Moro et al., 1991). Mixing term or preterm human milk with pasteurized concentrated human milk, suggests that the concentration of immunologic factors in the fHM will be higher than in non fortified human milk, leading to a potential "hyperimmune breast milk". Even so, the possible interaction between native and modified human milk components (denaturated proteins, oxidized FFA, etc.) needs to be assessed in order to establish the immunologic adequacy of fHM.

The fHM obtained was designated to be fed to preterm VLBW infants at volumes of 150 ml/kg/d. However several recent studies have used a volume equal to 180 ml/kg/d (Moro et al., 1991; Priolisi et al., 1992; Moro et al., 1995). The estimated nutrient content of fHM was calculated based on both volumes, separate calculations (Table 7). As can be seen by comparing the estimated daily requirements for VLBW infants (AAP, 1985) with the estimated nutrient content of 150 or 180 ml of fHM (Table 7), all fHM preparations ("low", "medium" and "high" fHM) had a nutrient content that was below the estimated requirements in terms of protein, calcium and phosphorus. Sodium content of 150 and 180 ml of fHM was within the estimated requirement. The caloric density was very high due to the high fat and lactose present in the fHM. Although the energy content of fHM may have been overestimated, its high fat content could represent a potential problem for the nutritional management of the VLBW infant (see section 5.3.1.2).
5.3.1 Protein, fat and energy

Heinig et al. (1993) reported that per gram of protein consumed, breastfed infants gained more weight and lean body mass than formula-fed infants. Human milk proteins seem to be more efficient in promoting growth than cow's milk protein. Requirements for the preterm infant are based on balance studies which used cow's milk protein-based formulas. Slower weight gain in breastfed infants compared to formula-fed infants seems to be due to the higher protein content of infant formulas (Position of the American Dietetic Association, 1993; Dewey et al. 1993). As a consequence, it is difficult to compare the rate of weight gain in breastfed and formula-fed newborns. The protein requirement for the preterm VLBW infant may be lower than the one recommended by the AAP (1985) and infants fed fHM may achieve adequate growth and development.

5.3.1.1 Prevention of hypoproteinemia

Human milk fortified with human milk proteins, fed at 2.6±0.4 g/kg/d at 2 weeks of age to preterm VLBW infants (gestational age 30±0.5) prevents hypoproteinemia without creating imbalances in the amino acid metabolism (Rönnholm et al., 1982). It can be argued that fortified human milk fed at 180 ml/kg/d ("medium" fHM) or at 150 ml/kg/d ("high" fHM) could potentially prevent hypoproteinemia in preterm VLBW babies. However, the high osmolality and energy intake by the newborn might interfere with the adequate growth of the VLBW infant (see section 5.3.2).
5.3.1.2 Growth of the VLBW infant

In this section, the estimated adequacy of the fHM to promote growth will be explored comparing the quantity of nutrients that are theoretically provided by fHM with intakes that have led to appropriate growth rates in different studies where preterm VLBW infants were fed fortified human milk (fortified either with human milk fractions or with a commercial human milk fortifier). It should be stressed that comparison is made only among protein, fat and energy content of the different fHM.

Several studies have shown that high intakes of protein (3-3.5 g/kg/d) and energy (about 110 kcal/kg/d) by VLBW infants were sufficient to achieve a desirable weight and length gain, without inducing metabolic stresses like metabolic acidosis, aminoaciduria etc. (Hagelberg et al., 1982; Lindblad et al., 1984; Garza et al., 1984; Senterre et al., 1984; Rönnholm et al., 1986; Moro et al., 1991). Studies by Kashyap et al. (1988) showed that in VLBW infants, a protein intake of 2.8 g/kg/d at an energy intake of 119 kcal/kg/d was sufficient to attain rates of weight gain and nitrogen retention that were in excess of the intrauterine rates without apparent signs of metabolic stress. The same authors were able to demonstrate higher rates of weight gain and nitrogen retention by feeding VLBW infants higher amounts of protein and energy.

According to the above referred estimates, even the "high" fHM fed at 180 ml/kg/d (protein 2.60±0.31 g/kg/d, energy 221±13 kcal/kg/d) will not support adequate rate of growth in preterm VLBW infants (Table 7). However, studies by Polberger et al. (1989) showed that in healthy VLBW, adequate weight gain was reached with 2.77 g/kg/d of human milk proteins. Also, Schanler et al. (1985a) feeding healthy preterm VLBW babies approximately 2.6 g of human milk protein/kg/d (125 kcal/kg/d, 6.6 g fat/kg/d) showed that babies gained weight, length, and head circumference at rates approximating intrauterine growth rates. Therefore, the "high" fHM at intakes of 180 ml/kg/d may be able to lead to adequate weight gain. However, the high fat content and the high osmolality of the "high" fHM may preclude its practical use.
Lower protein intakes may also promote adequate growth in VLBW infants. Järvenpää et al. (1983) fed preterm infants human milk at an estimate of at least 1.78-1.92 g of protein/kg/d and 126-136 kcal/kg/d. They found that at those protein intakes, the weight gain approached the intrauterine rate without evidences of metabolic stress (as judged from plasma levels of valine, phenylalanine and tyrosine). The "medium" fHM fed at volumes of 150 ml/kg/d and the "low" fHM fed at 180 ml/kg/d would provide between 1.78 and 1.85 g of protein/kg/d and approximately 145 kcal/kg/d to the infant (Table 7). According to the aforementioned results (Järvenpää et al., 1983), it can be stated that VLBW infants fed "low" and "medium" fHM may achieve adequate growth rates. However, because 45% of the milk intake of the babies was preterm human milk (Järvenpää et al., 1983), the exact protein and caloric intake is not known and was probably superior to the estimates made by these authors.

5.3.1.3 Protein to energy ratio and weight gain composition

Both protein and energy intake influences the rate of weight gain (Schulze et al., 1987; Polberger et al., 1989). According to Kashyap et al. (1988), the ratio of protein to fat of the newly synthesized tissue is related to the protein-to-energy ratio consumed by the newborn. The protein-to-energy ratio should be monitored because energy deficiency limit the efficient utilization of protein (Garza et al., 1984). Kashyap et al. (1988) suggests that protein intakes in excess of 3 g/100 kcal will not be utilized completely. Similar results were found by Polberger et al. (1989). According to their study protein intakes higher than 3 g/kg/d or energy intakes higher than 120 kcal/kg/d (protein-to-energy ratio >2.5 g/100 kcal) are not effective in promoting higher growth rates.

The fHM prepared in this study ("low", "medium" and "high" fHM) have a protein-to-energy ratio of 1.27, 1.22 and 1.17 g/100 kcal, respectively. This ratio is well below the estimated adequate ratio of 2.45-2.55 g/100 kcal (Itabashi et al., 1992). Itabashi et al. (1992) feeding VLBW infants with fortified preterm human milk (protein-to-energy ratio equal to 2.45-2.55 g/100 kcal)
showed that the rate of weight gain was approximating the intrauterine accretion rate, without any apparent metabolic disturbances. From the findings of the above studies (Kashyap et al., 1988; Itabashi et al., 1992), it can be speculated that proteins found in the fHM will be adequately utilized due to the high energy content.

High fat content of the different fHM may lead to an increase in fat deposition. It has been shown that fat deposited was closely related to energy intake (Chessex et al., 1981). Although, Polberger et al. (1989) found little or no influence of fat intake on growth rate, Schulze et al. (1987) reported that in VLBW infants (birth weight about 1500g) fed a formula containing high protein and high energy (3.5 g/kg/d, 149 kcal/kg/d), the greater rate of weight gain was associated with a greater rate of fat deposition. These results were confirmed by a subsequent study (Kashyap et al., 1990) showing that energy intake was predictive of weight gain in the form of fat. Nevertheless, Singania et al. (1989) showed that an intake of 200 kcal/kg/d did not lead to "visible complications" and that with high caloric intake, the rate of weight gain was similar to intrauterine growth rates. According to the authors of this study (Singania et al., 1989), it is therefore unnecessary to increase the protein intake of the preterm infant because of the protein sparing effect of a high caloric diet. Therefore, according to this hypothesis, fHM at intakes of 150 or 180 ml/kg/d (excluding the "high" fHM at 180 ml/kg/d \(^2\)) would lead to adequate rate of weight gain due to their high energy content and their relatively high protein content (Table 7).

However, it should be kept in mind that mineral, vitamin and iron supplements were given simultaneously to the preterm babies (Singania et al., 1989), thereby potentially enhancing the growth rate and confounding the results of the study. The effect of minerals like calcium and phosphorus on the growth of the human fetus is not known. Although regression analysis computed by Polberger et al. (1989) led to the conclusion that calcium did not have any influence on growth rate, there are no other published data on the effects of calcium alone on growth of premature infants. Moreover, in Singania's study (1989), supplemental calories were given as

\(^2\) Excluded due to its high osmolality and the high energy provided (>200 kcal)
medium-chain triglycerides (MCT). MCT are water miscible, thus, their absorption does not depend on the concentration of bile salts. MCT have been shown to be better absorbed than long-chain triglycerides in preterm infants (Faber et al., 1988). The results of Singhania et al. (1989) should be interpreted with caution and the fHM obtained in this study might not be adequate for the VLBW infant.

5.3.2 Lactose and osmolality

Although, according to Aurichhio et al. (1965), the preterm infant should be able to digest between 13 and 33 g of lactose per day, lactose malabsorption is present to some extent in most preterm infants. The developmental digestive immaturity of the enzyme lactase may produce excessive enteric gas following malabsorption and can result in abdominal distention (Kliegman, 1990). Unabsorbed lactose may play a role in the regulation of colonic flora leading to the establishment of a fecal flora with predominance of bifidobacteria lactobacilli and staphylococci (MacLean et al., 1980; Wharton et al., 1994).

Even if fHM is administered at a rate of 180 ml/kg/d, it will provide less than 30 g of lactose (Table 7) which should not result in lactose malabsorption. In addition, in the present study, the amount of lactose determined by the colorimetric method of Barnett and Tawab (1956) is probably an overestimation of the true lactose level of human milk (dHM, cHM and fHM).

Also, lactose enhances net calcium absorption (Ziegler et al., 1983; Allen, 1982). According to Allen (1982) lactose enhances the diffusional component of calcium transport rather than the active, vitamin D-dependent component. Several authors have demonstrated that lactose increased intestinal calcium absorption, especially in the ileum, where the calcium binding proteins are less abundant (Gleason cited by Allen, 1982). Schanler et al. (1990) showed that lactose intake was negatively correlated with fecal calcium and phosphorus excretion. Also, in the presence of lactose, calcium absorption was found to be enhanced by about 50% (Ziegler et al., 1983). Although the exact mechanism by which lactose increases calcium absorption is still not known, it
Discussion

has been demonstrated that lactose enhances net calcium absorption and retention in full term, healthy infants, and subsequently increases net phosphorus retention by decreasing phosphorus excretion (Ziegler et al., 1983). Thus, fHM being high in lactose, may enhance calcium retention and thereby improve bone mineralization in the VLBW infant.

Osmolality of gastric and duodenal contents is directly influenced by the osmolality of the diet (Billeaud et al., 1982). The osmolality of the diet should fall within the recommended range of gastric osmotic load which is around 450 mOsm/kg of water. Enteral feedings with higher osmolality may predispose the infant and in particular the VLBW infant to complications such as delayed gastric emptying and necrotizing enterocolitis (Billeaud et al., 1982; Koo et al. 1991). Even so, several milk formulas have been found to have high osmolality. Fomon et al. (1977) reported that Pregestimil [Mead Johnson Co.] had an osmolality of 670 mOsm/kg of water. Voyer et al. (1984) found a mean value of 388 mOsm/kg of water in 8 different samples of formula. It is also a clinical practice to fortify human milk with oil and sugars (Singhania et al., 1989; Martinez, personal communication) and, therefore, the osmolality of the final product may be higher than recommended. Since, hyperosmolar feedings (>700 mOsm/l) could induce mucosal injury (Kliegman, 1990) they should be avoided. The fHM preparations obtained in this study ("low", "medium" and "high" fHM) have very high osmolality (Table 6; Figure 14 and Appendices II, III, and IV), while, the "low" fHM and the "medium" fHM preparations have osmolality values that are within the accepted range for the VLBW infant.

5.3.3 Calcium, phosphorus and sodium

Apart from protein fortification, human milk needs to be fortified with both calcium and phosphorus as well as energy in the form of fat (Hagelberg et al., 1982) in order to meet the high requirements of the VLBW infant. The preterm VLBW infant is born with a deficit in phosphorus thereby limiting bone synthesis (Natal-Pujol et al., 1993). Intakes of calcium without appropriate intakes of phosphorus may lead to hypercalciuria (Natal-Pujol et al., 1993). Hypercalciuria found
in VLBW infant fed human milk seems to be due to the relative low phosphorus intake (Senterre et al., 1984). Deficient phosphorus intakes lead to increased calciuria because the absorbed calcium cannot be utilized. For every 2.2 molecules of calcium deposited in bone there is a need for a molecule of phosphorus. The deficiency of phosphorus is exacerbated if human milk is enriched with calcium and/or protein (Salle et al., 1986). If protein is added to human milk without adequate phosphorus addition, hypercalciuria will develop because increased protein turnover requires increased phosphorus utilization (Salle et al., 1993).

Although human milk has an adequate calcium to phosphorus ratio, the amount of those minerals is inadequate for optimal bone formation of preterm VLBW infants. According to the estimated intrauterine accretion rate (Fomon et al., 1977), calcium and phosphorus content of human milk is inadequate to support optimum bone mineralization. Thus, calcium and phosphorus need to be added to human milk.

5.3.3.1 Calcium absorption and retention

Calcium absorption from milk is influenced by factors such as the milk calcium-phosphorus ratio, fecal fat losses, intestinal secretions of endogenous calcium, intake and metabolism of Vitamin D, and postnatal age (Allen, 1982). Calcium absorption depends on dietary intakes of vitamin D (70% absorbed if vitamin D is added at a dose of 1200 IU/d). Calcium retention depends on phosphorus intake (Salle et al. 1993). Hence, adequate calcium retention depends on concomitant phosphorus and calcium fortification.

The best way to increase calcium absorption would be to either increase calcium and phosphorus intake and/or increase fat intake (Schanler et al., 1988). Predictions by Schanler and Garza (1988) estimated that intakes of 160 mg/kg/d of calcium and 94 mg/kg/d of phosphorus, are necessary to achieve intrauterine retention of 100 and 70 mg/kg/d, respectively. Due to better calcium and phosphorus bioavailability in human milk, Cooper et al. (1985), suggests that the amounts required to prevent biochemical evidences of rickets may be lower than the estimated
Discussion

requirements. Moreover, postnatal retention and accretion of calcium may be lower than in utero accretion due to the remodeling of the bones, the effect of hormones (PTH and 1,25-OH2D) and the effect of gravity (Salle, 1993). VLBW infants with adequate 25-hydroxyvitamin D may also have lower calcium and phosphorus requirements to prevent biochemical signs of rickets than the estimated intrauterine accretion rates (Cooper et al., 1985).

Preterm infants fed their own mother’s milk at volumes as high as 180 ml/kg/d had calcium retention rates that averaged only 23% of the intrauterine retention rates (Atkinson et al., 1983). Absorption of phosphorus averaged 90% of the intake of the unpasteurized preterm human milk (Atkinson et al., 1983), with the highest retention being only 66% of the intrauterine retention rate. Schanler et al. (1985b) showed that intakes of 50-60 mg of calcium/kg/d and 34 mg of phosphorus/kg/d by VLBW infants (gestational age 29 weeks, birth weight 1200 grams) led to inadequate calcium and phosphorus retention rates (respectively 20% and 40% of the estimated intrauterine accretion rate), accompanied by hypercalcemia, hypercalciuria, and high serum alkaline phosphatase activity. Ehrenkranz et al. (1989) fed premature VLBW infants their mother’s fresh milk fortified with a powdered protein-mineral fortifier (2 g protein/100 ml, 4 g fat/100 ml, 80 mg calcium/100 ml and 47 mg phosphorus/100 ml) at a volume approximating 150 ml/kg/d. Whereas infants’ weight gain, fat and nitrogen accretion were similar to intrauterine growth and accretion rates; calcium and phosphorus accretion rates were still lower than the intrauterine accretion rates (82 vs. 132 mg calcium/kg/d and 58 vs. 76 mg phosphorus/kg/d). Similar results were found by Raschko et al. (1987). They fed VLBW infants a 1:1 mixture of preterm human milk and liquid fortifier (approximately 1.8 g/100 ml of protein, 3.6 g of fat/100 ml, 103 mg of calcium/100 ml, and 52 mg of phosphorus/100 ml) at 165 ml/kg/d, thereby providing 120 kcal/kg/d. VLBW infants’ growth rate was 18.8 g/kg/d. Nitrogen retention was equal to the intrauterine estimates (363 vs. 325 mg/kg/d) (AAP, 1985) and fat absorption averaged 94% of the intake. Calcium and phosphorus intakes were 167 and 84 mg/kg/d, respectively, with retention of 51 and 67 %, respectively. Accretion rates were 85 mg of calcium/kg/d and 56 mg of phosphorus/kg/d which were still below the expected intrauterine accretion rates. Low serum phosphate, high serum
alkaline phosphatase, low urinary phosphorus excretion accompanied with calciuria demonstrated that VLBW infants were in a relative phosphorus depletion state. Greer et al. (1988) fed premature infants (gestational age 29 weeks, birth weight 1200 g) either preterm human milk or preterm human milk fortified with a commercial fortifier providing protein, calcium and phosphorus. Infants fed the fortified human milk received approximately 4.1 g of protein/kg/d, 136 mg of calcium/kg/d and 68 mg of phosphorus/kg/d. After 6 weeks, although calcium intake approached the estimated requirements, bone mineral content was still lower than the intrauterine value and relative phosphorus deficiency was still present as shown by the high activity levels of alkaline phosphatase. Still, infants fed the fortified human milk had improved bone mineral content compared to the non fortified human milk fed group. Also, growth rate was significantly higher in the fortified human milk group (17.3 vs. 13.4 g/kg/d), achieving the estimated intrauterine rate.

The fHM prepared in this study contained relatively low amounts of calcium and phosphorus (Table 6) compared to preterm formulas. Feeding fHM would provide low amounts of those minerals compared to the estimated requirements for the preterm VLBW infant (Table 7). Thus, feeding preterm VLBW infants fHM, even at intakes of 150 ml/kg/d or 180 ml/kg/d, will not lead to appropriate mineral retention and mineral supplementation will still be necessary.

5.3.3.2 Calcium and fat interaction

Schanler and Garza (1988), suggested that a 10% increase in fat intake (from 6.9 g/kg/d to 7.6 g/kg/d) would lower the amount of calcium required for adequate bone mineralization at 150 mg/kg/d. Our fHM preparations provide high amounts of fat (Table 7). From this, it could be speculated that, even though fHM will provide minerals in lower amounts compared to the estimated requirements, calcium absorption and retention will be high and their intake may be sufficient to reduce the risk of osteopenia.

On the other hand, high calcium intakes have been reported to decrease fat absorption in VLBW infants (Day et al. cited by Allen, 1982). A possible explanation seems to be the fact that
calcium combines with FFA and forms insoluble soaps in the intestinal lumen. Calcium soap formation seems to occur during hydrolysis of the emulsified fat droplets. The bioavailability of calcium from these soaps becomes lower as the chain length of the FA increases and as the degree of unsaturation decreases (Allen, 1982). This is of concern because Lavine et al. (1987) showed that human milk undergoes several changes during storage. Increased lipolysis by the lipoprotein lipase present in the milk leads to increased FFA, especially long chain unsaturated fatty acids. The increase in FFA content in banked human milk and possibly in the concentrated human milk (cHM) may lead to a decreased bioavailability hence a decreased absorption of calcium from our fortified human milk (fHM). It is not known whether any of these calcium soaps will be absorbed with micelles (Allen, 1982).

Since the milk used in this study was pasteurized, the activity of milk lipases may have been reduced (Björkstén et al., 1980) and the increase in FFA may therefore not be significant. It is recommended that studies on the mineral bioavailability of fHM should be undertaken to assess the quantity of minerals available to the preterm VLBW infant.

5.3.3.3 Calcium and phosphorus bioavailability

Calcium absorption is related to the amount ingested (Allen, 1982). High intakes of calcium would be readily available for retention. Since calcium retention is dependent on phosphorus retention, the calcium-to-phosphorus ratio should be kept as close to that found in human milk in order to optimize absorption, retention rates and bone mineralization.

Also, the form in which calcium and phosphorus are present in the concentrate is important to be determined. If present as simple salts, calcium might complex with phosphorus and precipitate due to the relatively alkaline environment of the small intestine. If calcium is bound to protein, the additional calcium and phosphorus present in the fHM would be bioavailable and would presumably contribute to achieve the intrauterine rate of accretion. Mineral interaction
(precipitation as insoluble calcium phosphate) and trace element bioavailability should be evaluated in the fortified human milk prior to routine administration to the VLBW infant.

In vivo bioavailability of calcium lactate and phosphorus salts added to fortified human milk were compared to bioavailability of calcium and phosphorus in a commercial formula by Schanler et al. (1985c). The intake of calcium and phosphorus were 112 mg/kg/d and 68 mg/kg/d, respectively. Calcium and phosphorus absorption were significantly higher in the fortified human milk than in the commercial formula (66 vs. 30% and 96 vs. 87% for calcium and phosphorus, respectively). Retention of 65 mg calcium/kg/d and 54 mg phosphorus/kg/d were achieved. They were higher than those found in infants fed with unfortified human milk (Schanler et al., 1985d) but still were below the estimated fetal accretion rates (120-140 mg calcium/kg/d and 70-75 mg phosphorus/kg/d). In another study by Schanler et al. (1988), VLBW infants were fed with fortified milk at levels of 150 ml/kg/d. The fortified milk composition (using pasteurized, lyophilized human skim milk fortifier) was as follows: energy 81 Kcal/100 ml, fat 4.5 g/100 ml, protein 1.9 g/100 ml, calcium 81 mg/100 ml (calcium lactate), phosphorus 46 mg/100 ml (phosphorus salts) and sodium 36 mg/100 ml. Rates of weight gain were similar to intrauterine rates (17-22 g/kg/d). The infant's energy, protein and fat intakes were 127 kcal/kg/d, 3 g/kg/d, 6.9 g/kg/d, respectively. Fat absorption was approximately 83%. The ratio of calcium/phosphorus retention approximated the intrauterine retention rates, however, net retention of calcium and phosphorus were 86% and 80% of the intrauterine estimate, respectively.

The different fHM prepared in this study ("low", "medium" and "high" fHM) have higher fat and energy content than donor human milk (dHM). The protein, calcium, phosphorus and sodium content are significantly lower than those used by Schanler et al. (1986). Therefore, retention of calcium and phosphorus will not meet the requirements of the VLBW infant. Table 7 displays the mineral content of 150 and 180 ml of the different fHM preparations. As can be seen comparing the nutrient content with the recommendations, even at high fluid intakes (180 ml/kg/d), mineral requirements are not met, and mineral supplementation will still be needed (Figure 15).
Schanler et al. (1990) showed that VLBW infants fed human milk fortified with human milk powder (40 mg/100 ml of calcium and 21 mg/100 ml of phosphorus) did not achieve the estimated intrauterine retention rates of these minerals. The "medium" fHM contains approximately 41 mg/100 ml of calcium and 20 mg/100 ml of phosphorus. It is inadequate, mineral-wise, to meet the requirements of the VLBW infant. The "high" fHM has higher calcium and phosphorus concentrations (calcium 53 mg/100 ml, phosphorus 26 mg/100 ml). But, due to its high osmolality, lactose and fat content (Table 6), it may not be suitable for the dietary management of the VLBW infant.

5.3.3.4 Sodium

Preterm VLBW newborns, due to their immature renal tubular function, have high sodium losses, leading to negative sodium balance and hyponatremia. Hyponatremia of prematurity can effectively be corrected by administering 3 mEq/kg/d of sodium (Roy et al., 1976). Studies by Al-Dahhan et al. (1984) have demonstrated that intakes of 4-5 mEq/kg/d from the 4th to the 14th postnatal day reduced the incidence of hyponatremia (37.5% vs. 13.6%), and improved growth in premature LBW infants (gestational age <34 weeks).

The AAP (1985) recommends sodium intake varying between 4-8 mEq/kg/d. However, large intakes of sodium (about 9 mEq/kg/d) may increase the extra cellular fluid volume and might precipitate heart failure in some infants (Wharton et al., 1987).

According to the results of this study and those found in the literature (Al-Dahhan et al., 1984), the different fHM preparations ("low", "medium" and "high" fHM) administered at either 150 ml/kg/d or at 180 ml/kg/d (Table 7) would theoretically be efficient in reducing the occurrence of hyponatremia of prematurity and improve the rate of weight gain without leading to edema, hypernatremia or circulatory overload.
6 Summary of Findings

6.1 Milk concentration process

Concentration of human milk (evaporation of the milk water and subsequent lactose crystallization and removal) resulted in a human milk concentrate (cHM) that had significantly higher protein, fat, calcium, phosphorus and sodium, as well as a higher osmolality and energy content compared to donor human milk (p<0.001).

However, the milk concentration process resulted in significant protein, fat and sodium losses. Fat tended to adhere to all the glassware used and therefore resulted in a lower than expected fat increment with possibly high losses of fat soluble vitamins such as vitamins A, D, and E.

The increment in the nutrient content of the cHM was lower than expected. Moreover, the increment level of each nutrient was highly variable which makes it difficult to predict the concentration of nutrients in the concentrated human milk.

Standardization of the concentration process was not achieved and more studies are needed to improve the human milk concentration technique. Nutrient losses needs to be reduced and lactose crystallization should be optimized in order to decrease the osmolality of the cHM.
6.2 Fortified human milk (fHM)

The addition of concentrated human milk (cHM) to donor human milk (dHM) resulted in a fortified human milk (fHM) that had a higher calcium, phosphorus, sodium and lactose content compared to donor human milk (p<0.001). The protein content of the fHM was also significantly higher compared to donor human milk (p<0.05 for the "low fHM" and p<0.001 for the "medium fHM" and "high fHM"). Fat content of the fHM was significantly higher than donor human milk only in the "high fHM" (p<0.05).

Yet, fHM was found to be inadequate for the optimal nutritional management of the preterm very low birth weight (VLBW) infant. The protein, calcium and phosphorus content of 150 and 180 ml of the different fHM preparations (low, medium and high fHM) were still below the estimated requirements of the preterm VLBW infant, and the high fat content and osmolality of the "high fHM" proscribe its use for the feeding of the preterm VLBW infant.
7 Conclusions and Recommendations

7.1 Conclusions

The purpose of this study was to assess the feasibility, in a practical setting, of an autologous/homologous fortification of human milk for the preterm VLBW infant using the human milk concentration method described by Martinez (1989). This autologous fortification would have the advantage of providing the high-risk newborn with adequate nutrition and antigens directly pointed against pathogens present in the mother's and infant's environment. In this study the autologous fortification of human milk was not achieved due to several practical reasons.

First, in order to prepare an autologous fortifier, adequate volumes of preterm human milk are needed to feed the newborn and for the concentration process. Because mothers who deliver at the Hospital das Clinicas de Ribeirão Preto leave the hospital soon after birth, it was impossible to obtain premature milk for the purpose of this study. Also, because of various socio-economic reasons (return to work, taking care of other children, long distance between home and the hospital, etc.), mothers generally do not provide adequate quantities of their own milk to their babies (Martinez, 1994 personal communication). Further, even if the proportion of mothers that elect to express their milk for their infant is high, the percent of mothers that actually produce sufficient volumes of bacteriologically safe milk is limited (Kashyap et al., 1990; Lucas, 1983).

Second, as shown by Dos Santos (1994), preterm human milk expressed from mothers during the first 2 months after birth seems to be inadequate to allow appropriate concentration. Early preterm milk contains high amounts of protein and anti-infectious components that seem to interfere with lactose crystallization. The high viscosity of preterm human milk may decrease the rate of conversion of beta-lactose to alpha lactose, thereby decreasing the formation of lactose crystals (Hunziker, 1949; Webb et al., 1974).
Conclusions and Recommendations

Third, as found during this study, the concentration process using small milk quantities led to high nutrient losses and, depending on the composition of the milk sample, the concentrated human milk produced had a highly variable nutrient content. The variable nutrient composition of the cHM prevented standardized fortification using fixed proportions of mother's milk and concentrated mother's milk.

Under these conditions, the most practical way to provide the VLBW infant with a nutritionally sound fortified human milk would be to prepare a concentrated human milk from pooled human milk and then add it to the mother's milk (homologous fortification). Yet, several other practical problems may hinder this practice.

First, as stated previously, mother's milk is not readily available and special actions should be taken in order to encourage preterm infant's mothers to express their milk and donate it for their baby (see section 7.2.1).

Second, with the increased risk of transmission of pathogens and viruses such as cytomegalovirus and the HIV virus, pooled human milk used to prepare the concentrated human milk may increase the risk of infection to the already high-risk newborn. Special care should be taken when handling and using donor human milk and pasteurization is definitely required (see section 7.2.2).

Third, as shown by this study, the preparation of a concentrated human milk is not well standardized thus leading to concentrated human milk having a highly variable nutritional composition. As mother's milk composition changes over time, it is premature to suggest human milk fortification with fixed proportions of non-concentrated and concentrated human milk. No single fortification is appropriate for all VLBW infants, because of individual requirements, absorption and nutrient retention rates (Greer et al., 1988). As a result, it is necessary to tailor the fortification according to the needs of each infant.

Fourth, this study demonstrated that minerals and probably protein supplementation of the fortified human milk, would still be needed in order to achieve the estimated nutrient requirement of the VLBW infant.
Conclusions and Recommendations

In conclusion, it is premature to encourage the clinical practice and use of the fortified human milk (fHM) for the preterm VLBW infant as prepared in this study.

7.2 Concentration and fortification processes: practical problems and recommendations

7.2.1 Quantity and quality of donor, mother and concentrated milk

The practice of giving VLBW infants their own mothers' milk is worth encouraging. Preterm human milk seems to be particularly useful for infants who are at risk of infection (Narayanan et al., 1981). The continuous expression of milk by the mothers of the preterm VLBW infant helps maintain lactation thereby increasing the chances of subsequent breastfeeding. Frequent stimulation of the breasts increases milk output so that adequate amounts of milk are available to feed the infant. Mothers giving birth prematurely are able to produce an average milk volume sufficient enough to feed their newborn (Dawodu et al., 1990; Hopkinson et al., 1992). Also, according to Garza et al. (1984), milk production of mothers delivering prematurely is higher than what their newborn can consume, as a consequence, surplus milk could be concentrated and used as milk fortifier.

The difficulties encountered during this study to obtain adequate milk samples and other practical considerations suggest that special infrastructures will have to be created and added to the already existing facilities of a milk bank. The policies and practices that should be implemented in all facilities providing maternity services and care for the newborn are summarized in the joint WHO/UNICEF statement "Ten steps to successful breast-feeding" (1989) (Appendix IX). According to that statement and to the problems encountered during this study, several suggestions can be made to promote and support breast-feeding, and to optimize the nutritional management of the preterm VLBW infant. These are:
Conclusions and Recommendations

1) mothers should be able to remain in the hospital (rooming-in) with their infants and regularly express their milk to feed their babies. Separation of the mother from the newborn soon after delivery has been shown to be an important risk factor impairing lactogenesis and subsequent galactopoiesis (Livingstone, 1994).

2) breast-feeding consultants are needed in order to help mothers initiating, establishing, and maintaining lactation while their baby is not able to suck. Milk production depends mainly on the stimulus received by the mother through the breast. Techniques of breast stimulation should be taught and initiated very early after delivery (Livingstone, personal communication). Early stimulation and frequency of feeding or expression are determinants of successful lactation (Livingstone, 1994). Moreover, milk yield can be increased by frequent drainage of the breasts thereby allowing the production of adequate volumes of breastmilk for the nutrition of the baby and for the concentration process described in this study (section 3.2).

3) routine laboratory assessment of protein, fat, carbohydrates, and minerals are needed in order to monitor the nutritional composition of the fortified human milk. Fortification carried without any knowledge of the composition of the milk and the fortifier used may lead to under- or overnutrition. Thus, constant monitoring is needed to avoid excessive nutrient load that could lead to edema and metabolic problems.

4) one full-time person/technician should be appointed to process and fortify each individual milk sample. Fortified human milk for a preterm infant should be prepared daily by mixing its own mother's fresh milk with an appropriate amount of concentrated human milk. The fortified human milk should then be thoroughly mixed and divided into smaller flasks, each containing the volume of milk needed for one feeding. If mother's fresh milk is not available in adequate amounts it should be complemented with banked pasteurized human milk. If the recipient's own mother's milk is not available at all, banked pasteurized human milk could be used. Methods of collection, handling and storage may greatly influence the composition of banked human milk. Several authors have demonstrated the variability in milk composition depending on the sampling scheme. Garza et al. (1986) point out that for each population with
distinct feeding patterns, an appropriate sampling scheme should be used. Once a specific sampling scheme is installed, the intra individual variability will be reduced and donor milk composition will be optimized (Michaelsen et al., 1990).

The cost associated with the implementation of such routines might be higher than the cost of commercial fortifiers. A cost-effective study should be undertaken. As stated by Senterre et al. (1984), "wherever human milk lactoengineering is not feasible (due to high costs related to the processing and monitoring techniques) or whenever human milk is not available, special commercial formulas adapted for the VLBW infant should be fed to the high-risk newborn". Nevertheless, priority should be given to the baby's own mother's milk. As shown by Guerrini (1994), human milk can be adequately supplemented in order to satisfy the infant needs for normal growth and development. More studies are needed to optimize and standardize the concentration and fortification processes described in this study.

7.2.2 Safety of human milk: to pasteurize or not to pasteurize?

In Brazil and in most developing countries where inadequate funds do not allow the purchase of special commercial formulas or fortifiers, it is usually the rule to feed small preterm babies with their own mother's milk or banked human milk (Mattar, 1994).

Heat treatment of milk has an adverse effect on milk proteins. Bile-salt-stimulated-lipase (BSSL) rapidly loses its activity when incubated at 50°C and is almost completely destroyed during pasteurization (Björkstén et al., 1980; Lyster et al., 1984). However, because of the high risk of transmission of viruses, like HIV or cytomegalovirus, it is advised that whenever donor milk is used for premature infants it should be heat treated because of the possibility of virus infection (Wharton et al., 1987). Homologous fortification of human milk (using own mother's fresh raw milk fortified with concentrated human milk made from pasteurized pooled human milk) would preserve the native anti-infective factors and enzymes (such as the BSSL), and would allow the
Conclusions and Recommendations

preterm VLBW infant to, not only be protected from pathogenic bacteria and other diseases (Narayanan et al., 1981), but also to be able to digest the fat provided by this fortified human milk.

Pasteurization of banked pooled human milk should be carried out prior to storage and concentration in order to reduce the risk of lipolysis and subsequently the production of lipid peroxides. However, pasteurization of human milk (62.5°C, 30 minutes) has been shown to be detrimental to several antimicrobial factors leading to a higher susceptibility to subsequent bacterial contamination (Räihä, 1985). As a result, it is very important that pasteurization be performed after the concentration process. A dual pasteurization before and after processing seems to be the only way to reduce the risk of bacterial and peroxidative damage to the VLBW infant fed fHM. Rapid high-temperature short-time treatment (72°C for 5 or 15 seconds) of human milk seems to be efficient in reducing the microbial content without significantly affecting the immunologic factors like sIgA, lactoferrin, and lysozyme (Goldblum et al., 1985) and should be promoted instead of the classic method of holder pasteurization (62.5°C, 30 minutes) which is commonly used in human milk banks.

7.2.3 Nutrient bioavailability, anti-infective properties and free radical content of fortified human milk

The problem with fortifiers such as the concentrated human milk (cHM) prepared in this study is that little is known about its bioavailability and the effects that the addition of this cHM would have on the native component of human milk. Denaturation of certain proteins or formation of new peptides during processing may interfere with the bioavailability of certain minerals and may have negative effects on the immunoglobulins and other anti-infective components of human milk. The milk concentration process may also lead to increased FFA facilitating the production of free radicals and potentially impairing the health of the VLBW infant. More studies are needed to determine the nutrient availability, the anti-infective properties, and the free radical content of the fortified human milk (fHM).
7.2.4 Fat losses during tube feeding

As shown by Greer et al. (1984), Narayanan et al. (1984), Stocks et al. (1985) and by Mehta et al., (1991), fat added to human milk tends to form a non-homogenous mixture due to the adherence of the fat globules to the feeding tube and syringe, and may lead to substantial fat losses during tube feeding, therefore decreasing the nutritional advantage of the fortified human milk. Ultrasonication of human milk has been shown to reduce fat losses during tube feeding (Martinez et al., 1987; Rayol et al., 1993). However, it may also cause denaturation of the milk proteins and dangerously increase the milk FFA content in amounts that may be detrimental for the premature infant (Hamosh, 1988).

Pasteurization of human milk at 62.5°C has been shown to completely inactivate human milk lipase (Björksten et al., 1980). However, due to some degree of lipolysis occurring during storage and processing, a high proportion of fat present in the concentrated human milk (cHM) may be in the form of FFA. FFA alters the surface tension of the milk and might decrease fat losses (Tarassuk cited by Lavine et al., 1989). During continuous pump infusion, Lavine et al. (1989) were able to show that human milk stored at 4°C for three days lead to lower fat losses than fresh human milk. However, human milk used in their study was not pasteurized and fat losses using the pasteurized high-fat fHM may be important. Further studies on the FFA content of the fHM and on the dynamics of delivery of milk fat during tube infusion should be undertaken in order to assess the risk or benefit of feeding the VLBW with a high fat fHM.
7.3 Technical suggestions

It has been shown in this study that concentration of donor human milk was not a predictable and reliable process due to high fat and protein losses, high viscosity of the evaporated human milk that interfered with lactose crystallization, and unexplained elevation of the osmolality. Some technical suggestions to optimize the concentration process include:

1) It would be desirable to concentrate large quantities of full-term pooled donor human milk in order to reduce the compositional variations of the cHM and the fHM. Pooled human milk would decrease variability (Michaelsen et al., 1990) and may, thereby, increase the predictability and reliability of the concentration process.

2) Concentration of human skim milk (delipidated human milk) could be performed, potentially reducing fat losses and milk viscosity. Concentration of human skim milk could easily be done by centrifugation of banked human milk prior to the concentration process. Centrifugation will allow the separation of human milk fat resulting into a cream phase and a skim milk fraction (delipidated human milk). The skim milk fraction could be concentrated leading to a high protein and minerals concentrate. The cream phase could then be used to increase the energy content of the newly produced fortified human milk, if necessary.

Careful assessment of both mother's milk and concentrated milk for macro and micronutrient content should be carried out prior to fortification in order to maintain an adequate level of these nutrients.
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Schanler R.J., Garza C., and Nichols B.L. Bioavailabilities of calcium and phosphorus are higher in fortified mother's milk compared to commercial formula (abstr). *Pediatric Research* 19:231A, 1985c.


Appendices
Appendix I: Macronutrient and mineral content of concentrated human milk (cHM)

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<th>Osmolality mOsm/kg (H2O)</th>
<th>Calcium mg/100 ml</th>
<th>Phosphorus mg/100 ml</th>
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Appendix II: Macronutrient and mineral composition of the "high fHM"

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Donor human milk fortified with concentrated human milk at a proportion of 2/3 donor and 1/3 concentrated milk.
Appendix III: Macronutrient and mineral composition of the "medium fHM"

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Donor human milk fortified with concentrated human milk at a proportion of 3/4 donor and 1/4 concentrated milk.

Fortified milk samples K and M were obtained mixing a contaminated milk sample containing very high amount of sodium, therefore artificially increasing the sodium concentration. Mean sodium concentration calculated after elimination of those two samples averaged 32.25 ± 2.49 mEq/l.
Appendix IV: Macronutrient and mineral composition of the "low fHM"

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Donor human milk fortified with concentrated human milk at a proportion of 4/5 donor and 1/5 concentrated milk.
Appendix V: Observed versus expected nutrient increment in the concentrated human milk

Comparison between the observed and the expected nutrient increment in the concentrated human milk samples (cHM).

The observed nutrient increment was determined dividing the nutrient content in the cHM by the nutrient content of the initial donor human milk (dHM). The expected value was estimated using the milk volume reduction after evaporation of the milk water.

The nutrient increment (or concentration factor) is expressed as: x times the initial nutrient content of donor human milk.
(P) protein, (F) fat, (L) lactose, (Ca) calcium, (P) phosphorus, (Na) sodium, (E) energy.

* p<0.005 observed vs. expected
** p<0.001 observed vs. expected
Appendix VIA: Composition of donor (dHM) and concentrated (cHM) human milk samples, and calculation of the concentration factor (cHM/dHM) for each nutrient as well as for the osmolality and the energy content.

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</table>
Appendix VIb: Composition of donor (dHM) and concentrated (cHM) human milk samples, and calculation of the concentration factor (cHM/dHM) for each nutrient as well as for the osmolality and the energy content.

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<th>Milk sample</th>
<th>Osmolality mOsm/kg (H2O)</th>
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Appendix VII: Protein, Fat and Calcium losses in the lactose pellet (a) after human milk evaporation, lactose crystallization and removal.

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<th>Milk samples</th>
<th>Protein (pellet) (g)</th>
<th>Protein (dHM) (g)</th>
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<th>Fat (pellet) (g)</th>
<th>Fat (dHM) (g)</th>
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<th>Calcium (pellet) (mg)</th>
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(a) Losses are expressed as percentage of the initial amount found in donor human milk (dHM). The amount found in the pellet was determined redissolving the lactose crystals in 10 ml of deionized water. The total amount found in the lactose pellet is given in grams (g) for protein and fat and in milligrams (mg) for calcium.
Appendix VIII

Method of Molybdenum-Blue:
Ammonium molybdate with inorganic phosphorous form a phosphomolybdate complex which react with the hydroquinone ascorbate (reduction) and form a colloidal solution of molybdenum-blue.

Modified Richterich method:

1 ml of Borate-pyrosulfite (20g of sodium tetraborate; 18g of sodium pyrosulfite adjusted to 1 liter)
0.1 ml of diluted milk ashes
0.250 ml of molybdic acid (ammonium molybdate 40.5 mM (5g of ammonium molybdate adjusted to 100 ml using 1N sulfuric acid (27.7 ml of H₂SO₄ 96% for 1 liter)
0.250 ml of hydroquinone ascorbate (0.5 ml of ascorbic acid plus 1 g of hydroquinone adjusted to 100 ml)
vortex, wait 15 min.
3 ml of carbonate-sulfite (7g of anhydrous sodium sulfite and 42g of anhydrous sodium carbonate for 1 liter)
vortex and wait 5 min. Read the absorption at 578 nm
Appendix IX: Ten steps to successful breast-feeding


Every facility providing maternity services and care for newborn infants should:

1. Have a written breast-feeding policy that is routinely communicated to all health care staff.
2. Train all health care staff in skills necessary to implement this policy.
3. Inform all pregnant women about the benefits and management of breast-feeding.
4. Help mothers initiate breast-feeding within a half-hour of birth.
5. Show mothers how to breast-feed, and how to maintain lactation even if they should be separated from their infants.
6. Give newborn infants no food or drink other than breast milk, unless medically indicated.
7. Practice rooming-in — allow mothers and infants to remain together — 24 hours a day.
8. Encourage breast-feeding on demand.
9. Give no artificial teats or pacifiers (also called dummies or soothers) to breast-feeding infants.
10. Foster the establishment of breast-feeding support groups and refer mothers to them on discharge from the hospital or clinic.