The effect of pasteurisation on the composition of expressed human milk from HIV positive mothers, and its adequacy in relation to the growth of their very low birth weight premature infants

by

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Declaration

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Date: 22 December 2008

ABSTRACT

Title: The effect of pasteurisation on the composition of expressed human milk from HIV positive mothers, and its adequacy in relation to the growth of their very low birth weight premature infants.

Objective: *Primary*: To investigate the effect of pasteurisation on the composition of expressed breast milk from HIV positive mothers and its adequacy in relation to the growth of their VLBW premature infants.

Methods: A descriptive, prospective case-controlled pilot study was performed in 3 regional state hospitals in the Western Cape, South Africa. The control and study groups consisted of 12 HIV negative and 11 HIV positive mothers, with their VLBW premature infants, respectively. All mothers (19 - 35 years old) belonged to the Xhosa ethnic group. All infants were born <34 weeks gestation and birth weight <1500g. Eight breast milk samples were collected on days 6, 7, 13, 14, 20, 21, 27 and 28 from all mothers during the first 28 days after birth. Breast milk was expressed by hand or pump from either the right or left breast. Half of each breast milk sample was kept raw. The remaining sample was Pretoria pasteurised, after which both samples were subdivided into 3 or 4 aliquots to determine energy, protein, carbohydrate, fat, folate, calcium, phosphorus, magnesium, sodium, potassium, iron, copper, zinc content and HI viral load (study group only). The mothers' dietary intake during pregnancy and lactation was obtained by means of a quantitative food frequency questionnaire and repeated 24-hour recall respectively. The infants' dietary intake was recorded daily. Mothers' anthropometric measurements taken at study entry and exit were weight, height and mid-upper arm circumference. All infants' daily weight, as well as length and head circumference at birth and on day 28 postpartum was obtained. Biochemical analysis was performed on the blood samples obtained at study entry and exit from all mothers and infants. For statistical analysis, Statistica® (release no 7, 2006) was used for repeated measures analysis of variance (ANOVA) to determine the effect of HIV, pasteurisation, milk expression and time on the composition of the breast milk.

Results: There was no significant difference in the studied macro- and micronutrient composition between raw or pasteurised expressed breast milk from HIV positive and HIV negative mothers with premature infants. A significant decline in breast milk protein ($p<0.01$), magnesium ($p=0.045$), potassium ($p=0.002$), zinc ($p<0.01$) and copper ($p=0.03$) content was observed for the whole study population over time, while folic acid content increased significantly ($p=0.012$) over time. The sodium/potassium ratio of both groups remained strongly indicative of the presence of sub-clinical mastitis. A significant ($p=0.03$) greater mean volume of milk was obtained with pump [42.5 Standard Deviation (SD) 18.1 ml], compared to hand expression [36.7 (16.7) ml]. Method of expression did not result in a significant difference in breast milk composition for any of the nutrients studied. Eight (35%) infants were born SGA while 21 (91%) infants were SGA on day 28.

The infants gained a mean of 16.37 (4.5) g/kg/day (i.e. 91% of the recommended growth rate of >18g/kg/day) from the day on which birth weight was regained. The control and study group infants respectively consumed a mean of 3.27 (1.36) and 3.21 (1.36) g protein/kg/day and 138.1 (33.8) and 142.3 (33.8) kcal total energy/kg/day. This corresponds to a PER of 2.6 (control group) and 2.5 (study group) respectively, which cannot provide for the increased growth needs of the SGA infant in need of catch-up growth.

Six infants experienced an incident of Grade 1 NEC which resolved and all completed the study. Two (18%) HIV-exposed infants were found to be HIV positive at 28 days post delivery. Four participating infants died. No adverse event or mortality was related to the study protocol as no intervention was undertaken.

Conclusion: HIV positive Xhosa mothers provide as nutritious breast milk to their VLBW premature infants as HIV negative Xhosa mothers. Maternal nutrient intake during pregnancy and lactation did not have a significant effect on the nutritional composition of breast milk, except for folate content.

OPSOMMING

Titel: Die effek van pasteurisasie op die samestelling van uitgemelkte borsmelk van MIV positiewe moeders en die borsmelk se voldoendheid in verhouding tot die groei van hul baie lae geboortemassa premature babas.

Doel: *Primêr*: Om die effek van pasteurisasie op die samestelling van uitgemelkte borsmelk van HIV postiewe moeders te bestudeer, asook die voldoendheid in verhouding tot die groei van hul BLGM premature babas.

Metodiek: 'n Beskrywende, prospektiewe gevalskontrole loodsstudie is uitgevoer in 3 streeks-staatshospitale in die Wes-Kaap, Suid-Afrika., Die kontrole- en studiegroep het bestaan uit 12 HIV negatiewe en 11 HIV positiewe moeders, met hul BLGM premature babas, onderskeidelik. Alle moeders (19 - 35 jaar oud) het behoort aan die Xhosa etniese groep. Alle babas was gebore op <34 weke gestasie en geboortemassa <1500g. Agt borsmelkmonsters is versamel op dae 6, 7, 13, 14, 20, 21, 27 en 28 van alle moeders gedurende die eerste 28 dae na geboorte. Borsmelk is met die hand of 'n pomp uitgemelk van óf die regter óf linkerbors. Die helfte van elke borsmelkmonster is rou gelaat. Die oorblywende borsmelkmonster is Pretoria gepasteuriseer, waarna beide monsters onderverdeel is 3 of 4 dele vir bepaling van energie, proteïen, koolhidrate, vet, folaat, kalsium, fosfaat, magnesium, natrium, kalium, yster, koper, sinkinhoud en MI virale lading (studiegroep alleenlik). Die moeders se dieetinname gedurende swangerskap en laktasie is verkry deur middel van 'n gekwantifiseerde voedselfrekwensie vraelys en herhaalde 24-uur herroep onderskeidelik. Alle babas se dieetinname is daagliks aangeteken. Moeders se antropometriese metings geneem met studie in- en afsluiting was gewig, lengte en mid bo-armomtrek. Alle babas se daaglikse gewig, asook lengte en kopomtrek by geboorte en dag 28 postpartum is verkry. Biochemiese ontleding is uitgevoer op die bloedmonsters wat by studie-insluiting en –afsluiting van alle moeders en babas verkry was. Beskrywende statistiek is gebruik om sosio-demografiese en antropometriese data te beskryf. Vir statistiese ontleding is Statistica® (vrystelling nr 7, 2006) gebruik vir herhaalde metings van variansie (ANOVA) om die effek van MIV, pasteurisasie, uitmelk en tyd op die borsmelksamestelling te bepaal.

Resultate: Daar was geen beduidende verskil tussen die bestudeerde makro- en mikronutriënt samestelling van rou of gepasteuriseerde uitgemelkte borsmelk van HIV positiewe en HIV negatiewe moeders met premature babas. 'n Betekenisvolle afname in borsmelk se proteïen- ($p<0.01$), magnesium- ($p=0.045$), kalium- ($p=0.002$), sink- ($p<0.01$) en koper- ($p=0.03$) inhoud is waargeneem vir die hele studiepopulasie oor tyd, terwyl foliensuurinhoud betekenisvol ($p=0.012$) toegeneem het oor tyd. Die natrium/kalium verhouding in beide groepe was deurgaans sterk aanduidend van die teenwoordigheid van sub-kliniese mastitis. 'n Beduidende ($p=0.03$) groter gemiddelde (SD) melkvolume is verkry met pomp [42.5 Standaard Deviasie (SD) 18.1) ml] in

vergelyking met hand uitmelk [36.7 (16.7) ml]. Uitmelkmetode het nie tot ‘n betekenisvolle verskil in borsmelksamestelling vir enige van die bestudeerde nutriënte gelei nie. Agt (35%) babas was KVD gebore, terwyl 21 (91%) babas KVD was op dag 28. Die babas het vanaf die dag dat geboortemassa herwin is, gegroei teen gemiddeld 16.37 (4.5) g/kg/dag (d.i. 91% van die aanbevole groeitempo van >18g/kg/dag). Die kontrole- en studiegroep babas het respektiewelik gemiddeld 3.27 (1.36) en 3.21 (1.36) g proteïen/kg/dag en 138.1 (33.8) en 142.3 (33.8) kkal totale energie/kg/dag ingeneem. Dit stem ooreen met ‘n PER van 2.6 (kontrolegroep) en 2.5 (studiegroep) respektiewelik, wat nie kan voorsien in die verhoogde groeibehoefte van ‘n KVD baba wat inhaalgroei benodig nie.

Ses babas het ‘n insident van Graad 1 NEK gehad wat opgeklaar het, sodat almal die studie voltooi het.

Twee (18%) MIV blootgestelde babas is MIV positief bevind op 28 dae na geboorte. Vier deelnemende babas het gesterf. Geen MIV positiewe status bevinding of enige van die sterftes was geassosieer met die studieprotokol nie, omdat geen intervensie onderneem was nie.

Gevolgtrekking: HIV positiewe Xhosa moeders voorsien hul BLGM premature babas met soortgelyke nutrisionele borsmelk as HIV negatiewe moeders. Maternale nutriëninname gedurende swangerskap en laktasie het nie ‘n beduidende effek op borsmelk se nutriëntinhoud gehad nie, behalwe vir folaatinhoud.

DEDICATION

This thesis is dedicated to every mother who was willing to take part in this study. I will never forget your selfless help. It was a privilege to work with you and getting to know you.

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LIST OF ABBREVIATIONS AND EXPLANATION OF TERMS

Achlorhydria: An abnormal condition characterised by the absence of hydrochloric acid in the gastric juice.[1]

APGAR Score: The evaluation of an infant's physical condition, usually performed 1 and 5 minutes after birth, based on a rating of five factors that reflect the infant's ability to adjust to extra-uterine life. Virginia Apgar (M.D.) developed the system for the rapid identification of infants requiring immediate intervention or transfer to an intensive care nursery. *Method:* The infant's heart rate, respiratory effort, muscle tone, reflex irritability and colour are scored from a low value of 0 to a normal value of 2. The five scores are combined and the totals at 1 minute and 5 minutes noted. *Outcome criteria:* A score of 0 to 3 represents severe distress, a score of 4 to 7 indicates moderate distress and a score of 7 to 10 indicates an absence of difficulty in adjusting to extra-uterine life. A 5 minute overall score of 0 to 1 correlates a 50% neonatal mortality rate.[1]

Bactericidal: Destructive to bacteria (also bacteriocidal).[1]

Bacteriostatic: Tending to restrain the development or the reproduction of bacteria.[1]

Catch-up growth: A growth rate which is accelerated to be faster than intra-uterine growth rate.[2, 3]

CI: Confidence Interval

DNA- or RNA PCR testing: Polymerase Chain Reaction testing is used for the determination of HIV-1 infection status

ELISA: Enzyme-linked Immunosorbent Assay: A test for determination of HIV-1 infection status

Exclusive breastfeeding: Defined as giving an infant no other food or drink (not even water), apart from breast milk (including cup feeding with expressed breast milk), with the exception of drops or syrup consisting of vitamins, mineral supplements or medicines, when medically prescribed.[4]

Extremely Low Birth Weight (ELBW): Any infant weighing less than 1000g at birth.[1]

Flash heating: 50 ml of breast milk in an uncovered jar is placed in 450 ml of water in an aluminium pot over a flame until the water begins to boil, after which the breast milk is then immediately removed from the water and heat and allowed to cool.[5]

Fore milk: The first milk obtained at the onset of suckling or breastfeeding. Contains less fat than hindmilk.[6]

HAART: Highly active anti-retroviral therapy

HELLP Syndrome: A condition of pregnancy characterised by raised liver enzymes, haemolysis and low platelet count with pre-eclampsia.[7]

HIV: Human Immunodeficiency Virus

Hind milk: Milk obtained later during the breastfeeding period, that is, at the end of the breastfeeding. This milk is usually high in fat and probably controls appetite.[6]

Hydrops Fetalis: Abnormal accumulation of fluid in the entire body of the neonate, in hemolytic disease due to antibodies present in the blood of the Rh-negative mother.[8]

IUGR: Intra-uterine growth restriction. Infant born with a birth weight and/or birth length and/or birth head circumference falling below the 10th percentile for gestational age on the perinatal growth chart.[9]

Kangaroo Mother Care: An intervention for the care of the low birth weight infant. It consists of four components: *Kangaroo position*: Stable LBW infants are nursed skin-to-skin between their mother's breasts. *Kangaroo nutrition:* Infants should be fed own mother's milk, either by breastfeeding on demand or by expresses breast milk via nasogastric tube or cup. *Kangaroo support:* Support the mother-infant dyad, both in hospital and after discharge, for optimal outcome. *Kangaroo discharge:* Infants may be discharged from hospital in the kangaroo position at any weight or gestational age, provided they are well and gaining weight on mother's milk and that there is adequate follow-up facilities.[10]

Low Birth Weight (LBW): Any infant weighing less than 2500g at birth.

Maillard-reaction: The chemical binding of essential amino acids, especially lysine, with various carbohydrates under the influence of heat.[11]

Mastitis: An acute inflammation of the interlobular connective tissue within the mammary gland in which para-cellular pathways between mammary alveolar cells open up, allowing inflammatory cells and extra-cellular fluid to enter the breast milk. Mastitis is usually unilateral and indicated by elevated breast milk sodium concentrations.[12, 13]

Mature breast milk: Breast milk composition as found after 21 days of lactation.[14]

Medium Chain Triglyceride (MCT): A fat with fatty acid chain lengths of between 8 and 12 carbons, which are short enough to be water soluble; requires less bile salt for solubilisation, is not re-esterified in the enterocyte and is transported as free fatty acid bound to albumin through the portal system.[3]

MTCT: Mother-to-child transmission of Human Immunodeficiency Virus

Nectrotising Enterocolitis (NEC): An acute inflammatory bowel disorder that occurs primarily in preterm or low-birth weight neonates. It is characterised by ischemic necrosis of the gastro-intestinal (GI) mucosa that may lead to perforation and peritonitis. The cause of the disorder is unknown although it appears to be a defect in host defences with infection resulting from normal GI flora rather than from invading organisms. Also called pseudo-membranous enterocolitis.[1]

OR: Odds ratio

PER: Protein-energy ratio

Premature infant: Any neonate regardless of birth weight, born before 37 completed weeks' gestation.[1]

Premature breast milk: Milk pumped within the first month postpartum by a mother who delivered at or before 36 weeks' gestation.[6]

Pretoria Pasteurisation: Milk is placed into a glass jar, the lid replaced and the jar then placed into an aluminium pot (1-liter capacity) containing 450 ml of water that has been brought to boiling point and removed from the heat source immediately before placement of the jar of milk into the pot for a period of 25-30 minutes.[15]

RR: Relative risk

Subclinical mastitis: A raised milk sodium/potassium ratio (>0.6), with severe sub-clinical mastitis indicated by a sodium/potassium ratio of >1.0. Possible causes of subclinical mastitis: poor lactation practice, micronutrient malnutrition, local (breast) infections and systemic infection.[16]

Small for gestational age (SGA): Any infant weighing less than the 10th percentile for gestational age at birth.[3]

Very Low Birth Weight Infant (VLBW): Any infant weighing less than 1500g at birth.[3, 17]

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CHAPTER 1: REVIEW OF RELATED LITERATURE

1.1 The Basics of HIV Infection in Mother-to-Child Transmission

Mother-to-child transmission (MTCT) is the transmission of Human Immunodeficiency Virus Type 1 (HIV-1) from an HIV-infected mother to her infant. MTCT can occur during pregnancy, labour and after birth, through breastfeeding.[1] It is estimated that 90% of all HIV-1 positive children acquire HIV-1 through MTCT, which corresponds to about 800 000 children becoming infected with HIV-1 through MTCT, according to current statistics.[18,19, 20]

The risk of infection is estimated to be 5-10% during pregnancy, 10-20% during labour and 10-20% through non-exclusive breastfeeding by untreated women who continue breastfeeding beyond the first year of life. In the absence of interventions to prevent MTCT, an estimated 25-45% of HIV-infected untreated mothers will pass the virus on to their infants (Table 1.1.)[4, 18, 19, 21, 22] Although antiretroviral therapy has been effective in reducing perinatal transmission of HIV-1, no therapeutic regimen has yet been developed that, throughout lactation, significantly reduces transmission via breastfeeding.[20]

Table 1.1 Estimated risk and timing of mother-to-child transmission of HIV in the absence of interventions

Timing	Rate of Transmission
During pregnancy	5 - 10%
During labour and delivery	10 - 15%
During breastfeeding	5 - 20%
Overall without breastfeeding	15 - 25%
Overall with breastfeeding to 6 months	20 - 35%
Overall with breastfeeding to 18 to 24 months	30 - 45%

(Adapted from references [18, 22, 23] and [24].)

In most HIV-infected women, HIV does not cross the placenta from the mother to the fetus. The placenta actually shields the fetus from HIV. However, this protection from the placenta may break down, if the mother:

- has a viral, bacterial, or parasitic infection during pregnancy,
- becomes HIV-infected during the pregnancy and hence develops a very high level of HIV for a short time, or
- has a severe immuno-deficiency associated with advanced acquired immuno-deficiency syndrome (AIDS).

In addition, malnutrition during pregnancy may indirectly contribute to MTCT.[4]

Infants of HIV-infected mothers are at great risk of becoming infected with HIV during birth. Most infants who acquire HIV during labour and delivery do so by swallowing, absorbing or aspirating maternal blood or cervical secretions that contains HIV.[4]
The first reports indicating the possibility of HIV transmission through breast milk were in breastfed infants of women who became HIV-infected through contaminated blood transfusions they had received after delivery. Following this, there were reports of infants with no known exposure to HIV who became infected through wet- nursing and through the consumption of pooled breast milk.[4]

The mechanism and routes of breastfeeding transmission remain unclear. HIV is expressed as either cell-free or cell-associated virus and it is unknown which one is most associated with transmission.[18, 25] The virus is also shed by epithelial cells in the mammary alveoli and ducts. The route of entry into the child is also unknown. The virus could be absorbed either in the infant's mouth, throat or intestine, although passage of the virus through the acid environment of the stomach (after neonatal achlorhydria) will reduce intestinal absorption. The risk of breast milk transmission of HIV may depend on:

- the size of the viral inoculum,
- the amount of cell-free and cell-associated HIV,
- the infant's individual susceptibility, and
- local specific immune responses to HIV.[26]

One study found the risk of MTCT of HIV-1 to be 14% in mothers with established HIV-1, compared with a 29% risk of transmission if the woman became infected while lactating.[27] The timing of transmission also has a major effect on the progression of HIV-1 disease, with in utero transmission being associated with a rapid course.[28]

The first prospective study to give quantitative estimations of breast milk infectivity was conducted in Nairobi, Kenya. This study found the probability of breast milk transmission of HIV-1 to be 0.00064 per litre of breast milk ingested and 0.00028 per day of breastfeeding respectively. Defining the above-mentioned probabilities a little differently, it equals one HIV-1 infection per 1500 litres of breast milk ingested (on average the infants in this study consumed 150 litres of breast milk) and 10 infections per 100 child years of breastfeeding respectively. Another correlation made indicated the probability of HIV-1 infection per litre of breast milk ingested by an infant to be similar in magnitude to the probability of heterosexual transmission of HIV-1 per unprotected sex act in adults.[29]

Breast milk infectivity was significantly higher for mothers with more advanced disease, as measured by prenatal HIV-1 RNA plasma levels and CD4 T-cell counts. Maternal viral RNA levels >43 000 copies/ ml (cohort median) was associated with a 4-fold increase in the risk of HIV-1 transmission, while prenatal CD4 T-cell counts of <400 X 10^6/litre (cohort median) had an almost 3-fold higher breast milk infectivity, both per litre of breast milk ingested and per day of breastfeeding. The main limitation of this study was that both the number of days of breast milk exposure for each child and the

estimated volume of breast milk ingested per day were based on maternal self-report, which may not have been completely accurate.[29] Rousseau et al[20] also found breast milk viral load to be inversely correlated with maternal CD4 T-cell count.

A trial conducted in Nairobi, Kenya identified seven correlates of overall transmission that were significantly associated with early (before 2 months of age) HIV-1 transmission, i.e. cervical HIV-1 DNA, vaginal HIV-1 DNA, cervical or vaginal ulcers, prematurity, breastfeeding, plasma virus load and immuno-suppression. Bleeding nipples were also associated with early infant HIV-1 infection. A possible mechanism suggested for an increased risk of HIV-1 transmission in the aforementioned circumstances might be due to the erosion of the normal maternal-infant barriers or the mobilization of inflammatory cells.[30]

Infant HIV-1 infection status is usually defined by using polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) testing. The sensitivity of DNA-PCR testing for a single infant specimen was found to be 95% and the specificity 98%.[31]

1.1.1 The incidence of premature birth in the presence of maternal HIV-1 infection.

Several associations have been made between the presence of maternal HIV infection and the incidence of premature delivery.[28] A study by Ryder et al[32] in Zaire found the incidence of prematurity to be 13% in HIV positive women and 3% in HIV negative women. This finding, however, is not consistent in HIV positive women in developed countries. Premature infants are more likely to be HIV-infected compared to term infants who were born to HIV positive women. Goedert et al[33] studied 687 HIV-infected women and found that among the 92 infants of these mothers born HIV-infected, the rate of transmission was 60% in premature infants and 22% in term infants. A European Collaborative Study[34] has found that the frequency of HIV positive mothers delivering prematurely and/or very low birth weight babies has increased significantly since the introduction of highly active anti-retroviral therapy (HAART) in Europe. Risk factors for premature delivery included baseline CD4 T-cell count below 200 (OR=2.36, p=0.0001) and protease inhibitor-containing HAART during pregnancy (OR=4.17, p=0.0001). Women already taking HAART when they became pregnant, or who commenced HAART in the first trimester, had a significantly higher risk of premature delivery.[34]

1.2 Factors Involved in Mother-to-Child Transmission of HIV-1

Factors affecting maternal-infant transmission can be divided into 2 categories: maternal and neonatal factors. (Table 1.2 and Table 1.3) Several of these factors (e.g. prematurity and maternal medication) can be classified as both maternal and neonatal, while other factors (e.g. nutrition) include both an intra-uterine and neonatal period.[28]

1.2.1 Maternal factors

Table 1.2 A summary of maternal factors associated with an increased risk of HIV-1 transmission through their effect on maternal infectivity

Factor	Description/ Elaboration
Viral factors	-Increased viral load -Breast milk concentration of cell-free and cell-associated HIV-1 -Viral strain: phenotype and genotype
Immunological or inhibitory factors	-Immuno-deficiency -Presence and function of HIV-1 specific antibodies or cytotoxic T-lymphocytes in breast milk -Presence of lipids, lactoferrin, lysozyme and other factors with either direct anti-viral action or viral inhibition through prevention of HIV-1 adhesion to CD4 T-cell receptors
Maternal stage of HIV/AIDS	-Zero conversion of mother during pregnancy or lactation -Advanced disease (low CD4, high CD8, or symptoms of AIDS)
Maternal nutritional status	-Severely malnourished mother
Anti-retroviral therapy	-No perinatal anti-retroviral therapy
Gynaecological factors	-Vaginal delivery -Premature rupture of membranes >4 hours -Episiotomy
Feto-placental factors	-Fetal scalp electrode -Chorioamnionitis
Breast and nipple abnormalities	-Cracked, bleeding, abscess, mastitis and sub-clinical mastitis
Presence of infection or disease	-Sexually transmitted diseases -Malaria -Tuberculosis -Hypertension and pre-eclampsia
Other factors	-Anaemia -Vitamin A deficiency -Maternal cigarette smoking -Maternal drug use -Maternal age (≥ 30 years)

(Adapted from references [19, 21, 28, 29, 30, 35, 36, 37] and [38].)

Several of the above factors remain largely speculative. The maternal immunological or inhibitory factors are discussed in Section 1.3.1.

Viral factors

Rousseau et al[20] found breast milk viral load to be the highest early after delivery (in colostrum and early breast milk) and confirmed that it can serve as a significant predictor of transmission. Each 10-

fold increase in breast milk viral load was associated with a 2.0-fold increase in the risk of transmission (p=0.002). Both maternal CD4 T-cell count and maternal plasma viral load were independent predictors of breast milk viral load and might serve as alternative indicators of women at risk of MTCT of HIV-1 via their breast milk. O'Shea et al[39] found that the predicted rate of transmission relative to maternal HIV-RNA load was 2% at 1000 copies/ml, 11% at 10 000 copies/ml and 40% at 100 000 copies/ml. Maternal HIV-RNA viral load was a stronger predictor of transmission than CD4 T-cell number.

Van de Perre et al[40] found, through multivariate analysis, the presence of infected cells in breast milk to be the factor most strongly associated with HIV-1 MTCT in those children surviving for more than 18 months. The frequency of viral shedding into the breast milk may occur on a continual or intermitted basis. It was not yet possible to identify a breast milk viral threshold level below which no transmission occurred. As this particular study only measured virus from one breast at a visit, it is not known whether virus levels are the same in both breasts.[20] Willumsen et al[41] confirms that viral load in milk, especially from one breast and one time-point only, is likely to be only marginally predictive of the risk for postnatal transmission and therefore recommends that milk samples must be obtained from both breasts and analysed separately.

Using multivariate analysis, Fawzi et al[36] found maternal CD4 T-cell count, viral load and clinical stage of HIV/AIDS significant predictors of intra-uterine, intrapartum and early breastfeeding transmission. Magder et al[37] confirmed that antenatal viral load and anti-retroviral therapy were associated with risk of both in utero and intrapartum transmission, while antenatal CD4 T-cell percentage was mostly associated with intrapartum transmission of HIV.

Maternal nutritional status

Adequate nutritional status may reduce vertical transmission by affecting several maternal or fetal and child risk factors for transmission including enhancing systemic immune function in the mother or fetus/child. This may reduce the rate of clinical immunological or virological progression in the mother, leading to a reduction in viral load and viral shedding in lower genital secretions and breast milk. Furthermore enhanced maternal nutritional status may reduce the risk of low birth weight or prematurity and contribute to the maintenance of fetal gastro-intestinal integrity.[42]

Breast and nipple abnormalities

A trial conducted by John et al[30] in 92 infected and 187 uninfected infants at 2 years of age in Nairobi, Kenya indicated that breastfeeding and mastitis were associated with increased transmission overall. The presence of plasma-derived components and inflammatory cells in breast milk during mastitis, such as HIV-1-infected lymphocytes, could raise the HIV-1 load from mother to child. Semba et al[38] examined breast milk HIV-1 load and mastitis as risk factors for MTCT of HIV-1 and found that women with elevated breast milk sodium concentrations consistent with mastitis had almost twice the rate of MTCT of HIV-1, compared with women who had normal breast milk sodium concentrations. The

proposed mechanism for an increased risk for the transmission of HIV through breastfeeding in the presence of mastitis is that during mastitis, inflammatory cells and extra-cellular fluid enter the breast milk and the concentration of cell-free HIV in breast milk is elevated.[38]

Vitamin A deficiency

In a study by Kreiss[35], an association was found between poor Vitamin A status and HIV-1 DNA in breast milk, but only in women with CD4 depletion. Severe Vitamin A deficiency was associated with a 20-fold increased risk of HIV-1 DNA shedding in breast milk. These results suggested that women with CD4 depletion or Vitamin A deficiency might be at an increased risk of transmitting HIV to their infants through breastfeeding.[35, 43]

Vitamin A supplementation was postulated to reduce MTCT of HIV-1 by affecting several maternal, fetal, and/or child risk factors for transmission. The effect of maternal Vitamin A supplementation on the gut function of infants of HIV-infected mothers was investigated. It was found that maternal vitamin A supplementation did not significantly affect infant gut permeability in the study group as a whole, but in the subgroup of mothers whose infants became infected, it might have prevented the deterioration of gut integrity. It appears that vitamin A has a major epithelial benefit on protection against gut damage and on restoring gut integrity after the acute infection has resolved.[44]

Contrary to the above-mentioned potential preventive effect of vitamin A supplementation on the risk of HIV-1 transmission, a study by Fawzi et al[45] found vitamin A supplementation to increase the transmission risk. A possible mechanism for this finding is the effect of high doses of β-carotene under highly oxidative conditions, which may result in eccentric cleavage oxidation breakdown products that might have adverse effects by interfering with retinoic acid availability at the tissue level. This study, however, did find multivitamin supplementation (of vitamins B-complex, C, E) of breastfeeding mothers reduced child mortality and HIV-1 transmission through breastfeeding among immunological and nutritionally compromised women. Multivitamin supplementation (of vitamins B-complex, C, E) furthermore significantly delayed the progression of disease among HIV-1 infected women. Adding Vitamin A to the multivitamin reduced the benefit, while supplementation with Vitamin A alone caused some negative effects with regard to some of the end-points examined. It is postulated that an adequate anti-oxidant vitamin status may also be associated with a reduced risk of mastitis, which furthermore is associated with a higher viral load in breast milk and a greater risk of vertical transmission.[45, 46]

A Cochrane review assessing the effect of vitamin A supplementation during pregnancy on the risk of MTCT, found no conclusive evidence that the antenatal and intrapartum use of vitamin A supplementation reduced the risk of MTCT of HIV-1 infection. To conclude: Antenatal and intrapartum vitamin A supplementation to reduce MTCT of HIV-1 is not recommended at present. The effects of such supplementation will become more precisely known as more trials report their findings.[47]

Presence of infection and disease:

Ascending vaginal infections may increase the risk of chorio-amnionitis which could lead to increased viral load in the amniotic cavity and intra-uterine transmission as well as premature rupture of membranes and prematurity. Sexually transmitted diseases, such as gonorrhea, are related to intra-uterine transmission, but not intrapartum or early breastfeeding transmission. Signs of lower genital infections were also associated with transmission. Women who had a history of tuberculosis were 2.32 times more likely to transmit HIV-1 infection during the intra-uterine period in univariate analysis.[36]

1.2.2 Neonatal factors

Table 1.3 A summary of neonatal factors that may influence susceptibility to HIV-1 transmission

Factor	Description/ Elaboration
Immunological factors	-Presence and function of salivary, gastro-intestinal or systemic HIV-1 specific antibodies or cytotoxic T-lymphocytes
Other factors	-Salivary inhibitors -Stomach pH (achlorhydria) -Oral stomatitis, ulceration, thrush, esophagitis, pharingitis, gastroenteritis -Prematurity, low birth weight -First-born of twins -Nutritional status -Feeding practices -High breast milk viral load -HLA type -No antiretroviral therapy for the infant

(Adapted from references [21, 28, 35, 36] and [37].)

Little is known about any of the above factors.

Immunological factors

The immune capacity of the neonate has no known effect on HIV-1 transmission but plays a major role in disease progression. While all divisions of the neonatal immune system are immature, the crucial defect for HIV is in the T-cell system. The main defects of the neonate's T-cells are the specialised functions of cytokine production, cytotoxic T-cell responses, delayed rejection of foreign cells and tropism for intracellular viral replication. The T-cell system is in general more susceptible to stress than the other divisions of the immune system. Neonatal stressors including intra-uterine or postnatal malnutrition may lead to T-cell depletion and decreased function. The antibody system of the neonate is largely dormant, replaced by passive trans-placental maternal immunoglobulin-G (IgG) and breast milk secretory Immunoglobulin-A (s-IgA). The lack of secretory IgA in the gastro-intestinal tract may allow HIV to traverse the mucosa and enter the infant's circulation.[28]

One of the first studies to look at the correlation between the presence of provirus or antibody in breast milk and the risk of transmission, found that the presence of HIV-1 DNA in the day 15 breast milk sample was associated with a 5,4 fold increased risk of infant infection. The study also found that, at 18 months of age, the presence of HIV-1 specific immunoglobulin M (IgM) in breast milk was protective and associated with an approximately 90% decreased risk of transmission.[35, 40]

A breastfeeding trial[31] was conducted in Nairobi, where 212 breast milk samples were collected from 107 HIV-1 positive women at standardised time-points during lactation. HIV-1 provirus was detected in 58% of the breast milk samples. The prevalence of HIV-1 DNA was 51% in colostrum and rose thereafter to a maximum of 71% at 6-9 months. The concentration of HIV-1-infected cells in breast milk ranged from 1 per 10^4 breast milk cells to over 3000 per 10^4 cells, which is over a 3-log difference. High concentrations of HIV-1-infected cells were maximal during the period 8-90 days after delivery. Factors associated with breast milk HIV-1 DNA were evaluated. It was found that women with CD4 counts below 400 had a 3.1-fold increased risk for HIV-1 DNA to be detected in their breast milk.[35, 36]

The neonatal gastro-intestinal (GI) function plays a significant role in mucosal transmission and a possible role in HIV-1 disease progression.[28] The at-risk infant is exposed to infected blood, vaginal secretions, amniotic fluid and breast milk. The newborn GI system has decreased gastric acidity, decreased enzyme activity, a thin GI mucosa, decreased mucus production and lack of secretory IgA, all of which may permit ingested HIV-1 to gain entry to the systemic circulation.[48]

Other factors

Salivary factors refer to the salivary epithelial cells' ability for cellular disruption and the formation of complexes between milk cells and the salivary cells. This process could alter both the infective and protective properties of milk and play an important role in oral transmission of cell-associated retroviruses.[49]

The portal of entry of HIV-1 into the infant mucosal surface is still an unknown component of HIV MTCT. HIV could enter into the epithelial sub-mucosa anywhere along the oral, pharyngeal, gastric or intestinal mucosa, either through defects caused by nutritional deficiencies, other infections or trauma.[19]

Prematurity may affect HIV-1 transmission but has no known effect on the rate of disease progression.[28] O'Shea et al[39] found an association between maternal viral load and prematurity: For every 10-fold increase in maternal HIV-RNA, the mean gestation at delivery decreased by 1.3 weeks. The risk of MTCT of HIV was investigated in Tanzanian women and it was found that babies born HIV negative at a gestational age ≤34 weeks, were 2.19 times more likely to become infected during the intrapartum and early breastfeeding periods than those born at or after 37 weeks. This finding suggests that prematurity increases the neonate's susceptibility to infection, rather than HIV infection

leading to a higher risk for prematurity. When using gestational age as a continuous variable, the multivariate risk of HIV transmission was reduced by 6% for every additional week of pregnancy.[36] Furthermore, prematurity has been associated with exaggerated immune deficiencies including profound T-cell defects and rapidly progressive HIV viral disease.[50]

The effect of low birth weight due to insufficient maternal and subsequent fetal nutrition on HIV transmission risk

Independent of HIV status, poor prenatal nutrition can result in intra-uterine growth restriction (IUGR) with poor growth for gestational age, thus leading to the delivery of low birth weight infants. Such infants have decreased cellular immunity with low T-cells, poor proliferative responses, small thymus size and an increased frequency of infection.[28] Moye et al[51] investigated the somatic growth of HIV positive infants and found that these infants' birth weight and length was on average 0.28kg less and 1.64cm shorter than that of HIV negative infants. While the weight of offspring of HIV positive mothers is slightly reduced, the frequency of IUGR and prematurity is not markedly enhanced.[28] Magder et al[37] on the other hand, found low birth weight (OR=2.8; 95%CI: 1.7-4.7) and prematurity (OR=2.4; 95%CI: 1.5-3.9) to be significantly associated with in utero transmission of HIV.

Nutritional status of the fetus and the neonate has a minimal effect on transmission but a significant effect in disease progression in HIV-1 positive older infants. Growth failure is a component of advanced HIV disease but not of asymptomatic or early disease. In the absence of complications, there is no evidence that HIV infection in and of itself leads to hypercatabolism and the main defects seem rather to be decreased energy intake and organ dysfunction.[52] Breastfeeding plays a major role in HIV-1 transmission but has no known effect on disease progression.[28]

1.3 Preventive Measures for Decreasing the Risk of Mother-to-Child Transmission through Breast Milk

The current guidelines of the World Health Organisation (WHO), United Nations Children's Fund (UNICEF), and the Joint United Nations programme on HIV/AIDS (UNAIDS) state:[53] "When replacement feeding is acceptable, feasible, affordable, sustainable and safe, avoidance of all breastfeeding by HIV-infected mothers is recommended. Otherwise exclusive breastfeeding is recommended during the first months of life.

To minimize HIV transmission risk, breastfeeding should be discontinued as soon as feasible, taking into account local circumstances, the individual woman's situation and the risks of replacement feeding (including infections other than HIV and malnutrition)."

The latest technical review on the optimal feeding of low-birth weight infants by the World Health Organisation [54] supports the above-mentioned recommendations on feeding infants of HIV positive women, stating that "there is no difference in the recommendations for normal and LBW infants."

The majority of infant HIV-1 infection can be prevented by following the three-pronged approach of antiretroviral therapy, caesarean section and complete avoidance of breastfeeding. These interventions, however, are difficult to implement in many of the resource-poor countries where there is no established infrastructure present. The efficacy of interventions to prevent HIV-1 transmission must be balanced against cultural acceptance, baseline infant mortality rates and the availability of resources (such as infant milk formula and clean water).[30, 55] A number of possible alternative feeding practices to breastfeeding in the presence of HIV have been proposed (Table 1.4).

Table 1.4 Possible alternatives to breastfeeding in HIV-infected mother-infant pairs

Alternative method	Comments
Use of wet-nurses or donor milk banks	Transmission of infection still possible, depending on status of donated milk
Heat treatment of milk	Impractical for sustained home use
Exclusive breastfeeding, with early abrupt weaning	It is not yet possible to specify an optimum time for weaning that would balance the advantage of breastmilk with a reduction in the risk of postpartum MTCT.
Reduced amount of breastfeeding mixed with replacement feeding	Preliminary data suggest this approach to be harmful rather than helpful
Antiretroviral therapy for mothers and exposed infants	Proven efficacy in developed countries, but expensive and not currently sustainable in the developing world.

(Adapted from references [19, 21] and [84].)

In developed countries the incorporation of the above-mentioned three-pronged approach, coupled with increased prenatal HIV-1 counselling and testing, has resulted in MTCT of HIV-1 rates of 2% or less.[21, 47]

The effect of anti-retroviral treatment on HIV-1 transmission risk:

Single-drug maternal prepartum, plus maternal intrapartum, plus infant postpartum anti-retroviral therapy can decrease peri-natal HIV transmission.[4] Recent clinical trails have demonstrated the efficacy of giving short-course antiretroviral treatment in reducing intra-uterine and intrapartum transmission.[36]

The disadvantage of peri-natal anti-retroviral therapy to the mother and infant includes a proposed increase in the incidence of premature delivery. The afore-mentioned finding was not supported by a meta-analysis of different studies. The meta-analysis included data on 3266 women with HIV infection delivering infants from 1990 to 1998. The results showed no difference in the rates of premature delivery (<37 weeks), low birth weight (<2500g), very low birth weight (<1500g), or low APGAR score (<7) in the 1143 women/infants who did not receive anti-retroviral therapy, compared to the rates of the 2123 women/infants treated with anti-retrovirals during pregnancy.[56]

1.3.1 Factors present in breast milk with anti-retroviral activity

Breast milk contains non-specific factors that may have direct activity or cause inhibition of binding of the HIV to the CD4 T-cell receptor.[35] Table 1.5 indicates these factors which are present in breast milk.

Table 1.5 Factors present in breast milk that may influence the vertical transmission of HIV-1 from mother to infant

Breast milk component	Action on HIV
Secretory Immunoglobulin A (sIgA), Immunoglobulin G (IgG), Immunoglobulin M (IgM)	Provides antigen-specific immunity.[25, 57] IgA and IgG antibodies to viral components such as gp-120 and IgG antibodies against HIV envelope protein have been identified in breast milk. It is proposed that IgM and IgA anti-HIV-1 in breast milk may protect against postnatal transmission of the virus.[40]
Glycosaminoglycan	A non-immunologic factor that inhibits the binding of the glycoprotein (gp-120) envelope of the virus to the host's receptor cells.[57, 58]
Free fatty acids and monoglycerides	Inactivate enveloped viruses by causing disintegration of the viral envelope.[25] Antiviral activity is enhanced by lipolysis of the milk fats by lipoprotein lipase,[59] but Chantry et al[60] found inherent lipolytic activity of fresh breast milk to be inadequate for the destruction of HIV-1.
Anti-oxidants (i.e. glutathione, cysteine, ascorbate, α-tocopherol and β-carotene)	Glutathione and its precursor cysteine may inhibit the activation of nuclear factor-kappa B (NF-κB) which is the HIV transcription factor.[61] The importance of the anti-oxidant content of breast milk in modulating the outcome of gene expression in the HIV-infected cell needs to be studied. Maintaining an optimal anti-oxidant status in the mother may be one intervention that can reduce virus expression.[62]
Secretory leucocyte protease inhibitor	Is a serine protease inhibitor and has antiviral activity, but has not been found to be a major determinant in preventing HIV transmission through breast milk.[25, 57]
Lactoferrin	Bacteriostatic through its iron-sequestering properties, bactericidal through the action of lactoferricin B (a peptide derived from lactoferrin) which inhibits HIV replication (shown in vitro). [25, 57]
Lysozyme	Bactericidal through lyses of susceptible bacteria by cleaving peptidoglycans off bacterial cell walls.[57]
Lactadhedrin	Inhibits rotavirus replication.[57]

Table 1.5 Factors present in breast milk that may influence the vertical transmission of HIV-1 from mother to infant (cont.)

Breast milk component	Action on HIV
Mucins	Inhibit bacterial adhesion to mucosa.[57]
Complement factor	Effect not known.[63]
Growth factors, e.g. Epidermal growth factor and transforming growth factor β	May enhance gut epithelial barrier, thus maintaining its integrity and hindering passage of virus.[64]
Chemokines, i.e., Interleukin 8 (IL-8) and Regulated on activation of normal T-cell expressed and secreted (RANTES)	IL-8 influences the neutrophil activity. RANTES inhibit HIV infection in vitro by interacting with the HIV-1 co-receptor on the CD4 lymphocytes.[57]
Oligosaccharides, e.g. lactodifucotetraose, lacto-N-fucopentaose I,II,III, monofucosyllacto-N-hexaose III, Lewis X-and Lewis Y sugar epitopes	Inhibit bacterial and viral binding to mucosa.[25, 57] Lewis x motif present in human milk possibly binds to DC-SIGN and prevents the capture and subsequent transfer of HIV-1 to CD4 T-lymphocyte cells.[65]

1.3.2 Exclusive breastfeeding

Coovadia et al has recently assessed the HIV transmission risks and survival associated with exclusive breastfeeding and other types of infant feeding.[66] The mentioned study found that infants receiving mixed feeding (i.e. fluid such as infant milk formula and/or complementary food, together with breastfeeding) before or after 14 weeks of life are at a significantly higher risk (almost double the risk) of MTCT through breastfeeding compared to exclusively breastfed infants.[66] This is attributed to the lower exposure of exclusively breastfed infants to bacterial contaminants and food antigens, which can damage the gut lining. Other liquids and foods may compromise intestinal integrity, resulting in small lesions in the immature gut, through which HIV can pass to infect the infant. Mothers who breastfeed exclusively might also follow practices that minimise breast inflammation, mastitis and cracked or bleeding nipples.[4] The effect of exclusive breastfeeding on MTCT of HIV was also investigated by Rollins et al[67], who examined two infant-associated physiologic indicators, i.e. gut permeability and urinary neopterin excretion, and found that neither appeared to explain which infants

became HIV-infected or why exclusive breastfeeding may be protective against postnatal HIV transmission.

John et al[30] suggests the following preventive measures for decreasing the risk of MTCT:
Breastfeeding HIV-1 positive mothers who develop mastitis (as defined by increased sodium levels in breast milk), bleeding nipples, or breast abscess should be discouraged from breastfeeding from the affected breast during the period of infection or bleeding.
A study conducted in HIV-1-infected lactating South African women determined breast milk sodium/potassium (Na/K) ratio and found a possible association with HIV-1 viral load, suggesting that in early lactation exclusive breastfeeding may protect against HIV transmission by decreasing the prevalence of sub-clinical mastitis and by preventing the increased viral load associated with raised milk Na/K ratio.[16]

In South Africa Coutsoudis et al[68] found that at 6 months of age, there was a substantially lower risk of transmission among infants who had been exclusively breastfed during the first 3 months, compared with infants who had received mixed feeding. The Zvitambo trial[69] in Zimbabwe recently found that exclusive breastfeeding in the first 3 months of life was associated with only a 1.3% risk of HIV-1 transmission from 6 weeks to 6 months of life. This was significantly lower than the transmission risk of 4.4% associated with mixed feeding. A possible explanation for this finding includes that damage to the intestinal mucosa from early introduction of food other than breast milk leads to delayed closure of the enterocyte junctions in the intestinal mucosal barrier. Another suggestion is that intestinal immune activation from early introduction of foreign antigens or pathogens may enhance HIV-1 transmission to the infant.[70]

Clinical trials are currently evaluating how effective exclusive breastfeeding for 6 months followed by rapid weaning is in reducing the transmission of HIV-1.[18] One such trial, is the ongoing ZEBS study in Zambia, which objective is to determine whether abrupt versus gradual weaning has a lower risk of HIV transmission.[18, 209] The PROMISE for Exclusive Breastfeeding (EBF) research consortium is running a randomized controlled trial of the safety and efficacy of EBF promotion by peer-counsellors among both HIV-uninfected and HIV-infected mothers in Burkina-Faso, Uganda, Zambia and South Africa.[18]

1.3.3 Pasteurisation of breast milk

Hospitals often use pasteurisation of manually or pump expressed breast milk successfully for sick and low birth weight infants, regardless of HIV status, who are at greater risk for infections when they receive artificial feeding and who may otherwise require special types of formula. There is little documented experience on the long-term (i.e. several months) use of breast milk expression and pasteurisation for exclusive breastfeeding, and also no evidence whether adequate milk supply can be sustained in the absence of the suckling stimulus.[53]

1.3.3.1 Effect of pasteurisation on breast milk composition

Pasteurisation of breast milk refers to a technique where containers with breast milk are heated in a water bath to a temperature of 56 – 62.5°C for a period of time (usually 30 minutes), after which the milk is cooled.[71] Pasteurisation does alter the nutrient concentrations found in breast milk and have been studied previously (Table 1.6 - Table 1.7).

A possible disadvantage of feeding pooled pasteurised breast milk to premature infants is a slower rate of weight gain, which could be attributed to the pasteurisation process, causing destruction of the heat-labile milk lipase.[11, 72] In most instances these premature infants would receive donor breast milk from lactating women who gave birth to term infants. The composition of the term donor breast milk differs from the preterm breast milk and might be a further cause, in addition to the increased energy and nutrient requirements of the premature infant, of the slower rate of weight gain experienced by these premature infants.

Table 1.6 The effect of pasteurisation on the macronutrient composition of breast milk

Nutrient/ Component	Effect of Pasteurisation
Protein:	Heating denatures milk protein, alter their electrophoretic pattern and may increase digestibility through better availability of the protein molecule for enzymatic cleavage. Denaturation of proteins is likely to lower their allergenicity. The Maillard-reaction might occur between free amino acids and lactose. [11, 72, 73]
Nitrogen	Absorption not affected.[11, 72, 74]
Immunoglobulins: Secretory A G M	 0-30% destroyed[11, 25, 71, 74, 75] 34% destroyed[71, 74] Mostly destroyed[63]
B- and T-cell lymphocytes	Some destroyed[75]
Bifidus growth factor	0-30% destroyed[74]
Transforming growth factor-α and β2	Well-preserved[76]
Lactoferrin	38% - 60% destroyed[11, 71, 74, 75, 77]
Lysozyme	0-30% destroyed[11, 71, 75]
Carbohydrate: Lactose	 Little or no effect[78]
Oligosaccharides	Stable during pasteurisation[63]
Amylase enzyme	15% reduction in activity[79]
Fat: Total fat content	 Remained the same[63, 80]
Saturated, poly- and mono-unsaturated fatty acid distribution	Not affected[79, 80]

Table 1.7 The effect of pasteurisation on the micronutrient composition of breast milk

Nutrient/ Component	Effect of Pasteurisation
Free fatty acids	Increased, due to lipolysis[75]
Triglycerides	6% decrease in levels, due to lipolysis[75]
α-Linolenic acid (Ω-3) Linoleic acid (Ω-6)	Not destroyed, but highly susceptible to oxidation due to their large amount of double bonds, which is counteracted by the high anti-oxidant activity found in breast milk.[79]
Long-chain poly-unsaturated fatty acids (Arachidonic acid, Docosa-hexanoic acid)	Not affected[79]
Lipase enzymes: Lipoprotein lipase Bile-salt activated lipase	Both types of lipase are totally inactivated.[72, 75, 79] Intestinal absorption of milk lipids decreased by 27% in very low birth weight infants fed pasteurised breast milk.[80]
Vitamin A	Little or no effect [80, 81] vs 10% increase in levels[77]
Vitamin D	Little or no effect[80, 81]
Vitamin E	20% decrease found with Pretoria pasteurisation method[77]
Vitamin K	Little or no effect[80, 81]
Thiamine (B1)	45% increase in levels found after Pretoria pasteurisation[77]
Riboflavin (B2)	15% decrease in levels[81] vs 6% increase in levels[77]
Niacin (B3)	Little or no effect[80, 81]
Pyridoxin (B6)	Little or no effect[77]
Cobalamin (B12)	31% decrease in levels[81]
Pantothenic acid	36% decrease in levels[81]
Folic acid	43 % increase in levels found after Pretoria pasteurisation[77]
Ascorbic acid (C)	32% decrease found with Pretoria pasteurisation[77]
Minerals and trace elements: Sodium, Potassium and Chloride	Little or no effect on absorption and retention[72]
Calcium, Phosphorous, Magnesium	Little or no effect on absorption and retention[72, 78]
Copper	Little or no effect[78]
Iron	Little or no effect[78]
Zinc	Redistribution of the zinc concentrations found in breast milk i.e. decreased zinc in the fat- and increased zinc in the whey fraction. Possible alterations in the zinc-binding pattern, which may reduce zinc bioavailability in the premature infant.[78]

1.3.3.2 *Effect of pasteurisation on HIV transmission*

HIV is a fragile virus that is easily inactivated by heat. The time required to reduce HIV infectivity by 1 log in culture medium at 56°C is 121 seconds. Holder pasteurisation (62.5°C for 30 minutes, followed by rapid cooling) of breast milk has been shown to destroy reverse transcriptase activity, thus inhibiting the transmission of HIV-1. The intrinsic factors of human milk that may be responsible for the inhibition of HIV-1 transmission were found to survive pasteurisation and actually be enhanced at higher temperatures.[62, 82]

The advantage of pasteurisation at the above-mentioned temperatures is that it preserves most of the protective factors in the milk, including secretory IgA. Pasteurisation of breast milk at 56°C for 15 minutes allows preservation of activity of 90% of the protective factors and effectively destroys pathogenic bacteria such as Escherichia coli.

During 1999 a new method was developed which allowed HIV-infected women to pasteurise their own expressed breast milk at home, thus offering their infants many of the benefits of breast milk without the risk of HIV transmission associated with ordinary breastfeeding and without the cost of infant milk formula.[82]

This method needed to make use of a passive transfer of heat to the breast milk, to prevent the milk from becoming too hot and destroying the immunological components in the milk. This method also needed to repeatedly be able to heat the milk to within a narrow temperature range in non-laboratory conditions without using a thermometer or thermostat. The heat source chosen was a container of water which had been heated to boiling point. Heating the water to boiling point in an electrical kettle was later added as an alternative to the original method, as more of the mothers have access to electricity. Although the boiling point of water varies according to altitude and barometric pressure, it remains in a narrow range unless it is at an extremely high altitude. By varying the volume of water and sizes, shapes and materials of the milk and water containers, it is possible to control the peak temperature of the milk and the time for which it remains above a certain temperature. The ideal temperature range was considered to be between 56 and 62.5°C. The process described was called Pretoria Pasteurisation and was developed by investigators from Pretoria University, South Africa.[82]

A study was undertaken to test the reliability of the Pretoria Pasteurisation technique under different conditions. The testing was done on cow's milk as surrogate for human milk for both practical and ethical reasons. The process was repeated 10 times for each set of starting values and found to be reliable, with a narrow 95% CI for each set of starting conditions tested. Despite the variation of the volume (50-150ml) and starting temperature (between ambient and 37°C) of the milk, milk temperature remained between 56 and 62.5°C for 10 to 15 minutes. It is unknown whether this length of time is sufficient to inactivate all cell-free and cell-associated HIV in the breast milk of infected women. Heat inactivation is necessary for the inactivation of cell-associated HIV, while a reduction in

the titre of cell-free HIV is achieved by leaving breast milk to stand for 30 minutes at room temperature.[82]

One of the concerns regarding the expression of breast milk is potential contamination with bacteria from women's hands. Pasteurisation of breast milk at 56°C for 15 minutes, however, was found to be effective in destroying common pathogens including Escherichia Coli and Staphylococcus aureus.[60] The investigators concluded that the process needed refining and further testing, but had the potential to offer an alternative method of infant feeding to women who do not have access to safe infant milk formula feeding and may reduce the vertical transmission of HIV in resource-poor communities, while retaining most of the benefits of breastfeeding.[82]

A follow-up study was devised to determine whether the Pretoria Pasteurisation technique effectively inactivated HIV in human milk. Samples of expressed breast milk were obtained from a group of HIV positive and -negative mothers. The samples of milk from the HIV negative mothers were inoculated with high titres of both cell-free and cell-associated HIV. Each sample was then divided into a control- and study portion after which the study portion underwent Pretoria Pasteurisation, followed by inoculation into lymphocyte co-culture of both portions for a period of 35 days. Results indicated viral RNA was present in the breast milk of 80% of the known HIV positive mothers. Evidence of viral replication was shown in nine of the 51 control specimens, with no evidence of viral replication detected in any of the 51 study specimens following Pretoria Pasteurisation. It was concluded that Pretoria Pasteurisation effectively inactivates HIV in human milk as well as in culture medium and could therefore be considered as a feeding option for the infants of HIV positive women.[15]

The findings of Jeffery et al[15] confirmed those of previous studies indicating that the process of HIV-1 inoculation and pasteurisation (62.5°C for 30 minutes in a water bath) effectively inactivated the infectivity of both cell-free HIV-1 and cell-associated HIV-1, with no virus being recovered even after repeated sub-culturing to rescue the virus. Pasteurisation reduced the infectious titre of cell-free HIV-1 and cell-associated HIV-1 by more than 5 logs and 6 logs respectively.[60, 83]

The present culture-based methods of evaluating the efficacy of inactivating HIV-1 in breast milk are insensitive, slow, technically demanding and lack precision. A study was therefore conducted to test a new approach for measuring HIV inactivation based upon the destruction of reverse transcriptase activity that is sensitive, rapid and precise. Two pasteurisation methods, i.e. Pretoria Pasteurisation and Flash-heating (method described on page xvi), were tested for their ability to inactivate 3 logs or more of HIV-1 reverse transcriptase activity. Results indicated that Flash-heating appeared to be superior to the Pretoria pasteurisation method in eliminating residual reverse transcriptase activity.[5, 77] The authors of this study do recommend that their study's results must be verified by a larger sample of naturally infected breast milk samples of HIV positive mothers from developing countries.[77]

Additional advantages of feeding pasteurised expressed breast milk is that it remains nutritionally superior to other replacement feeds, maintains some immunological protection, avoids risk of allergy and costs very little.

The success of feeding pasteurised breast milk may however be hindered by continued avoidance of suckling the infant, as well as contending with family and community pressures. Pretoria Pasteurisation is useful for feeding premature infants in the hospital setting, while its' use in a domestic setting and for term infants require further investigation. The latter has been experienced at the Cato Manor site in Durban, South Africa, where mothers exposed to heat-treated breast milk as a safe feeding alternative, had a very low uptake of this feeding method. One of the main reasons given by the mothers for not implementing this method was that it was impractical due to the fact that heat-treatment of breast milk is a time-consuming process, especially when formula feeding is readily available as a feeding alternative. [84]

1.4 The Effect of Maternal Nutritional Status on Breast Milk Composition

Walker et al[85] addressed the question of a mothers' lactation capacity in the light of an inadequate dietary intake in 1952 already. It was found that even under very adverse conditions (such as concentration camps and prisoners of war) breastfeeding may be maintained with a varying degree of success. Unfortunately data on the yield and composition of the breast milk produced under the mentioned circumstances was not available. In 1978 Jelliffe et al[88] investigated this further when he evaluated the volume and composition of human milk in poorly nourished communities. It was found that breast milk volume varied greatly, but seemed to be the lowest in communities with poor levels of nutritional status and inadequate living conditions. The investigators also found that the breast milk composition of poorly nourished women was surprisingly good, although breast milk composition may have been affected by inadequate maternal nutrition, depending on the severity and length thereof, as well as the mother's previous nutritional status.[88]

Information on the breast milk composition of black South African mothers was investigated because the high cereal diet, although seldom deficient in energy, was inadequate in various respects according to accepted standards. For the nutrients determined, no correlation was apparent between the nutritional status of the mother and the breast milk composition. It was suggested that either the role of diet in lactation is over-emphasised, or alternatively, the body has a great capacity to adapt itself to an inferior diet.[85]

As research in this field continued, results contradictory to the above-mentioned became evident. The WHO declared in 1985 that the composition and volume of breast milk may be affected by inadequate maternal nutrition, depending upon its severity, length and the mother's previous nutritional status.[86] The vitamin content of breast milk is affected by factors such as maternal vitamin intake and

subsequent maternal vitamin nutritional status. In general, when maternal vitamin status is low, breast milk contents are correspondingly lower and respond rapidly to maternal intake. Water-soluble vitamins are particularly influenced, especially ascorbic acid and riboflavin. Breast milk vitamin D and K content, even in well-nourished women, may not provide adequate amounts for the infant, which confirms the supplementation of these two vitamins in infants.[65] The levels of vitamin A and the fatty acid pattern also reflect the mother's diet and stores. Contrary to their organic counterparts, the inorganic constituents (i.e. minerals) in breast milk do not correlate with amounts in the maternal diet or maternal serum.[87] The protein content of human milk in poorly nourished women usually falls within or just below normal limits.[88]

The effect of maternal malnutrition seems to be specific rather than non-specific, as confirmed when some of the antimicrobial proteins in breast milk were investigated. It was found that mean concentrations of IgA, lysozyme and complements C3 and C4 in malnourished Chinese women where only half of the levels found in well-nourished women[89], while a study conducted in Caucasian and Aboriginal women in Australia found significantly higher lactoferrin concentrations in women with a weight-for-height greater than 90%.[90] These findings mostly contradict a previous study that observed no difference in milk lysozyme or IgA contents between well and malnourished mothers. Only IgA levels were found to be higher in the colostrum of the well-nourished mothers.[91]

A few years later the influence of deprived maternal nutrition on the antibacterial factors of human milk at different stages of long-term lactation was investigated in urban and rural mothers in Zaire and compared with European (Belgian) mothers. As lactation progressed, lactoferrin levels decreased by 33% and 55% in the urban and rural mothers respectively, whereas secretory Immunoglobulin A remained unchanged and lysozyme increased steadily. There was more lysozyme and s-IgA in the rural milk, contrasting with the poorer maternal nutrition. The investigators were thus unable to demonstrate any correlation between maternal nutritional status and milk concentration of the three mentioned antibacterial proteins investigated, but it was determined that the main factor influencing the amount of antimicrobial proteins effectively fed to infants is the milk yield. The milk yield, however, is influenced by the maternal nutritional status.[92]

The aspect of genetic differences in lactation ability in different human groups has never been explored adequately. This might be an important factor linked to variation in diet and exposure to different forms of environmental and psychosocial stress between different ethnic groups.[87, 88] This review concluded that the adequacy of breast milk as the sole source of food for the infant is related to the mother's diet during pregnancy, to maternal energy reserves in the form of subcutaneous fat, to fetal stores and to the iron obtained from placental transfusion.[88]

The fatty acid distribution but not total fat content of breast milk may partially reflect the mother's diet. A diet high in poly-unsaturated fatty acids and low in energy can affect the milk-fat concentration and composition found in breast milk.[87, 93] Table 1.8 gives an indication of some of the factors that may affect milk fat content and composition.

Table 1.8 Factors influencing breast milk fat content and composition[87]

Factor	Influence
Duration of gestation	Shortened gestation increases the long-chain poly-unsaturated fatty acids secreted
Stage of lactation	Phospholipid and cholesterol contents are highest in early lactation
Parity	High parity is associated with reduced endogenous fatty acid synthesis
Volume	High volume is associated with low milk fat content
Feeding	Human milk fat content progressively increases during a single nursing (fore-milk versus hind milk)
Maternal diet	A diet low in fat increases endogenous synthesis of medium chain fatty acids (C6 to C10)
Maternal energy status	A high weight gain in pregnancy is associated with an increase in milk fat

1.5 The Composition of Preterm versus Term Breast Milk

Three distinct phases in breast milk composition are present during the first month of lactation. Colostral milk is defined as breast milk produced during the first 7 days of lactation. Transitional milk is produced from lactation day 8 to 21. Mature breast milk is found after 21 days of lactation.[14] These three phases in breast milk composition remain, irrespective of the gestational age of the infant at birth.

There is, however, a further difference in breast milk composition due to the gestational age (i.e. preterm or term) of the infant at birth. A recent study analysed the nutritional content of breast milk from human milk banks to determine the level of supplementation required when this donor breast milk is fed to low birth weight infants. Differences in the composition of pasteurised mature and premature breast milk have been studied (Table 1.9). Preterm milk has been noted to have increased amounts of nitrogen, total protein, immuno-proteins, total lipids, medium-chain fatty acids, total energy, some vitamins and minerals, as well as trace elements. The long-chain poly-unsaturated fatty acids (especially docosa-hexaenoic acid and arachidonic acid) found in both term and preterm milk has been implicated in optimal brain development and retinal maturation. The degree of prematurity and whether infants are born appropriate or small for gestational age may also play a role in milk composition. Some studies have not found a difference between term and preterm milk composition, but no studies have found lesser concentrations of nutrients in preterm milk at similar stages of lactation. The lack of agreement between studies may reflect small sample size, greater inter-

individual variability of milk composition in preterm milk, or variation in milk sample collection methods and inclusion of wide ranges of gestational age.[74]

In addition, preterm milk seems to have a higher concentration of growth factors and hormones to aid in the development of the gastro-intestinal tract and other organs. Preterm milk contains more infection-fighting cells, immuno-globulins like secretory Ig-A, anti-inflammatory factors and immune-modulators than term milk. Both term and preterm milk seem to have the same trend of a decrease in nutrient and immunologic factor concentrations as lactation progress.[74]

The above-mentioned compositional differences were also found by previous studies. Gross et al[95] found the breast milk of mothers delivering preterm contained significantly higher concentrations of protein[96, 97], sodium and chloride, while concentrations of lactose were significantly lower than in milk produced by mothers delivering at term. During the first four weeks of lactation the lactose, lipid and energy concentrations of the preterm milk increased, while the nitrogen content decreased steadily to reach a composition similar to that of term milk.[96] The energy concentration of milk produced by mothers with term and premature infants was similar, as were the concentrations of potassium, calcium, phosphorus and magnesium. It appears that preterm milk approximates more closely the nutritional needs of the preterm infant than does term breast milk.[95]

Table 1.9 Compositional difference of pasteurised mature and premature breast milk (as found by one study)[94]

Nutrient	Unit	Mature milk	Premature milk
Fat	g/100 mL	2.56 (± 0.8)	2.48 (± 0.76)
Lactose	g/100 mL	8.6 (± 0.9)	6.56 (± 1.41)
Protein	g/100 mL	1.07 (± 0.22)	1.72 (± 0.4)
Energy	kcal/100 mL	61.67 (± 8.92)	55.44 (± 8.00)
Calcium	mg/100 mL	17.88 (± 5.56)	22.03 (± 9.39)
Magnesium	mg/100 mL	2.15 (± 0.39)	2.16 (± 0.26)
Potassium	mg/100 mL	35.53 (± 7.54)	44.37 (± 12.83)
Sodium	mg/100 mL	16.27 (± 5.92)	37.98 (± 11.34)
Zinc	mg/100 mL	0.46 (± 0.26)	0.72 (± 0.26)
Phosphorus	mg/100 mL	9.98 (± 1.72)	8.47 (± 2.43)

Although differences in nutrient composition of preterm and term milk remain uncertain, study authors agree that these differences disappeared after the first month of lactation and that variability in nutrient content (protein, fat and minerals) is higher in preterm than in term milk.[98]

1.6 Breast Milk Expression Techniques and their Influence on Breast Milk Volume and Composition

Manual expression of breast milk by hand compared with suction usually produces less volume and fat.[99] It is known that the adequacy of breast emptying influences milk composition as a result of differences between fore- and hindmilk, with a higher fat concentration in the latter. Collection methods that differ in their breast milk emptying efficiency may therefore yield milk of varying composition.[100] A limited number of studies in mothers of term infants have shown differences in milk volume and composition according to the method (i.e. manual or hand pump) of breast milk expression used.[101] Few studies, however, have quantitated the proposed compositional differences which are expected between different milk expression techniques under controlled conditions.[102] Simultaneous pumping of the breasts is more effective at producing milk than sequential pumping and breast massage has an additive effect, improving milk production in the immediate period after preterm delivery.[101, 103] Fat content seem to be higher when simultaneous pumping is used compared to sequential pumping.[103]

1.7 Optimal Feeding of the Premature Infant

1.7.1 Enteral nutritional needs of the premature infant

Two successive periods are described in the nutrition of premature infants, namely:
1) The early adaptive or "transition" period, which lasts from birth to the second week of life, and
2) The "stable-growing" period, which lasts up to discharge from the neonatal unit (i.e. ±55 days postpartum).[104]

Protein intake and protein to energy ratio are the main determinants of growth and body composition during the stable, growing period. Nutritional deprivations arise in the early adaptive period as well as from several neonatal events and illness; therefore additional recommendations are suggested to promote early catch-up growth before discharge. These recommendations (Table 1.10.) are in line with previous guidelines from other Committees (AAP, ESPGHAN, CPS LSRO/ASNS).[104]

Table 1.10: Suggested enteral and parenteral protein intake during the transition period[104]

Protein intake during the transition period:
To achieve a zero nitrogen balance: • 0.75 g/kg/day in healthy, growing preterm infants (if on enteral feeding of 110 kcal/kg/day). • 1.0 – 1.5 g/kg/day during the first days of life when energy supply is limited (parenteral feeding when only "maintenance" energy intake of 50 – 60 kcal/kg/day is provided). • 1.5 – 2.0 g/kg/day during the first days of life if concomitant catabolic conditions are present.
To achieve a positive nitrogen balance: 2.5 – 3.0 g/kg/day during the first days of life when energy supply is limited (i.e. ±50 - 60 kcal/kg/day).
Progressive increase in energy and protein supplies to recommended values for conceptional age.[104, 105]

Human milk is the recommended form of enteral nutrition for premature infants. The milk could be from the infant's mother or expressed milk from donor mothers.[84] Human milk has non-nutrient advantages for premature infants, primarily through the delivery of immuno-protective and growth factors to the immature gastro-intestinal tract mucosa.[106]

In 1981 it was postulated by Anderson[96] that premature infants fed their own mothers' milk at volumes of 150-200 ml/kg/day would be provided with sufficient quantities of protein, energy, sodium, chloride, potassium and magnesium to meet their requirements in the early weeks of life.

Current premature infant feeding regimens promote weight gain due to body fat rather than gain in lean body mass. This is due to a contrast between the usual nutrient supply to the fetus (high amino acids and sufficient glucose) and what the low birth weight infant is fed (high intakes of lipid and glucose but low protein intake). It is therefore suggested that the nutritional requirements of the low birth weight infant and the outcomes likely from current feeding practices must be reconsidered. Replicating the body composition of the fetus of the same post-conceptional age as the premature infant is a more desirable nutritional goal than simply achieving the fetal rate of weight gain. Measuring actual body composition of very premature infants is difficult and few data are available.[2]

The amino acid and energy intakes necessary to support intrauterine rates of weight gain and protein accretion, whether administered via the enteral or parenteral route, are ±3g/kg/day and ±90kcal/kg/day. Initially most infants lose 10-20% of their initial weight and do not regain birth weight before at least two weeks of age. Even with intakes sufficient to support intra-uterine rates of nutrient accretion, the premature infant will remain behind the fetus of the same post-conceptional age for some time. An additional allowance for "catch-up" growth therefore needs to be provided to the premature infant.

Modern preterm milk formulas and supplemented human milk provide protein intakes of 3.3-3.6g/kg/day and an energy intake of ±120kcal/kg/day, which was previously considered to be sufficient to support growth and protein accretion rates that exceed intrauterine nutrient requirements; thus providing additional nutrients to achieve the recommended "catch-up" growth.[2, 107, 108, 109, 110]

Unfortunately, most infants fed the mentioned intakes still remain below the tenth percentile of intra-uterine standards at discharge. It is therefore clear that most premature and/or low birth weight infants could benefit from a higher protein intake.[2] Recent recommendations reflect this likely need for a higher protein content of human milk fortifiers and preterm formulas, with a recommended maximum protein content of 3.6g/100kcal/day (i.e. 4.3g/kg/day at an energy intake of 120kcal/kg/day) considered ideal for a healthy, growing premature infant.[104, 111] Caution, however, is advised when prescribing a high-protein diet, as excessive protein intakes have been reported to result in adverse neuro-developmental outcomes and are associated with evidence of metabolic stress such as acidosis and elevated blood urea concentrations.[112] There is furthermore an absence of data

evaluating long-term and adverse effects of protein supplementation additional to fortification of breast milk or a preterm infant milk formula.[113]

The above-mentioned recommendation on energy intake is confirmed by other research, which found little or no evidence that an energy intake of more than 120kcal/kg/day is desirable, as it will most likely only result in higher rates of fat accretion.[85] For the ELBW infant at least 130kcal/kg/day of enteral intake seems necessary to achieve a positive energy balance of 25 – 30kcal/kg/day. Up to 150kcal/kg/day may be required for this population group to achieve an energy balance closer to that of a healthy, growing, more mature premature infant.[104]

Contrary to the beneficial effect of "catch-up" growth in the short term, there remains the concept of "beneficial under-nutrition". This refers to the phenomenon of improved life expectancy following long periods of low growth rate due to relative under-nutrition. In humans, rapid rates of growth in infancy reportedly increases the risk for cardiovascular disease, hypertension, obesity and non-insulin dependant (Type 2) Diabetes Mellitus later in life, while slower growth appears to be protective against the later development of cardiovascular disease.[114]

Long-term prospective studies are needed to resolve the dilemma between potentially favourable effects of slower growth rates on future cardiovascular health versus demonstrated adverse effects of poor growth on neuro-developmental outcome.[115]

Excessive or inadequate intakes of essential fatty acids and iron are of particular concern in the premature infant, as both are crucial to normal central nervous system development, with the potential for long lasting effects that extend beyond the period of dietary insufficiency.[116]

Current knowledge on the premature infant's nutritional needs have led to the consensus recommendations for the ELBW premature infant and for the VLBW premature infant (Table 1.11 - Table 1.12 respectively).[117]

Table 1.11 Consensus Recommendations: Enteral Nutritional needs of the stable/ growing ELBW (<1000g) premature infants[117]

Nutrient	Unit	Enteral	
		Per kg/day	**Per 100 kcal**
Water	ml	160 - 220	107 - 169
Energy	**kcal**	**130 - 150**	**100**
Protein	**g**	**3.8 - 4.4**	**2.5 - 3.4**
Carbohydrate	g	9 - 20	6.0 - 15.4
Fat	g	6.2 - 8.4	4.1 - 6.5
Linoleic (Ω 6)	mg	700 - 1680	467 - 1292
Linolenic (Ω 3)	mg	1 - 4% of cal	110 - 440
C18:2 / C18:3		5 - 15 : 1	5 - 15 : 1
Arachidonic acid	mg	≥28	≥22
Docosahexaenoic acid	mg	≥21	≥16
Vitamin A (ug)	IU	700 – 1500 (210-450)	467 – 1154 (140-346)
Vit A in lung disease#	IU	1500 – 2800 (450-840)	1250 - 2333
Vitamin D	IU	150-400 (aim 400)	100 - 308
Vitamin E	IU	6 - 12 (max 25)	4 – 9.2
Vitamin K	ug	8 - 10	5.3 - 7.7
Vitamin C	mg	18 - 24	12 – 18.5
Thiamin (B1)	ug	180 - 240	120 - 185
Riboflavin (B2)	ug	250 - 360	167 - 277
Niacin (B3)	mg	3.6 - 4.8	2.4 - 3.7
Piridoxine (B6)	ug	150 - 210	100 - 162
Pantothenate	mg	1.2 - 1.7	0.8 - 1.3
Biotin	ug	3.6 - 6	2.4 - 4.6
Folate	ug	25 - 50	17 – 38
Vitamin B_{12}	ug	0.3	0.2 - 0.23
Sodium	mg	69-115 (up to 161*)	46 - 88
Potassium	mg	78 - 117	52 - 90
Chloride	mg	107 - 249	71 - 192
Calcium	mg	100 - 220	67 - 169
Phosphorus	mg	60 - 140	40 - 108
Magnesium	mg	7.9 - 15	5.3 - 11.5
Iron	mg	2 - 4	1.33 - 3.08
Zinc	ug	1000 - 3000	667 - 2308
Copper	ug	120 - 150	80 - 115
Selenium	ug	1.3 - 4.5	0.9 - 3.5
Chromium	ug	0.1 - 2.25	0.07 - 1.73
Manganese	ug	0.7 - 7.5	0.5 - 5.8
Molybdenum	ug	0.3	0.20 - 0.23
Iodine	ug	10 - 60	6.7 - 46.2
Taurine	mg	4.5 - 9.0	3.0 – 6.9
Carnitine	mg	~2.9	~1.9 - 2.2
Inositol	mg	32 - 81	21 - 62
Choline	mg	14.4 - 28	9.6 - 21.5

**: May require up to 160mg/kg/day in case of late hyponatremia.[104]*

#: Reference for this recommendation: 118

Conversion factors[3]: *Vit A: 3.33 IU = 1µg retinol; Vit D: 1 IU = 0.025µg cholecalciferol (Vit D_3); Vit E: 1.49IU = 1 mg d-α tocoferol*

Table 1.12 Consensus Recommendations: Enteral Nutritional needs of the stable/ growing VLBW (>1000g) premature infants[117]

Nutrient	Unit	Enteral	
		Per kg/day	**Per 100 kcal**
Water	ml	135 - 190	104 - 173
Energy	**kcal**	**110 - 130**	**100**
Protein	**g**	**3.4 - 4.2**	**2.6 - 3.8**
Carbohydrate	g	7 - 17	5.4 - 15.5
Fat	g	5.3 - 7.2	4.1 - 6.5
Linoleic (Ω 6)	mg	600 - 1440	462 - 1309
Linolenic (Ω 3)	mg	1 - 4% of cal	110 - 440
C18:2 / C18:3		5 - 15 : 1	5 - 15 : 1
Arachidonic acid	mg	≥24	≥22
Docosahexaenoic acid	mg	≥18	≥16
Vitamin A (ug)	IU	700 – 1500 (210-450)	538 – 1364 (162-409)
Vit A in lung disease#	IU	1500 – 2800 (450-840)	1250 - 2333
Vitamin D	IU	150-400 (aim 400)	115 - 364
Vitamin E	IU	6 - 12 (max 25)	4.6 - 10.9
Vitamin K	ug	8 - 10	6.2 - 9.1
Vitamin C	mg	18 - 24	13.8 – 21.8
Thiamin (B1)	ug	180 - 240	138 - 218
Riboflavin (B2)	ug	250 - 360	192 - 327
Niacin (B3)	mg	3.6 - 4.8	2.8 - 4.4
Piridoxine (B6)	ug	150 - 210	115 - 191
Pantothenate	mg	1.2 - 1.7	0.9 - 1.5
Biotin	ug	3.6 - 6	2.8 - 5.5
Folate	ug	25 - 50	19 - 45
Vitamin B_{12}	ug	0.3	0.23 - 0.27
Sodium	mg	69-115 (up to 161*)	53 - 105
Potassium	mg	78 - 117	60 - 106
Chloride	mg	107 - 249	82 - 226
Calcium	mg	100 - 220	77 - 200
Phosphorus	mg	60 - 140	46 - 127
Magnesium	mg	7.9 - 15	6.1 - 13.6
Iron	mg	2 - 4	1.54 - 3.64
Zinc	ug	1000 - 3000	769 - 2727
Copper	ug	120 - 150	92 - 136
Selenium	ug	1.3 - 4.5	1.0 - 4.1
Chromium	ug	0.1 - 2.25	0.08 - 2.05
Manganese	ug	0.7 - 7.5	0.5 - 6.8
Molybdenum	ug	0.3	0.23 - 0.27
Iodine	ug	10 - 60	7.7 - 54.5
Taurine	mg	4.5 - 9.0	3.5 - 8.2
Carnitine	mg	~2.9	~2.2 - 2.6
Inositol	mg	32 - 81	25 - 74
Choline	mg	14.4 - 28	11.1 - 25.5

**: May require up to 160mg/kg/day in case of late hyponatremia.*[104]

#: Reference for this recommendation: 118

Conversion factors[3]***:*** *Vit A: 3.33 IU = 1µg retinol; Vit D: 1 IU = 0.025µg cholecalciferol (Vit D_3); Vit E: 1.49IU = 1 mg d-α tocoferol*

1.7.2 Breast milk fortifiers

1.7.2.1 *Why are breast milk fortifiers needed?*

Preterm infants consuming fortified human milk show improved weight, length and head circumference gains.[119] When fed unsupplemented breast milk, these infants do not grow at the intra-uterine growth rate (of 15g/kg/day). Even if the total protein, calcium, phosphorus, sodium and zinc contents of a reasonable amount of breast milk were absorbed and completely retained, the amounts retained would still not be sufficient to support intra-uterine accretion rates.[2, 17, 109, 120, 121, 122, 123] Undoubtedly human milk still remains the optimal choice of feed for the term and preterm infant, but to meet the special nutritional needs of the very low birth weight infant, breast milk fortification is required and recommended.[75, 118]

When very low birth weight infants are fed pasteurised expressed own or donor breast milk, the decrease in some of the water-soluble vitamin levels (such as vitamin C, B6 and folic acid) necessitates the need for vitamin supplementation.[81] A further concern that vitamin A, ascorbic acid and riboflavin concentrations decline in human milk under conditions of light exposure as found during milk expression and passage through feeding tubes, confirms the proposed need for breast milk fortification when feeding premature infants.[124, 125]

Multi-nutrient fortifiers are available in South Africa to add to human milk. Fortification of human milk with calcium and phosphate may improve bone mineral content. Protein and energy supplementation of human milk increases the rate of weight gain and head growth, at least in the short term. Long-term follow-up studies are needed to determine if nutrient fortification of human milk improves neuro-developmental outcomes in premature infants.[106, 126]

Osteopenia of prematurity is an acquired condition associated with feeding premature infants unfortified human milk. Low dietary mineral intake is recognised as the major cause of metabolic bone disease in premature infants.[127, 128] Even when VLBW infants are fed at high rates (e.g. 200ml/kg/day), unfortified human milk can provide at most about one-third of the in utero level of calcium and phosphorous absorption.[17] Expert committees therefore all recommend the routine supplementation of calcium and phosphorus for VLBW premature infants fed human milk, but these recommendations vary greatly: from 70 – 192mg calcium/ 100kcal (i.e. 56 – 155mg/ 100 ml) and from 50 -117mg phosphorus/ 100kcal (i.e. 40 – 95 mg/ 100 ml).[17, 111, 118, 128, 129, 130]

It is recommended that a human milk fortifier is added to the breast milk of all premature infants with a birth weight of less than 2000g and/or gestational age younger than 35 weeks'.[120]

The duration of fortification of breast milk in the premature infant remains unclear. The desirability of promoting catch-up growth and bone mass in preterm infants after hospital discharge has been

proposed by previous studies.[131, 132] Without additional supplementation after hospital discharge, it was found that low birth weight infants took two years for their bone mineral content to catch up with similar infants fed infant milk formula.[120] Findings furthermore show that preterm infants are highly responsive to mineral intake during the early post-discharge period.[128, 133, 134, 135]

To prevent the occurrence of iron deficiency anaemia, iron supplementation is recommended when full enteral feeds have been established and the absence of infection has been confirmed.[107] The current recommendation for iron supplementation in premature infants is 2-4mg/kg/day which must be started from as early as two weeks to no later than 2 months after birth.[116, 126, 136]

The optimal method of fortification remains undetermined. Various methods of cow's milk based human milk fortification have been developed. The focus of these commercial fortifiers is to increase the content of energy, protein, calcium, phosphorous, sodium, vitamins and other minerals. Ideally the preferred fortification method would be an individualised approach, with the addition of separate vitamins, minerals, protein, carbohydrates and fat for each infant. However, for most nurseries the latter approach is not practical and provides little benefit to the use of commercial multi-nutrient fortifiers.[17, 121]

The ideal composition of breast milk fortifiers remains unknown.[119] Using complex modelling based on analytic data on 6000 breast milk samples, it was calculated that a protein supplement of approximately 0.7g/100ml is an optimal goal for human milk fortification.[137] When comparing different protein sources for the fortifiers, it was found that both bovine-derived and human-milk-derived protein sources were equally well utilised.[126] Fortifiers must furthermore aim to increase energy and carbohydrate content with 10kcal and 2g per 100ml breast milk respectively, as well as increase the calcium: phosphorus ratio and sodium intake.[138]

1.7.2.2 *Safety considerations with breast milk fortification*

With breast milk manipulation, feeding tolerance and hydration status need to be monitored as milk osmolality and/or potential renal solute load will increase. Milk osmolarity should be <400 mOsm/L of milk or an osmolality of <450 mOsm/kg of water. Potential renal solute load of up to 30 – 35 mOsm/100kcal has been suggested.[127, 139]

A case-control study from Finland evaluated the frequency of and risk for necrotising enterocolitis (NEC) in preterm infants and found that fortified human milk feedings were an overall risk factor for all cases of NEC, but not for severe NEC cases (Bell grades II-III). Usually only the more severe cases of NEC have a major impact on the ultimate outcomes of infants. This, however, was not a blinded, controlled trial and there was a large weight difference between patients with severe NEC and controls (926g versus 1440g, respectively).[17, 140] Outcomes of a randomised, controlled study found

that premature infants who consumed fortified human milk had a non significant higher incidence of NEC compared to control infants consuming preterm formula.[141]

Contrary to the above-mentioned findings, other investigators have reported a beneficial effect of human milk, even when fortified, over preterm milk formula. Infection, including NEC, was less frequently observed in infants who received fortified human milk.[137, 142] Possible explanations for this increased protection in the group fed human milk, are the presence of high immunoglobulin A levels in the human milk and the likely effect of more frequent skin-to-skin contact between mother and infant (i.e. kangaroo mother care).[142] A recent Cochrane review evaluating 13 studies on multi-nutrient fortification of human milk, confirmed that fortified human milk was not associated with significant adverse events, including NEC. The authors concluded that multi-nutrient fortification of human milk for premature infants is therefore an appropriate strategy at this time.[17, 119] The ideal fortifier, when mixed with breast milk, should not result in precipitation and sedimentation of mineral salts or reduce availability of the nutrients.[120, 137]

When the effect of fortification on the osmolality of human milk was evaluated for five different commercial breast milk fortifiers, it was found that human milk and human milk fortifier interact to induce a rapid increase in osmolality, which was higher than would be expected from composition alone. A possible explanation for this finding is the amylase activity in human milk, which induces hydrolysis of the dextrin content of the human milk fortifier and leads to small osmotically active molecules of oligosaccharides.[143] Substitution of some of the carbohydrate with fat could reduce the osmolality of the fortifier and further reduce the proposed increase in osmolality due to hydrolysis of the carbohydrate fraction.[144]

Bacterial colony counts increase over time with storage of fortified human milk. When fortified human milk was evaluated under simulated nursery conditions, bacterial colony counts were not significantly different after 20 hours' storage at refrigerator temperature. However, a small increase (with approximately one log difference) from 20 to 24 hours when maintained at incubator temperature did occur. These data suggest caution in the handling of fortified human milk.[126]

1.8. Motivation for the Study

One of the major mechanisms of the protection conferred through breastfeeding is by passive transfer of antibodies, immune-competent cells and cytokines. For mothers with HIV-1-related immune-compromise, it is unknown whether breastfeeding would confer the same magnitude of protection. No study to date has definitively evaluated the degree of protection that breast milk affords infants of HIV-1-infected mothers.[145] However, it has been shown that exclusive breastfeeding is protective against postnatal MTCT early in lactation (i.e. before 6 months of age) and that mixed feeding (i.e. breast milk

plus food-based fluid, solid food or infant milk formula) during the first month of life, significantly increases the risk of postnatal MTCT nearly 6-fold.[208]

Studies on nutritional intervention in HIV-infected infants and children are scarce.[28] Furthermore surprisingly little is known about the effect of breastfeeding on the health of HIV-1 infected mothers.[26] One [146] of two studies showed a 3.2 fold higher risk of death within two years among HIV-1-infected women who breastfed compared to those who formula-fed their infants, while the second study[147] found no additional risk. These 2 studies are however different in design with the first having a randomised and the second an observational design. Limitations in the data of both studies were that no detailed information on the mode, duration and quantity of breastfeeding and associated mortality risks were provided. The mothers in the two studies were also of different health status, thus suggesting a cautious interpretation of the findings. The findings are however important and additional research on this issue is required.[148]

WHO, UNICEF and UNAIDS recommend manually expressed, heat-treated breast milk as one alternative to breastfeeding for HIV positive mothers in developing countries.[149] Rollins et al[84] confirm that reliable knowledge about alternative approaches on how to make breastfeeding safe in the context of HIV is of scientific interest and also public health importance.

Very few studies have been performed regarding the effect of different methods of milk expression in mothers with preterm infants, a group in whom differences in breast milk composition due to the method of milk expression used, would be important.[101]

A much more complete knowledge of the nutrient content of milk from mothers giving birth prematurely is needed. Knowledge of the composition of preterm newborn mother's milk could be an important step for formulation of the most suitable diet for premature infants.[98]

The application of pasteurisation of HIV-infected breast milk must be studied further with regard to safety aspects and the maintenance of the protective elements in breast milk.[77]

In view of the lack of information currently available, the aim of the present study was to investigate the effect of pasteurisation on the composition of expressed human milk from HIV positive mothers, and its adequacy in relation to the growth of their very low birth weight premature infants.

CHAPTER 2: METHODOLOGY

2.1 Aim

The aim of the study was to investigate the effect of pasteurisation on the composition of expressed human milk from HIV positive mothers, and its adequacy in relation to the growth of their very low birth weight premature infants.

2.2 Objectives

2.2.1 Primary objectives

1. To determine the nutrient composition of expressed human milk of both HIV negative and HIV positive mothers' who delivered premature infants in Tygerberg Academic Hospital, situated in the Western Cape Region of South Africa.
2. To determine the effect of different milk expression techniques (hand versus pump) on the nutritional composition of preterm human milk.
3. To determine whether pasteurisation affects the nutritional composition of expressed human milk of both HIV positive and HIV negative mothers.
4. To determine the effect of maternal nutrient intake during pregnancy and lactation on the nutritional composition of breast milk.

2.2.2 Secondary objectives

To assess the adequacy of currently used feeding regimen protocols in achieving an optimal short-term growth rate in the premature infant in the region.

2.3 Null Hypothesis (N_0)

1. There is no difference in the nutrient composition of expressed human milk from HIV positive and HIV negative mothers of premature infants.
2. The milk expression technique (hand versus pump) used has no effect on the nutrient composition of expressed human milk of mothers of premature infants.
3. Pasteurisation has no effect on the nutrient composition of expressed human milk in HIV negative and/or HIV positive mothers of premature infants.
4. Maternal nutrient intake during pregnancy and lactation has no significant effect on the nutritional composition of breast milk.

2.4 Study Design

A descriptive, prospective case-controlled pilot study was undertaken. This was a pilot study, since no South African data currently exists with which this study's findings can be compared. If this study provided significant results, the latter would serve as a motivation for conducting a larger study in this field.

2.5 Study Site and Population

2.5.1 Study site

For the past four years an average of 860 low birth weight and premature infants were seen annually at Tygerberg Academic Hospital. Karl Bremer Hospital's Kangaroo Mother Care (KMC) unit treated an average of 300 premature infants annually. Eersterivier Hospital is a new hospital operating since September 2002. The majority of the premature infants treated at Karl Bremer and Eersterivier Hospitals are transfers from Tygerberg Academic Hospital. As Tygerberg Academic Hospital was the only recruiting hospital, the VLBW premature infant numbers of this hospital were used to determine the potential patient numbers for this study. The rate of HIV positive mothers as detected with the PMTCT program in Tygerberg Academic Hospital (calculated for the period: 01/04/2002 – 30/09/2003) was 6%. [150]

2.5.2 Study population

Only mothers of the Xhosa ethnic group were included in the study. The reason for the exclusion of other ethnic groups was based on the consequence that more detailed stratification of dietary and other data, due to such differences as ethnicity and subsequent different dietary intake patterns, might make the data too diverse to draw any conclusions. Furthermore, the decision to include only Xhosa mothers in the study was based on the number of subjects required for the study, since the selected population group has the highest incidence of HIV. An explanation of the above-mentioned follows:

Actual numbers (for the year 2003) from ward J2B in Tygerberg Academic Hospital (TBH) indicate a minimum of 76 very low birth weight (<34 weeks gestational age) premature infants of the Xhosa ethnic group born per year. Based on the current HIV positive rate of approximately 6% for the above-mentioned group, a minimum of 5 mothers per year would qualify for inclusion into the study (HIV positive) group. At least 71 mothers per year will qualify for inclusion into the control group.

Mothers furthermore had to be willing to stay in the hospital's Kangaroo Mother Care unit for four weeks (28 days) in order to be included in the study. A mother was only requested to stay in hospital if it was medically indicated for her infant to remain in hospital. Mothers and infants were allowed to be transferred from Tygerberg Academic Hospital to Karl Bremer Hospital or Eersterivier Hospital during this four-week period, as the latter two hospitals also formed part of the study.

2.5.3 Inclusion criteria

Control group:

Consenting in writing, consecutive HIV negative mothers, who gave birth to a singleton premature infant, were included. All mothers had to be able to express one full breast of milk per sample-collection day, to ensure that the breast milk composition was a reflection of fore and hind milk, as well as enable optimal analysis of all the nutrients studied. For the analysis of all control group participants' breast milk composition, 22 ml of the breast milk sample obtained was used. All participating mothers were between the ages of 19 to 35 years and belonged to the Xhosa ethnic group. All included premature infants had a gestational age of less than 34 weeks and a birth weight of less than 1500g. All participants were recruited in the neonatal ward J2 and later, upon conversion, ward G2 of Tygerberg Academic Hospital and followed up while they remained in the Kangaroo Mother Care (KMC) Units of Tygerberg Academic, Karl Bremer or Eersterivier Hospital. All consenting HIV negative mothers underwent a rapid-test determination after entry to the study, for confirmation of their prenatal HIV negative status.

Study group:

Consenting in writing, consecutive HIV positive mothers, who gave birth to a singleton premature infant and had decided to feed their infants pasteurised expressed breast milk, were included. Mothers were informed of the feeding options for their infants when they attended an antenatal clinic. All mothers who did not attend antenatal clinics were informed of the feeding options for their infants postpartum. All mothers had to be able to express one full breast of milk per sample to ensure that the breast milk composition was a reflection of fore and hind milk, as well as enable optimal analysis of all the nutrients studied. For the analysis of all study group participants' breast milk composition, 26 ml of the breast milk sample obtained was used. All participating mothers were between the ages of 19 to 35 years and belonged to the Xhosa ethnic group. All included premature infants had a gestational age of less than 34 weeks and a birth weight of less than 1500g. All participants were recruited in the neonatal ward J2 and later, upon conversion, ward G2 of Tygerberg Academic Hospital and followed up while they remained in the Kangaroo Mother Care (KMC) Units of Tygerberg Academic, Karl Bremer or Eersterivier Hospital. All consenting HIV positive mothers had to have a confirmed HIV positive rapid-test result, as obtained from the PMTCT-program during the prenatal period. Most HIV positive mothers were on the PMTCT treatment regimen and if anti-retroviral medication was prescribed, only mothers receiving Nevirapine® and Zidovudine® (AZT) were included. HIV positive mothers with a CD4 count of less than 200 were only included when they were not clinically sick and able to express breast milk for their infants. These mothers

were also informed that their infant had an increased risk for HIV, due to their advanced stage of AIDS.

2.5.4 Exclusion criteria

HIV negative or HIV positive lactating mothers with the following diseases/conditions present:

- Mothers known with substance (alcohol and drug) abuse.
- Mothers with known psychiatric disorders on medication therapy.
- Mothers on anti-neoplastic therapy.
- Mothers with renal disease treated with corticosteroids and immunosuppressive agents.
- Mothers on other medications that were contra-indications for breastfeeding (radiopharmaceuticals, ergot and its derivatives (e.g. methysergide), chloramphenicol, phenylbutazone, atropine, thiouracil, iodides and mercurials).[151]
- All HIV positive mothers on highly active anti-retroviral (HAART) therapy.
- Mothers delivering premature infants with severe malformations and/or developmental disabilities.
- Mothers participating in any other clinical trial.

2.5.5 Study plan

A chronological presentation of the study plan:

Day 0 - 5: Screening of all new premature infants admitted to ward J2B (and later G2) for identification of possible participants. (Day of birth = Day 0) During this period, all infants were fed either own raw or own pasteurised expressed breast milk, with the exception of one study group infant who received a semi-elemental infant milk formula in small quantities during days 1 to 3 after birth.

Day 6 after mother gave birth:

Actions on/to the mother	Actions on/to the infant
5ml Blood drawn for determination of Serum: Albumin, Transferrin, Ferritin, Hb, Hct, sIg A, Ig G, Vit A and CRP	1,5 ml blood drawn for determination of Serum: Albumin and Pre-albumin RNA-PCR determined only for infants with HIV positive mothers
Anthropometric measurements: Weight, height, upper arm circumference	Anthropometric measurements: Weight, length, head circumference
Finger prick for rapid test to confirm HIV status. Rapid test done once if result obtained indicated the mother was HIV negative. Rapid test done twice if result obtained indicated the mother was	

HIV positive.	
1ml of blood sample sent for CD4 and DNA-PCR determination in HIV positive mothers only	
Around 12h00 of Day 6: A full sample of breast milk expressed, either by hand or hand pump (it was the method not used the previous day) was obtained, of which 22ml of the control group mothers' and 26ml of the study group mothers' breast milk were used for nutrient analysis. Right or left breast chosen at random. Milk sample divided into 2 samples of at least 11 ml (HIV negative group) or 13 ml (HIV positive group) each. "Raw" milk sample was subdivided into 3 or 4* aliquots of 1 to 5ml each. Samples then either frozen or kept "fresh" before sent for analysis to different laboratories. Second milk sample (at least 11ml or 13ml) Pretoria-pasteurised before the sample was subdivided into 3 or 4 aliquots of 1 to 5ml each. All samples were either frozen or kept "fresh" before sent for analysis.	

Day 7 after delivery:

Actions on/to the mother	**Actions on/to the infant**
Around 12h00: A full sample of breast milk expressed, either by hand or hand pump (it was the method not used the previous day) was obtained, of which 22ml of the control group mothers' and 26ml of the study group mothers' breast milk were used for nutrient analysis. Sample division as for day 6. Right or left breast not used the previous day.	

During week 1 of study participation:

Actions on/to the mother	**Actions on/to the infant**
Socio-demographic and obstetric history assessment.	
Quantitative food frequency questionnaire was completed.	

During week 1 to 4 of study participation:

Actions on/to the mother	Actions on/to the infant
3 x 24-hour dietary intake during hospital recorded.	Daily monitoring of input and output.

Days 13, 20 and 27 (around 12h00):

Actions on/to the mother	Actions on/to the infant
A full sample of breast milk expressed, either by hand or hand pump (it was the method not used the previous day) was obtained, of which 22ml of the control group mothers' and 26ml of the study group mothers' breast milk were used for nutrient analysis. Right or left breast chosen at random. Sample division as for day 6.	

Days 14, 21 and 28 (around 12h00):

Actions on/to the mother	Actions on/to the infant
A full sample of breast milk expressed, either by hand or hand pump (it was the method not used the previous day) was obtained, of which 22ml of the control group mothers' and 26ml of the study group mothers' breast milk were used for nutrient analysis. Right or left breast not used the previous day. Sample division as for day 6.	

Day 28 (last day of study participation):

Actions on/to the mother	Actions on/to the infant
5ml Blood drawn for determination of Serum: Albumin, Transferrin, Ferritin, Hb, Hct, sIg A, Ig G, Vit A and CRP	1,5 ml blood drawn for determination of Serum: Albumin and Pre-albumin RNA PCR determination repeated in infants with HIV positive mothers
Anthropometric measurements: Wt and Uac	Anthropometric measurements: Wt, length, head circumference

Abbreviations used: Hb = Haemoglobin, Hct = Haematocrit, CRP = C-reactive protein, Wt = Weight, Ht = Height and Uac = upper-arm circumference.

* 3 Milk aliquots each for HIV negative and HIV positive mothers, but a fourth aliquot was needed for viral load determination of the milk of the HIV positive mothers.

2.5.6 Questionnaires

A total of 3 questionnaires were used in the study to collect the required information. All questionnaires were face validated and translated into the three languages of the region (i.e. English, Afrikaans and Xhosa). All socio-demographic and dietary intake data were obtained with the help of an interpreter, who was working in the recruitment Kangaroo Mother Care Unit (Ward G2) of Tygerberg Academic Hospital.

Socio-demographic Questionnaire

Study participants were interviewed once during the study period to determine background information such as level of income, level of education, previous as well as current medical and obstetric history, dietary habits and social circumstances.

Quantitative Food Frequency Questionnaire

A pre-tested Quantitative Food Frequency Questionnaire (QFFQ) that was used during the National Food Consumption Survey in 1999[152] to determine dietary intake of South African children, 1-9 years of age, was adapted for use in this study population of Xhosa women. The QFFQ was used to determine their dietary intake during pregnancy. Food models of a few basic foods as well as eating and drinking utensils were used as aids in the completion of the QFFQ.

24-Hour Dietary Recall Questionnaire

Intake during lactation was determined by repeated 24-hour recalls during the 28-day study period. The aim was to determine dietary intake for two week days and one weekend day for the 4 weeks of follow-up for each study participant. Most of the 24-hour recall data obtained was that of the hospital diet they received while they remained in the Kangaroo Mother Care Units of Tygerberg, Eersterivier or Karl Bremer Hospital.

Dietary intake of infants

Dietary intake of all study infants was recorded on feed monitoring forms. Both intravenous and enteral intake was noted, together with data on output and vital signs. Direct calorimetry could not be performed to determine total enteral energy intake and the latter was calculated by using the following values: 3.6 kcal/g FM85® Human milk fortifier (Nestlé) and 8.5 kcal/ml Medium Chain Triglyceride (MCT) oil (Nutricia). The amount of fortifier and volume MCT oil administered was recorded daily on the infants' feed monitoring form in their nursing file. Breast milk energy content was dietarily determined from the actual breast milk protein, fat and carbohydrate composition chemically analysed for days 6, 7, 13, 14, 20, 21, 27 and 28.

2.5.7 Anthropometric status

2.5.7.1 Mothers

Anthropometric determinations performed at study entry for all mothers were: weight, height and mid-upper arm circumference. At study exit the weight and mid-upper arm circumference measurements were repeated. All measurements were performed by the investigator or research assistant, both of whom are registered dietitians and were standardised against each other.

Weight was determined by using an electronic scale (Precision Health Scale: Model UC-321; Manufacturer: A&D Instruments Ltd), with specifications: maximum capacity: 150 kg, minimum display and increments: 0.05 kg. The weight was determined as follows:[153]

1. Set the slide switch to “WEIGHT”.
2. Press the measurement switch gently.
3. Step on the scale gently.
4. The weight can be read after the O mark is displayed.
5. Step off the scale.

Height measurements were taken on a portable height measuring stick (Leicester Height Measure, Manufacturer: Chasmors Weighing Equipment Ltd). Specifications: A precision instrument for measuring children and adults from a minimum height of 75cm to a maximum height of 205cm. Height was determined as follows: [154]

1. Subjects stand barefoot on the “feet” section with his/her heels together and touching the backstop. The spine at pelvis and shoulder level should touch the upright. Shoulders should be relaxed, arms to the side. Remove all headgear.
2. Lower the measuring arm onto the head and position the head so that an Imaginary horizontal line runs between the ear-hole (external meatus of the ear) and the lower border of the eye socket.
3. Ask the subject to stand up straight.
4. Read off the metric height to the last completed millimetre. Do not round up.
5. Measurements were repeated and the values averaged.

Mid-upper arm circumference was measured using a standard non-stretchable measuring tape with 1cm and 1mm dimensions. The mid-point of the arm was determined: [155]

1. Measurement was performed on the right arm.
2. Patient stood upright with the feet together.
3. The right arm was bent at the elbow to form a 90° angle.
4. The palm of the hand faced upward.
5. The mid-point between the corner of the acromion and the point of the olecranon, in the posterior position was determined.

6. Measurements were repeated and the values averaged.

After determination of the mid-point of the arm, the mid-upper arm circumference was measured:

1. The measurement was measured on the right arm.
2. The measurement was taken in a horizontal plane on the same level as the mid-point of the arm.
3. Patient stood upright with the arm hanging relaxed along the side.
4. The soft tissue was not compressed.
5. Measurement was taken to the nearest 1 mm.
6. Measurements were repeated and the values averaged.

2.5.7.2 Infants

Birth weight, length and head circumference were noted from the medical file for each study participant. Daily weights were recorded from each participants nursing file. Any drastic weight change was queried and the weight repeated under supervision of the investigator or research-assistant. The scales used for weight determination were all Salter Model 914 scales, with a maximum capacity of 20 kg and reader precision to the nearest 10 g. Weight was determined as follows: [155]

1. An electronic scale (a pan-type for infants < 2 years) was used, with an accuracy of 0.001kg (< 2 years).
2. Before measurements were taken, zero calibration of the scale was done.
3. The infant was placed in the middle of the scale, without any clothes or nappy. If a nappy was worn, the weight was corrected by subtracting the weight of the nappy (i.e. the clean nappy was weighed separately).
4. The baby had to lie still before the reading was taken.
5. The baby had not to have held onto anything for support.
6. The measurement was read to the nearest 0.001kg.
7. Measurements were repeated and the values averaged.

At study exit, the length and head circumference were measured again to determine growth during the 28-day follow-up period. Length measurements were performed by the investigator or assistant, with the help of the infant's mother. A portable length mat with 0.5 and 1 cm dimensions was used to determine length. A description of the length measurement follows: [155]

1. A portable plastic length mat with a solid headboard and a moveable footboard was used, with 0.5 cm increments.
2. Two people performed the measurement.

3. All socks and headgear was removed.
4. One person held the crown of the head against the headboard and with the Frankfort plane forming a 90^{o} angle with the length mat. This person also ensured that the head, shoulders and buttocks touched the length mat.
   The other person kept the legs straight against the length mat and slid the foot-board against the bottom of the feet with the toes pointing upwards. In difficult cases one of the infant's legs was pressed down at the knee to flex the foot up.
5. The measurement was read to the nearest 0.5 cm.
6. Measurements were repeated and the values averaged.

A standard non-stretchable measuring tape with 0.5 cm and 1mm dimensions were used for the two determinations respectively. A description of how head circumference was determined: [155]

1. A flexible, non-stretchable measuring tape was used with 1 cm increments.
2. The infants were measured whilst being held upright by the mother.
3. All headgear was removed.
4. The head was held in the Frankfort plane.
5. The tape was positioned just above the eyebrows (i.e. supraorbital ridges), above the ears and around the back of the head (i.e. occiput) so that the maximum circumference was measured.
6. The tape was on the same plane on both sides of the head.
7. The measurement was read to the nearest mm.
8. Measurements were repeated and the values averaged.

2.5.8 Blood sampling and variables measured

2.5.8.1 Mothers

A sample of blood (5 ml) was obtained from each participant at study entry and exit. All blood samples obtained were drawn by either a qualified medical doctor or, in case of personnel shortage, a final year medical student under supervision of a qualified medical doctor. The African Micronutrient Research Group Laboratory of the Division of Human Nutrition: Tygerberg Academic Hospital analysed the blood for the following constituents: Pre-Albumin, Albumin, Transferrin, Ferritin, Immunoglobulin A, Immunoglobulin G, C-reactive Protein and Vitamin A. National Health Laboratory Services (Tygerberg Academic Hospital) determined the haemoglobin and haematocrit content of all mothers, as well as the CD4-determinations of the HIV positive group. The Division of Virology: Stellenbosch University determined the blood viral load of all HIV positive mothers.

Methods used for determination of the above-mentioned serum constituents are described:

Pre-Albumin, Albumin, Transferrin, Ferritin, Immunoglobulin A, Immunoglobulin G and C-reactive protein was determined by immunochemical methods. In an immunochemical reaction, the proteins in the sample form immune complexes with specific antibodies, which scatter a beam of light. The intensity of the scattered light is proportional to the concentration of the protein and compared to the standard curve of known concentration. The instrument used was the BN ProSpec (Manufacturer: Dade Behring). As all analysis was performed in one batch, no specific coefficients of variance apply. The quality control measures of the methods used in the laboratory in which the samples were analysed with coefficients of variation (CV) for the different constituents (indicated in brackets): Pre-Albumin (CV= 1.7%), Albumin (CV= 4.3%), Transferrin (CV= 2.5%), Ferritin (CV= 5.3%), Immunoglobulin A (CV= 3.4%), Immunoglobulin G (CV= 2.4%) and C-reactive protein (CV= 2.9%).

Vitamin A levels in the blood were determined with high-performance liquid chromatography. Serum or plasma is first de-proteinised by precipitation, after which the fat-soluble components, including vitamins A and E, are extracted with hexane. After evaporation of the hexane, the residue is dissolved in methanol and aliquots used for quantitative determination by high-performance liquid chromatography (HPLC). These operations are conducted in subdued light on account of the sensitivity of vitamin A to certain wavelengths of light.

Instruments used for vitamin A determination: Waters HPLC system, two pumps (Model M45), auto-sampler (Model 717+) and automatic gradient controller (Model 680), a linear programmable UV-VIS detector (Model SSI 525), Pentium 3 computer with a 20Gb hard drive and 64Mb RAM, EZChrom Elite/Client Server (version 2.3) Chromatography Data System (Scientific Software Inc., USA) under Windows 95® software (Microsoft) were used with a Supelco LC-18 HPLC column (250 x 4.6mm; 5µ beads). (Manufacturer: Microsep). The above-mentioned method is internationally published and accepted as correct, while accuracy, precision, reproducibility and selectivity (validation criteria) have been established.[156]

Haemoglobin was determined by lyses of red blood cells to release the haemoglobin. The haem ion is oxidised to the ferric state which then combines with the cyanide in the reagent. Optical readings were taken at 546nm and plotted. The coefficient of variance for the test was 0.898 (Of QC as on 04/05/2007). (Instrument: Advia, Model: 2120, Manufacturer: Siemens.)[157]

Haematocrit was determined from the haemoglobin value, where haematocrit = (Red cell count X Mean Corpuscular Value) ÷ 10. The coefficient of variance for the test was 1.272 (Of QC as on 04/05/2007).[157]

CD4 T-Cell lymphocyte count determination involves the addition of monoclonal reagent to human whole blood. The fluorochrome-labeled antibodies bind specifically to antigens on the surface of leucocytes. The stained samples are then treated with FACS Lysing Solution to lyse erythrocytes. An aliquot of the stained sample is then introduced to a flow cytometer in which it passes through the path of a laser beam. The stained cells fluoresce after they are excited by the laser beam and the emitted light is collected and processed by the cytometer. These cells also react with the laser beam by scattering the light. The BD Facs Calibur uses the MultiTEST reagent panel which is a four-colour direct immuno-fluorescence reagent to identify total T-cell, T-Helper, T-Suppressor, Natural Killer and B-cell levels. The percentage (%) coefficient of variance for the respective cells was 7.02 for the helper/inducer lymphocytes, 6.79 for the suppressor/ cytotoxic cells and 6.34 for the total T-cells lymphocytes. (Instrument: Flow Cytometer, Model: Facs Calibur, Manufacturer: Becton Dickenson) [158]

The complete blood collection procedure used during the study and described above is summarised in Table 2.1.

Table 2.1: Summary of blood collection and analysis procedure for all mothers

Blood collection day	Blood constituent	Volume needed for analysis	Type of tube to be used for sample	Sample frozen or kept fresh
Days 6, 28	Haemoglobin, Haematocrit	2 ml	EDTA (PURPLE Top)	Fresh
Days 6, 28	Serum: Albumin, Pre-albumin, Ferritin, Transferrin, Immunoglobulin A, Immunoglobulin G, Vitamin A C-reactive Protein	4 ml	RED top	Fresh
For HIV positive mothers only: Days 6, 28	CD4 T-Cell count	2 ml	EDTA (PURPLE top)	Fresh
For HIV positive mothers only: Days 6, 28	Viral load	2 ml	EDTA (PURPLE top)	Fresh

2.5.8.2 Infants

A sample of blood (±0.7ml) was obtained from each participant at study entry and exit. The Analytical Metabolic Research Group of the Division of Human Nutrition: Tygerberg Academic Hospital analysed the blood for the following constituents: Pre-Albumin and Albumin. The Division of Virology: Stellenbosch University determined the DNA Polymerase Chain Reaction (PCR) of all HIV-exposed infants, which required a further 0.5 ml blood (Table 2.2).

Table 2.2: Summary of blood collection and analysis procedure for the infants

Blood collection day	Blood constituent	Volume needed for analysis	Type of tube to be used for sample	Sample frozen or kept fresh
For infants with HIV positive mothers only: Day 28	HIV-1 DNA-PCR	0.5 ml	EDTA (PURPLE top)	Fresh
Days 6, 28	Serum: Albumin, Pre-albumin	0.5 - 1 ml	RED TOP	Fresh

2.5.9 Breast milk determinations

Breast milk was collected in sterilised glass containers (375 ml capacity; 60mm cross-section) with metal lids (R&S Enterprises) during the mornings of the following days after birth: days 6 and 7, 13 and 14, 20 and 21, 27 and 28.

All mothers received a hand pump (Avent Isis® breast pump; Item nr: AVT0203080020; Distributor: Fountain Medical, Greenside, Johannesburg) which was used for expression of the milk samples on 4 of the 8 allocated breast milk collection days. All mothers were taught by the investigator or research assistant, who were standardised regarding the pump cleaning procedure, how to use and clean a hand pump. The decision on whether the hand or pump, as well as left or right breast, had to be used on a specific expression day was determined by means of random selection (i.e. flip of a coin).

A breast milk sample of at least 22 ml for the control group and 26 ml for the study group had to be obtained with every milk expression to be able to determine all of the specified constituents. For the “raw” milk sample, a total volume of 11 ml for the control group and 13.5 ml for the study group was required. The milk sample was then divided into 3 or 4 aliquots of “raw” breast milk with a volume of 1 to 5 ml each. The latter variation in milk

volume of the samples was in accordance with the volume requested by each participating laboratory for analysis of the specified milk components (Table 2.3). Three milk aliquots were needed for the HIV negative and four aliquots for the HIV positive mothers. The extra aliquot was needed for viral load determination in the “raw” breast milk samples of the HIV positive mothers.

For the pasteurised milk sample, a total volume of 11 ml for the control group and study group was required. The breast milk was pasteurised according to the Pretoria pasteurisation technique. This method entailed the heating of water to boiling point in an electric kettle. The boiled water was then immediately poured into an aluminium pot with a capacity of one litre (Brand name of pot: Hart) and filled until 1cm below the rim of the pot. The breast milk in the glass jar, with its lid screwed on tightly, was then placed in the centre of the pot and left for 27 minutes 30 seconds, which is the mean of the recommended standing time of 25 – 30 minutes. [82]

The temperature of each milk sample was recorded before and at the end of the pasteurisation process with a previously calibrated thermometer (810-363 ETI Max/Min water resistant thermometer; Manufacturer: Electronic Temperature Instruments Ltd, West Sussex, United Kingdom), to ensure the correct end-temperature was reached. The temperature of the water at the start and end of the pasteurisation process were also recorded for each pasteurised sample.

After pasteurisation, the milk samples were handled in exactly the same manner as the “raw” breast milk samples, with the milk sample divided into 3 aliquots with a volume of 1 to 5 ml each.

All breast milk samples were frozen at -20° C and later delivered to the specific laboratories. Only the samples for macronutrient determination, which had to be stored in a test tube with preservative, were refrigerated and delivered for analysis within a maximum of 14 days. All samples were stored in rigid polypropylene test tubes. Each test tube received a label which clearly indicated the time and date of collection as well as an encoded number allocated to the particular mother for the purposes of confidentiality.

Breast milk samples were collected from the mothers in both groups and analysed (Table 2.3) for macronutrient and micronutrient content, as well as HIV viral load, in the appropriate laboratories (Table 2.4).

The macronutrients that were determined for all 8 days of sample collection were carbohydrate, fat and protein content. The energy content of each sample was calculated by using the following conversion factors: 4 kcal for every gram of carbohydrate or protein and 9 kcal for every gram of fat contained in 100ml breast milk.[159]

The micronutrients that were determined for all 8 days of sample collection were the vitamin folic acid and the minerals calcium, phosphorus and magnesium, trace minerals iron, copper and zinc and electrolytes sodium and potassium.

Table 2.3: Summary of the milk collection procedure followed for pasteurised and unpasteurised human milk

Milk collection day	**Milk constituent**	**Volume needed for analysis**	**Type of tube to be used for sample**	**Sample frozen or kept fresh**
Days 6, 7, 13, 14, 20, 21, 27, 28	Macronutrients: Fat, Protein, Carbohydrate	5 ml	5ml Cryo Vial	Preserved with tablet. Can be stored for 2 weeks.
All 8 days of analysis	Folic Acid	1 ml	5 ml Tube with BLUE top	Frozen
All 8 days of analysis	Minerals: Ca, PO_4, Mg, Fe , Zn, Cu	5 ml	5 ml Cryo Vial	Frozen
	Electrolytes: Na, K			
One sample per week ie day 6 **or** 7, 13 **or** 14, 20 **or** 21, 27 **or** 28 **Collection from HIV positive mothers' milk only #**	HIV viral load (RAW milk)	2 - 3 ml	14 ml Conical Vial	Must be kept fresh and delivered within 2 hrs of sample collection
Minimum volume of milk/sample:		**13 – 14 ml**		

#: Virology receives breast milk samples from the HIV positive group only and for 4 of the 8 days (one viral load determination per week). One raw or pasteurised milk sample thus subdivided into 3 or 4 test tubes.

Table 2.4: Laboratories participating in the analysis of the different milk constituents:

Laboratory	Milk constituent(s) analysed
Agricultural Research Council (ARC) Animal feeding and Products Institute: Dairy Laboratory: Elsenburg, Stellenbosch.	Carbohydrate, Protein and Total Fat
National Health Laboratory Services, Tygerberg Academic Hospital: Haematology Laboratory	Folic acid
ARC/ Department of Agriculture: Western Cape, Stellenbosch	Calcium, Phosphorous, Magnesium, Iron, Zinc, Copper, Sodium and Potassium
Division of Virology: Stellenbosch University	HIV viral load determination

2.5.10 Laboratory techniques used for analysis of the different milk constituents

2.5.10.1 Macronutrients

Protein, fat and lactose content were determined with an Infra Red Analyser (Model: Milkoscan 133B; Manufacturer: Foss Instruments). FOSS instruments are high-capacity, fully automatic, mid-range infrared spectrophotometers used for the determination of compositional parameters in milk. The instrument is calibrated according to the following wet-chemistry reference methods:

-Kjeldahl method for determination of nitrogen content. (Brussels: International Dairy Federation Standard 20B: 1993)

-Rose Gottlieb gravimetric method for determination of fat content (Brussels: International Dairy Federation Standard 1C: 1987) as well as

-Laboratory manual methods of analysis of milk and its products for determination of lactose content (Washington DC: Milk Industry Foundation, 1959 and Brussels: International Dairy Federation Standard 28A:1974).

The coefficient of variance for protein is 0.0117%, for fat 0.0056% and for lactose 0.0027%. All coefficients of variance were determined by in-house testing at the ARC-Elsenburg Analytical Services Laboratory.

2 5.10.2 Micronutrients

Vitamin

Folate was quantitatively determined using the ADVIA Centaur® System, which is a competitive immuno-assay using direct chemi-luminescent technology. Folate in the breast milk sample competes with acridinium ester-labelled folate in the Lite Reagent for a limited amount of biotin-labelled folate binding protein. Biotin-labelled folate binding protein binds to avidin that is covalently coupled to paramagnetic particles in the solid phase. In the ADVIA Centaur® Folate assay the sample is pre-treated to release the folate from endogenous binding proteins in the sample. An inverse relationship exists between the amount of folate present in the breast milk sample and the amount of relative light units detected by the system. The sensitivity and assay range of the ADVIA Centaur® Folate detects folate concentrations from 0.35 up to 24 ng/ mL (54.36 nmol/L) are measured, with a minimum detectable concentration of 0.35 ng/ mL (0.79 nmol/ L).[160]

Minerals

Calcium, phosphate, magnesium, iron, copper and zinc were all measured by using inductively coupled plasma (ICP) mass spectrometry. This method entails that a known volume of sample is evaporated to dryness and ashed at 550°C; a minimum quantity of 10% HCl is added to dissolve the ash and made up to a final volume of 50 ml with distilled water. The individual minerals were determined by direct aspiration on ICP. ICP model used: IRIS HR Plasma Spectrometer (Manufacturer: Thermo Jarrell Ash Corporation).

Electrolytes

Sodium and potassium were determined by the same method as described above for mineral analysis, i.e. inductively coupled plasma (ICP)-mass spectrometry. The ICP model used was IRIS HR Plasma Spectrometer (Manufacturer: Thermo Jarrell Ash Corporation).

2.5.10.3 Viral load: blood and breast milk

Circumstances outside the control of the laboratory are the reason why there was a change in the method of viral load determination in June 2006. The performance characteristics of both methods are similar. Both amplify a virus-specific sequence of the RNA genome. A description of both methods follows:

Test used until June 2006:

Abbott Realtime™ HIV-1 Test, Ref 2G31 (Manufacturer: Abbott)

Principles of the procedure: [161]

The Abbott Realtime HIV-1 assay uses reverse transcription polymerase chain reaction (RT-PCR) to generate amplified product from the RNA genome of HIV-1 in clinical specimens. An RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR and serves as an internal control to demonstrate that the process has proceeded correctly for each sample. The amount of HIV-1 target sequence that is present at each amplification cycle is measured through the use of fluorescent–labelled oligo-nucleotide probes on the Abbott m2000rt™ instrument. The amplification cycle at which fluorescent signal is detected by the Abbott m2000rt is proportional to the log of the HIV-1 RNA concentration present in the original sample.

Volume of whole blood or breast milk used for analysis: 0.2 ml

Interpretation of results:

Limits of Detection (LOD) of test: 150 copies/ ml

Target detected if: 2.18 to 7.00 Log (>150 copies/ ml to <10 million copies/ ml))

Upper limit of quantitation exceeded if: >7.00 Log (10 million copies/ ml)

Specificity of test: 100%

Test used from June 2006:

NucliSens EasyQ® HIV-1, version 1.1 (Manufacturer: bioMérieux bv)

Principles of the procedure:[162]

NucliSens EasyQ® HIV-1 consists of a two-step process, namely nucleic acid amplification combined with a homogenous detection step. This process requires isolated nucleic acids as starting material. A short description of these processes follows:

1. Nucleic acid isolation: A specimen of human plasma is added to NucliSens Lysis Buffer containing guanidine thiocyanate and Triton X-100. NucliSens Lysis Buffer inactivates RNases and DNases present in the specimen. A synthetic calibrator is added at a known concentration which functions as an internal standard for the isolation, amplification and detection procedure. Silica is added to initiate the isolation process. Nucleic acids present in the NucliSens Lysis Buffer will bind to the silica dioxide particles under the high salt conditions. The silica is then washed before eluting the nucleic acids from the solid phase prior to using them in the amplification and detection procedure.
2. Nucleic acid amplification and detection: NucliSens EasyQ HIV-1 utilises a combined process of nucleic acid amplification and homogeneous detection with molecular beacons. For nucleic acid, amplification primers are used that are specific for sequences found in wild type HIV-1 RNA and the synthetic NucliSens EasyQ HIV-1 calibrator RNA.

The detection process uses two different target-specific molecular beacons, i.e. DNA oligo-nucleotides. One nucleotide is specific for the wild type HIV-1 amplicon and one for the calibrator amplicon. The use of two fluorescent dyes allows the synthesis of target calibrator RNA to be followed individually. Kinetic analysis of the fluorescent signals revealing the transcription rates of both types of the afore-mentioned can be used to derive the quantity of HIV-1 RNA in the original specimen. Quantitation of the viral load is then possible using a data reduction algorithm in the HIV-1 assay software.

Volume of whole blood used for analysis: 1.0 ml

Volume of breast milk used for analysis: 0.5 ml

Interpretation of results:

Limit of detection at a 95% detection rate, is: 357 IU/ml for a 1 ml input

Linear range: 50 – 3 000 000 IU/ml

Cut-off: 25 IU/ml

Specificity of test: 99.7% (95% confidence limits: 98.5 – 100%)

2.5.10.4 Dioxy-ribonucleic acid polymerase chain reaction (DNA-PCR)

This test was performed on a whole-blood sample of all HIV-1 exposed premature infants at the end of the follow-up period of 28 days to determine their HIV-1 status.

Test used: *Amplicor® HIV-1 DNA Test, version 1.5 (Manufacturer: Roche)*

Principles of the procedure: [163]

The Amplicor® HIV-1 DNA test, version 1.5 (v 1.5) is a qualitative in vitro test for the detection of HIV-1 DNA in human whole blood. The test uses amplification of target DNA by the Polymerase Chain Reaction (PCR) and nucleic hybridisation for the detection of HIV-1 DNA in human whole blood.

The Amplicor® HIV-1 DNA test, (v 1.5) is based on four major processes: sample preparation; PCR amplification of target DNA using HIV-1-specific complementary primers; hybridisation of the amplified products to oligo-nucleotide probes specific to the target(s); and detection of the probe-bound amplified products by calorimetric determination.

Volume of whole blood used for analysis: 150 µL

Program used for Amplification: Applied Biosystems GeneAmp PCR System 9600 for the Amplicor® HIV-1 DNA test, v 1.5.

As it is a qualitative test, coefficient of variance determination does not apply.

2.5.11 Ethics approval

The study was approved by the Committees for Human Research of the Faculty of Health Sciences of the University of Stellenbosch, Tygerberg Academic Hospital, Eersterivier Hospital and Karl Bremer Hospital. (Project number: N04/04/070) The guidelines for Applied Good Clinical Practice were adhered to during the entire study period. All participating mothers were informed about the study in their first language (Xhosa) or an alternative second language (English or Afrikaans), if they were fluent in the latter. All study participants were given a patient information leaflet in Xhosa, English or Afrikaans that explained the study. (Refer to Appendix A for the English copy.) All prospective participants were given until the following day to think about participation before they signed the written consent form in their preferred language. All participants received a copy of this consent form.

All HIV negative mothers had to be willing to undergo a rapid test for confirmation of their HIV negative status, as determined during the prenatal period. For the study group participants, only an HIV positive mother who had decided to feed her infant pasteurised breast milk in the hospital was eligible to partake in the study.

Confidentiality was maintained throughout the study. All mothers anonymously took part in the study, as the mothers were allocated an encoded number that was used for the identification of her questionnaires and milk samples. The HIV status of the mother and her infant was treated with confidentiality and was not revealed to any persons other than the patients' doctors, nursing staff and the investigator and / or research assistant. Both the control and study groups' milk samples were collected, pasteurised and analysed in exactly the same manner, preventing conclusions about the mother's HIV status to be made on the basis of the group she formed part of.

No feed was withheld from any infant due to milk sample collection and, if the infant needed to be fed at the time of sample collection, the infant was fed first with either human milk from the breast not used for the collection or expressed human milk collected during a previous feeding. The infants of the HIV negative mothers were never fed pasteurised breast milk, but all received fortified (if indicated) unpasteurised expressed breast milk while in hospital. All infants of HIV positive mothers received fortified (if indicated) pasteurised expressed breast milk.

During and after the mothers' and infants' participation in the study, all mothers and infants received the appropriate medical care required. HIV positive mothers and all

infants who tested HIV positive at exit from the study received the prescribed treatment for their condition as stipulated by the regional protocol for the treatment of HIV/ AIDS. There was no interference with regard to the latter by this study's actions. All infants found to have a positive DNA-PCR result at exit from the study, were formally referred to their nearest PMTCT Baby Clinic for further management and follow-up.

2.5.12 Data analysis

All socio-demographic data were imported into an Excel spreadsheet. All data was double checked by the investigator on two different occasions. Data was double checked by the investigator when importing data from the received results to the participant file and again when the data was imported into the Excel spreadsheet. The data was transferred to Statistica® (release nr 7, 2006) for analysis and interpreted by means of descriptive statistics.

All dietary intake data were analysed with Foodfinder® 3 (version 1.0.0) Dietary Analysis software program, [164] to determine average daily nutrient intake for the two time periods for each study participant.

Anthropometric reference standards used were Body Mass Index for interpretation of the mother's weight in relation to their height and mid-upper arm circumference, which is an indicator of pre-pregnancy nutritional status. The cut-off values used for BMI were according to the WHO guidelines, where the normal BMI range falls between 18.5 and 24.99. Overweight is classified as a BMI between 25 and 29.99, while the obese grades 1 to 3 correspond with a BMI between 30 to 34.99, 35 to 39.99 and ≥40 respectively. Underweight is classified as a BMI < 18.5.[165] The percentiles used for mid-upper arm circumference, weight and height of the lactating women were those for African-American females >20 years of age as no South African data in this regard is available.[155]

All participating infants' weight, height and head circumference measurements at birth as well as on day 28 of life were plotted on the Fenton-Babson growth chart (Appendix B).[166] Weight loss since birth as well as growth velocity after birth weight was regained were determined. Growth velocity was regarded as the gain in weight an infant experiences from the lowest recorded weight until the current weight, divided by the number of days between these two points in time. Birth weight was classified according to two classification systems, i.e. small-for-gestational age (SGA), appropriate-for-gestational-age (AGA) and large-for-gestational-age (LGA) as well as extremely low birth weight

(ELBW) or very low birth weight (VLBW).[3, 167] To determine the extent of growth retardation, all infants' weight, length and head circumference at birth and at 28 days of life were used to determine whether the infants had symmetrical or asymmetrical growth retardation.[3]

Descriptive statistics were used to describe the dietary intake and biochemical assessments of the premature infants.

2.5.13 Statistical analysis

Data was analysed by the investigator with the help of a statistician from the Centre for Statistical Consultation: Stellenbosch University. Descriptive statistics were used for the analysis of the socio-demographic, food frequency and dietary intake data of all mothers, as well as to describe the dietary intake, anthropometric and biochemical assessments of the premature infants.

For statistical analysis, repeated measures analysis of variance (ANOVA) was used to determine the effect of HIV, pasteurisation, milk expression and time on the composition of the breast milk.

Confidence intervals were determined in the analysis of the data. A p-value of <0.05 was considered to indicate a significant difference between groups.[168]

CHAPTER 3: RESULTS

3.1 Study Population

Data collection commenced from 1 February 2005 and was completed on 12 December 2006. The total number of patients screened during the data-collection period amounted to 251. Forty-two of the screened mother-infant pairs were recruited according to the inclusion and exclusion criteria, of which 23 (55%) successfully completed the study. Written consent was obtained from all participants, after which they were allocated to the control group or study group, depending on their HIV status. The control group (HIV negative) consisted of 12 and the study group (HIV positive) 11 mother-infant pairs. The process of screening and participant inclusion are presented diagrammatically (Figure 3.1).

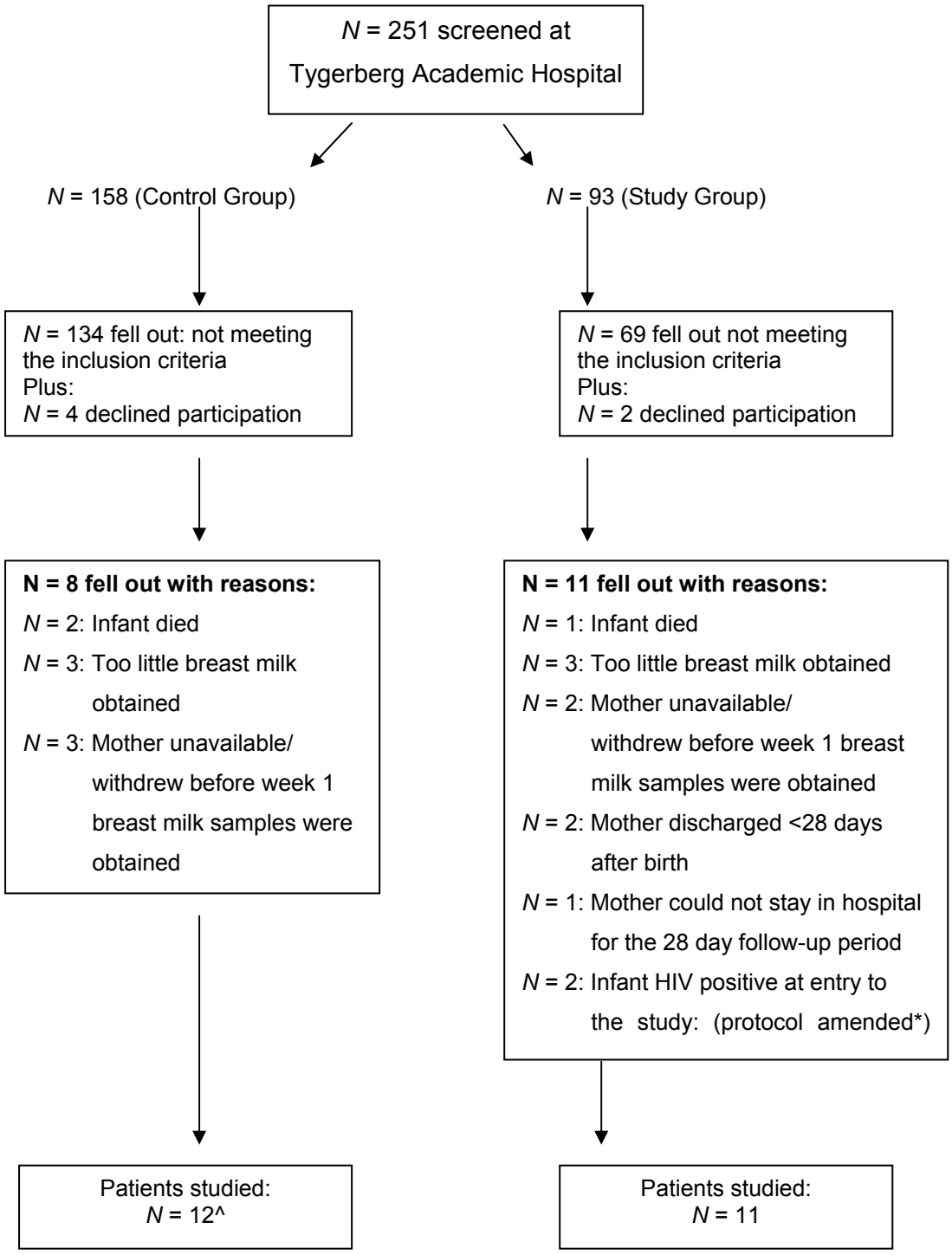

Figure 3.1: A box diagram describing the screening of the control and study group participants

*: The protocol was amended after consultation with Prof. Mark Cotton and his team from the Unit for Infectious Diseases: Tygerberg Academic Hospital on 16 May 2005, to exclude DNA-PCR testing during the first week of life, because the accuracy of this determination is questionable when performed so early. It was concluded that DNA-PCR testing at exit from the study must continue. All study infants found to be HIV positive upon DNA-PCR testing were referred to the appropriate PMTCT Baby Clinic for further management and follow-up.

^: Recruitment of HIV negative mothers was suspended on 24 July 2006 due to financial constraints. Only HIV positive mothers were recruited for the remainder of the data-collection period to ensure a fairly similar size in group was obtained.

3.2 Socio-demographic Variables

Results pertaining to socio-demographic and obstetric information that were not statistically or clinically significant are summarised and presented in Appendix C.

The mean age of the HIV negative mothers was 26.5 (3.8) years and did not differ significantly from that of the study group (HIV positive) mothers, i.e. 25.8 (3.8) years. High School education (i.e. grade 8-12) was the mean level of education obtained by the mothers. The mothers in the two study groups were well-matched, except for one determinant which differed significantly (p=0.04) between the groups, i.e. "the average age of weaning the previous child/children from the breast" (Appendix C).

Only some mothers had a history of risk factors that could have contributed to the premature deliveries observed in this study population (Table 3.1).

Table 3.1: Risk factors for premature delivery identified in the control and study group

Risk factors for premature delivery	Control Group	Study Group
Alcohol consumption during pregnancy	2	1
Smoking during pregnancy	2	2
Maternal Infection	1	1
Maternal Hypertension	6	3
HELLP Syndrome^	1	1
HIV positive status	0	11

^HELLP Syndrome: Haemolysis, elevated liver enzymes and low platelet count.

From an obstetric perspective, pre-eclampsia toxaemia and fetal distress were the two most frequent known reasons for premature delivery in this study population (Table 3.2).

Table 3.2: A comparison of the reasons for premature delivery for the control and study group

Reasons for premature delivery*	Number (%) of Control group mothers affected	Number (%) of Study group mothers affected
Pre-eclampsia	3 (25%)	0
Pre-eclampsia Toxaemia	5 (67%)	5 (45%)
Fetal Distress	7 (58%)	4 (36%)
Prolonged Rupture of Membranes	0	3 (27%)
HELLP^ Syndrome	1 (8%)	1 (9%)
Reason unknown	2 (17%)	1 (9%)

*More than one reason was indicated for the premature delivery in some cases.

^HELLP Syndrome: Haemolysis, elevated liver enzymes and low platelet count.

3.3 Dietary Variables

3.3.1 Dietary habits of the mothers

Information on the dietary habits of the control and study group mothers was obtained from the socio-demographic questionnaire and revealed that their dietary patterns and changes thereof during pregnancy were unremarkable (Table 3.3). These results and other variables of interest (eg. alcohol consumption, smoking, caffeine intake and pica), of which none revealed remarkable findings, are discussed in more detail in Appendix D.

Table 3.3: A summary of all dietary related practices during pregnancy for the control and study group

Dietary practices during pregnancy*	Number of control group mothers (*N*=12) affected	Number of study group mothers (*N*=11) affected	p-value* (cg vs sg)
Deviation from diet usually consumed	7	3	0.12
Food cravings	9	9	-
Food aversions	7	6	-
Food intolerance	1	0	-
Caffeine consumption	8	9	-
Artificial sweetener consumption	0	0	-
Vegetarian diet	0	0	-
Food restrictions	2	2	-
Fasting	1	1	-
Micronutrient supplementation	8	7	-
Pica	2	6	0.053

*: Chi-square test used for determination of p-value.

The only dietary practice that showed a trend towards a significant difference between the control and study group was that of pica (p = 0.053).

3.3.2 Dietary intake

3.3.2.1 Nutrient analysis of dietary intake of the mothers during pregnancy and lactation

Dietary intake was determined for two time-periods, i.e. during pregnancy and during the first month of lactation.

No significant difference in intake of any nutrient was observed between the control and study groups. When the average daily intake of the two groups was compared to the pregnancy-related DRI or AI, the mean for the group for energy, protein, vitamin A was significantly higher than the recommended DRI whereas the intake of folic acid and iron was significantly lower. Iron and folic acid supplementation was not taken into consideration when determining dietary iron intake during pregnancy. Results obtained from the socio-demographic questionnaire indicated that 15 (65.2%) of the 23 participants received iron supplementation during pregnancy, while nine (39.1%) mothers received a folic acid supplement as well.

The nutritional intake during pregnancy for both groups, as obtained by means of the quantitative food-frequency questionnaire (Appendix E) is presented in Table 3.4.

The nutritional intake during the first 28 days of lactation for both groups was obtained by means of repeated 24-hour dietary recall and indicated no significant differences in intake of any of the studied nutrients between the control and study groups. When the mean (SD) daily intake of the whole group was compared to the lactation-related DRI or RDA/AI, the mean for the group for protein ($p<0.01$) and iron ($p=0.01$) was significantly more, while energy, folic acid and vitamin A intake was significantly less ($p<0.01$). Mothers did not use any form of micronutrient supplementation while lactating. (Refer to Appendix F for these results.)

The compositional difference in the macronutrient content of the diets used in the Kangaroo Mother Care Units of the three hospitals, were compared to the Dietary Reference Intake/ Adequate Intake for lactating women. The high-protein diet of TBH was the only diet among the institutions in the study which provided sufficiently in relation to the recommended (DRI) energy needs of the lactating woman (Table 3.5). The energy content of the normal diets provided at ERH and KBH was significantly less than the DRI recommendation and hence inadequate to sufficiently provide in the energy needs of the lactating woman. The protein content of ERH and KBH compared well with the DRI for lactating woman, while the high protein diet of TBH provided nearly double the recommended protein intake.

Table 3.4: A comparison of the mean (SD)[#] dietary intake during pregnancy between the control and study group, as well as with the Dietary Reference Intake (DRI) (10 – 51 years) or when the latter was unavailable, Recommended Dietary Allowance (RDA) or Adequate Intake (AI) for pregnant women

Nutrient	Unit per day	Control Group	Study Group	p-value[b]	Mean daily intake (cg+sg)#	DRI: Pregnant women (10 - 51 yrs)	p-value* (cg vs DRI)	p-value* (sg vs DRI)
Energy	kJ	14 521.8 (4036.7)	12 962.8 (4036.7)	0.62	13 742.3 (4036.7)	10 460^	<0.01*	0.04*
Total Protein	g	97.86 (29.9)	95.14 (29.9)	0.83	96.5 (29.9)	60.0^	<0.01*	<0.01*
Total Carbohydrate	g	454.0 (130.6)	417.20 (130.6)	0.51	435.6 (130.6)	N/A		
Total Fat	g	124.3 (38.8)	101.0 (38.8)	0.17	112.7 (38.8)	N/A		
Saturated Fat	g	39.95 (16.4)	28.72 (16.4)	0.12	34.3 (16.4)	N/A		
Poly-unsaturated Fat	g	33.95 (10.5)	29.28 (10.5)	0.30	31.6 (10.5)	N/A		
Mono-unsaturated Fat	g	38.94 (13.1)	34.34 (13.)1	0.41	36.6 (13.1)	N/A		
Total dietary Fibre	g	29.56 (10.1)	30.16 (10.1)	0.89	29.9 (10.1)	N/A		
Vitamin A (RE)[a]	µg	1282.3 (1137.9)	1784.4 (1137.9)	0.30	1533.4 (1137.9)	770	<0.01*	0.01*
Folic acid	µg	352.1 (156.1)	368.1 (156.1)	0.81	360.1 (156.1)	600	<0.01*	<0.01*
Iron	mg	15.44 (4.9)	13.64 (4.9)	0.39	14.5 (4.9)	27.0	<0.01*	<0.01*

#: Abbreviations: SD: Standard deviation; cg= control group; sg= study group; N/A: Not available

^: RDA/AI used where no DRI was available

[a]: RE= Retinol Equivalents

[b]: Mann-Whitney U test used to determine level of significance. A p-value <0.05 indicates a level of significance is reached.

*: One-sample T-test used to determine level of significance. A p-value <0.05 indicates a level of significance is reached.

Table 3.5: The mean (SD)* compositional difference in macronutrient content of the diets used in the Kangaroo Mother Care Units of the three hospitals and a comparison to the Dietary Reference Intake (DRI) (19 – 31 years) or when the latter was unavailable, the Recommended Dietary Allowance (RDA) or Adequate Intake (AI) for lactating women

Nutrient	Unit	Tygerberg Academic Hospital (High-protein diet) [169]	Eersterivier and Karl Bremer Hospital (Normal diet) [169]	Mean (SD)# hospital diet composi-tion	Mean (SD)# actual daily intake of mothers during lactation	DRI: Lactating women (19- 31 yrs)
Energy (kJ/ day)	kJ/ day	11 027	8904	9966 (1501)	9694.2 (1874)	11 300^
Total Protein	g/ day	111 [17%*]	71 [13.5%*]	91 (28.3) [15.5%*]	75.5 (16.8) [14%]	65.0^
Total Carbohydrate	g/ day	294 [45%*]	265 [50.5%*]	280 (20.5) [47.5%*]	311.3 (61.4) [56.5%]	
Total Fat	g/ day	109 [38%*]	84 [36%*]	97 (17.7) [37%*]	72.5 (17.7) [29.5%]	

#: (SD) = Standard deviation ^: RDA/ AI used where no DRI was available

*: Value in [brackets] indicates percentage of total energy.

As intake during pregnancy and the first 28 days of lactation followed the same trend and no significant difference in intake for any of the studied nutrients was observed between the control and study group, the pooled group's nutritional intake during pregnancy and the first 28 days of lactation were compared. The dietary intake of all nutrients analysed decreased significantly from pregnancy to early lactation. The diet provided to the mothers at Eersterivier and Karl Bremer Hospitals had an optimal macronutrient distribution, but energy content was too low, while protein content was higher than the recommended DRI for lactating women. Mean vitamin A and folic acid content was significantly lower than the respective AI's of 1300 µg/day and 500 µg/day. Mean iron content was significantly higher than the RDA for lactating women (i.e. 9 mg/day) and expected, due to the high protein content of the diets. (Refer to Appendix G for a tabularised version of these results.)

3.3.3.2 Analysis of the dietary prescription and intake of the Infants

The dietary prescription and intake of all participating infants included both intravenous and enteral prescription and the intake was noted on the same feed monitoring form with data on fluid output and vital signs.

Enteral intake of raw or pasteurised EBM was the enteral feeding initiated in all study participants. Two (9%; one control and one study group infant) of study participants started with 2-18 ml/kg EBM on day one of life, while 16 (70%) of all study infants started EBM on day 2 of life. Of the infants starting on day 2 of life, 7 (58%) were in the control group, while 9 (82%) of study group participants started with pasteurised EBM. Three (25%) control group participants initiated enteral feeding on day 3 of life, while the remaining two (9%; one each control and study group) participants started on day 4 of life. The difference in initiation of enteral feeding was due the availability of EBM, the stability and gastro-intestinal functioning of the infant.

The enteral intake of the control and study group infants (Figure 3.2) increased from day one to day eight postpartum and stabilised at 160 ml/kg by day eight, afterwhich it remained at this level of intake throughout the 28-day study period. The differences between the groups were not significant. Total fluid intake (intravenous plus enteral fluid in weeks) (Figure 3.3) was also not significantly different between the two groups, although on day 7 a trend (p=0.06) for higher fluid intake in the control group was recorded.

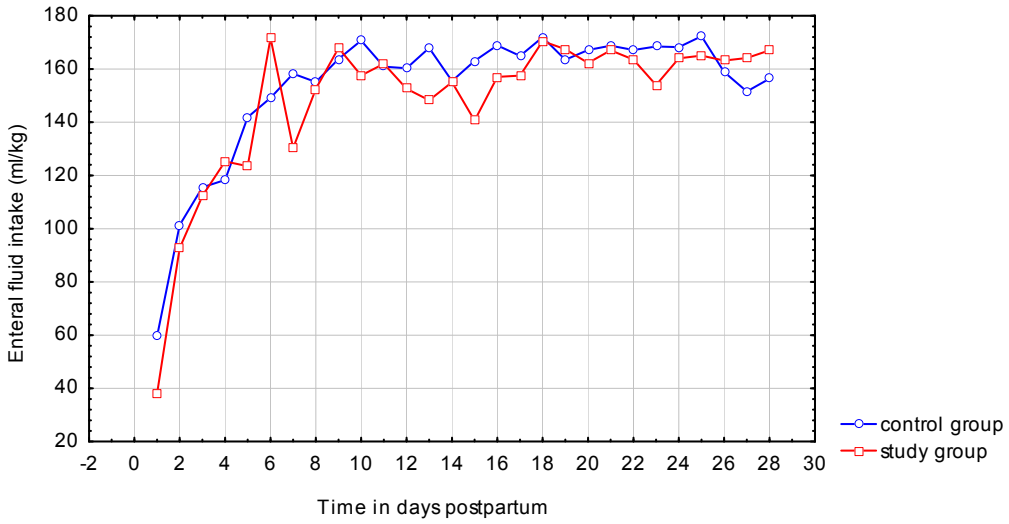

Figure 3.2: A scatterplot indicating enteral fluid intake of the control and study group over the first 28 days of life

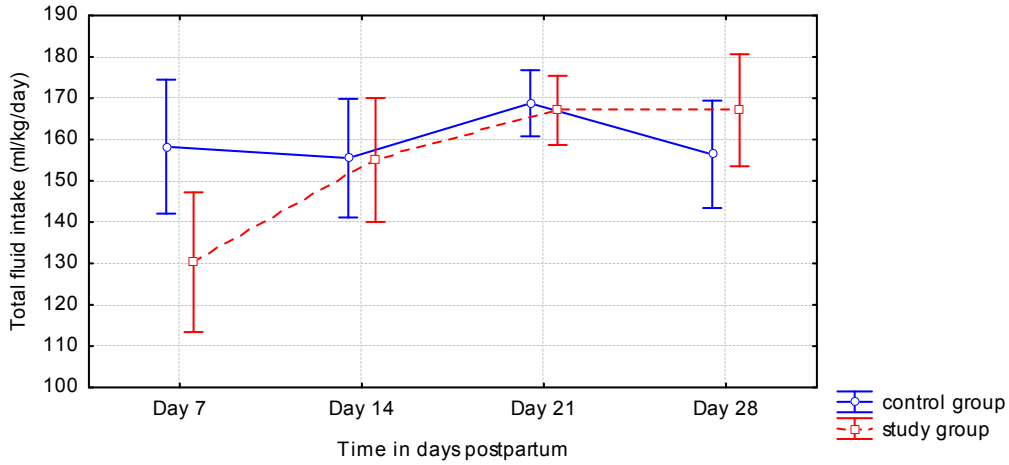

Figure 3.3: Total fluid intake (IV plus enteral) of the control and study groups at the end of each study week

Fluid intake differed significantly (p=0.013) over some of the study days between the control and study group, while energy and protein intake for days 7, 14, 21, and 28 did not differ significantly between the two groups (Table 3.6).

Table 3.6: Mean (SD)* actual fluid, energy and protein intake of all infants on days 7, 14, 21, and 28

Nutrient	Unit per day	Day 7		Day 14		Day 21		Day 28		p-value [c]
		Cg^	Sg#	Cg^	Sg#	Cg^	Sg#	Cg^	Sg#	
Total[A] fluid intake[a]	ml/kg	158.3 (27.0)	130.3 (27.0)	155.5 (23.9)	155.0 (23.9)	168.8 (13.3)	167.0 (13.3)	156.4 (21.6)	167.1 (31.6)	0.013*
P-value: cg vs sg		0.06		1.00		1.00		0.94		
Energy intake[b]	kcal /kg	45.5 (33.8)*	30.1 (33.8)*	112.9 (33.8)*	98.2 (33.8)*	135.9 (33.8)*	136.6 (33.8)*	140.3 (33.8)*	148.0 (33.8)*	0.48
P-value: cg vs sg		0.27		0.30		0.96		0.58		
Protein intake[b]	g/kg	1.32 (1.35)	1.30 1.33)	3.48 (1.35)	2.64 (1.36)	3.36 (1.35)	3.06 1.35)	3.18 (1.36)	3.35 (1.36)	0.63
P-value: cg vs sg		0.97		0.19		0.65		0.78		

Abbreviations used: *SD: Standard deviation; ^Cg: control group; #Sg: study group

[A]: Total fluid intake includes IV maintenance fluid (i.e. 10% Neonatalyte®), total parenteral nutrition and expressed breast milk intake.

[a]: Tests used for analysis: Repeated measures Analysis of Variance (ANOVA)

[b]: Tests used for analysis: Variance Estimation, Precision and Comparison (VEPAC)

[c]: P-value determined with inclusion of both group and sample collection day effect for the whole follow-up period.

*: Significance level: p<0.05.

Enteral intake was the main contributor towards these infants' energy and protein intake, as the nutrient contribution from intravenous intake (including intravenous fluid and TPN) was found to be less than 10% of total energy and 0% of protein intake. Only 3 of the 23 study participants received TPN at some stage during the follow-up period. All infants, however, did receive intravenous maintenance fluid (i.e. Neonatalyte®). The mean (SD) energy contribution from intravenous fluid intake for the 28-day follow-up period was 13.0 (33.3) kcal/kg/day for the control group and 14.9 (33.4) kcal/kg/day for the study group, which was a non-significant (p=0.48) difference between the groups. Intravenous energy contribution was initially 30 – 40 kcal/kg/day during the first week of life, after which the energy contribution declined drastically during the second week of life to reach an IV energy intake of 0-10 kcal/kg/day during the third week of life. None of the participating infants received any IV fluid during the fourth week of life (Figure 3.4).

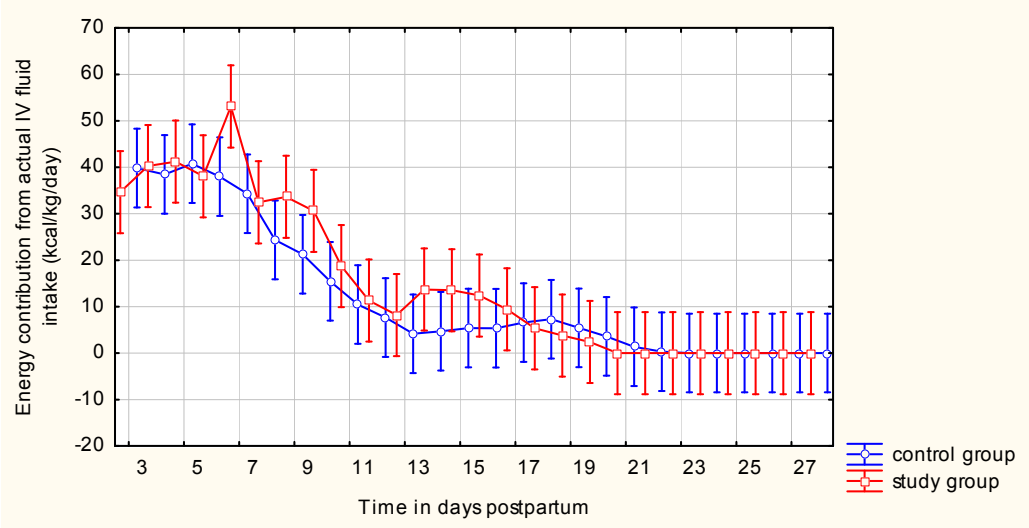

Figure 3.4: Energy contribution from actual intravenous fluid intake for the control and study group infants (P-value = 0.86)

Expressed breast milk was the feed of choice for enteral feeding. Only four infants (all from the study group) received preterm (i.e. Prenan®, Nestlé) or a specialised infant milk formula (i.e. Alfaré®, Nestlé). The main reason for infant milk formula use was the unavailability of pasteurised EBM. As the amounts of infant milk formula actually consumed were at minimal enteral nutrition levels (i.e. <1% total energy and protein) only, the energy and protein composition of the infant milk formula consumed were omitted in the determination of actual energy and protein intake.

As the breast milk protein, fat and carbohydrate composition were chemically determined for days 6, 7, 13, 14, 20, 21, 27 and 28 during the follow-up period, each infant's actual intake could be determined for the mentioned days. The actual intake data was used to determine mean weekly energy and protein intake for each infant. To determine actual

energy and protein intake as accurately as possible, the raw breast milk composition was used for all control group participants, while pasteurised breast milk composition was used for study group participants, as this scenario best simulates their dietary intake in hospital. Energy content was dietarily determined from the chemically determined macronutrient content by means of the following energy conversion factors: 1 gram protein = 4 kcal; 1 gram carbohydrate = 4 kcal; 1 gram fat = 9 kcal. [159]

Mean [95%CI] actual enteral energy intake of the control group was 100.1 [95% CI, 82.6 - 117.7] kcal/kg versus 97.2 [95% CI, 74.6 – 119.8] kcal/kg for the study group and did not differ significantly (p=0.83) between the groups over the 28-day follow-up period (Figure 3.5).

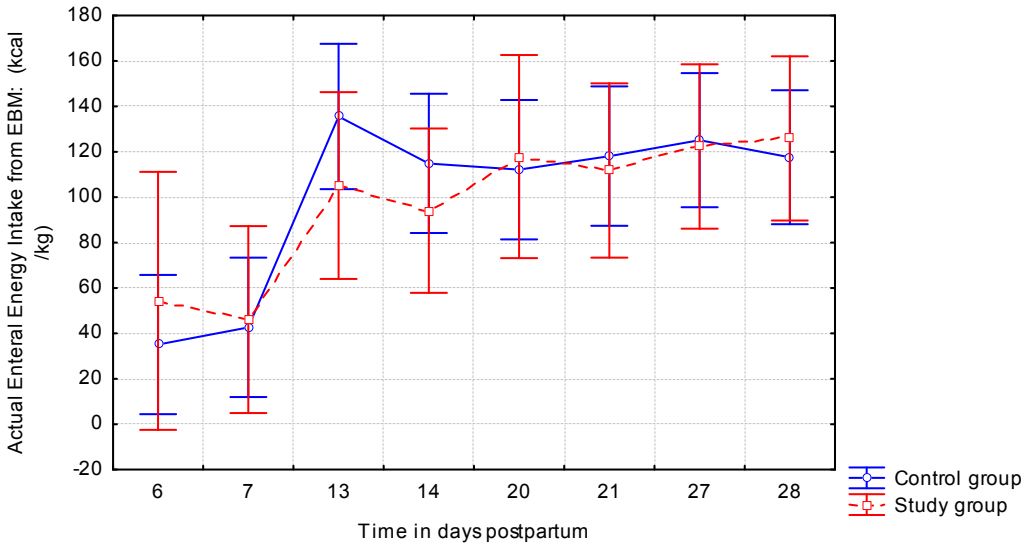

Figure 3.5: Mean [95% CI]* actual enteral energy intake for the control and study group during the first 28 days of life [*95% CI: 95% Confidence Interval]

Enteral energy and protein intake, however, did increase significantly (p<0.01) over time, which was to be expected, as enteral nutrient intake was built up to full feeds, with fortification and supplementation also added during the second to third week of life when full volume feeds were reached. It is important to note that the milk composition analysed could not be a reflection of the composition of colostrum, as the first milk sample were only collected on day 6 after birth from all participating mothers. To establish whether the nutrient prescription was actually consumed, a comparison was made between mean actual intake of energy and protein and the prescribed energy and protein intake for the 28-day follow-up period. Both mean actual energy and protein intake was significantly lower than that prescribed at some days of observation with a trend for significance for lower actual intakes over the four week period (Table 3.7).

Table 3.7: A comparison of the mean (SD)* prescribed with mean (SD)* actual enteral energy and protein intake in the control and study group over the 28-day follow-up period

Mean prescribed and mean actual intake for sample collection days	Energy^ (kcal/kg)		p-valueº		Protein^ (g/kg)		p-valueº	
	Cg^	Sg#	Cg vs Sg	Pr vs Ac	Cg^	Sg#	Cg vs Sg	Pr vs Ac
Mean prescription for day 6	40.1 (42.4)	27.4 (42.4)	0.7	0.9	1.3 (1.2)	0.8 (1.2)	0.7	0.7
Mean actual intake for day 6	33.1 (41.8)	31.8 (36.8)			1.1 (1.2)	1.2 (1.0)		
Mean prescription for day 7	50.7 (42.4)	32.4 (42.4)	0.5	0.9	1.7 (1.2)	0.9 (1.2)	0.3	0.8
Mean actual intake for day 7	43.0 (41.8)	39.4 (39.1)			1.3 (1.2)	1.1 (1.1)		
Mean prescription for day 13	127.1 (42.4)	87.4 (42.4)	0.03	0.7	3.7 (1.2)	2.7 (1.2)	0.02º	0.5
Mean actual intake for day 13	127.3 (41.2)	93.6 (39.1)			3.9 (1.2)	2.8 (1.1)		
Mean prescription for day 14	123.0 (42.4)	96.4 (42.4)	0.2	0.44	3.5 (1.2)	3.0 (1.2)	0.2	0.4
Mean actual intake for day 14	109.3 (41.8)	96.1 (40.6)			3.3 (1.2)	2.8 (1.2)		
Mean prescription for day 20	144.8 (42.4)	138.8 (42.4)	0.9	0.006º	4.2 (1.2)	3.7 (1.2)	0.7	0.04º
Mean actual intake for day 20	111.5 (41.8)	114.0 (38.4)			3.3 (1.2)	3.4 (1.1)		
Mean prescription for day 21	145.3 (42.4)	144.7 (42.4)	0.9	0.002º	4.2 (1.2)	3.8 (1.2)	0.5	0.004º
Mean actual intake for day 21	117.3 (41.8)	112.0 (39.9)			3.3 (1.2)	3.1 (1.1)		
Mean prescription for day 27	144.0 (42.4)	142.5 (42.4)	0.8	0.03º	4.1 (1.2)	3.7 (1.2)	0.6	0.14
Mean actual intake for day 27	125.1 (42.4)	120.5 (40.6)			3.6 (1.2)	3.5 (1.2)		
Mean prescription for day 28	142.6 (42.4)	144.7 (42.4)	0.8	0.01º	4.0 (1.2)	3.8 (1.2)	1.0	0.02º
Mean actual intake for day 28	117.6 (42.4)	124.0 (40.6)			3.2 (1.2)	3.4 (1.2)		
Mean prescription: week 1- 4	114.7 (64.8)	101.8 (64.8)	0.3	0.06º	3.3 (1.9)	2.8 (1.9)	0.15	0.3
Mean actual intake: week 1- 4	98.0 (63.1)	91.4 (57.1)			2.9 (1.9)	2.7 (1.7)		

*SD: Standard deviation; ^Cg: control group; #Sg: study group; Pr: Prescribed intake; Ac: Actual intake

^: Tests used for analysis: Variance Estimation, Precision and Comparison (VEPAC)

º: p-value (difference between prescribed and actual intake) reached statistical significance, i.e.: $p<0.05$

3.3.3.3 Feeding tolerance and output data of the infants

All participating infants were continuously monitored by the medical personnel for signs of gastro-intestinal intolerance (i.e. vomiting or a gastric aspirate of >2ml/kg/day) as well as output data (i.e. number of wet nappies and stools as well as stool appearance) (Table 3.8). No significant difference was observed between the two groups for any of the above-mentioned indicators of gastro-intestinal tolerance. Urine and stool output remained within normal limits, while the number of incidents of vomiting or a gastric aspirate of >2ml/kg over the 28 days was at an acceptably low level.

Table 3.8: A comparison of the number of incidents of vomiting and gastric aspirates >2ml/kg/day, as well as output data between the control and study group infants for the first 28 days of life [means (SD)[#]]

Indicator	Unit	Control group	Study group	P-value*
Incidents of vomiting/ gastric aspirate per study participant for the 28-day follow-up period	no^/ day	0.1 (0.1)	0.1 (0.1)	0.72
Wet nappies	no/ day	10.0 (0.8)	10.0 (0.8)	0.98
Stools	no/ day	1.9 (0.4)	2.2 (0.4)	0.10

[#]SD: Standard deviation; ^no: Number

*: Mann-Whitney U test used for determination of p-values

3.4 Anthropometric Data

3.4.1 Mothers

Weight, height, mid-upper arm circumference was measured in all participating mothers at entry to the study, while weight and mid-upper arm circumference were repeated at study exit (Table 3.9). No significant difference was observed for any anthropometric indicator between the two groups. All anthropometric indicators remained stable during the 4-week follow-up period. Mean weight of the women was between the 50th and 75th percentile for women 20-39 years of age[155] through-out the follow-up period. Mean height of the mothers was between the 15th to 25th percentile for women, 20-39 years of age.[155] Mean BMI remained in the range classified as overweight.[165] Mean mid-upper arm circumference remained in the 25th to 75th percentile range for women, 20-39 years of age.[155]

Table 3.9: A comparison of the anthropometric data of the control and study group mothers at entry to (week 1) and exit from (week 4) the study [means (SD)[#]]

Measure-ment	Unit	Control group: Week 1	Study group: Week 1	p-value *	Control group: Week 4	Study group: Week 4	p-value*
Weight	kg	74.1 (14.0)	67.5 (14.0)	0.67	73.1 (14.1)	67.6 (14.1)	0.78
Height	m	1.58 (0.07)	1.57 (0.07)	0.68	N/A	N/A	-
BMI	kg/ m^2	29.8 (5.4)	27.5 (5.4)	0.73	29.4 (5.4)	27.5 (5.4)	0.83
Mid-upper arm circum-ference	cm	30.9 (4.0)	30.3 (4.0)	0.99	31.1 (4.2)	30.6 (4.2)	0.99

[#]SD: Standard deviation

*: Mann-Whitney U test used for determination of p-values

N/A: Not applicable: Height was measured once only, as all mothers were older than 19 years of age and have thus reached their height potential before study participation.

3.4.2 Infants

The mean (SD) gestational age at birth was 28.7 (1.6) weeks for the control group infants and 30.4 (1.6) weeks for the study group infants, which differed significantly (p=0.02). Anthropometric data obtained from all the study infants were birth weight, length and head circumference. All infants were weighed daily by trained nursing personnel if the infant's medical condition allowed it (e.g. not on ventilator support such as continuous positive airways pressure). Length and head circumference were measured at the end of the 28-day follow-up period. All anthropometric indicators increased significantly ($p<0.05$) within the 28-day follow-up period. Weight, length and head circumference growth did not differ significantly between the control and study groups from birth to day 28 postpartum (Table 3.10).

Table 3.10: A comparison of the anthropometric data of the control and study group infants at birth and exit from (week 4: day 27 or 28) the study [means (SD)[#]]

Measurement	Unit	Control group: At birth	Control group: w4	p-value^ (cg[a])	Study group: At birth	Study group: w4	p-value^ (sg#)
Weight	g	966 (131)	1273.8 (186)	<0.01*	1066 (131)	1352.5 (186)	<0.01*
Length	cm	34.8 (2.5)	38.5 (1.5)	0.00036*	35.1 (2.5)	39.5 (1.5)	0.00019*
Head circumference	cm	25.8 (1.2)	28.8 (1.2)	0.00018*	26.5 (1.2)	29.1 (1.2)	0.00018*

Abbreviations: [#]SD: Standard deviation; [a]Cg: control group; #Sg: study group; w4: week 4

^: Repeated measures analysis of variance: Mann-Whitney or Bonferroni test used for determination of p-values.

*: $p<0.05$ indicates a significant difference

Birth weight was regained on average at day 12 to 13 of life. Weight gain velocity from the day that birth weight was regained until day 28 was determined to assess the rate of catch-up growth obtained by these infants and how this compared with the recommended growth velocity for improved neuro-developmental outcome (Table 3.11).[170] No significant difference ($p>0.05$) was observed between the control and study group, as well as in comparison with the recommended growth velocity of >18 g/kg/day.

Table 3.11: Mean (SD)* weight gain velocity from the day that birth weight was regained by the control and study, as well as pooled group of infants during the 28-day follow-up period, compared with the recommended growth velocity for improved neuro-developmental outcome

Weight gain velocity	Unit	Cg	Sg	Cg+ Sg	Rec. gr. vel.[170]	P-value: Cg vs Sg	P-value: Cg vs Rec. gr. vel.[170]	P-value: Cg vs Rec. gr. vel.[170]	P-value: Cg+Sg vs Rec. gr. vel.[170]
Weight gain	g/kg/ day	16.75 (5.0)	15.95 (4.0)	16.37 (4.5)	>18.0	0.68	0.41	0.12	0.10

Abbreviations used: *SD= Standard deviation; Cg=Control group; Sg= Study group; Rec. gr. vel.: Recommended growth velocity

Data on the weight accretion pattern of the control and study group for the first 28 days of life are presented in Figure 3.6. Although the study group participants' mean weight remained non-significantly (p=0.32) higher than that of the control group during the follow-up period, both groups followed the same pattern of weight accretion as determined by non-linear estimation.

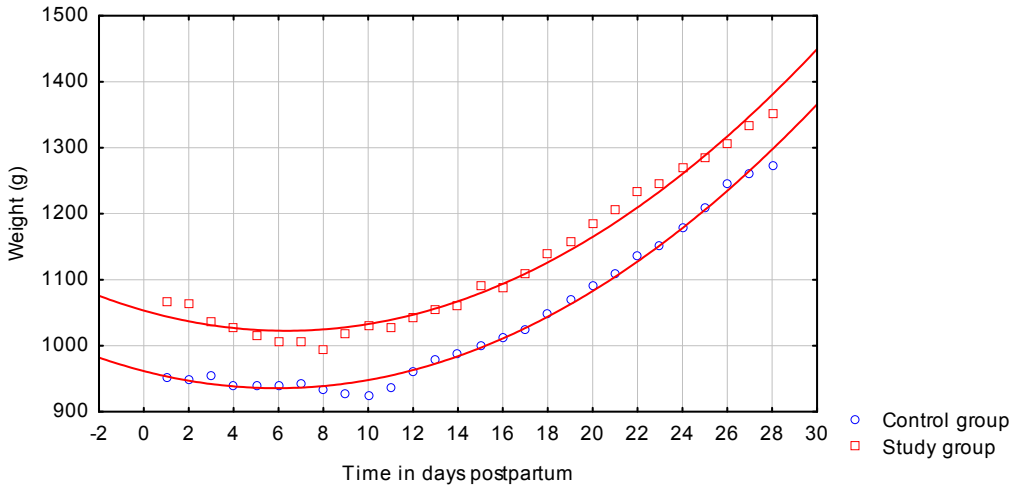

Figure 3.6: The weight accretion pattern observed for the control group and study group during the study period. Both groups followed a similar growth curve.

The growth of the extremely low birth weight (ELBW; *N=10*) and low birth weight (LBW; *N=13*) infants was compared and is presented in Figure 3.7. The p-value of 0.43 indicates that both groups followed the same growth pattern and no significant difference was observed in the growth velocity of the ELBW versus the LBW infants.

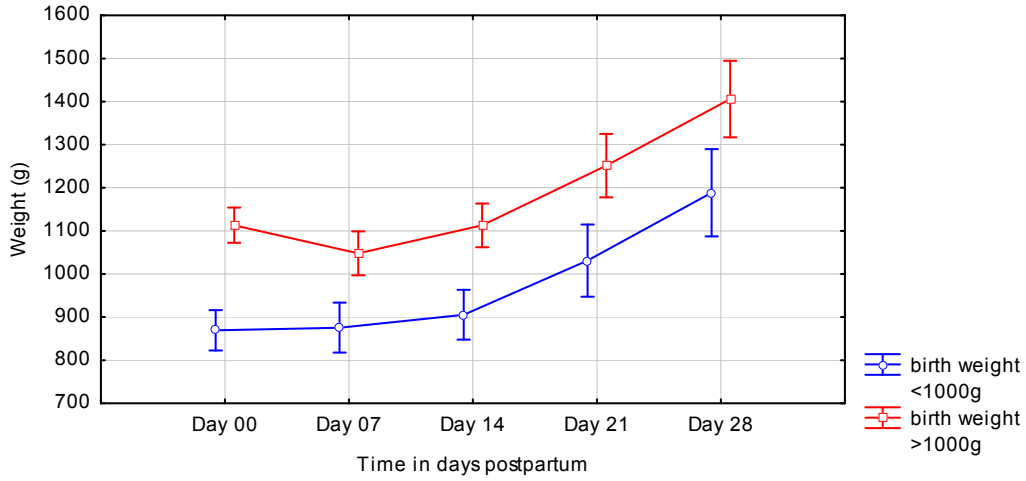

Figure 3.7: A comparison of the growth of extremely low birth weight infants (<1000g birth weight) with low birth weight infants (>1000g) [P-value = 0.43]

Total length accretion for the control and study group respectively was significant ($p<0.01$) over time from a mean (SD) birth length of 34.8 (2.5) cm and 35.1 (2.5) cm to 38.5 (1.5) cm and 39.5 (1.5) cm at 28 days after birth, averaging a 4.1 cm (±1.0 cm per week) increase during the 28-day follow-up period for the pooled group. Head circumference increased significantly ($p<0.01$) during the 28-day follow-up period for the control and study group respectively from a mean (SD) value of 25.8 (1.2) cm and 26.5 (1.2) at birth to 28.8 (1.2) cm and 29.1 (1.2) cm at day 28, averaging a 0.69 cm gain/week for the pooled group. Length and head circumference gain never differed significantly between the two groups at any time point, as reflected by the respective p-values of 0.4 and 0.3.

3.5 Biochemical Parameters

3.5.1 Mothers

Blood biochemical determination (Table 3.12) revealed a decreased albumin value of 29.4 (4.3) g/L and 28.7 (4.3) g/L for the control and study group respectively that increased significantly ($p<0.01$) over time to 36.6 (5.4) g/L and 34.0 (5.4) g/L, which nearly reached the normal range of 37 – 53 g/L[171] during the 28-day follow-up period.

Pre-albumin and hemoglobin levels did not differ significantly ($p>0.05$) between the two groups and also did not change significantly ($p=0.178$ and $p=0.47$ respectively) over time. Hematocrit and ferritin levels did not differ significantly ($p>0.05$) between the control and study group, but increased significantly ($p<0.01$) in both groups over time. Transferrin and immunoglobulin A (IgA) levels did not differ significantly ($p>0.05$) between the two groups and also did not change significantly ($p=0.799$ and $p=0.674$ respectively) over time.

A comparison between the control and study group mean immunoglobulin G (Ig G) values showed a significant ($p=0.0002$) difference in the mean (SD) week 1 value of 11.6 (3.9) vs 25.2 (3.9) g/L and the week 4 value of 14.2 (5.4) vs. 29.9 (5.4) g/L values for the two groups respectively.

C-reactive protein levels were moderately raised for both groups at initiation of the study, with the levels decreasing significantly ($p<0.01$) over the follow-up period to reach near-normal levels at the end of the 28-day study period.

Vitamin A levels remained within the normal range for the control and study group, although a significant ($p=0.017$) decrease in serum vitamin A levels of both groups was observed over the 28-day follow-up period (Table 3.12).

Table 3.12: A comparison of mean (SD)^ week 1 and week 4 serum values for specified blood constituents for the control and study group mothers

Determinant (Serum)	Unit	Normal values[171]	Week 1 value		p-value w1: Cg vs Sg	Week 4 value		p-value w4: Cg vs Sg	p-value wk1: vs wk 4 (Cg +Sg)
			Cg^	Sg#		Cg^	Sg#		
Albumin	g/L	37 - 53	29.4 (4.3)	28.7 (4.3)	0.9	36.6 (5.4)	34.0 (5.4)	0.6	<0.01 *
Pre-albumin	g/L	0.2 - 0.4	0.28 (0.08)	0.26 (0.08)	0.9	0.26 (0.05)	0.23 (0.05)	0.7	0.178
Haemoglobin	g/dL	12.0 -15.0	11.9 (2.1)	10.9 (2.1)	0.7	12.5 (1.95)	11.2 (1.95)	0.4	0.47
Haematocrit	L/L	0.36 -0.46	0.36 (0.06)	0.34 (0.06)	0.9	0.39 (0.05)	0.35 (0.05)	0.3	0.01*
Ferritin	µg/L	4.5 - 170	100.4 (89.7)	68.4 (89.7)	0.7	33.9 (25.1)	40.2 (25.1)	1.0	0.01*
Transferrin	g/L	2.0 - 3.6	2.94 (0.61)	2.86 (0.61)	1.0	3.08 (0.64)	2.68 (0.64)	0.5	0.799
Immunoglobulin A	g/L	0.7 - 4.0	3.2 (1.2)	3.7 (1.2)	0.8	3.2 (1.5)	3.9 (1.5)	0.6	0.674
Immunoglobulin G	g/L	7.0 - 16.0	11.6 (3.9)	25.2 (3.9)	<0.01 *	14.2 (5.4)	29.9 (5.4)	<0.01 *	<0.01 *
C-reactive protein	mg/L	≤ 3	14.3 (10.1)	13.5 (10.1)	1.0	4.4 (7.6)	7.7 (7.6)	0.8	<0.01 *
Vitamin A	µg/ dL	>20	72.0 (22.6)	58.0 (22.6)	0.3	59.6 (16.1)	51.4 (16.1)	0.7	0.017 *

^SD: Standard Deviation; ^Cg: control group; #Sg: study group; W1: week 1; W4: week 4
*p-value<0.05, indicating a significant difference: Repeated Measures of Variance (ANOVA) test used to determine p-values.

CD4 T-cell lymphocyte count of the study group mothers showed a trend towards a significant decrease from a mean [95%CI] week 1 value of 562.4 [95% CI, 314.1 - 810.8] µl to 446.3 [95% CI, 262.8 - 629.9] µl at the end of week 4. The log value of the serum

viral load of the HIV positive mothers remained above the normal value and increased significantly (p=0.009) over the 4-week follow up period (Table 3.13).

Table 3.13: Mean [95% CI] CD4 and Viral Load determinations of the study group (HIV positive mothers) only

Determinant (Serum)	Unit	Normal values[171]	Week 1 value	Week 4 value	p-value
CD4 T-cell Count	µl	700 - 1100	562.4 [314.1 - 810.8]	446.3 [262.8 - 629.9]	0.056
Viral Load	Log values	<2.18	3.58 [2.82 - 4.35]	4.55 [4.08 - 5.02]	0.009*

^[95% CI]: 95% Confidence Interval

*p-value<0.05, indicating a significant difference: Repeated Measures Analysis of Variance (ANOVA) test used to determine p-values.

3.5.2 Infants

Albumin and pre-albumin values were determined at the end of week 1 and week 4 for each infant to get a reflection on their nutritional status as well as an indication of whether the current feeding regimen provided them with sufficient nutrients. Table 3.14 reveals the mean values for the pooled group, as no significant difference in mean values were found between the control and study group infants (p=0.7 for week 1 and p=1.0 for week 4 mean values of the control and study group for albumin and p=0.8 for week 1 and p=0.4 for week 4 mean values for pre-albumin). Both albumin and pre-albumin levels remained stable and below the normal ranges throughout the study period.

Table 3.14: A comparison of mean [95% CI] week 1 and week 4 serum values for specified constituents for the infant study population as a whole

Determinant (Serum)	Unit	Normal values[172]	Week 1 value	Week 4 value	p-value
Albumin	g/L	35 - 52	31.1 [28.4 -33.7]	31.0 [27.9 – 34.0]	0.91
Pre-Albumin	g/L	0.2 - 0.4	0.08 [0.05 – 0.1]	0.08 [0.05 – 0.1]	0.73

^[95% CI]: 95% Confidence Interval

*p-value<0.05, indicating a significant difference: Repeated Measures Analysis of Variance (ANOVA) test used to determine p-values.

HIV status of study group infants:

DNA-Polymerase chain reaction results as determined on day 27 or 28 of the follow-up period indicated that 2 (18.2%) out of the 11 HIV-exposed infants were HIV positive, while the remaining 9 (81.2%) infants were HIV negative.

3.6 Breast Milk Determinations

Breast milk samples were collected from the mothers in both groups, and analysed for macronutrient, micronutrient and immuno-protein content, as well as HIV viral load. As direct (i.e. bomb calorimetric method) calorimetric determinations of energy intake were not possible, all energy intake determinations in this section were calculated theoretically using the following energy conversion factors: 1 gram protein = 4kcal; 1 gram carbohydrate = 4kcal; 1 gram fat = 9 kcal.[159]

3.6.1 Macronutrients

The week 1 macronutrient composition of the control group was compared to the study group (Table 3.15) and revealed no significant difference in energy, protein, carbohydrate or fat content between the two groups and between the raw and pasteurised breast milk samples. The corresponding week 4 analysis found a similar result, i.e. no significant difference in energy, protein, carbohydrate or fat content was observed between the two groups and between the raw and pasteurised breast milk samples for week 4 (Table 3.16).

As no significant compositional differences were observed between the groups and milk processing method (i.e. raw or pasteurised), the results were pooled to obtain a clearer picture of the breast milk macronutrient composition, as well as to determine mean [95% CI] week 1 and week 4 breast milk macronutrient composition for this study's population (Table 3.17). Protein was the only nutrient to show a significant ($p<0.01$) decrease over the 4-week follow-up period for the pooled group, which is a confirmation of the protein results obtained for both groups (Tables 3.15 and 3.16).

Table 3.15: Mean [95% CI] macronutrient composition of the Raw and Pasteurised breast milk samples compared between the control and study groups for week 1

Nutrient	Unit per 100 ml	Control group (w1) (Raw)	Control group (w1) (Past.)	P-value (raw vs past.)*	Study group (w1) (Raw)	Study group (w1) (Past.)	P-value (raw vs past.)*	P-value* groups: raw samples	P-value* groups: past. samples
Energy	kcal	65.9 [59.9 – 71.9]	65.4 [57.8 – 72.9]	1.00	65.1 [53.9 – 76.3]	69.8 [55.7 – 83.9]	0.54	1.0	0.99
Protein	g	2.15 [1.85 - 2.45]	2.15 [1.85 - 2.46]	1.00	1.88 [1.27 - 2.48]	1.88 [1.27 - 2.49]	0.99	0.99	0.99
Carbohy-drate	g	6.66 [6.29 - 7.03]	6.87 [6.46 - 7.27]	0.51	6.92 [6.18 - 7.66]	7.07 [6.27 - 7.87]	1.00	1.00	1.00
Fat	g	3.26 [2.68 - 3.84]	3.13 [2.43 - 3.82]	0.99	3.32 [2.16 - 4.48]	3.78 [2.39 - 5.17]	1.00	0.99	1.00

Abbreviations: w1= week 1

^95% CI: 95% Confidence Interval

*: General Linear Models (Tukey test) used to determine p-value: level of significance: p<0.05

Table 3.16: Mean [95% CI] macronutrient composition of the Raw and Pasteurised breast milk samples compared between the control and study groups for week 4

Nutrient	Unit per 100 ml	Control group (w4) (Raw)	Control group (w4) (Past.)	P-value (raw vs past.)*	Study group (w4) (Raw)	Study group (w4) (Past.)	P-value (raw vs past.)*	P-value* groups: raw samples	P-value* groups: past. samples
Energy	kcal	61.6 [55.1 - 68.1]	61.5 [54.9 – 68.1]	1.00	59.9 [47.7 – 72.0]	64.3 [52.0 – 76.6]	0.63	1.00	1.00
Protein	g	1.51 [1.20 – 1.82]	1.53 [1.20 – 1.86]	1.00	1.42 [0.79- 2.04]	1.38 [0.72 - 2.03]	1.00	1.00	1.00
Carbohy-drate	g	6.63 [6.22 – 7.03]	6.74 [6.43 – 7.06]	0.98	6.23 [5.42 – 7.03]	6.66 [6.03 – 7.29]	0.41	0.9	1.00
Fat	g	3.21 [2.60 - 3.82]	3.16 [2.55 – 3.76]	1.00	3.26 [2.04 - 4.47]	3.57 [2.37 - 4.77]	0.99	1.00	0.99

Abbreviations: w4= week 4

^95% CI: 95% Confidence Interval

*: General Linear Models (Tukey test) used to determine p-value: level of significance: p<0.05

Table 3.17: A comparison of the mean [95% CI] pooled (both group and milk processing method pooled) week 1 and week 4 values for energy, protein, carbohydrate and fat

Nutrient	Unit per 100ml	Pooled Group (week 1)	Pooled Group (week 4)	P-value (week 1 vs week 4)
Energy	kcal	66.5 [56.6 – 76.5]	61.8 [52.1 – 71.6]	0.52
Protein	g	2.01 [1.53 -2.49]	1.46 [0.95 -1.96]	0.000161*
Carbohydrate	g	6.88 [6.28 -7.48]	6.56 [6.00 – 7.12]	0.47
Fat	g	3.37 [2.39 - 4.35]	3.30 [2.35 - 4.25]	0.99

^95% CI: 95% Confidence Interval

*: General Linear Models (Tukey test) used to determine p-value: level of significance: $p<0.05$

3.6.2 Micronutrients

The breast milk micronutrient content of the control and study group did not differ significantly over time for folic acid (p=0.1), calcium (p=0.93), phosphorus (p=0.36), magnesium (p=0.53), sodium (p=0.73), potassium (p=0.2), iron (p=0.96), zinc (p=0.92) and copper (p=0.37) (Table 3.18).

The mean ([95% CI] calcium content of the control group's pasteurised breast milk samples decreased significantly over time (i.e. 27.0 [18.2-35.8] vs 23.1 [19.4-26.7] mg/100ml), while the raw samples' calcium content remained stable. In the study group the mean [95% CI] calcium content decreased significantly only in the raw samples over time (i.e. 29.3 [19.2-39.4] vs 24.4 [20.2-28.6] mg/100ml).

The phosphorus content presented a definite time effect, where the control group's mean [95% CI] phosphorus content decreased significantly (p=0.026) for the raw breast milk samples, from 15.15 [11.5-18.8] to 12.90 [10.1-15.7] mg/100ml, as well as for the pasteurised samples, from 16.3 [12.2-20.4] to 12.6 [9.9-15.3] mg/100ml; p=<0.001. In the study group samples, the time effect was only significant (p=0.033) for the mean [95% CI] raw breast milk samples, i.e. 15.93 [11.5-20.3] to 13.29 [9.9 -16.6] mg/100ml, while the pasteurised breast milk samples still presented with a decrease in phosphorus content over time although the latter decrease was not found to be significant (p=1.00).

Magnesium content in all breast milk samples remained fairly constant and no significant effect of time, group or processing method on the breast milk composition was observed.

A significant difference was observed for the control group only, where the mean sodium content of the raw and pasteurised breast milk samples decreased significantly ($p<0.001$ in both cases) over time. The study group did however show a non-significant ($p=0.23$) decrease in sodium levels in the raw samples over time, while the sodium content of the pasteurised samples remained fairly stable.

Time had a definite effect on breast milk potassium content, with the latter decreasing in the breast milk samples of both groups as well as in the raw and pasteurised samples. The decrease in mean [95% CI] potassium content was however only significant for the control group samples as reflected by a p-value of <0.001 for the raw, from 72.35 [60.8-83.9] to 55.60 [45.5-65.7] mg/100ml and pasteurised samples, from 71.80 [63.4-80.2] to 53.80 [43.6-64.0] mg/100ml). The study group presented with the same trend of a decrease in raw and pasteurised breast milk potassium content, although it was not found to be significant ($p=0.154$).

Iron content in all breast milk samples remained fairly constant and no significant effect of time, group or processing method of the breast milk was observed.

Zinc values decreased significantly ($p<0.001$) during the first 28 days of lactation in both control and study group participants and in both raw and pasteurised breast milk samples. The extent of the decrease in zinc content when the two groups were compared did not differ significantly ($p=0.92$).

A significant difference was observed for the study group over time in the raw samples, where mean [95% CI] copper content of the breast milk decreased from 0.099 [0.08-0.12] to 0.07 [0.05-0.09] mg/100ml, ($p=0.0096$) while a similar trend, although not significant, was observed in the pasteurised samples. The control groups' copper content remained stable during the follow-up period for both the raw and pasteurised samples.

Table 3.18: A comparison of the mean [95%CI]# micronutrient content of raw and pasteurised breast milk of the control and study group over the 4-week study period

Breast milk micronutrient content	Unit	Control group (week1)	P-value (cg: R vs P)	Study group (week1)	P-value (sg: R vs P)	P-value (cg vs sg)#	Control group (week4)	P-value (cg: R vs P)	Study group (week4)	P-value (sg: R vs P)	P-value (cg vs sg)#	P-value (cg: w1 vs w4)#	P-value (sg: w1 vs w4)#
Folic acid: R	µg/L	10.4 [6.9-13.9]	0.249	7.4 [3.9-10.9]	0.998	0.999	11.1 [5.6-16.6]	0.421	14.2 [8.7-19.7]	0.960	0.999	1.000	0.184
Folic acid: P	µg/L	16.8 [10.3-23.4]		9.9 [3.4-16.4]		0.924	16.9 [10.6-23.1]		17.7 [11.5-23.9]		0.999	1.000	0.069
Calcium: R	mg	25.2 [16.7-33.6]	0.61	29.3 [19.2-39.4]	0.71	1.00	23.2 [19.7-26.7]	1.00	24.4 [20.2-28.6]	1.00	1.00	0.53	0.0006*
Calcium: P	mg	27.0 [18.2-35.8]		27.2 [16.7-37.8]		1.00	23.1 [19.4-26.7]		24.1 [19.8-28.5]		1.00	0.001*	0.131
Phosphorus: R	mg	15.15 [11.5-18.8]	1.00	15.93 [11.5-20.3]	1.00	1.00	12.90 [10.1-15.7]	1.00	13.29 [9.9-16.6]	1.00	1.00	0.026[a]	0.033[a]
Phosphorus: P	mg	16.30 [12.2-20.4]		15.50 [10.6-20.4]		1.00	12.60 [9.9-15.3]		14.00 [10.7-17.3]		1.00	<0.001 [a]	1.00
Magnesium:R	mg	4.25 [3.7-4.8]	1.00	3.79 [3.1-4.5]	1.00	1.00	3.50 [3.0-4.0]	1.00	3.29 [2.7-3.9]	1.00	1.00	1.00	1.00
Magnesium :P	mg	4.70 [3.9-5.5]		3.50 [2.6-4.4]		1.00	3.60 [3.1-4.1]		3.36 [2.7-4.0]		1.00	0.19	1.00

#: Abbreviations: 95% CI: 95% Confidence Interval; cg= control group; sg= study group; w1= week 1; w4= week 4; R: Raw; P: pasteurised

*: General Linear Models (Tukey test) used to determine p-value: *level of significance: $p<0.05$

[a]: General Linear Models (Bonferroni test) used to determine p-value: *level of significance: $p<0.05$

Table 3.18: A comparison of the mean [95%CI][#] micronutrient content of raw and pasteurised breast milk of the control and study group over the 4-week study period (cont.)

Breast milk micronutrient content	Unit	Control group (week1)	P-value (cg: R vs P)	Study group (week1)	P-value (sg: R vs P)	P-value (cg vs sg)#	Control group (week4)	P-value (cg: R vs P)	Study group (week4)	P-value (sg: R vs P)	P-value (cg vs sg)#	P-value (cg: w1 vs w4)#	P-value (sg: w1 vs w4)#
Sodium: R	mg	47.60 [32.3-62.9]	0.998	45.64 [27.4-63.9]	0.729	1.00	36.30 [21.5-51.1]	1.000	37.79 [20.1-55.5]	0.895	1.00	0.0008*	0.23
Sodium: P	mg	50.15 [35.1-65.2]		39.93 [21.9-58.0]		0.999	36.30 [22.3-50.3]		42.64 [26.0-59.3]		0.999	0.0001*	0.99
Potassium: R	mg	72.35 [60.8-83.9]	1.00	60.71 [46.9-74.6]	1.00	1.00	55.60 [45.5-65.7]	1.00	51.86 [39.8-63.9]	1.00	1.00	0.000*	0.154
Potassium: P	mg	71.80 [63.4-80.2]		59.50 [49.5-69.5]		1.00	53.80 [43.6-64.0]		50.64 [38.4-62.9]		1.00	0.000*	0.154
Iron: R	mg	0.171 [0.09-0.25]	1.00	0.198 [0.10-0.29]	1.00	1.00	0.194 [0.12-0.27]	1.00	0.178 [0.09-0.27]	1.00	1.00	1.00	1.00
Iron: P	mg	0.211 [0.15-0.28]		0.170 [0.09-0.25]		1.00	0.199 [0.12-0.28]		0.164 [0.07-0.26]		1.00	1.00	1.00
Zinc: R	mg	0.488 [0.39-0.59]	0.921	0.454 [0.33-0.58	1.000	1.000	0.334 [0.26-0.40]	0.999	0.314 [0.22-0.40]	0.999	1.000	0.0001*	0.0001*
Zinc: P	mg	0.517 [0.43-0.61]		0.459 [0.34-0.58]		0.999	0.349 [0.28-0.42]		0.336 [0.24-0.43]		1.000	0.0001*	0.0002*
Copper: R	mg	0.106 [0.09-0.12]	0.327	0.099 [0.08-0.12]	0.970	1.00	0.100 [0.08-0.12]	0.999	0.070 [0.05-0.09]	1.000	0.613	0.999	0.0096*
Copper: P	mg	0.122 [0.11-0.14]		0.089 [0.07-0.11]		0.450	0.105 [0.09-0.12]		0.070 [0.05-0.09]		0.403	0.202	0.374

See previous page for an explanation of the abbreviations and type of tests used for significance testing.

Sodium/Potassium ratio

Sodium/potassium ratio remained above the cut-off of 0.6 throughout for both the control and study group, which is indicative of the presence of sub-clinical mastitis.[16] A significant difference (p=0.04) in the sodium/potassium ratio was observed between the week 1 and week 4 pasteurised breast milk samples of the study group only (Figure 3.8 and Table 3.19).

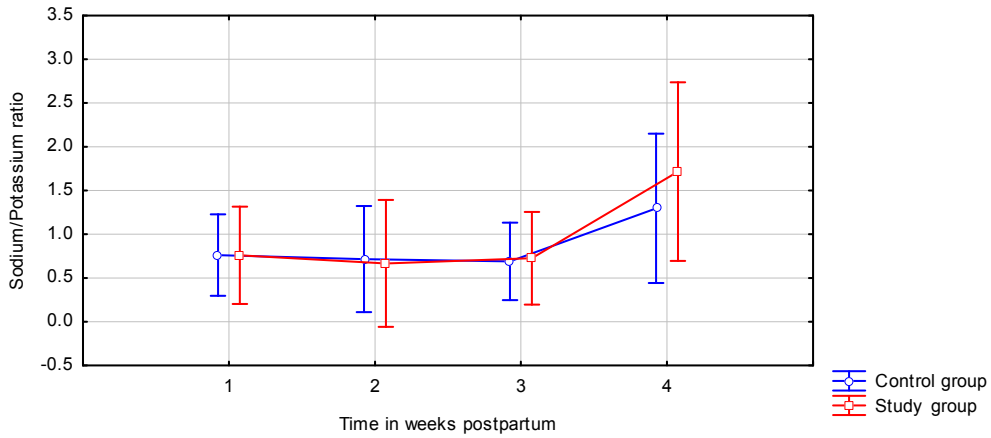

Figure 3.8: Mean [95% CI] sodium/potassium ratio of the control and study group over the 4 weeks of follow-up. No significant difference (p=0.71) was observed between the groups over time

Table 3.19: The mean [95% CI] ratios of Sodium/Potassium for the control and study group during week 1 and week 4 in the raw and pasteurised breast milk samples. Normal value: <0.6[16]

Sodium/ Potassium (Na/K) ratio in breast milk samples	Control group (w1)	Study group (w1)	P-value (cg vs sg)#	Control group (w4)	Study group (w4)	P-value (cg vs sg)#	P-value (cg: w1 vs w4)#	P-value (sg: w1 vs w4)#
Na/K ratio: R	0.75 [0.41-1.08]	0.81 [0.41-1.21]	1.00	0.71 [0.31-1.10]	0.89 [0.41-1.36]	1.00	1.00	1.00
Na/K ratio: P	0.78 [0.45-1.11]	0.71 [0.32-1.10]	1.00	1.88 [0.43-3.34]	2.54 [0.81-4.3]	0.98	0.36	0.04*
P-values (R vs P)	1.00	1.00		0.27	0.09			

#: Abbreviations: [95% CI] = 95% Confidence Interval; cg = control group; sg = study group; w1= week 1; w4 = week 4; R = Raw; P = pasteurised

*: General Linear Models (Tukey test) used to determine p-value: *level of significance: p<0.05

As no significant difference was observed between the mean raw and pasteurised breast milk samples of control and study group mothers for any nutrient analysed, the weekly breast milk results obtained were pooled to better observe the effect of time on breast milk composition. The mean [95% CI] weekly results obtained are presented in table 3.20.

A significant decline in breast milk protein, magnesium, potassium, zinc and copper content was observed for the whole study population over time, while folic acid content increased significantly over time. Energy, carbohydrate, fat, calcium, phosphorus, sodium and iron content remained fairly stable over the 28-day follow-up period.

The present study's nutrient composition of premature breast milk at day 27-28 of life was compared with published data (Table 3.21).[94, 173] This study found the mean [95% CI] energy content (theoretically determined using the factors 4 kcal/g protein or carbohydrate and 9 kcal/g fat) of raw and pasteurised breast milk to be 61.8 [52.1-71.6] kcal/ 100ml at 27 – 28 days post-delivery. Anderson[96] found (raw) preterm milk to contain 71 (2) kcal/100 ml at 28 – 29 days post-delivery, while Schanler[173] found the mean (SD) energy content of 69 (0.5) kcal/100ml preterm milk at 22 – 28 days. Mean [95% CI] breast milk energy content for the control versus study group over the 28-day follow-up period was 65.9 [60.0-72.0] kcal/100ml and 63.4 [52.0-74.8] kcal/100ml respectively, which did not differ significantly (p=0.67). Bortolozo et al[94] found a low mean (SD) energy content of 55.44 (8) kcal/100ml in the pasteurised breast milk samples of the preterm milk they analysed.

The carbohydrate content of the current study remained fairly stable during the follow-up period and day 27-28 mean [95% CI] carbohydrate content compares well with previous data, i.e 6.56 [6.00-7.12] g/100ml vs a mean (SD) carbohydrate content of 6.7 (0.4) g/100ml [173, 196] and 6.56 (0.14) g/100ml found by Bortolozo et al[94] in pasteurised premature milk, although the current study's carbohydrate content is lower than the mean of 7.3 g/100ml found for preterm human milk at 2-4 weeks.[104,186]

The mean [95% CI] fat content of 3.30 [2.35-4.25] g/100ml found by the current study at 27-28 days of lactation, were lower than the mean (SD) fat content determined by other studies as 3.6 (0.7) g/100ml[173, 196] and 3.5 g/100ml[104, 186], but were higher than the mean (SD) fat content found by Bortolozo et al in pasteurised premature milk.

The mean [95%CI] breast milk folic acid content of 1.5 [1.0-2.0] µg/100ml (or 15.0 [10.0-20.0]µg/L) at 27 – 28 days of lactation was found to be lower than reported by other

investigators. Schanler et al[173] reported folic acid content to average at 33 µg/L in preterm breast milk (22 - 28 days), which is more than double the value found with this study.

A comparison of mean [95%CI] phosphorus content 13.2 [10.2-16.2] mg/100ml at 27-28 days of life with previous studies, show a similar (i.e. 14.5mg/100ml[104, 195]) or slightly lower (i.e. mean (SD)= 8.47 (2.43)[94] and mean [95%CI]=9.3 [7.3-11.3] mg/100ml[54, 173]) phosphorus content.

Breast milk mean [95% CI] magnesium content decreased significantly (p=0.045) from 4.06 [3.46-4.66] mg/100ml to 3.44 [2.86-4.01] mg/100ml during the 28-day follow-up period. The mean [95% CI] 27-28 day magnesium value of 3.44 [2.86-4.01] mg/100ml is much higher than found with other studies. Schanler et al[173] found the mean (SD) breast milk magnesium content of 2.4 (0.7) mg/100ml, while Bortolozo et al[94] found mean (SD) magnesium content to be 2.16 (0.26) mg/100ml in pasteurised preterm breast milk samples.

Sodium content decreased during the follow-up period from a mean [95% CI] value of 45.83 [29.3-62.4] mg/100ml to 38.26 [22.6-53.9] mg/100ml in the pooled group analysis. A comparison with previous studies who determined preterm breast milk sodium content after the first month of lactation, the current study's population group had a very high value of 38.26 [22.6-53.9] mg/100ml compared to a mean (SD) of 20.2 (4.6) mg/100ml.[173] The mean sodium content of the current study did compare well to the mean (SD) pasteurised premature breast milk sodium content of 37.98 (11.34) mg/100 ml, found by Bortolozo et al. [94]

The pooled analysis showed a decrease in potassium content in this study population from a mean [95% CI] value of 66.09 [55.6-76.6] mg/100ml (week 1) to 52.98 [42.1-63.9] mg/100ml (week 4). This decrease in potassium over time was significant (p=0.002). Compared to other studies determining preterm breast milk composition after the first month of lactation, mean [95% CI] potassium content was found to be higher in this population group, i.e. 52.98 [42.1-63.9] vs 48.9 [35.9 -61.9] and mean (SD)= 44.37 (12.83) mg/100ml).[54, 94, 104, 173]

Iron content in all breast milk samples remained fairly constant and no significant effect of time, group or processing method of the breast milk was observed. Mean [95% CI] iron content at 27-28 days of lactation in the current study was 0.184 [0.10-0.27]

mg/100ml, which was higher than previous published data, i.e. 0.04[54, 173] and 0.09[104,195] mg/100ml at ±14 - 28 days postpartum.

A significant (p=0.00004) decrease in mean zinc values were found in the whole study population over time. Zinc values decreased from a mean [95% CI] of 0.48 [0.37-0.59] mg/100ml to 0.33 [0.25-0.41] mg/100ml at the end of week 4. These zinc values are much lower than the mean (SD) of 0.72 (0.26) mg/100 ml reported by another study looking at pasteurised preterm breast milk zinc content. The only disadvantage of the latter comparison is that the exact time after birth when the breast milk samples was collected for the preterm milk is not specified.[94] Another study, however, did find a similar mean (SD) zinc content of 0.22 (0.09) mg/100ml in preterm breast milk, 22 - 30 days after birth, which compares well to the current study's findings. [104]

Copper content significantly (p=0.03) decreased from a mean [95% CI] of 0.10 [0.09-0.12] mg/100ml to 0.09 [0.07-0.11] mg/100ml over time in the pooled group. One other study which determined the copper content of preterm breast milk at 3 to 4 weeks post delivery had a lower mean (SD) value of 0.051 (0.02) mg/100ml[173], while Picciano et al found a higher breast milk copper content of 0.2 – 0.4 mg/100ml.[87]

Table 3.20: Mean [95% CI][#] pooled breast milk composition of the study population as a whole over the 4-week study period

Nutrient	Unit per 100ml	Pooled group mean: wk1	Pooled group mean: wk2	Pooled group mean: wk3	Pooled group mean: wk4	P-value (time effect)*
Energy	kcal	66.5 [56.6-76.5]	63.1 [52.0-74.2]	67.2 [51.1-83.2]	61.8 [52.1-71.6]	0.44
Protein	g	2.01 [1.53-2.49]	1.71 [1.03-2.39]	1.62 [0.99-2.26]	1.46 [0.95-1.96]	0.000004*
Carbohydrate	g	6.88 [6.28-7.48]	6.71 [5.88-7.54]	6.60 [5.89-7.31]	6.56 [6.00-7.12]	0.158
Fat	g	3.37 [2.39-4.35]	3.17 [2.01-4.32]	3.80 [2.25-5.34]	3.30 [2.35-4.25]	0.45
Folic Acid	µg	1.11 [0.69-1.54]	1.61 [1.12-2.10]	1.59 [1.03-2.15]	1.5 [1.0-2.0]	0.012*
Calcium	mg	27.16 [17.8-36.6]	23.79 [19.0-28.5]	23.87 [19.1-28.6]	23.69 [19.8-27.6]	0.14
Phosphorous	mg	15.72 [11.5-20.0]	15.49 [12.4-18.6]	13.91 [10.8-17.0]	13.20 [10.2-16.2]	0.10
Magnesium	mg	4.06 [3.46-4.66]	3.87 [3.04-4.70]	3.86 [3.18-4.54]	3.44 [2.86-4.01]	0.045*
Sodium	mg	45.83 [29.3-62.4]	35.31 [16.6-54.0]	36.42 [23.1-49.7]	38.26 [22.6-53.9]	0.21
Potassium	mg	66.09 [55.6-76.6]	58.72 [46.2-71.2]	56.78 [46.3-67.1]	52.98 [42.1-63.9]	0.002*
Iron	mg	0.187 [0.12-0.26]	0.171 [0.11-0.23]	0.156 [0.10-0.21]	0.184 [0.10-0.27]	0.48
Zinc	mg	0.48 [0.37-0.59]	0.44 [0.36-0.52]	0.37 [0.31-0.44]	0.33 [0.25-0.41]	0.00004*
Copper	mg	0.10 [0.09-0.12]	0.08 [0.07-0.10]	0.09 [0.07-0.11]	0.09 [0.07-0.11]	0.03*

[#]: 95% CI: 95% Confidence Interval Abbreviations: wk = week

*: Repeated measures of analysis or General Linear Models used (Tukey or Bonferroni test) to determine p-values. Significance level: $p<0.05$

Table 3.21: A comparison of the mean [95% CI][#] composition of premature breast milk in the present study to published data

Nutrient	Unit per 100ml	Current study: Premature milk (27-28 days)#	Premature milk (22 – 30 days)^ [173, 196]	Preterm human milk (2 - 4 weeks postpartum) [104,195]	Pasteurised premature milk^ [94]
Energy	kcal	61.8 [52.1-71.6]	69 (5)	66	55.44 (8.00)
Protein	g	1.46 [0.95-1.96]	1.5 (0.1)	1.6	1.72 (0.4)
Carbohydrate (Lactose)	g	6.56 [6.00-7.12]	6.7 (0.4)	7.3	6.56 (1.41)
Fat	g	3.30 [2.35-4.25]	3.6 (0.7)	3.5	2.48 (0.76)
Folic Acid	µg	1.5 [1.0-2.0]	3.3	3.1	ND
Calcium	mg	23.69 [19.8-27.6]	28.8 (5.2)	25.3	22.03 (9.39)
Phosphorus	mg	13.20 [10.2-16.2]	9.3 (2.0)	14.5	8.47 (2.43)
Magnesium	mg	3.44 [2.86-4.01]	2.4 (0.7)	3.3	2.16 (0.26)
Sodium	mg	38.26 [22.6-53.9]	20.2 (4.6)	28	37.98 (11.34)
Potassium	mg	52.98 [42.1-63.9]	48.9 (13.0)	50	44.37 (12.83)
Iron	mg	0.184 [0.10-0.27]	0.04	0.09	ND
Zinc	mg	0.33 [0.25-0.41]	0.22 (0.09)	0.37	0.72 (0.26)
Copper	mg	0.09 [0.07-0.11]	0.051(0.02)	0.038	ND

#: Values indicated as means for week 4 [95% Confidence Interval].

Repeated measures of analysis or General Linear Models used for statistical analysis of data of the current study.

ND: Not determined

^: Values indicated as means plus standard deviation.

3.7 Hand versus Pump Expression

The mean (SD) breast milk volume obtained per expression was 41.7 (15.0) ml for the control group and 38.3 (15.0) ml for the study group, which did not differ significantly (p=0.6) between the two groups. The method of expression, however, did indicate a difference in breast milk yield, with a significant (p=0.03) greater volume of milk (16% more) being obtained when the breast was expressed with a hand pump (mean [95% CI] volume=42.5 [24.4-60.6] ml) when compared with hand expression (mean [95% CI] volume=36.7 [20.1-53.3] ml). The Pretoria pasteurisation process was performed by the investigator or research assistant throughout and remained exactly the same for both groups during the study period. The results of the main measuring points are shown in Table 3.22.

Table 3.22: Means (SD)* of main measuring points recorded during the pasteurisation process for the whole group (Duration of pasteurisation: 27 minutes 30 seconds.)

Pasteurisation process: Measuring points	Unit	Mean value (SD)*
Volume of breast milk pasteurised	ml	21.9 (4.5)
Starting temperature of the water	°Celcius	91.8 (1.5)
Starting temperature of the breast milk	°Celcius	25.0 (15.5)
End-pasteurisation temperature of the water	°Celcius	55.5 (6.7)
End-pasteurisation temperature of the breast milk	°Celcius	51.0 (6.6)

*SD: Standard deviation

The breast milk's end temperature indicated that the pasteurisation temperature zone (56 – 62.5°Celcius) would have been reached and maintained for a period of 10-15 minutes, which is sufficient to decrease HI viral load with an average of 3 logs, as proposed by Jeffery et al.[15]

The effect of the hand versus pump expression on mean [95%CI] breast milk nutrient composition during the first 28 days of lactation was determined. No significant difference (p>0.05) between hand and pump expression was observed for the control and study groups for any of the nutrients studied (Table 3.23). Only protein content in the control group differed significantly (p=0.01) between the hand and pump expressed samples. The difference in mean [95%CI] protein content of 1.7 [1.5-2.0] mg/100ml in the hand versus 1.8 [1.6-2.1] g/100ml in the pump expressed samples were not observed for the study group and is furthermore of no clinical significance.

Table 3.23: A comparison of the effect of expression technique (i.e. hand versus pump) on mean [±95%CI]# breast milk nutrient composition during the first 28 days of lactation

Nutrient	Unit/ 100 ml	Hand expressed breast milk		P-value (hand: cg vs sg)*	Pump expressed breast milk		P-value (pump: cg vs sg)*	P-value (hand vs pump: cg)*	P-value (hand vs pump: sg)*
		Cg#	Sg#		Cg#	Sg#			
Energy	kcal	66.5 [61.1-71.8]	67.2 [59.8-74.6]	0.9	64.2 [58.9-69.6]	64.9 [57.4-72.3]	0.9	0.3	0.4
Protein	g	1.7 [1.5-2.0]	1.9 [1.5-2.2]	0.5	1.8 [1.6-2.1]	1.8 [1.5-2.1]	0.9	0.01*	0.6
Carbohydrate	g	6.8 [6.5-7.0]	6.7 [6.3-7.0]	0.7	6.7 [6.5-7.0]	6.5 [6.2-6.9]	0.4	0.7	0.3
Fat	g	3.6 [3.1-4.1]	3.7 [2.9-4.4]	0.9	3.3 [2.8-3.9]	3.5 [2.8-4.2]	0.7	0.2	0.6
Folic Acid	µg	14.6 [11.3-17.8]	14.0 [10.0-17.9]	0.8	14.5 [11.3-17.7]	14.5 [10.4-18.7]	1.0	0.9	0.7
Calcium	mg	22.8 [18.8-26.7]	21.0 [15.8-26.2]	0.6	22.7 [18.8-26.6]	20.9 [15.7-26.1]	0.6	0.9	0.9
Phosphorus	mg	15.4 [13.2-17.5]	12.5 [9.6-15.3]	0.1	14.6 [12.5-16.8]	11.7 [8.8-14.6]	0.1	0.1	0.3
Magnesium	mg	3.8 [3.3-4.3]	3.4 [2.7-4.1]	0.4	4.0 [3.5-4.5]	3.4 [2.6-4.1]	0.2	0.1	0.8
Sodium	mg	35.9 [24.5-47.2]	34.7 [19.5-49.9]	0.9	38.2 [27.0-49.4]	35.6 [20.0-51.1]	0.8	0.5	0.9
Potassium	mg	62.5 [54.2-70.8]	53.3 [42.3-64.3]	0.2	61.6 [53.3-69.9]	50.8 [39.7-61.9]	0.1	0.5	0.2
Iron	mg	0.18 [0.06-0.30]	0.17 [0.01-0.33]	0.9	0.2 [0.08-0.32]	0.24 [0.08-0.41]	0.6	0.5	0.2
Zinc	mg	0.43 [0.35-0.50]	0.36 [0.26-0.46]	0.3	0.40 [0.33-0.48]	0.34 [0.24-0.44]	0.3	0.6	0.7
Copper	mg	0.10 [0.08-0.12]	0.08 [0.05-0.10]	0.2	0.10 [0.08-0.12]	0.08 [0.05-0.10]	0.1	0.9	0.6

#: Abbreviations: [95% CI]: 95% Confidence Interval; cg= control group; sg= study group; w1= week 1; w4= week 4; past.= pasteurised

*: p-value determined by Level of Significance Detection testing

3.8 Viral Load: Breast Milk

Breast milk viral load was determined weekly in the raw breast milk samples of all study group mothers (i.e. HIV positive). Lower than detectable levels of virus were found in all raw breast milk samples analysed for 45% (n=5) of the 11 study group participants. The mean (SD) viral load for the remaining mothers was 1579 (1747) copies/ml, with a respective mean (SD) log value of 2.84 (0.7).

A log value of <2.18 (i.e. <150 copies/ml) corresponds to a lower than detectable level of HI viral load being present. The mean breast milk viral load of the study group mothers indicated a low number of viral copies present in the breast milk if the upper levels for viral load quantification amounts to a log value of 7 (i.e. 10 million copies/ml).[161]

3.9 Adverse Events, Serious Adverse Events and Mortality

3.9.1 Incidents of Necrotising Enterocolitis (NEC)

There were 6 (26%) infants (3 control and 3 study group infants) who experienced an incident of necrotising enterocolitis during the follow-up period. All incidents were classified as presumed or Grade I NEC according to the Bell classification system, which is displayed in table 3.24.[174] None of these participants were excluded from the study.

Table 3.24: Management of NEC based on the Bell Classification system[174]

Stage	Clinical findings	Radiological findings	Treatment
I: Presumed NEC	-Mild abdominal distension -Feeding intolerance -Vomiting	Non-specific: -Sausage-shaped bowel loops -Asymmetrical bowel loops - Thickened bowel wall - Possible ascites	-NPM for ± 3 - 5 days -IV fluids -Antibiotics (for at least 2 - 7 days: An individualised approach was followed.)

The first control group infant experienced one episode of Grade I NEC on day 10 of life. He was treated conservatively by keeping him NPO for 6 days, after which feeds were initiated slowly on day 17 of life. TPN was administered from day 14 to 20. The second control group infant had an episode of presumed NEC on day 6. The patient was kept NPO for

approximately 14 hours, after which enteral feeds were started slowly on day 7 of life. The third control group infant had an episode of Grade I NEC on day 5. He was kept NPO for two days after which small volume enteral feeds were restarted on day 7 of life. The latter two infants received IV fluid (10% NNL) but no TPN support during the presumed NEC incidents.

The first study group infant experienced an episode of presumed NEC on day 5 of life. He was kept NPO for one day and enteral feeds were started slowly on day 7 of life. The second study group infant was made NPO on day 12 of life due to symptoms suggestive of Grade I NEC. Enteral feeds in the form of pasteurised expressed breast milk were initiated slowly on day 13 again. The last study group infant had an episode of presumed NEC or sepsis on day 5 of life. He was kept NPO on day 6 after which enteral feeds in the form of pasteurised expressed breast milk were restarted slowly on day 7.

Three (50%) of the six infants experiencing symptoms of presumed NEC did so on day 5 of life, one infant on day 6, while the remaining two infants experienced the symptoms on days 10 and 12 respectively. In the present study the expected time for NEC to present seems to be at the end of the first week of life and during the second week of life.

3.9.2 Serious adverse events

Two of the study group infants (HIV-exposed) tested HIV positive at the end of the study period. The exact time of HIV transmission could not be determined in any of the two cases. In the first case the method of delivery was a normal vaginal delivery. No blood or breast milk viral load results were available for the mother. The only known result was a CD4 count of 746 µL at the end of the study period, which is classified as within normal limits (i.e. 700 – 1100 µL).

In the second study group infant (HIV-exposed) the method of delivery was via a caesarean section. The infant's mother did have a very high blood HI viral load level at the end of the fourth week of follow-up, i.e. 110 000 copies/ml; log value=5.84 versus the mean [95% CI] group value of 446.3 [262.8 - 629.9]; log value=4.55 [4.08 - 5.02]). However, at the corresponding time the HI viral load in her raw breast milk sample had dropped from 1600 copies/ml (log value=3.2) at the end of the third week to 99 copies/ml (log value= 2.0) at the end of the fourth week of follow-up.

Infant case number two's mother did not receive any antenatal antiretroviral (ARV) therapy according to the provincial ARV protocol, while no such information was available for case number one's mother. ARV's, especially the single-dose Nevirapine these mothers must have received according to the PMTCT protocol at the time the study was conducted, are known to decrease the risk of HIV transmission by lowering the breast milk viral load early during lactation.[207]

As ARV's are known to provide prophylaxis from MTCT through breastfeeding[18], the lack of appropriately and timeously administered ARV therapy in the second case could have contributed to the infant's sero-conversion.

3.9.3 Mortality

Four participating infants (three control group and one study group) died. One infant died before inclusion, two died during study participation and one died 6 days after successful completion of the study. The reasons for the infants' death were cardio-respiratory failure (*N*=1), grade 3 Intra-ventricular haemorrhage due to hypoxic ischemic encephalopathy (*N*=1) and severe prematurity (*N*=1), with the reason for death of the fourth infant (*N*=1) indicated as sepsis following aspiration together with severe prematurity. All deaths were reported to the Division of Human Nutrition Research and Human Research Committee of the Faculty of Health Sciences of Stellenbosch University.

None of the deaths were deemed to be related to the study protocol since there was no intervention undertaken in the present study.

CHAPTER 4: DISCUSSION

This is the first time this work has been done in South Africa and there is little such data in the peer reviewed literature at present. The main findings which will be discussed are the non-significant difference in the studied macro- and micronutrient composition between raw or pasteurised expressed breast milk from HIV positive and HIV negative mothers with premature infants. The decline in breast milk protein, magnesium, potassium, zinc and copper content observed for the whole study population, as well as the increase in folic acid content over time. The sodium/potassium ratio of both groups remained strongly indicative of the presence of sub-clinical mastitis. A significant greater mean volume of milk was obtained with pump compared to hand expression. Method of expression did not result in a significant difference in breast milk composition for any of the nutrients studied. The poorer than recommended growth rate due to a lower than recommended energy and protein intake, was a contributory factor to the finding that most of the infants were SGA on day 28 postpartum.

4.1 Dietary Intake of the Mothers during Pregnancy and Lactation

The same trends were observed for the control and study group mothers with regard to dietary intake of the studied nutrients during pregnancy and lactation. It was expected that the mothers would have the same level of nutrient intake during the first month of lactation, as they mostly lodged in the respective hospitals' Kangaroo Mother Care units and predominantly consumed the standardised hospital diet. The composition of the hospital diet did differ between the tertiary hospital (i.e. Tygerberg Academic Hospital) and the secondary hospitals (i.e. Eersterivier and Karl Bremer Hospital).

The actual intake of the mothers during lactation as obtained with repeated 24 hour-dietary recall correlated well with regard to energy intake to the hospital diets' mean compositional data. When compared to the RDA (no DRI available) for energy and protein for lactating women only the energy content of Tygerberg Academic Hospital's diet fell within the recommendation. Protein content of the normal diet closely resembled the recommendation, while Tygerberg Academic Hospital's diet provided 70% more protein than recommended by the RDA for lactating women. Actual protein intake, however, was 33% less than the proposed dietary composition of Tygerberg Academic Hospital's high protein diet, while the reported protein intake at the other two hospitals correlated well with the calculated nutrient composition of their hospital diet. Macronutrient composition of the high protein diet furthermore needs adjustment with regard to the macronutrient distribution,

as the current distribution falls outside the guidelines for an optimal diet, i.e. 10 - 15% protein, 50 - 55% carbohydrate and <30% fat.[175] The so-called ward (i.e. normal) diet's composition is more in accordance to the percentage distribution proposed for an optimal diet. It is interesting to note that the reported intake of the lactating mothers indicated an optimal macronutrient distribution.

A significantly lower intake of all reported nutrients during lactation was observed for the group as a whole. This finding was expected as the mothers consumed a standardised diet during the first month of lactation while they remained in the hospital to provide kangaroo care. Furthermore, the difference in intake during pregnancy and lactation is explained by the method used to determine dietary intake (i.e. semi-quantitative food-frequency questionnaire versus repeated 24-hour dietary recalls). In general, the 24-hour recall tends to underestimate mean intakes in the elderly and children, but produces valid mean intakes for other population groups.[176] With all dietary assessment methods it needs to be remembered that there is not, and probably never will be a method that can estimate dietary intake without error.[177]

The macronutrient distribution of the mothers during pregnancy correlates well with their intake during lactation and closely resembles the optimal diet's macronutrient distribution. Although most mothers reported that the hospital diet differed from their usual intake, self-selection produced an intake similar to their home diet, except that the total amount consumed was significantly less than their intake during pregnancy. The amounts consumed during lactation, however, could be a more accurate reflection of their usual non-pregnant dietary intake, or the difference in intake could be due to underreporting of additional food items consumed during lactation. The latter could explain the anthropometric findings of their maintenance of weight during the first month of lactation, which is not problematic in the short-term, as it indicates that sufficient energy was consumed to allow adequate lactation.

4.2 Dietary Intake of the Infants

Average total fluid intake remained within the recommended guidelines throughout the follow-up period. Expressed breast milk (raw or pasteurised) was initiated within the first four (median day 2) days of life at 2 – 18 ml/kg/day in all participating infants. Mean energy and protein intake increased drastically between days 7 to 14, with a more gradual increase

found between days 14 to 21 and 21 to 28. This was in accordance with the change in fluid composition, where these infants were gradually weaned from receiving mainly intravenous fluid (i.e. Neonatalyte®) to full enteral feeds of expressed breast milk. The latter transition was usually completed within the first two to three weeks of life. Fortification of the breast milk commenced when full volume enteral feeds were reached (i.e. at least 150 ml/kg/day) according to the hospital protocol for the management of premature infants. Additional energy and protein provided by the fortifier was the reason for the increase in energy and protein intake between days 14 to 21 and 21 to 28.

A comparison of actual versus prescribed energy and protein intake revealed a significant difference (i.e. p=0.0056 for energy and p=0.066 for protein), where actual intake was found to be lower than prescribed intake over the 4-week follow-up period. A possible reason for the latter finding was that the fortification and supplementation was not always administered according to the prescription, due to medical procedures where the infant had to be kept nill per mouth for a period of time during the day or nursing personnel not making the additions to the infant's feed as prescribed. As the fortifier contained more energy relative to protein and the medium chain triglyceride oil contained only energy, the difference between the energy prescription and actual intake was greater than between protein prescription and actual intake.

Knowing that SGA infants are born with a body composition that is lower in protein and fat content relative to AGA infants, most clinicians currently recommend increased protein and energy intakes per kg body weight compared to normally grown infants in order to achieve catch-up growth.[170, 178] The following table indicates the revised recommended protein intake and protein: energy ratio (PER) for stable, growing preterm infants according to post-conceptional age and the need for catch-up growth.[104]

Table 4.1: The recommended protein intake and protein: energy ratio (PER) for stable, growing preterm infants according to post-conceptional age and the need for catch-up growth[104]

Post-conceptional age	Without need for catch-up growth (AGA)	With need for catch-up growth (SGA)
26 – 30 weeks: 16 – 18 g/kg/d Lean Body Mass 14% protein retention	3.8 – 4.2 g/kg/day PER: ~3.3 NPE: 125 – 139 kcal/kg/day (TE: 140 -156 kcal/kg/day)	4.4 g/kg/day PER: ~3.4 NPE: 125 – 139 kcal/kg/day (TE: 143 -157 kcal/kg/day)
30 – 36 weeks: 14 – 15 g/kg/d Lean Body Mass 15% protein retention	3.4 – 3.6 g/kg/day PER: ~2.8 NPE: 95 – 101kcal/kg/day (TE: 109 -115 kcal/kg/day)	3.8 – 4.2 g/kg/day PER: ~3.3 NPE: 125 – 139 kcal/kg/day (TE: 140 -156 kcal/kg/day)
34 – 40 weeks: 13 g/kg/d Lean Body Mass 17% protein retention	2.8 – 3.2 g/kg/day PER: 2.4 – 2.6 NPE: 67 – 83 kcal/kg/day (TE: 78 -96 kcal/kg/day)	3.0 – 3.4 g/kg/day PER: 2.6 - 2.8 NPE: 78 – 95 kcal/kg/day (TE: 90 -109 kcal/kg/day)

Abbreviations: PER = Protein: Energy Ratio, NPE: Non-Protein Energy, TE: Total Energy

When applying Table 4.1 to the studied infants, it is clear that they all fell into the post-conceptional age group: 30 - 36 weeks at 3 to 4 weeks of life. Table 3.6 indicates that the control and study group infants daily consumed a mean (SD) of 3.27 (1.36) and 3.21 (1.36) g protein/kg, 138.1 (33.8) and 142.3 (33.8) kcal total energy/kg, which corresponds to a non-protein energy/kg of 125.0 kcal/kg and 129.5 kcal/kg for the respective groups. The obtained PER of 2.6 and 2.5 for the control and study group may in part be sufficient to provide in the growth needs of AGA infants without the need for catch-up growth, but will not provide in the increased growth needs of the SGA infant in need of catch-up growth.

At birth 8 (35%) of the 23 infants were born SGA while 21 (91%) of the 23 infants were found to be SGA at the end of the 28-day follow-up period. This finding reveals that the current feeding regimen used in the three participating hospitals is not optimal. Total energy and non-protein energy intake falls below or at the low end of the recommended range (Tables 3.6 and 4.1) while protein intake seems to be at a level which is too low to achieve adequate catch-up growth in the ELBW and SGA infants.

It is suggested that non-protein energy and total energy intake must be increased with a further 20 – 34 kcal/kg/day (i.e. 16 – 24% increase) and 24 – 37 kcal/kg/day (i.e. 17 – 24%

increase) respectively to achieve adequate catch-up growth in the ELBW and SGA infant. Total protein intake should be increased with 0.6 – 0.8 g/kg/day (20 - 25% increase in protein intake), i.e. from a mean (SD) of 3.24 (1.36) g/kg/day to 3.8 – 4.2 g/kg/day for this population group of ELBW and VLBW infants. These findings are similar to those of Ernst et al[170] who proposed that if the ELBW infants in their study received 0.5 g protein/kg/day more than their observed protein intake of 2.9 – 3.0 g/kg/day, intra-uterine weight and head circumference growth rates could be achieved. The author furthermore confirmed a previous recommendation that most ELBW infants could achieve catch-up growth if they received 140 kcal energy/kg and 3.2 g protein/kg/day from the day that birth weight was regained. When the latter is applied to the current study, protein intake appears to be acceptable, while energy intake must be increased. As 91% of the studied infants were SGA at 28 days after birth, it might be that a protein intake higher than 3.2 g/kg/day is needed. The current study's suggestion of a protein intake of 3.8 – 4.2 g/kg/day is in accordance with another author, who proposed that intra-uterine protein accretion rates approximate 3.85 g/kg/day.[179] It furthermore confirms the latest protein recommendations from the American Academy of Pediatrics, i.e. 3.8 – 4.4 g/kg/day for stable, growing ELBW and 3.4 -4.2 g/kg/day for VLBW infants. [104]

4.3 Anthropometric Findings: Mothers

The mothers did not lose a significant amount of weight in the first month of lactation and remained anthropometrically stable during this period.

4.4 Anthropometric Findings: Infants

The study group infants were on average 92 grams heavier, 0.3 cm taller and had a 0.7 cm bigger head circumference at birth. At the end of the study period, the study group's average weight was 79 grams heavier, length 1 cm taller and head circumference 0.27 cm bigger than the control group participants. The main reason for these differences was due to the significant difference (p=0.02) in gestational age at birth between the two groups. The study group's mean (SD) age at birth was 30.4 (1.6) weeks, which was 1.7 weeks later than the mean age of control group participants (i.e. 28.7 (1.6) weeks gestational age). The same trend in growth, however, was experienced in both groups for all three anthropometric indicators and no significant difference was observed between the two groups.

Weight loss after birth averaged 3.5%, which was lower than the expected 5 – 15% usually found in premature infants.[105, 180] The lower than expected weight loss could be due to aggressive fluid therapy, as observed by other studies as well.[181] Birth weight was regained on average at day 12 to 13, which fell within the recommended range of between days 10 - 14 for premature infants.[180]

Weight gain velocity of all participating infants averaged at a rate of 9.9 g/kg/day for the whole 28-day period. The recommended rate of weight gain is 15 g/kg/day which reflect third trimester intra-uterine accretion.[180, 182] The infant population gained at only 66% of the intra-uterine growth rate, which indicates that catch-up growth estimates were not reached with the feeding regimen that was followed. Ehrenkrantz[183] recommends a growth rate of >18g/kg/day as infants growing at this rate experience the least neurological impairment. The control and study group infans gained at a mean (SD) of 16.75 (5.0) and 15.95 (4.0) g/kg/day (i.e. 93% and 89%) of the recommended growth rate of >18g/kg/day from the day birth weight was regained.

The latter findings emphasise the effect of suboptimal nutrition and specifically the PER as a contributing factor to the inability to achieve optimal catch-up growth as well as predisposing these infants to neuro-developmental impairment, i.e. defined as cerebral palsy, Bayley II Mental Developmental Index <70, Bayley Psychomotor Developmental Index <70, deaf/ hearing loss requiring amplification in both ears or bilateral blindness. [183]

Classification of the infants according to weight reveals that 25% (*N* =3) of the 12 control and 45% (*N* =5) of the 11 study group participants were small-for-gestational age (SGA) at birth. All other participating infants were born appropriate-for-gestational age (AGA). At completion of the follow-up period (day 28 of life), 91.7% (*N*=11) control group and 90.9% (*N* =10) study group participants were classified as SGA.

An alternative weight-based classification[105, 167] of the infants indicates that 58.3% (*N* =7) of the 12 control and 27.3% (*N* =3) of the 11 study group participants were born with an extremely low birth weight (i.e. <1000g). All other participating infants were born with a birth weight between 1000 and 1500 grams (i.e. very low birth weight). A comparison of the growth of the ELBW with the LBW infants shows a similar growth pattern, which emphasises the inability of the current treatment and feeding regimen to enable the smallest infants to effectively catch-up in the first month of life.

All three anthropometric indices (i.e. weight, length and head circumference) are used to classify the extent of growth restriction, which varies between asymmetric with a good prognosis (if head circumference falls above the 10th percentile) to symmetric with a poor prognosis.[105] At birth a total number of 4 (17%) (i.e. 2 control group and 2 study group) infants had a symmetric growth restriction as reflected by weight, length and head circumference all falling below the tenth percentile on the Fenton Tanis growth chart for preterm babies.[166] (Appendix B: Fenton Tanis growth chart.) After the 28-day follow-up period, a total of 13 (57%) (5 control group and 8 study group infants) were classified as having a symmetric growth restriction. Symmetric growth restriction increased by 40% (*N* =9) during the first 28 days of life. Only one (control group) infant had no growth restriction upon study completion, while the remaining study participants (*N* =9; 39%) presented with asymmetric growth restriction with a good prognosis, i.e. head circumference fell above the 10th percentile.

The difference in weight between the control and study group was never found to be significant. An unexpected finding was that the study group (HIV-exposed group) had a higher mean (SD) birth weight [1066 (131) g vs 966 (131) g] and higher mean (SD) gestational age [30.4 (1.6) vs 28.7 (1.6) week] at birth. The mean birth weights of the control and study group were not found to be significant, although the p-value of 0.12 (as determined by analysis of covariance, with the effect of gestational age removed) does indicate a tendency towards a difference. A possible explanation for the higher mean birth weight of the study group could be due to better follow-up of the study group mothers which had been included antenatally into the Prevention-of-Mother-to-Child-Transfer (PMTCT) program.

Total length accretion was an average of 4.04 cm (1.0 cm per week) during the 28-day follow-up period. The recommended rate of length accretion of 0.8 – 1.0 cm per week[180] was thus achieved by the infant study population.

Head circumference increased significantly during the follow-up period from a mean (SD) value of 26.2 (1.2) cm at birth to 28.9 (1.2) cm at day 28, averaging 0.69 cm/week. The infant study populations' head circumference increase falls well within the recommended range of 0.5 – 0.8 cm/week.[180] To minimise the risk of neuro-developmental impairment, Ehrenkrantz suggests a mean rate of head growth of >0.9cm/week. Compared to the latter, the infants in this study achieved a mean head circumference growth of 76% of the recommendation.[183] As 40% more infants had symmetric growth restriction, including a

head circumference below the 10th percentile on day 28 of life, there is an urgent need to re-look the nutritional support these infants receive with specific focus on the protein/energy ratio provided.

4.5 Biochemical Indices: Mothers

Biochemical analysis revealed the mean (SD) pre-albumin, transferrin and immunoglobulin A levels remained within their respective recommended ranges and did not alter significantly during the 4-week follow-up period.

An expected finding was a significantly ($p<0.01$) decreased albumin value that increased over time and nearly reached the normal range of 37 – 53 g/L[171] during the 28-day follow-up period. Albumin decrease due to the expansion of plasma volume by 45 - 50% during pregnancy and this effect remained during early lactation.[87] In this population group, albumin values were moderately decreased with the first determination, which was at the end of week 1 after delivery. The albumin values, however, did increase during the next 3 weeks to reach near-normal values at the end of week 4 after delivery, indicating that the effect of the pregnancy-induced plasma volume expansion had diminished.

Haemoglobin levels were slightly decreased, which is expected in early lactation due to red cell mass and plasma volume expansion which occurred during pregnancy. Red cell mass expands less than plasma volume (15 – 20% vs 45 – 50%) in pregnancy, which results in haemoglobin and haematocrit concentration decreasing during the first and second trimester, while these values gradually start increasing in the third trimester of pregnancy. The Institute of Medicine[184] found haemoglobin to decrease to 11.6 g/dl in the second trimester and to increase to 12.5 g/dl at 36 weeks gestational age. Mean gestational age of this study's 2 participating groups, however, was 29 and 31 weeks which corresponds to early third trimester; hence the finding that haemoglobin levels were still decreased.

Haematocrit levels increased significantly ($p=0.01$) through the 28 day period, although values remained at the lower end of the normal range of 0.36 – 0.46 L/L.[171] The reason for the initial decrease was due to the expansion of plasma volume associated with pregnancy. The decreased week 1 value still reflected the effect of the pregnancy, with a normalisation of haematocrit values during the 28 day follow-up period.

Ferritin values remained within the very wide normal range of 4.5 – 170 µg/L.[171] The ferritin values, however, did decrease significantly (p=0.01), because of the fact that ferritin is an acute phase protein, which decreases synergistically to a decrease in C-reactive protein levels. Ferritin values were initially also higher due to the effect of iron supplementation which most mothers (*N*=15) received during pregnancy, as well as mean (SD) maternal dietary iron intake, which were significantly higher (p=0.00097) during pregnancy [i.e. 14.5 (4.9) mg iron/day] than during lactation [i.e. 10.44 (2.6) mg iron/day].

C-reactive protein (CRP) values were moderately raised due to the presence of infection. Sub-clinical mastitis in lactating mothers is one known infection associated with raised acute phase proteins.[185]

Vitamin A levels were found to be within the recommended range, but decreased significantly (p=0.017) during the follow-up period, possibly due to the significantly lower mean (SD) vitamin A intake observed during lactation compared to pregnancy [i.e. 1031 (430.8) µg/day vs 1533 (1137.9) µg/day].

Mean immunoglobulin G (Ig G) values delivered an interesting finding. The control group's mean Ig G values remained within normal limits, while the study group's mean Ig G values remained raised. Possible reasons for the raised study group Ig G values are the presence of HIV, which cause changes in the ratio of helper to suppressor cells; specifically affecting CD4 T-cell lymphocyte population.[171]
CD4 T-cell lymphocyte count of the study group mothers revealed a trend towards a significant decrease (p=0.056) in CD4 T-cell lymphocyte count, possibly due to the direct effect of the HI virus on the CD4 cells[186], which is an indication of progression of the HIV-1 disease. Blood viral load of the study group mothers correspondingly increased as the CD4 T-cell lymphocyte count decreased. The increase in the mean [95%CI] viral load from a log-value of 3.58 [2.82 - 4.35] to 4.55 [4.08 - 5.02] at the end of week 4 was significant (p=0.009).

4.6 Biochemical Indices: Infants

Serum Albumin has long been used as a measure of protein status. However, the long half-life (±12.5 - 21 days)[187] of this protein limits its usefulness in assessing nutritional status.[188] Normal albumin levels remain undefined for the premature infant, with albumin levels increasing significantly with gestational age (e.g. mean serum albumin= 19g/L for <30

weeks gestational age to a mean of 31g/L at term).[189, 190, 191] Hypoalbuminaemia occurs in a number of clinical situations, including prematurity, the acutely sick infant, respiratory distress syndrome, chronic lung disease, necrotising enterocolitis, hydrops fetalis (see p xvi for definition) and oedema.[189]

Plasma pre-albumin concentration is a reliable indicator of protein nutritional status in healthy, growing VLBW infants due to its' short half-life of ±2 days.[187, 191, 192] In the studied infant population, pre-albumin levels remained low for the 28-day study period. This finding indicates an insufficient protein intake. Table 3.6 (p 65) compared to Table 4.1 (p 96) indicates that protein intake between days 21 - 28 reached only the lower end of the recommended intake levels for AGA (30-36 weeks gestational age) infants without the need for catch-up growth, but not for SGA (30-36 weeks gestational age) infants with the need for catch-up growth. The fact that pre-albumin levels were below the recommendation at the end of the follow-up period, may indicate that the effect of the 2 to 3 week delay in reaching adequate protein intake levels is too big, which delays the achievement of optimal protein nutritional status. This corresponds well with the finding of a protein: non-protein energy ratio (PER) of 2.6 and 2.5 for the control and study group respectively, which was below the recommended PER of 3.3 that is needed to achieve catch-up growth in the SGA infant. Due to this study's short follow-up period of 28 days, the actual time when pre-albumin levels reached the normal range, could not be determined. This finding serves as supporting evidence for the delayed catch-up growth that these VLBW and ELBW premature infants experienced.

4.7 Breast Milk Composition

4.7.1 Macronutrient composition

4.7.1.1 Energy

The reason for the much lower mean breast milk energy content found with the current study could be due to the lower fat content, but may also not be an accurate reflection of energy composition as the latter was not determined directly by bomb calorimetric methods. Very few studies have researched the difference in energy content of breast milk using direct measurement (i.e. bomb calorimetry) and calculation based on the known conversion energy factors for protein, fat and carbohydrate. One Japanese study, however, did compare the energy content of colostrum, transitional and mature breast milk of mothers delivering term infants.[193] The study found that measured energy content of breast milk was

lower than the calculated energy based upon standard tables of food composition. Should the latter be true for the current study, it could mean that the calculated energy content found could still be an over-estimation of the actual breast milk energy content. Such a finding could furthermore explain the inability of the premature infants to catch-up in growth.

Pasteurisation decreased the breast milk fat content mildly, but not significantly. The latter could be due to the effect of heat, which destroys lipase enzymes, releasing fat from their emulsified state and making it possible that fat could be lost due to adhesion to the wall of the container used.

4.7.1.2 Protein

Breast milk protein content of the studied population decreased significantly (p=0.000161) during the first 28 days of lactation. This is in accordance with the results obtained from a similar study which investigated the compositional changes in preterm breast milk over the first 28 days of lactation.[96]

4.7.1.3 Carbohydrate and Fat

An unexpected finding with the current study was that carbohydrate and fat content remained stable throughout the 28-day follow-up period. Anderson et al[96] found that during the first four weeks of lactation, the lactose, lipid and energy concentrations of the preterm milk increased, while the nitrogen content decreased steadily to reach a composition similar to that of term milk. Although the extent of differences in nutrient composition of preterm and term milk remains uncertain, study authors agree that these differences disappeared after the first month of lactation and that variability in nutrient content (protein, fat and minerals) is higher in preterm than in term milk. [98]

4.7.2 Micronutrient composition

4.7.2.1 Folic acid

Folic acid in breast milk bears a relation to maternal serum concentration in overtly deficient women and increases with progression of lactation or remains stable.[87] Average dietary folic acid intake of all participating mothers was significantly ($p<0.01$) lower than the recommended DRI for pregnant and lactating women. Only 9 (39.1%) of the 23 participants received folic acid supplementation during pregnancy and none took folic acid supplements during lactation. Maternal serum folic acid concentration was not determined, but it is suggested that serum folic acid and consequently breast milk levels would be low due to the continued low folic acid intake observed. This low folic acid intake is confirmed by the

United States Third National Health and Nutritional Examination Survey (NHANES III)[194], which found mean intake of folate to be above the RDA for all groups, except for pregnant and lactating women. As these mothers consumed less than 60% of the DRI for folate (i.e. mean (SD) folate=191.6 (43.9) µg/day versus the recommended 500µg/day) for lactating women, they would benefit from folate supplementation if the low fotale content of the hospital diet could not be increased.[6]

4.7.2.2 Sodium

The general trend towards a decrease in sodium levels in preterm milk over the first 28 days of lactation is an expected finding, which is confirmed by previous studies.[94, 96, 97] Mature human milk is known to contain a very low sodium content of 5 – 6 mmol/L (i.e. 11.5 - 13.8 mg/100ml).[197, 198] Raised breast milk sodium concentrations of >12 mmol/L (i.e. >27.6 mg/100ml) are regarded as sensitive indicators of sub-clinical mastitis.[16, 38, 185]

In the control and study group mothers, mean [95% CI] sodium values remained above the levels indicative of the presence of mastitis. No mother, however, reported the presence of mastitis upon questioning (socio-demographic questionnaire). As the questionnaire was completed once during the study period, it could be that the presence of mastitis was missed. A more valid reason for the non-reporting of the presence of mastitis is the fact that sub-clinical mastitis was present and not mastitis. Sub-clinical mastitis is a condition which is diagnosed only by biochemical analysis[16, 26], while mastitis is the condition which presents clinically as breast pain, chills, myalgia and fever.[13] Both forms are most likely due to the stasis of milk in the breast tissue which develops from poor breastfeeding technique, local mammary gland involution as a result of reduced milk production (as seen during weaning), or possibly due to an inability to empty breasts completely (as seen when expressing milk by hand). Micronutrient deficiencies or systemic infection has also been identified as possible etiological factors in the development of mastitis.[13, 16] It is worth noting that sodium concentrations do not differ between fore- and hind- milk and maternal sodium intake does not influence breast milk sodium levels.[12, 198, 199, 200]

4.7.2.3 Sodium/Potassium ratio

Sub-clinical mastitis is defined as a raised milk sodium/potassium (Na/K) ratio >0.6, with a ratio >1.0 classified as severe. Sub-clinical mastitis is associated with an increased risk of HIV transmission.[12, 38] The proposed mechanism by which sub-clinical mastitis may increase breast milk viral load, is through disruption of the mammary epithelial tight junctions which may allow plasma constituents such as sodium to leak into the alveolus and

inflammatory chemokine production can increase the influx of leukocytes. If sub-clinical mastitis can increase breast milk viral load, it may contribute to increase the risk of post-natal HIV transmission. The presence of sub-clinical mastitis is also associated with poor infant growth in both HIV-infected and HIV- uninfected mothers.[201] In the current study population, the infants did not grow at the desired rate of ≥15g/kg/day during the first four weeks of life, but growth velocity improved drastically when full enteral feeds were reached during the third week of life. Despite the improved growth velocity, 91.3% (21 out of 23) of the infants were SGA at the end of the 4-week follow-up period. The main reason for the poor growth experienced in this study population is primarily due to insufficient nutrient supply early in life. The possible contributory effect that sub-clinical mastitis may have in the poor growth observed is not quantifiable at present.

The sodium/potassium ratio in the breast milk of the mothers whose infants were found to be HIV positive remained high for most days of breast milk sample collection. Na/K ratios in the first mother increased after the first week to a ratio of 1.6 (average ratio of 0.97 for all samples), which started to decline again from the third week of lactation. Unfortunately, the first mother's viral breast milk and blood viral loads could not be determined, but her CD4 T-cell lymphocyte count of 746 at the end of the study implies that her blood and breast milk viral load could not have been very high due to the inverse relationship which exists between HI viral load and CD4 T-cell lymphocyte count.

The second mother's breast milk Na/K ratio was found to be 2.7 on day 7 after birth (average ratio of 1.23 for all samples). At the same time (end of week 1) her blood viral load was 1200 copies/ml (log value= 3.08), while breast milk viral load was lower than the detectable level. The Na/K ratio decreased to an average of 0.7 during the second and third week. At the end of the fourth week blood viral load was very high at 110 000 copies/ml (log value= 5.04), while breast milk viral load had decreased from 1600 copies/ml (log value= 3.2) to 99 copies/ml (log value= 2.0) at the end of the third week.

It is not known when the aforementioned two infants became sero-positive. The presence of sub-clinical mastitis might, together with high maternal and breast milk viral load, have contributed to the post-natal HIV-infection of the second infant in the first month of life. Not even the effect of a proposed decrease in breast milk viral load of at least 3 logs due to the pasteurisation of the breast milk seem to have been able to prevent transmission in the latter case. Unfortunately, no result for reverse transcriptase activity could be determined in the pasteurised breast milk samples of the HIV positive mothers. Reverse transcriptase is

an easy method for determination of the presence of live HIV by measuring the activity of the reverse transcriptase enzyme, which is needed for HIV replication. If pasteurisation is correctly performed, all or most of the reverse transcriptase enzymes are supposed to be destroyed, leaving the breast milk a safe (i.e. HIV transmission-free) food source for the infant.[77]

The same trend in Na/K ratios were always observed in both the raw and pasteurised breast milk samples on a specific collection day.

4.8 Expression Technique: Hand versus Pump

A significant (p=0.03) greater volume of milk (16% more) was obtained when the breast was expressed with a hand pump compared to hand expression. This finding was expected and confirmed the results of a previous study[99] which compared manual to hand pump expression. Fewtrell et al[101] compared hand pump (Avent Isis®) expression to electric pump expression and concluded that the manual pump showed greater milk flow which may be due to the more physiologic pump design. Jones et al[103] found the average volumes expressed with an electric pump in mothers who delivered preterm (mean gestational age= 29.97 weeks) were 51.32 g (sequential breast pumping with no massage) to 125.08 g (simultaneous breast pumping with breast massage). The fat concentration of the milk was not affected by the increase in volume achieved by the interventions. Another study[99] compared hand to electric pump expression and obtained mean (SD) volumes of 52 (7) ml with an electric pump, which was 90% greater than that obtained by hand expression, i.e. 27 (3) ml. These mean values were significantly ($p<0.001$) different. Despite a 90% difference in milk volumes, significant differences in fat concentration were not observed.

When the nutrient composition of the hand- and pump-expressed breast milk was compared between the two groups, no significant difference in composition was found for any of the nutrients studied. This study also confirms the results obtained by Garza et al[99] regarding fat concentration. No significant difference was observed regarding breast milk fat concentration and expression method even though total volume expressed were 16% more with pump than manual (hand) expression.

It can be concluded that hand expression produces expressed breast milk with a composition similar to pump-expressed breast milk. Pump expression by hand, however, does deliver a significantly greater milk yield when compared to manual (hand) expression.

4.9 Viral Load: Blood and Breast Milk

Women are more likely to transmit HIV to their infant if their breast milk contains detectable levels of the virus.[53] In this study nearly half (45%; *N*=5) of the HIV positive mothers' raw breast milk samples did not have detectable viral levels while the remaining 6 mothers had a mean (SD) breast milk viral load of 1579 (1747) copies/ml (log value= 2.84), which indicates a low number of viral copies present in the breast milk The mean blood viral content of the HIV positive mothers, however, did increase significantly (p=0.009) over the 28-day follow-up period with nearly 1 log, while CD4 T-cell count showed a definite trend towards a decrease. Breast milk viral load remained lower than maternal blood viral load, indicating that the risk for HIV-transmission in this population group was relatively low. One study[20] has found a nearly 3-fold increase in HIV transmission for every 10-fold increase in cell-free or cell-associated viral load in breast milk. O'Shea et al previously found the predicted rate of transmission relative to maternal HIV-RNA load was 2% at 1000 copies/ml, 11% at 10 000 copies/ml and 40% at 100 000 copies/ml. Maternal HIV-RNA viral load was a stronger predictor of transmission than CD4 T-cell number. [39]

CHAPTER 5: RECOMMENDATIONS AND CONCLUSIONS

5.1 Null Hypothesis Accepted

5.1.1 There is no significant difference in the macronutrient (i.e. protein, carbohydrate, fat and energy) and micronutrient (i.e. folic acid, calcium, phosphorus, magnesium, sodium, potassium, copper, iron and zinc) composition of expressed human milk from HIV positive and HIV negative mothers with premature infants.

The mean energy content of the breast milk of the population as a whole over the 28-day follow-up period, however, was found to be lower than what is expected (i.e. 64.6 kcal/100 ml vs 66.7 kcal/ 100ml).[104] The low energy content is due to the protein, fat and carbohydrate content which were all lower than found by previous studies.[96, 104, 173]

Folate content was approximately half of the content found by other studies (i.e. 1.5 µg/100 ml vs ±3.2 µg/100 ml) and is a direct consequence of the low maternal folate intake during the first 28 days of lactation. Sodium content was nearly double the mean values found by previous studies (i.e. 38.3 mg/100 ml vs 20 – 28 mg/100 ml), [104, 173] while potassium content was slightly raised (i.e. 53 mg/100 ml vs 49 – 50 mg/100 ml).[104, 173] The presence of sub-clinical mastitis is the main reason for the raised breast milk sodium values found in this study. Breast milk calcium content was lower than found by previous studies (i.e. 23.8 mg/100 ml vs 25.3 – 28.8 mg/100 ml), [104, 173] while phosphorous (13.2 mg/100 ml vs 9.3 – 14.5 mg/100 ml) [104, 173] fell within the range and magnesium (3.4 mg/100 ml vs 2.4- 3.3 mg/100 ml)[104, 173] was at the higher end of the range. Iron and copper content were nearly double that found by previous studies, i.e. 0.18 mg/ 100 ml vs 0.04 – 0.09 mg/100 ml[104, 173] and 0.09 mg/100 ml vs 0.04 – 0.05 mg/100 ml respectively.[104, 173] Zinc content of the breast milk fell within the range of previous findings (i.e. 0.33 mg/100 ml vs 0.22 – 0.37 mg/100 ml).[104, 173]

Pasteurisation has no significant effect on the energy and macronutrient composition as well as the micronutrient content, i.e. folic acid, calcium, phosphorus, magnesium, sodium, potassium, copper, iron and zinc of expressed human milk in HIV negative and/or HIV positive mothers with premature infants.

HIV positive Xhosa mothers thus provide as nutritious breast milk to their very low birth weight premature infants as HIV negative Xhosa mothers.

When compared to the enteral recommendations of the ELBW and VLBW premature infant[104], the macro- and micronutrient content of the breast milk of both the HIV negative and HIV

positive mothers are not able to solely provide in the increased needs of these infants; hence fortification is indicated.

5.1.2 The milk expression technique (hand versus pump) used has no effect on the mean nutrient composition of expressed human milk of mothers with premature infants, as proven by level of significance detection testing.

Milk volume yielded, however, did differ between hand and pump expression. Hand pump expression yielded a 16% greater volume of breast milk compared to hand (manual) expressing, which was significant ($p=0.03$). It is suggested that mothers delivering ELBW and VLBW premature infants with a gestational age less than 33-34 weeks rather use a hand pump, specifically if long term expression is expected, as in the case of the HIV positive mothers. Along with the use of a hand pump in a hospital setting, comes the responsibility to ensure these mothers are taught how to use and clean these hand pumps effectively. When single-user hand pumps are used, all mothers must be taught how to thoroughly clean their pump and be aware of the fact that pumps may never be shared.

5.1.3 Pasteurisation has no effect on the nutrient composition of expressed human milk in HIV negative and/or HIV positive mothers of premature infants.

No significant difference was observed for any of the nutrients studied in the raw and pasteurised breast milk samples of the HIV negative and HIV positive Xhosa mothers participating in this study. It is concluded that HIV positive mothers are able to provide their ELBW and VLBW premature infants with breast milk of similar nutritional quality that that of HIV negative mothers with ELBW and VLBW premature infants. Furthermore, pasteurised breast milk of confirmed HIV negative mothers are suitable to use as donor breast milk when mothers with premature infants are unable to provide their own infant with breast milk.

5.1.4 Maternal nutrient intake during pregnancy and lactation does not have a significant effect on the studied nutrients in breast milk, with the exception of folic acid.

The macronutrient distribution of the diet consumed by the mothers during pregnancy correlates well with their intake during lactation and closely resembles the optimal diet's macronutrient distribution. The total amount of energy, vitamin A, folic acid and iron consumed during lactation, however, was significantly less while protein intake was significantly more than their intake during pregnancy as well as the Dietary Reference Intakes (DRI) and/or

Adequate Intake (AI) or, when not available, the Recommended Dietary Allowance (RDA) for lactating women.

As the folic acid content of the breast milk samples was found to be much lower than that found with previous published data[154], it is suggested that the low maternal folic acid intake during lactation could be a contributing factor to the observed low breast milk folic acid content at 28 days postpartum. As all of these infants received folic acid supplementation in the fortifier currently used (i.e. FM 85® from Nestlé), a folic acid deficiency is not expected in the infant population. The enteral recommendation for folic acid intake is 25 – 50 µg/kg/day for ELBW and VLWB infants.[104] FM 85® contains 40 µg folic acid/ 5g fortifier. Most infants would receive 6 - 12 g fortifier daily, corresponding to 48 - 96 µg folic acid/ day, which would provide in the folic acid needs of almost all VLBW and ELBW infants.

The diets analysed during lactation mostly reflect the respective KMC unit's diet, as all mothers lodged in the hospital during the 28-day follow-up period. It can be concluded that these diets all need adjustment with regard to their nutrient content. Considering the diets available in the participating hospitals, the most suitable diet would be a high energy diet. Currently only a high protein diet (that is also high in energy) is available at the tertiary hospital (i.e. TBH) with no such option available at the secondary hospitals. The two secondary hospitals only provide a normal diet. It is recommended that the secondary hospitals (i.e. KBH and ERH) consider changing their current diet provided to KMC mothers from a normal diet to a large portion of the normal diet (i.e. providing a third more energy and nutrients than the normal diet). Tygerberg Academic Hospital would also better provide in the needs of the lactating mothers if a large portion of the normal diet is provided rather than the high protein diet, which is too high in protein. The portions of protein provided, however, could remain the same as for the normal diet, as protein content was found to be in accordance with the DRI for lactating women. Vitamin A content will meet the needs of the lactating women if dietary intake is increased by a third, but folic acid content would still only provide about 50% of the recommended RDA for lactating women. More vegetables and fruit (which are currently provided at a very low level of not even one fruit per day) could be included to increase the folic acid content of the current diet. Iron and energy content will compare well with the DRI/AI for lactating women if the proposed high energy diet is provided.

5.2 Evaluation of the Current Infant Feeding Regimen at the Three Participating Hospitals

At birth 8 (35%) of the 23 infants were born with a birth weight below the 10th percentile (i.e. SGA), while 21 (91%) of the 23 infants were found to be SGA at the end of the 28-day follow-up period. Symmetric growth restriction, where weight, length and head circumference all fall below the 10th percentile, increased from 17% (*N*=4) at birth to 57% (*N* =13) at 28-days of life.

A mean of enteral intake of 3.4 g protein/kg, 118.9 kcal total energy/kg and 105.3 kcal non-protein energy/kg, which corresponds to a PER of 3.2, was found at weeks 3 to 4 of life. The observed PER is sufficient to provide in the growth needs of AGA infants without the need for catch-up growth, but does not provide in the increased growth needs of the SGA infant in need of catch-up growth.

There is an urgent need to re-look the nutritional support these infants receive with specific focus on the protein/energy ratio provided. It is suggested that non-protein energy and total energy intake must be increased with a further 20 – 34 kcal/kg/day (i.e. 16 – 24% increase) and 24 – 37 kcal/kg/day (i.e. 17 – 24% increase) respectively to achieve adequate catch-up growth in the ELBW and SGA infant. Total protein intake should be increased with 0.4 – 0.8 g/kg/day (10 - 20% increase in protein intake), i.e. from 3.4 g/kg/day to 3.8 – 4.2 g/kg/day for this population group of ELBW and VLBW infants. Increasing the energy and protein content of the fortifier could be considered, but will still not address the early malnutrition these infants experience during the first two weeks of life.

It is still not known which extra-uterine post-natal growth is the optimal for the very premature infant. The American Academy of Pediatrics suggests that post-natal growth should approximate that of a normal fetus with the same post-menstrual age as the aim[118] The current study confirms the findings of other studies that the latter recommendation is nearly impossible to achieve at present.[202] After birth, preterm infants are in a completely different environment and receive completely different nutrition than during intra-uterine life.[202]

It has not been proven that achievement of a high growth rate early in post-natal life produces good long-term results with regard to health outcomes such as cardiovascular health, risk of overweight, insulin resistance and Diabetes Mellitus.[203] On the other hand, it has been shown that early malnutrition at a vulnerable period of brain development has been shown to result in a decreased number of brain cells as well as deficits in behaviour, learning, and memory.[204, 205] Inadequate nutrition may directly result in brain injury, it may also interfere

with overall health by reducing immune competence, resistance to infection, and the energy and nutrients available to support recovery from acute and chronic lung disease and to support an adequate rate of growth and development, let alone catch-up growth, during the hospitalisation.[183]

5.3 Recommendations

5.3.1 Recommendations for feeding practices in the neonatal ward

The current study found that actual energy intake was significantly less than prescribed energy intake, with a similar trend found with protein intake. Care should be taken when breast milk fortification and/or supplementation is administered. It must be ensured that the correct dosage is given as over-administration may be just as problematic as under-administration. It must be ensured that the secondary hospitals specifically are well-informed of the correct procedures by providing them with the latest protocol for breast milk fortification and supplementation. All doctors, dietitians and nursing personnel working in the neonatal ward must be well-informed about the role of and the correct procedures for the prescription and administration of breast milk fortification and supplementation.

Frequent screening for sub-clinical mastitis, which may be associated with poor infant growth in both HIV-infected and HIV-uninfected mothers is recommended.[201] Lactation practice must be monitored closely in all mothers expressing breast milk either by hand or hand pump for an extended time period. Regular (i.e. at least weekly) determination of serum sodium/ potassium ratio of all lactating mothers who are expressing breast milk on a regular basis, is an easy and effective screening method for determination of the presence of sub-clinical mastitis. Immediate therapeutic intervention in mothers with sub-clinical mastitis might contribute to better infant growth with its subsequent advantages of earlier hospital discharge, improved immunity and neuro-developmental outcome due to an increased growth rate velocity and earlier achievement of goals for catch-up growth.

5.3.2 Recommendations for the fortifier composition and administration

The current breast milk fortifier added to premature EBM was used as the gold standard from which the current study's EBM content was subtracted to obtain a proposed fortifier suitable to provide in the needs of this study population (Table 5.1). Appendix I give a breakdown of the calculations.

Table 5.1: A comparison of the composition of the breast milk fortifier (BMF) currently used with the proposed fortifier when added to 150ml of preterm expressed breast milk (EBM)

Nutrient	Unit	Current BMF^ (per 7.5g which is added to 150ml premature EBM)[104]	Proposed content of a BMF to be added per 150ml of this population's EBM
Fluid	ml	-	-
Energy	kcal	27	30
Protein	g	1.5	1.5
Carbohydrate	g	5.03	6.08
Fat	g	0.3	0.45
Folic Acid	µg	60	62.25
Calcium	mg	112.5	114.78
Phosphorus	mg	67.5	67.95
Magnesium	mg	3.6	2.97
Sodium	mg	30	16.95
Potassium	mg	63	53.7
Iron	mg	1.95	1.835
Zinc	mg	1.2	1.19
Copper	mg	0.06	0

^: FM 85® from Nestlé

The nutrient content of the proposed fortifier compares remarkably well to that of the fortifier currently used. The question remains why the infants studied still did not grow optimally although they received a fortifier able to provide in the enteral needs of all the nutrients studied.

A possible explanation of the current problem is that during the first two weeks of life an energy and protein deficit develops due to the current feeding regimen which is unable to prevent negative nitrogen balance. The main reason for the latter is the inability of the current feeding regimen to provide all of these infants with total parenteral nutrition from birth. South

Africa is a developing country and although infrastructure is in the forefront in African terms, financial constraints and overpopulation in the most neonatal wards, such as those in Tygerberg Academic Hospital, makes it nearly impossible to provide all of the 30 – 40 ELBW and VLBW premature infants who occupy these wards at any given stage, with parenteral nutritional support. The enteral route, with intravenous fluid administration, is the only option for nutritional support of this vulnerable patient population.

The current enteral practice, however, is not optimal, as reflected in the slower growth velocity these infants experience. To prevent negative nitrogen balance, a premature infant requires at least 50 – 60 kcal/kg energy when receiving 1 – 2 g protein/kg.[104] Feeding on breast milk only, a 1 kg premature infant requires at least 75 ml/kg breast milk. This volume of breast milk is usually achieved by day 6 – 7 of life, when no complications occur. Fortification of the breast milk is showing a definite improvement in short-term growth. It is proposed that the problem with the current feeding regimen might be that fortification is administered too late. As these premature infants do not have the parenteral nutrition support which can be administered from day one of life, catabolism is a certain consequence. It takes at least one week for most premature infants to adjust to extra-uterine life. During this transitional period it is recommended to give the safest feed possible, i.e. breast milk and gradually build up the feeding volume by 10 – 20ml/kg/day (i.e. 6.2 – 13.4kcal/kg/day), from day one of life until full enteral feeds (i.e. 150ml/kg/day) are reached. To restrict the energy and protein deficit which develops in the first 2-3 weeks of life to the minimum, negative nitrogen balance must be converted to positive nitrogen balance as soon as possible.

It is proposed that a more aggressive approach with regard to enteral feeding should be followed. Fortification is started too late to prevent the growth deficit that results in more than 90% of infants becoming SGA. It is therefore suggested that a randomised, controlled trial must be conducted, where breast milk fortification is started at the beginning of the stable, growing period (i.e. ± day 6 – 7 of life and receiving ± 75ml/kg/day) in the study group, while the control group commence with fortification when a feeding volume of 150ml/kg/day is reached. The risk of feeding intolerance and necrotising enterocolitis is of major concern, but more studies are finding no increased incidence of necrotising enterocolitis with more aggressive enteral feeding regimens.[206]

As the gradual volume increase of breast milk leads to continual energy increase, fortification will have to be built up slowly. It is suggested that the recommended daily energy increase for supplementation of 10 – 15 kcal/kg/day be adhered to. A gradual introduction of fortification

early in the second week of life, will increase protein intake by ±0.5 g/kg/day over 2 - 3 days, which will result in a much earlier achievement of the ideal protein intake of >2.5 – 3.0g/kg/day, which is the minimum protein required for a positive nitrogen balance.[104] Although negative nitrogen balance is unavoidable, the aim is to at least restrict the amount of time the premature infant experiences a negative or zero nitrogen balance. Earlier conversion to a positive nitrogen balance should result in better short-term growth with the result of a lower incidence of SGA infants at discharge, as well as the long-term effect of improved neuro-developmental outcome. The proposed enteral feeding regimen for ELBW and VLBW is presented in figure 5.1.

The future of effective fortification might lie with different fortifier compositions. If fortification needs to start as early as possible (i.e. at ±75 ml/kg/day), a fortifier which increases protein content with at least 0.5 g/kg/day, while still having a low osmolarity and renal solute load would be ideal. When full volume enteral feeds are reached, the fortifier could be changed to "full-strength", i.e. adding more energy and micronutrients; thus containing a higher osmolarity and renal solute load.

It is known that an ELBW infant in need of catch-up growth requires a higher energy and protein intake to achieve adequate catch-up growth, when compared to an AGA VLBW premature infant. A fortifier with a composition adjusted to the higher needs of the ELBW infant would provide better in their increased needs and hence in achievement of catch-up growth. Using the same fortifier for an AGA VLBW infant might cause unnecessary fat accretion. The AGA VLBW would require a fortifier with a similar protein and micronutrient content, while energy content might have to be lower than that of the SGA ELBW infants' fortifier requirement.

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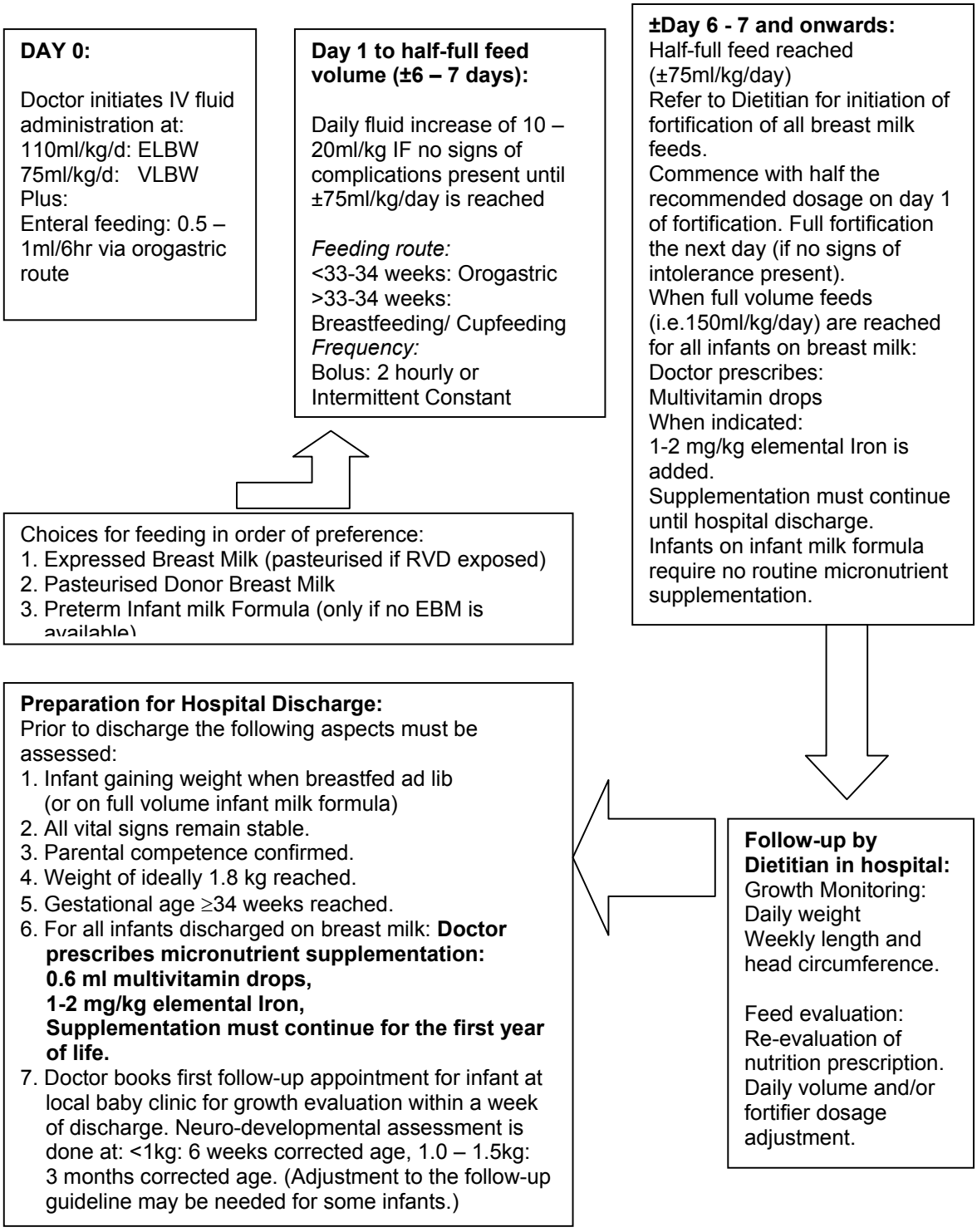

Figure 5.1: Flow diagram for the enteral feeding of the ELBW and VLBW premature infant

5.3.3 Recommendations for post-discharge feeding of VLBW infants on exclusive breastfeeding

As more than 90% of the current study population were SGA at 28 days of life, it is expected that most of these infants would still be in need of catch-up growth upon discharge (i.e. at a weight of ±1.8kg) from the hospital. When ad lib breastfeeding is the feed to be administered upon discharge, it means that the VLBW infant would have to consume at least 172 - 203 ml/kg breast milk to achieve an energy intake of 110 – 130 kcal/kg, while a daily intake of 234 ml/kg would result in an energy intake of 150 kcal/kg. It is known that the VLBW infant is able to consume such amounts of breast milk upon discharge. Although energy consumption might be sufficient post-discharge, the other nutrients might be deficient. Determination of the nutrient content of the current study's breast milk composition reveals that protein, carbohydrate, folic acid, calcium, phosphorus, magnesium, sodium, iron and zinc might be too low to achieve an adequate growth rate.

It is suggested that this population group should be discharged on a breast milk fortifier which would provide in their needs and enable catch-up growth (Table 5.2). Due to the sub-clinical mastitis present in these mothers, the sodium content was much higher than expected; which implies that the suggested sodium content of the fortifier might be even higher than proposed, should sub-clinical mastitis not be present. Routine iron supplementation provides all ELBW and VLBW infants with an additional 1- 2mg iron/kg/day. It needs to be considered if the fortifier should contain iron. A study would have to be conducted to determine how long the fortification must continue post-discharge.

Table 5.2: Proposed composition of a breast milk fortifier suited to provide 110 - 130 kcal/kg to VLBW Xhosa premature infants, HIV-exposed or unexposed post-discharge

Nutrient	Unit	Preterm breast milk composition (mean at 2-4 weeks of life) per 110 kcal	Preterm breast milk composition (mean at 2-4 weeks of life) per 130 kcal	Deficit needed to be added to EBM to meet enteral recom-mendations of 110-130kkal/kg#	Enteral recommendations of the stable, growing premature VLBW infant (per kg/day)[104]
Fluid	ml	171.6	202.80	-	135 - 190
Energy	kcal	110	130.00	-	110 - 130
Protein	g	2.75	3.25	0.65 - 0.95	3.4 – 4.2
Carbohydrate	g	11.34	13.41	0 – 3.6	7 – 17
Fat	g	5.84	6.91	0 – 0.3	5.3 – 7.2
Folic Acid	µg	2.75	3.25	22.25 – 46.75	25 - 50
Calcium	mg	40.87	48.30	59.13 – 171.7	100 - 220
Phosphorus	mg	24.41	28.84	35.6 – 111.2	60 -140
Magnesium	mg	6.39	7.56	1.51 – 7.44	7.9 - 15
Sodium	mg	63.08	74.55	5.92 – 40.45	69 - 115
Potassium	mg	96.59	114.16	0 – 2.84	78 - 117
Iron	mg	0.29	0.35	1.71 – 3.65	2 – 4
Zinc	mg	0.65	0.77	0.35 – 2.23	1 - 3
Copper	mg	0.15	0.18	0	0.12 – 0.15

#: Amounts are calculated by subtracting the EBM contents of column nr 3 and number 4 from the minimum and maximum values of the enteral recommendation in column 6.

Practical administration of fortifier in HIV negative breastfeeding mothers

It is recommended that the HIV negative mother expresses 10 - 20ml breast milk in a sterilised cup prior to a breastfeeding session. The prescribed single dosage of fortifier is then thoroughly mixed with the breast milk and fed to the infant with the cup. Ad libitum breastfeeding is then resumed. This process is repeated with every breastfeeding session or for at least 6 – 8 sessions of the day, whichever is the most appropriate and feasible.

5.3.4 Recommendations regarding the use of donor breast milk banks in South Africa

No significant difference was found between the nutrient composition of raw and pasteurised breast milk of HIV negative mothers of premature infants with the current study. These findings confirm the validity of the use of pasteurised donor breast milk as a safe and nutritionally superior feeding alternative when own mother's breast milk is unavailable to a VLBW or ELBW premature infant.

As most women donating breast milk will have delivered term infants, the composition of the breast milk might not be exactly matching the specific needs of the recipient premature infant. It is therefore recommended that donor breast milk must be fortified according to the fortification protocol of the neonatal unit.

It is concluded that donor breast milk banks have a definite and essential role to play in the feeding of premature infants (whether HIV-exposed or not) in South Africa.

When an institution decides to open a donor breast milk bank, it is imperative that all potential donating mothers must be tested for HIV-1. The most stringent hygienic procedures must be used during the pasteurisation process and record-keeping of information regarding the donor and the respective recipient infant is essential. Informed consent from the woman upon becoming a donor, as well as from the recipient infants' parents or legal guardian, must be obtained at all times. All aspects of the donation process must be done according to a standardised protocol as the legal implications, should a problem occur, would be great.

It is strongly recommended that the Directorate: Health of the public sector in South Africa provide any institution wishing to open a donor breast milk bank with standardised regulations and protocols. Furthermore, all operating donor breast milk banks must be routinely assessed by a governing authority to ensure that all donor breast milk banks are operating correctly. It is suggested that the assessment of Donor breast milk banks must form part the Baby-friendly Hospital Initiative.

5.4 Shortcomings of the current study

Financial constraints was the main reason why the study could not accomplish all the objectives it aimed to determine. Aspects affected most were essential fatty acid, immuno-protein and vitamin A composition of the breast milk which could either not be analysed or the results obtained.

Unavailability of the test kits and a laboratory equipped to do the analysis in South Africa was the reasons why it was not possible to determine the effect of pasteurisation on the viral load of the breast milk of the HIV positive mothers. The objective was to determine the activity, if any, of the reverse transcriptase enzyme in the milk of the HIV positive mothers after pasteurisation. If no activity of the reverse transcriptase enzyme could be found, it would confirm previous findings that pasteurisation substantially decreases the HIV viral load of breast milk, hence decreasing the risk of MTCT of HIV. [15, 77]

5.5 Recommendations for future research

This was a pilot study with a small sample size. It is recommended that this study is performed on a larger scale to confirm the results obtained with the current study. The determination of reverse transcriptase activity in the pasteurised breast milk samples of the HIV positive group to objectively confirm HI viral destruction is strongly recommended with future study.

This study collected samples, but due to financial and technical difficulties, could not determine or obtain results regarding the essential fatty acid, immuno-protein and vitamin A composition of the breast milk of the studied population.

Total fat content is not affected by breast milk pasteurisation. As essential fatty acids are of such great importance for neurological and retinal development as well as immunity, a study to determine the effect of pasteurisation on breast milk essential fatty acid, including the longer chain derivatives (i.e. arachidonic and docosa-hexaenoic acid) composition is recommended.

Maintaining a high level of immunity is of the utmost importance in the premature and LBW infant and even more so for the HIV-exposed premature infant. Recent developments in the methodology used to determine breast milk immuno-protein (e.g. secretory immunoglobulin A, lactoferrin and lysozyme) content after pasteurisation makes it possible to determine if there is a difference in the immuno-protein content of the breast milk of HIV positive and HIV negative mothers. It is recommended that a study be done to compare the immuno-protein content of raw and

pasteurised breast milk of HIV positive and HIV negative mothers with premature infants, as data regarding this topic is scarce.

The effect of pasteurisation on breast milk vitamin A content in HIV negative and HIV positive mothers with premature infants must be determined.

Breast milk energy content, as determined indirectly by using conversion factors, indicated a lower than expected energy composition at the end of the study period. It would be interesting to determine breast milk energy content after the first month of life in the current study population. Discrepancies between direct (bomb calorimetry) and indirect methods (calculation with conversion factors) of determining energy content of breast milk directs towards a comparative study of breast milk energy content determined by the two methods.[193]

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APPENDICES

APPENDIX A: WRITTEN SUBJECT INFORMATION

The effect of pasteurisation on the composition of expressed human milk from HIV positive mothers, and its adequacy in relation to the growth of their very low birth weight premature infants.

TRAIL CODE NUMBER: **NO4/04/070**

1. INVITATION

You are invited to take part in a research study. Participation is voluntary. Before you decide whether you would like to take part, you must understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your doctor, if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part or not.

2. WHAT IS THE PURPOSE OF THE STUDY?

It has been shown that low-grade heating (pasteurisation) of breast milk kills the Human Immuno-deficiency Virus (HIV) that might be present in the milk of mothers who are HIV positive. Pasteurisation of breast milk enables an HIV positive mother to feed her preterm infant her own milk that has been expressed and pasteurised. The problem is that we do not know in full what the effect of pasteurisation is on the levels of the nutritional- and protective substances in breast milk. Furthermore the effect of different milk expression techniques (hand expression versus hand pump expression) on the breast milk composition is not known.

We do know that premature infants have higher nutritional needs than full term babies, because they have to catch up in their growth to reach that of their full term peers. Breast milk can unfortunately not meet all of the preterm infant's nutritional needs. We therefore require the use of a human milk fortifier, which is a powder that is added to your breast milk while your baby is treated in the hospital. The human milk fortifier currently used in the Kangeroo Mother Care unit where your baby is being cared for, is however too expensive to send the baby home with a supply thereof.

This study would like to determine the amounts of nutritional and protective substances in the pasteurised human milk of mothers who gave birth to preterm babies at Tygerberg- or Eersterivier Hospital. If the effect of pasteurisation is known, recommendations can be made on whether it is worthwhile to give pasteurised breast milk to premature babies of HIV positive mothers. If the effect of the method of human milk expression is known, recommendations can be made on whether milk expression is a worthwhile option in cases where mothers cannot

breastfeed due to her HIV positive status. We would also like to make suggestions on the composition of a human milk fortifier suitable for these babies. The ideal would be if such a fortifier could be manufactured locally and given to premature infants on discharge, to help these babies achieve adequate catch-up growth at home.

3. WHY HAVE I BEEN CHOSEN?

You have been chosen, because you gave birth to a very small and premature baby, and have been admitted to the Kangeroo Mother Care Unit of Tygerberg or Eersterivier Hospital and are able to express a sample of breast milk.

4. DO I HAVE TO TAKE PART?

It is your own decision whether you would like to take part or not. If you decide to take part, you will be given this information sheet to keep and asked to sign a consent form.
If you decide to take part in the study, you are still free to withdraw at any time and without giving a reason. This will not affect in any way the standard of care you or your baby receives in the hospital.

5. WHAT WILL HAPPEN TO MY BABY AND I IF I TAKE PART?

You will have to express one full breast of milk on the following days after your baby has been born: days 3 (optional), 6, 7, 13, 14, 20, 21, 27 and 28. This means that you will have to remain in hospital for the four weeks that your baby will still be treated. During your stay in hospital, you will be provided with three meals per day and all the facilities available in the Kangaroo Mother Care Unit.

You will be asked to express your breast milk by hand or with a hand pump that will be provided to you. Your milk sample will be divided into two even samples. One sample will be subdivided into 4 - 5 even parts and frozen immediately, while the second sample will be pasteurised, after which it will be subdivided into 4-5 even parts and frozen. The milk samples will then be sent away to the different laboratories for analysis.

You will be asked to answer a few questions regarding your pregnancy history. You will also have to answer a few questions on your dietary intake during pregnancy and now, while breastfeeding. Your height, weight and mid-upper arm circumference will be measured twice during your stay in the hospital.

You must be willing to undergo one rapid test to confirm your HIV status before any milk samples can be collected. The rapid test is the same test you had at the clinic, while you were pregnant.

You must be willing to give two blood samples of about one teaspoon each (one at the beginning and one at the end of your participation in the study) to confirm your nutritional status.

Your baby will be weighed and his/her length and head circumference determined at the beginning and end of the study. Only two small samples of blood of 1 ml (one drop) each will be collected at the beginning and at the end of the study for determination of your baby's HIV- and nutritional status. For this study no other tests will be done on your baby.

6. WHAT ARE THE POSSIBLE DISADVANTAGES OR RISKS OF TAKING PART?

You will have to repeat the rapid test for HIV that was previously performed on you. You and your baby must provide one sample of blood at the beginning and end of the study. During your stay in the hospital you will have to express one of your breasts in full on 8 occasions and provide the expressed breast milk for analysis.

7. WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

The acknowledgement that you were a valuable contributor in providing the medical team with valuable data, that might help them in their search for the best way of feeding premature infants. All participating mothers will furthermore be allowed to keep the hand pump after her completion of the study. During your stay you will daily receive three balanced meals in the hospital and may use all the facilities provided in the Kangaroo Mother Care Unit.

8. WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available about the topic that is being studied. If this happens, your investigator(s) will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your investigator(s) will arrange for your and your baby's care to continue as before in the hospital. If you decide to continue in the study you will be requested to sign an updated consent form.

9. WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

All milk samples obtained in the study will be analyzed to determine the nutritional composition of the breast milk of the study population. The composition of breast milk that has been pasteurised will also be ascertained. The nutrient and immunological deficits (if any) will be identified in the breast milk samples.

Knowledge of the breast milk composition will enable the investigator to make recommendations on the composition of a human milk fortifier suitable for the South African population's premature infants. We would also like to determine whether donor milk banks for the provision of pasteurised breast milk, would be beneficial to premature babies of HIV positive mothers.

10. WHAT IF SOMETHING GOES WRONG?

It is unlikely that something will go wrong, because the only invasive measures undertaken will be the rapid test for HIV status determination and the two blood collections of about one teaspoon each. Only two drops of blood will be drawn from your baby over the period of one month. If you are not able to express milk at the planned collection time, you can try to express milk again later. The investigator will inform you of the next appropriate time to express the breast milk. Throughout the study standard procedures will be used, thus making the chances that something will go wrong, very slim.

11. WILL MY PARTICIPATION IN THE STUDY BE KEPT CONFIDENTIAL?

Some of the milk samples will be kept by the different laboratories for 10 years, but these milk samples cannot be traced back to you, because your name will not be put on any of your milk samples. Only the investigator and research-assistant will know which sample belongs to which mother. The investigator and research-assistant will treat all information gathered as strictly confidential and no information that can identify you or your baby will be released to any person not directly associated with the study.

12. WHAT WILL HAPPEN TO THE RESULTS OF THE STUDY?

The information obtained /collected will be handled with complete confidentiality and anonymity, but will be published in scientific journals and presented at congresses. Under no circumstances will your baby's or your name be revealed, because the results will only give the data of the study group as a whole.

13. WHO IS ORGANISING AND FUNDING THE RESEARCH?

The project is being carried out by the University of Stellenbosch and Tygerberg Academic Hospital, as well as at Eersterivier Hospital. The principle investigator is Ms E. van Wyk and the study leaders Professors GF Kirsten and D. Labadarios.

14. WHO HAS REVIEWED THE STUDY?

The study has been reviewed by the Research Ethics Committees of the University of Stellenbosch, Tygerberg Academical- and Eersterivier Hospitals. The study complies with the standards of good clinical practice of the Medical Research Council (MRC) and ICH and according to the Declaration of Helsinki (2000 version).

15. CONTACT FOR FURTHER INFORMATION

For any further information or questions about this study, please contact:

_____ _____

Investigator's signature Date

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Research Ethics Committee
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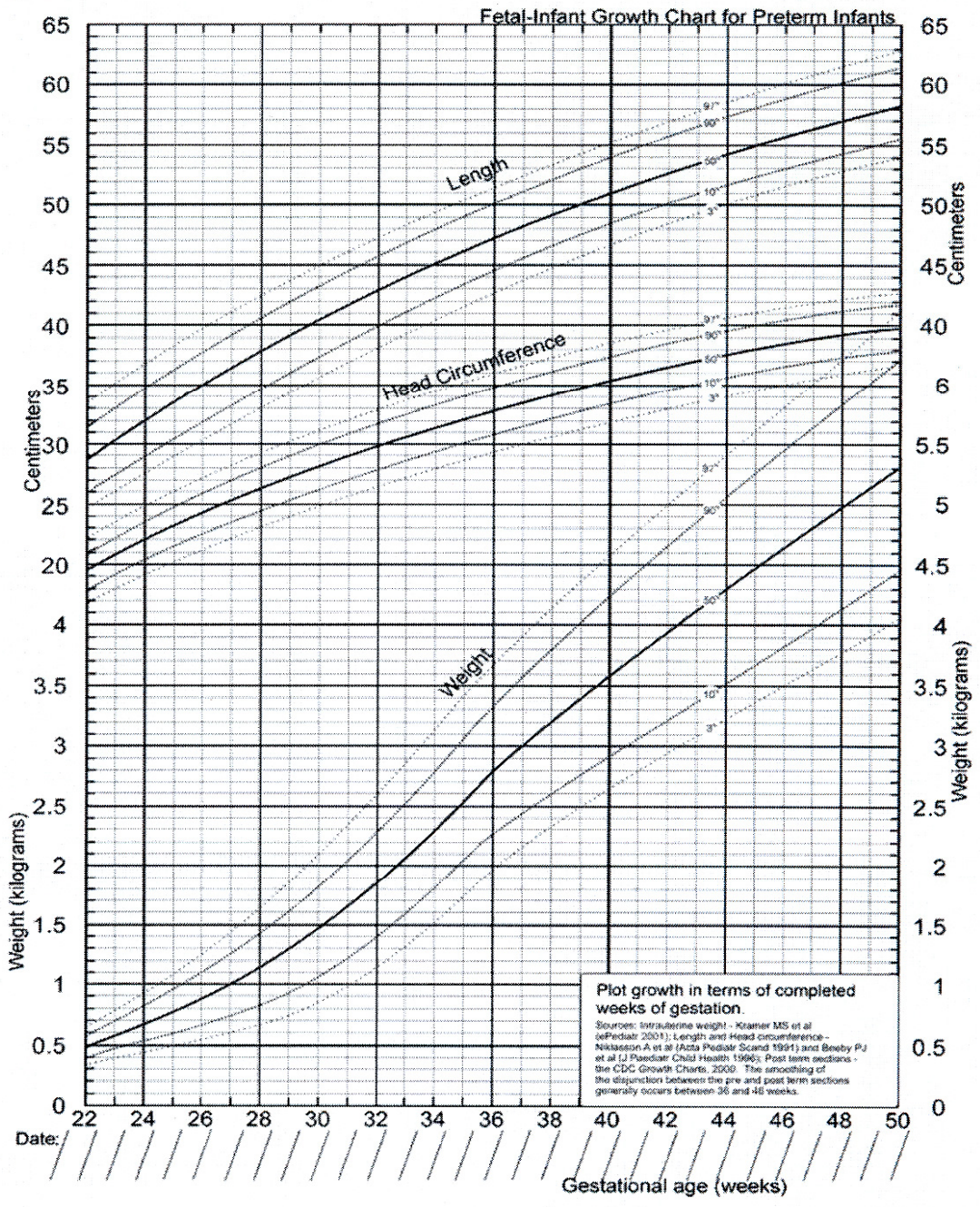
APPENDIX B: Fetal-Infant Growth Chart for Preterm Infants[166]

Ref: Fenton Tanis[i]. *BMC Pediatrics* 2003,



[i] Fenton Tanis R: A new growth chart for preterm babies: Babson and Benda's chart updated with recent data and a new format. *BMC Pediatrics* 2003, 3:13

APPENDIX C:
Socio-demographic descriptors or variable [means and (Standard Deviation (SD)] of the mothers included in the control and the study groups

Determinant	**Unit**	**Number of control group affected (*N*=12)**	**Number of study group affected (*N* =11)**	**p-value (identified below)**
Age of mother	years	26.5 (3.8)	25.8 (3.8)	0.67[a]
Level of Education[#]	-	3 (0.7)	3 (0.7)	0.74[a]
Age of infant at birth	weeks gestation	28.7 (1.6)	30.4 (1.6)	0.21 [a]
Number of mothers with no income	-	7	7	-
Average income of household	R/month	1331.3	1624.7	0.63
Parity (including this pregnancy)	-	2.1	2.1	-
Number of children (including this child)	-	2	1.8	-
Number of previous miscarriages	-	1	2	-
Number of previous premature deliveries	-	3	1	-
Number of mothers with other children who initiated breastfeeding previously	-	8 (out of 8)	5 (out of 8)	0.1^
Average duration of exclusive breastfeeding with previous children	months	0.5 - 5	1 – 3	-
Average age when previous child was weaned from the breast	years	2.1	0.77	0.04*
Number of mothers experiencing medical problems during this pregnancy	-	9	6	-
Number of mothers diagnosed with hypertension	-	8	6	-
Number of mothers diagnosed with gestational Diabetes Mellitus	-	2	0	-

Socio-demographic descriptors or variable [means and (Standard Deviation (SD)] of the mothers included in the control and the study groups (cont.)

Determinant	Unit	Number of control group affected (N=12)	Number of study group affected (N =11)	p-value (identified below)
Number of mothers experiencing nausea at some stage during pregnancy	-	8	6	-
Number of mothers reporting vomiting at some stage during pregnancy	-	7	4	-
Number of vaginal deliveries	-	2	6	-
Number delivered by Caesarean Section	-	10	5	-
Number of mothers reporting some form of exercise during pregnancy	-	7	6	-
Number of mothers using contraceptives before pregnancy	-	7	7	-
Number of mothers using medication during pregnancy	-	6 (50%)	7 (64%)	-
Number of mothers visiting a traditional healer	-	1 (8%)	1 (9%)	-
Number of mothers with food insecurity	-	0	2 (18%)	-

[#]**Level of education:** Level 3 refers to high school education (grade 8 - 12).

[a]: Mann-Whitney U test used to determine the p-value.

^: Fischer exact test used for determination of p-value.

*: Level of difference is significant (p-value <0.05).

APPENDIX D: A description of information obtained with the socio-demographic questionnaire

Alcohol consumption:

Beer was the alcoholic beverage consumed by all three mothers. Frequency of consumption was once for the study group mother and 1-2 times per month and on weekends for the two control group mothers respectively. Amount of beer consumed was one cup for the study group mother and 4 – 6 small bottles and 3 – 4 glasses for the two control group mothers respectively. No clinical signs of Fetal Alcohol Syndrome were reported for any participating infant.

Smoking and drugs:

The two control group mothers reporting alcohol consumption were also the two mothers that smoked in the control group. The two study group mothers who reported smoking during pregnancy did not report any alcohol consumption while pregnant. The amount of cigarettes smoked daily was low for all four mothers (i.e. 1 – 4 cigarettes/day).

None of the participating mothers reported the use of any type of recreational drugs during pregnancy.

Medication:

Anti-hypertensive medication and analgetics, i.e. aspirin or paracetamol was the medications used by most of the mothers who took medication during their pregnancy. Five (45.5%) of the HIV positive mothers reported that they used anti-retroviral medication during pregnancy. The anti-retroviral medication consumed was either a non-nucleoside protease inhibitor (Nevirapine®) and/or a nucleoside protease inhibitor (Zidovudine®). The anti-retroviral medication regimen used was not uniform for all HIV positive mothers, because the regional Prevention of Mother-to-Child Transmission (PMTCT) protocol was changed during the data-collection period. All mothers received either one or both of the mentioned anti-retroviral medicines. None of the participants received highly active anti-retroviral (HAART) therapy.

Exercise:

Walking was the type of exercise performed by most mothers. Only one mother reported taking part in organised sport (netball). The average amount of time spent exercising was 49 minutes for the control and 26 minutes for the study group. Six (46.2%) of the 13 mothers exercised daily, 2 (15.4%) exercised 3-4 times per week, while 3 (23.0%) exercised 1-2 times per week. Two (15.4%) mothers did not indicate their frequency of exercise. All exercising mothers indicated their exercise was done at a low intensity level, while the mother taking part in organised sport felt very out of breath after exercising.

Consulting a traditional healer:

Two mothers consulted a traditional healer while they were pregnant. One mother received ointment, while the other mother received a medicine mixture of which she had to consume 125ml for 28 days.

Food insecurity:

Two mothers experienced periods of food insecurity (defined as “not having enough money to buy food due to loss of income or unemployment”) during their pregnancy. Both mothers experienced the food insecurity during the last week of every month for their whole pregnancy.

Stress:

Two (16.7%) control group and three (27.3%) study group mothers experienced social stress (e.g. death in the family, domestic violence) for a period of time during their pregnancy. Economical stress due to insufficient household income was experienced by 41.7% (*N*=5) control and 18.2% (*N*=2) study group mothers. Emotional stress (e.g. feelings of depression, worrying about pregnancy outcome) was reported by 33.3% (*N*=4) control and 36.4% (*N*=4) study group mothers.

Diet consumed during pregnancy:

Three mothers reported eating less than normal during their pregnancy. Three mothers reported a higher milk or amasi (fermented milk) intake, two mothers a higher fruit intake and one mother consumed more porridge and peanut butter during her pregnancy. Two mothers avoided meat and/or chicken for the whole or a part of their pregnancy.

Dietary cravings:

Fruit in general or certain types of fruit was the craving reported by most mothers (*N*=10; 55.6%). Three mothers craved amasi and a further two mothers crumbly maize meal porridge with amasi. Three mothers craved more starch in the form of bread or pasta, and one mother craved salt-and vinegar potato crisps. Red meat was craved by two mothers, while pork and chicken was craved by one mother respectively. Two mothers craved carbonated cold drinks and two mothers craved certain types of vegetables (i.e. spinach, turnip and cabbage). Two mothers craved fat in the form of margarine or peanut butter.

Food aversions:

Mothers were asked whether there were certain foods they could not eat during pregnancy, because the food made them feel uneasy or ill. Red meat (*N*=4; 17.4%) and chicken (*N*=7; 30.4%) was the food avoided by most mothers during their pregnancy. Samp and rice was avoided by two mothers respectively, while eggs, tomato and onion, beans, crumbly porridge and peanut butter was avoided by one mother respectively.

Food intolerance and allergies:

No mother reported a diagnosed food allergy. Only one mother reported symptoms of abdominal cramps after ingestion of peas, which may indicate possible food intolerance.

Caffeine consumption:

Instant coffee was the source of caffeine consumed by most mothers (*N*=13; 76.5%). Ten (58.8%) mothers consumed Ceylon tea, while 6 (26.1%) mothers used only Rooibos tea (caffeine-free).

Food restrictions:

Food restrictions was practiced out of own choice. Two mothers did not eat pork, one not eggs and one mother did not eat apples and peaches.

Fasting:

Two mothers reported fasting during their pregnancy. One mother fasted for two days per month during her pregnancy. The second mother fasted once a week for six of the months she was pregnant. No specific reasons were provided for their fasting by the two mothers.

Micronutrient supplementation:

Iron was the supplement used by most mothers (*N*=15; 65.2%). Nine (39.1%) of all participating mothers received a folic acid supplement with their iron supplement at the antenatal clinic. One mother added a calcium supplement and another mother a Vitamin B complex supplement to the iron and folic acid that both of them received from their antenatal clinic. A major deviation from the antenatal protocol, where all mothers are supposed to receive iron and folate supplementation during pregnancy occurred. Possible reasons for this finding were late booking of pregnancy, as well as poor attendance at the antenatal clinics by the mothers.

Pica:

This was the only dietary habit that showed a trend towards a significant difference (p=0.053) between the control and study group, with 55% (*N*=6) the study group participants and only 17% (*N*=2) of the control group participants reporting pica during pregnancy. Ice and ice suckers was the substance consumed most (*N*=6; 75%) by mothers reporting pica. Ice was consumed daily by five of the mothers and twice a week by the remaining mother. Ice consumption varied between a few months to the full duration of the pregnancy. One mother ate soil daily for two months and another mother charcoal daily for one month. Pica might be a sign of iron deficiency anaemia, which is expected in pregnancy when there is a lack of good dietary sources of iron and/or insufficient iron supplementation.

Diet consumed during lactation:

The hospital diet differed from their normal dietary intake at home for 18 (78.3%) of the 23 mothers. The major difference was that they ate a more traditional (Xhosa) diet at home, compared to the more westernised hospital diet. Comparing the amount of food served in hospital to how much they usually consumed at home, revealed that 7 (30.4%) mothers ate the same amount, 6 (26.1%) mothers ate more and 7 (30.4%) mothers ate less in hospital than their usual intake at home. Three (13%) mothers did not indicate how their intake differed.

APPENDIX E: Quantitative Food Frequency Questionnaire[152]

ADAPTED FOOD CONSUMPTION SURVEY: MOTHERS (19 –31 YRS OLD): DETERMINING DIETARY INTAKE DURING PREGNANCY

Subject Number: | | | | | | | **Birth Date:** DD MM YY | | | | | | **Interview Date:** DD MM YY | | | | | |

Province:_____ EA.: _____ Interviewer: _____ Interviewer Code: _____

QUANTITATIVE FOOD FREQUENCY QUESTIONNAIRE

Greeting

Thank you for giving up your time to participate in this survey. We would like to find out what mothers 19 to 31 years old and living in this area, usually eat and drink while they are pregnant. This information is important to know as it will tell us if what mothers eat during their pregnancy has an effect on the composition of their breast milk.

Please think carefully about the food and drinks you have consumed while you were pregnant. I will now go through a list of foods and drinks with you and I would like you to tell me, while you were pregnant:

- if you ate these particular foods,
- how the food was prepared,
- how much of the food you ate at a time, and
- how many times a day you ate it (if you ate it every day), or how many times a week or a month it was eaten?

To help you to describe the amount of a food, I will show you models of different amounts of the food. Please say which model is the closest to the amount eaten, or if it is smaller, between sizes or bigger than the models. Amounts must be reported as cups (c), tablespoons (T), serving spoons (SP) or teaspoons (t).

- **THERE ARE NO RIGHT OR WRONG ANSWERS.**
- **EVERYTHING YOU TELL ME IS CONFIDENTIAL.**
- **IS THERE ANYTHING YOU WANT TO ASK NOW?**
- **ARE YOU WILLING TO GO ON WITH THE QUESTIONS?**

QUESTION	YES	NO		REMARKS / OTHER			
1. Did you follow any special diet while you were pregnant?	1	2	Don't Know	If yes, please specify:			
				Diabetic	Slimming	Allergies	Other
				1	2	3	4

2. Did you eat breakfast while you were pregnant?	Regularly (4 or more times a week)	Sometimes (1-3 times a week)	Never
	1	2	3

INSTRUCTIONS TO FIELDWORKERS:
CIRCLE THE CHOSEN ANSWER AND FILL IN THE AMOUNT AND TIMES EATEN IN THE APPROPRIATE COLUMNS.

I will ask you about the type and the amount of food you have been eating during your pregnancy.
Please tell me if you ate the food, how much you ate and how often you ate the food.

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/ NEV
PORRIDGE	Maize-meal Porridge Brand name:	Stiff (Pap) –Plain	**3400**	1c stiff = 250 g 1T = 75g						
		- Enriched	**4278**							
		Soft (Slappap) – Plain	**3399**	1c soft = 250g 1T = 75g						
		- Enriched	**4277**							
		Crumbly (Phutu) – Plain	**3401**	1 c crumbly = 140 g 1T = 30g						
		- Enriched	**4279**							
	Sour Porridge	Maize with Vinegar Maize Fermented Mabella with Vinegar Mabella Fermented	**P0001** **P0002** **P0003** **P0004**	½c = 125g 1c = 250g						
	Mabella Porridge/Cornrice	Stiff	**3437**	½ c = 125g						
		Soft	**3437**							
	Maltabella Porridge	Stiff	**3241**	½ c = 125g						
		Soft	**3241**							
	Oats Porridge	Brand Name:	**3239**	2c = 125g						
	Nutrition Scheme products (i.e. enriched mealie meal porridge, eg. Khulani or Philani Mealie meal)	Stiff Soft		4 scoops =50 g						
	Other Cooked Cereals	Specify Type:								
	Milk on Porridge **(Circle type usually used)**	None								
		Whole/Fresh	**2718**	little = 30g med = 60g much = 125g						
		Sour	**2787**							
		2%	**2772**							
		Fat Free / Skim	**2775**							
		Milk Blend	**2771**							
		Soy Milk	**2737**							
		Condensed (Whole, Sweet)	**2714**	1t = 10g						
		Condensed (Skim, Sweet)	**2744**							
		Evaporated Whole	**2715**	1t = 3g						
		Evaporated Low Fat	**2827**							
		Non-Dairy Creamer	**2751**	1t = 4g						

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/ NEV
	Is sugar added to porridge? (Circle type usually used)	None								
		White	**3989**	1t sugar = 6g						
		Brown	**4005**							
		Syrup	**3988**	1t honey/syrup = 15g						
		Honey	**3984**							
		Sweetener: Type	**P0016**							
	Is fat added to porridge? (Circle type usually used)	None								
		Animal Fat (Butter)	**3479**	1t marg/oil = 5g						
		Hard Margarine	**3484**							
		Soft Margarine (PM)	**3496**							
		Soft Margarine (Med)	**3531**							
		Sunflower Oil	**3507**							
		Peanut Butter	**3485**	1t = 12g						
BREAKFAST CEREALS	Breakfast Cereals	Specify types usually eaten		**(See Manual)**						
	Milk on Cereal	Specify Type		**(See Manual)**						
	Is sugar added to cereal?	Specify Type		**(See Manual)**						
	Is fat added to cereal?	Specify Type		**(See Manual)**						

How many times a week did you eat porridge or breakfast cereals at any time of day (not only breakfast): _____

I am now going to ask about starchy foods:

STARCHES	Samp/Maize Rice	Samp, White	**3250**	1T = 55g; 1 SP = 125g; ½ c = 125g						
		Maize Rice	**3250**							
		Sweetcorn Boiled	**3725**	1T = 25g; 1 SP = 45g; ½ c 65g						
	Samp and Beans	Specify Ratio:	**3402**	1T = 50g 1SP = 125g ½ c = 125g						
	Samp and Peanuts	Specify Ratio:	**P0013**							
	Rice: Specify Brands Names	White	**3247**	1T = 25g; 1SP = 60g; ½ c = 65g						
		Brown	**3315**							
	Stamped Wheat		**3249**	1T = 30g; 1SP = 80g; ½ c=80g						
STARCHES	Pastas	Macaroni	**3262**	1T = 35g; 1SP = 70g; ½ c = 90g						
		Spaghetti Plain	**3262**							
		Spaghetti and Tomato Sauce	**3258**	1T =45g; 1SP =80g; ½ c=125g						
		Other: Specify								

	Do you add fat to any of these starchy foods?	Yes ____ No ____ If yes, specify types, amounts and to which food?		**(See Manual)**						
	How many times a week did you eat the above starchy foods? _____									
BREADS AND SPREADS	**Now we come to bread and bread spreads:**									
	Bread/Bread Rolls	White	**3210**	Wh+Br 10mm = 30g Wh + Br 20mm = 60g Wh + Br 30mm = 100g ½ loaf = 400g						
		Brown	**3211**							
		Whole Wheat	**3212**	Ww 10mm = 35g						
	Other Breads (Specify Types)	Raisin	**3214**	m/s = 30g; L/s = 50g						
		Maize Meal	**3278**							
		Sweetcorn	**3379**							
		Rye	**3213**							
		Pumpernickel	**3283**							
		Other								
	How many times per week did you eat bread? _____									
	Dumpling	(Depends on specific areas)		**(See Manual)**						
	Vetkoek	(Depends on specific areas)		8 cm diam = 60g						
	Provita		**3235**	6g						
	Crackers	Cream Crackers	**3230**	8g						
		Refined (eg. Tuc)	**3331**	4g						
		Wholewheat	**3391**	8g						
	Pizza	(Specify Toppings)		**(See Manual)**						
	Hot Dogs	(Specify Sausage)		**(See Manual)**						
	Hamburgers	(Specify Meat)		**(See Manual)**						

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/ NEV
BREADS AND SPREADS	**Did you spread any of the following spreads on your bread?** **Fat Spreads: (Tick box)**	Butter	**3479**	1t = 5g						
		Butro	**3523**							
		Animal Fat (Beef Tallow)	**3494**							
		Lard	**3495**							
		Hard Margarine	**3484**							
		Soft Margarine (PM)	**3496**							
		Soft Margarine (Med)	**3531**							
	PeanutButter		**3485**	1t = 12g						
	Sweet Spreads	Jam	**3985**	1t = 15g						
		Syrup	**3988**							
		Honey	**3984**							
	Marmite/ OXO(or Bovril/ Fray Bentos)	Marmite	**4030**	thin = 2g; med = 4g; thick=7g						
		Oxo (or Bovril/ Fray Bentos)	**4029**							
	Paste	Fish Paste	**3109**	thin = 5g; med = 7g; thick = 10g						
		Meat Paste	**2917**							

	Cheese (Specify Types)	Cheddar	**2722**	grated: med = 10g; thick = 15g
				cubes = 30g;
				slice = 8g;
				cheezi = 20g						

		Gouda	**2723**							
		Cottage Low-Fat Cheese	**2760**	med = 20g; thick = 30g						
		Cream Cheese	**2725**	thin = 10g; med = 20g						
		Other								
	Cheese Spreads (Specify Types)		**2730**	med = 12g; thick = 25g						
	Atchar		**3117**	1T = 14g; 1SP = 60g						
	Other Spreads (Specify Types)									
You are being very helpful. Can I ask you about protein foods? These are: meat, beans, chicken, fish and eggs.										
CHICKEN		Boiled with skin	**2926**	Breast + skin = 125g Thigh =80g Drumstick = 42g Foot = 30g Wing = 30g						
		Boiled without skin	**2963**							
		Fried in batter/crumbs	**3018**							
		Fried – not coated	**2925**							
		Roasted/grilled with skin	**2925**							
		Roasted/grilled without skin	**2950**							
	Chicken Bones Stew	(Specify ingredients)	**P0048**							
	Chicken Heads		**2999**							

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/ NEV
	Chicken Stew	With Vegetables	**3005**	1SP = 90g; ½ c = 125g						
		With Tomato & Onion	**2985**							
	Chicken Feet		**2997**	Foot = 30g						
	Chicken Offal	Giblets	**2998**	stomach = 20g						
	Chicken Liver		**2970**	Liver = 30g						
	Chicken Pie	Commercial or homemade	**2954**	med = 150g						
RED MEAT	**Beef**	Roasted with Fat	**2944**	120 x 60 x 5 = 35g						
		Roasted, Fat Trimmed	**2960**	120 x 60 x 10 = 70g						
		Rump, Fried with Fat	**2908**	S/s 130 x 70 x 15 = 125g						
		Rump, Fried, Fat Trimmed	**2959**	L/s 165 x 70 x 30 = 270g						
		Stewed/Boiled With Fat (Cabbage)	**3006**	1SP = 105g; ½ c = 125g						
		Stewed/Boiled Without Fat (Vegetables)	**2909**							
		Mince With Tomato and Onion	**2987**	1T=40g;1SP=85g; ½ c=100g						
		Other Preparation Methods:								
	Mutton	Fried/Grilled: With Fat	**2927**	Loin chop = 60g; Rib chop = 40g						
		Fried/Grilled: Without Fat	**2934**							
		Stew: Plain	**2974**	1SP = 105g; ½ c = 125g						
		Stew: Irish (Vegetables)	**2916**							
		Stew: Curry	**3039**							
		Stew: Greenbean	**3040**							
		Other Preparation Methods:								
	Pork	Fried/Grilled: With Fat	**2930**	Chop: 115 x 80 x 20 = 100g						
		Fried/Grilled: Without Fat	**2977**	Schnitzel: 115 x 80 x20 = 110g						
		Roast With Fat	**2958**	Roast: 110x 65 x 5 = 30g						
		Roast Without Fat	**2978**	1SP = 105g; ½ c = 125g						
		Other Preparation Methods:								
	Goat	Fried/Grilled: With Fat	**P0008**	120 x 60 x 5 = 35g						
		Fried/Grilled: Fat Trimmed	**P0009**	120 x 60 x 10 = 70g						
		Stewed (Plain)	**4281**	1SP = 105g ½ c = 125g						
		Stewed (With Vegetables)	**4282**							
		Other Preparation Methods:								

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/ NEV
MEAT: GENERAL	**Offal**	"Vetderm" Fried	**P0023**	1SP = 105g; ½ c = 125g						
		Liver: Beef (Fried)	**2920**	80g						
		Liver: Sheep (Fried)	**2955**	55g						
		Kidney (Beef)	**2923**	85g						
		Kidney (Sheep)	**2956**	30g						
		Tripe, Beef, Cooked in Milk	**2951**	1SP = 105g; ½ c = 125g						
		Heart (Beef)	**2968**	60g						
		Heart (Sheep)	**2969**	60g						
		Lung (Beef)	**3019**	60g						
	Wors/Sausage	Fried	**2931**	Thin x 200mm = 45g; Thick x 165mm = 90g						
	Bacon	Fat	**2906**	1 rasher = 10g						
		Lean	**2915**							
	Cold Meats	Polony	**2919**	Slice 5mm thick = 8g Comm slice = 16g						
		Ham	**2967**	Med slice = 25g						
		Viennas	**2936**	100mm = 30g; 150mm =40g						
		Other								
	Canned Meats	Bully Beef	**2940**	138 x 85 x 3 = 20g; ½ c = 100g						
		Other (Specify)								
	Meat Pie	Bought (Steak & Kidney)	**2957**	120g						
		Other (Specify)								
	Legumes (Specify dried beans/peas/legumes)	Stews (Bean, Potato & Onion)	**3178**	1T=60g; 1SP = 120g; ½c=125g						
		Soups: Commercial	**3165**	½ c = 125g						
		Split Pea	**3157**	1T=35g; 1SP = 80g; ½ c = 130g						
		Lentil	**3153**							
		Beef & Vegetables	**3159**							
		Bean	**3145**							
		Legume Salad	**3174**	1T=40g; 1SP=105g; ½ c=135g						
	Soya Products e.g. Toppers / Imana	(Specify)	**3196**	1SP = 85g; ½ c = 120g						

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/ NEV
FISH	**Fried Fish (Fresh or Frozen, Fried in Sun Oil)**	With Batter/Crumbs	**3094**	Small 50 x 55 x 30 = 60g; Med 100 x 55 x 30 = 120g						
		Without Batter/Crumbs	**3084**							
	Canned Fish	Pilchards in Brine	**3055**	1 Pilchard = 75g						
		Pilchards in Tomato Sauce	**3102**							
		Pilchards, Mashed	**3102**	1 SP = 85g; ½ c = 100g						
		Sardines in Oil	**3104**	Ss = 7g; L/s = 25g						
		Sardines in Tomato Sauce	**3087**							
		Tuna in Oil	**3093**	¼ c = 50g						
		Tuna in Brine	**3054**							
		Other (Specify)								
	Pickled Fish/Curried Fish		**3076**	1 SP = 95g; ½ c=140g						
	Did you remove fish bones before eating canned fish? Yes ___No __									
	Fish Cakes	Fried: Oil/Butter/Margarine	**3098**	65 x 15mm = 50g						
	Fish Fingers	Fried: Oil/Butter/Margarine	**3081**	85mm = 35g						
EGGS	**Eggs**	Boiled/Poached	**2867**	1 egg = 50g						
		Scrambled in Oil	**2889**	1T = 35g; 1SP = 80g; ½c=115g (approx. 2 eggs)						
		In Butter	**2886**							
		In Margarine	**2887**							
		Fried in Oil	**2869**	1 egg = 52g						
		In Butter	**2868**							
		In Margarine	**2877**							
		In Bacon Fat	**2870**							
		Curried	**2902**	1 egg + sauce (1T) = 75g						

How many times a week did you eat meat, beans, chicken, fish or eggs? _____

Are there any other foods in this category that you ate? If yes, please list these foods/dishes					YES			NO
FOOD	**DESCRIPTION**	**CODE**	**AMOUNT USUALLY EATEN (HHM)**	**AMOUNT USUALLY EATEN (g)**	**TIMES EATEN**			
					Per day	**Days per week**	**Per month**	**Seldom/ Never**

	FOOD	**DESCRIPTION**	**CODE**	**QUANTITY (g/ml)**	**AMOUNT USUALLY EATEN (HHM)**	**AMOUNT USUALLY EATEN (g)**	**P/D**	**D/W**	**P/M**	**SEL/NEV**
We now come to vegetables:										
VEGETABLES	Cabbage	Boiled, Nothing Added	**3756**	1T=30g; 1SP=55g; ½ c=80g						
		Boiled with Potato, Onion and Fat	**3813**	1T=35g; 1SP=75g; ½ c=80g						
		Fried, Nothing added	**3812**	1T=30g; 1SP=55g; ½ c=80g						
		Boiled, then fried with potato, onion	**3815**	1T=35g; 1SP=75g; ½ c=80g						
		Other								
	Spinach/Marog/Imifino/ Amaranth Leaves Other Green Leafy Vegetables: List Names	Boiled, nothing added	**3980**	1T=40g; 1SP=105g; ½ c=90g						
		Boiled, fat added	**3898**	1T=40g; 1SP=105g; ½ c=90g						
		Boiled with Onion, Potato and Fat	**3901**	1T=50g; 1SP=105g; ½ c=110g						
		Boiled with Peanuts	**P0015**	1T=55g; 1SP=120g; ½ c=105g						
		Other:								
	Tomato and Onion "Gravy"/ Relish/Chow/Sheshebo	Home Made with Sugar	**3910**	1T = 35g; 1SP = 75g; ½ c = 140g						
		Home Made, no Sugar	**3925**							
		Canned	**4192**							
	Pumpkin (Specify Type)	Boiled, nothing added	**4164**	1T = 45g; 1SP = 85g; ½ c = 105g						
		Cooked in Fat and Sugar	**3893**							
		Other								
	Carrots	Boiled, Sugar and Fat	**3818**	1T = 25g; 1SP=50g; ½ c = 85g						
		With Potato/Onion (HM)	**3822**	1T=35g; 1SP=70g; 1/2 c=105g						
		Raw, Salad (Sugar added)	**3721**	1T = 25g						
		Chakalaka	**P0046**							
		Other								

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/NEV
VEGETABLES	**Mealies/Sweet Corn**	On Cob	**3725**	1T =30g; 1SP = 60g; ½ c =95g						
		Off Cob – Creamed, Sweet Corn	**3726**	1T = 55g; 1SP = 125g; ½ c = 135g						
		Off Cob – Whole Kernel Canned	**3942**							
		Other								
	Beetroot	Cooked (No Sugar)	**3698**	1T=40g; 1SP = 70g; ½ c = 80g						
		(With Sugar)	**3699**							
		Salad (Grated)	**3699**	1T = 25g; 1SP = 65g						
	Potatoes	Boiled/Baked with Skin	**4155**	S/s = 60g; m/s = 90g						
		Without Skin	**3737**							
		Mashed (WM)	**3876**	1T=50g; 1SP = 115g; ½ c = 125g						
		Roasted	**3878**	1 med = 70g						
		French Fries/Potato Chips	**3740**	½ c = 50g; med = 80g						
		Salad	**3928**	1T = 45g; 1SP = 105g; ½ c = 120g						
		Other								
	Sweet Potatoes	Boiled/Baked with Skin	**3748**	1T = 50g; 1SP = 110g; ½ c = 145g						
		Without Skin	**3903**							
		Mashed (With Sugar)	**3749**							
		Other								
	Green Beans	Green, Frozen	**4123**	1T = 25g; 1SP=60g; 1/2 c=80g						
		Cooked, Potato & Onion (HM)	**3792**	1T = 40g; 1SP = 75g; ½ c =120g						
		Other								
	Peas	Green, Frozen, Boiled	**4146**	1T=30g; 1SP = 65g; ½ c = 85g						
		Green, Frozen with Sugar, Boiled	**3720**							
		With Sugar and Butter	**3859**							
	Green Peppers	Raw	**3733**							
		Cooked	**3775**							
	Brinjal/Egg Plant	Cooked	**3700**	1 slice = 20g (70 mm) + batter = 30g						
		Fried in Oil	**3802**							
		Stew (oil, onions, tomato)	**3798**	1T=50g; 1SP=100g;1/2c=130g						

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/NEV
	Mushrooms	Raw	**3842**	1T=30g; 1SP = 65g; 1/2c = 80g						
		Sauteed in brick margarine	**3839**							
		Sauteed in oil	**3841**							
	Onions	Sauteed in Sun Oil	**3730**	1T = 50g						
	Salad Vegetables	Raw Tomato	**3750**	Med = 120g; slice = 15g						
		Lettuce	**3723**	1 med leaf = 30g						
		Cucumber	**3718**	Med slice = 10g; thick = 15g						
		Avocados	**3656**	¼ avo (80 x 50mm) = 40g						
	Other Vegetables: Specify									
VEGETABLES	**If you fry vegetables or add fat, specify type of fat usually used**	Butter	**3479**	1t = 5g						
		Butro	**3523**							
		Animal Fat (Beef Tallow)	**3494**							
		Lard	**3495**							
		Hard Margarine (Brick)	**3484**							
		Soft Margarine (Tub, PM)	**3496**							
		Soft Margarine (Med)	**3531**							
DRESSINGS	**Mayonnaise/Salad Dressing**	Mayonnaise – Bought	**3488**	1t = 10g 1T = 40g						
		- Home-made	**3506**							
		Cooked Salad Dressing	**3503**	1t = 5g; 1T = 15g						
		Salad Dressing, low-oil	**3505**							
		Salad Dressing, French	**3487**							
		Oil – Olive Oil	**3509**	1t = 5g; 1T = 15g						
		- Sunflower Oil	**3507**							
		- Canola	**4280**							

How many times a week did you eat vegetables? _____

How many times would this be fresh? _____ Canned _____ Frozen _____

I will now ask you about fruit

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/NEV
	Apples	Fresh	**3532**	1T=60g; ½ c = 120g; 1 med = 150g (52 x 66)						
		Canned, Pie, Unsweetened	**4216**							
	Bananas		**3540**	1 med = 75g						
	Oranges/Naartjies		**3560**	Med (7cm) = 180g						
	Grapes		**3550**	Med bunch = 230g; ½ c = 90g						
	Peaches	Fresh	**3565**	1 med = 150g (60 x 65)						
		Canned in Syrup	**3567**							
	Apricots	Fresh	**3534**	1 med = 35g						
		Canned in Syrup	**3535**							
	Mangoes	Fresh	**3556**	135mm = 350g						
		Canned in Syrup	**3633**							
	Pawpaw		**3563**	Wedge 165 x 26 x 27 = 90g						
	Pineapple	Raw	**3581**	1 slice (85 x 10mm) = 40g						
		Canned in Syrup	**3648**							
	Guavas	Fresh	**3551**	Med (6cm) = 95g						
		Canned in Syrup	**3553**							
	Pears	Fresh	**3582**	1 med (80 x 65mm) = 165g						
		Canned in Syrup	**3583**							
	Wild Fruit and Berries: **(Specify Type)**									
	Dried Fruit (Also as Snacks)	Raisins	**4232**	1 handful = 27g						
		Prunes (Raw)	**4230**	1T = 50g; ½ c = 110g; 1 = 12g						
		Prunes (Cooked with Sugar)	**3564**							
		Peaches (Raw)	**3568**	1 med = 150g (60 x 65)						
		Peach (Cooked with Sugar)	**3569**							
		Apples (Raw)	**3600**	1T=60g; ½ c = 120g; 1 med = 150g (52 x 66)						
		Dried Fruit Sweets	**3995**	**(See Manual)**						
		Other								
	Other Fruit									

How many times a week did you eat fruit? _____

How many times would this be fresh _____ Canned _____ Frozen _____

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/NEV
DRINKS	**Tea**	Ceylon	**4038**	Teacup = 180ml; mug = 250ml						
		Rooibos	**4054**							
	Sugar Per Cup of Tea	Specify Type: White	**3989**	1t sugar = 6g						
		Brown	**4005**							
	Milk per Cup of Tea	Fresh/Long Life Whole	**2718**	20ml – tea in cup 35ml – tea in mug 40ml – coffee in cup 75ml – coffee in mug						
		Fresh/Long Life 2%	**2772**							
		Goat	**2738**							
		Fresh/Long Life from (skimmed)	**2775**							
DRINKS		Whole Milk Powder Reconstituted (Specify Brand)	**2831**	1t = 4g						
		Skimmed Milk Powder, reconstituted (Specify Brand)	**2719**	1t = 4g						
		Milk Blend, reconstituted (Specify Brand)	**2771**	20ml – tea in cup 35ml – tea in mug 40ml – coffee in cup 75ml – coffee in mug						
		Whitener/non-dairy creamer (Specify Brand)	**2751**	1t = 4g						
		Condensed Milk (Whole)	**2714**	1t = 10g						
		Condensed Milk (Skim)	**2744**							
		Evaporated Milk (Whole)	**2715**	1t = 3g						
		Evaporated Milk (Low-Fat)	**2827**							
		None								
	Coffee		**4037**	Teacup = 180ml; mug= 250ml						
	Sugar per Cup of Coffee	Specify Type: White	**3989**	1t sugar = 6g						
		Brown	**4005**							
	Milk per Cup of Coffee	Specify Type		**(See Manual)**						
DRINKS	**Milk as such:** **What type of milk did you drink as such?**	Fresh/Long Life/ Whole	**2718**	To drink ½ c = 125ml Tea cup = 180ml Mug = 250ml Small glass = 150ml Medium glass = 250 ml Large glass = 500 ml						
		Fresh/Long Life/2%	**2772**							
		Fresh/Long Life/Fat Free (skimmed)	**2775**							
		Goat	**2738**							
		Sour/Maas	**2787**							

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/NEV
	Milk drinks. Specify Brands, including milk supplements and type of milk used	Nestle Drinking Chocolate	**4287**	1t = 5g						
		Malted Milk Beverage, no Sugar (eg Milo)	**2735**	1t = 5g						
		Flavoured Milk:	**2774**	Carton = 250ml; S/s plastic = 350 ml						
		Other								
DRINKS	**Yoghurt**	Drinking Yoghurt	**2756**	S/s = 175ml Yogisip = 350ml ½ c = 125g						
		Thick Yoghurt: Plain, Fat-Free	**2778**							
		WM Plain	**2757**							
		- Fruit, Low Fat	**2732**							
		Other								
	Squash	Sweeto, Sixo	**3982**	Small glass = 150ml Medium glass = 250 ml Large glass = 500 ml S/s bottle = 350ml L/s bottle = 500ml S/s can = 350ml						
		Oros/Lecol with Sugar	**3982**							
		Artificial Sweetener	**3990**							
		Kool Aid	**3982**							
		Other								
	Fruit Juice	Fresh/Liquifruit/Ceres/Purity	**2866**	1 Liquifruit s/s = 250ml 1 Liquifruit L/s = 500 ml S/s bottle = 350ml L/s bottle = 500ml S/s can = 350ml						
		"Tropica"/mixture with milk	**2791**							
	Fruit Syrups	Average	**2865**	1t = 5g						
		Guava Syrup	**2864**							
	Fizzy Drinks (e.g. Coke, Fanta)	Sweetened	**3981**	S/s bottle = 350ml L/s bottle = 500ml S/s can = 340ml						
		Diet	**3990**							
	Magou/Motogo		**4056**	1 carton = 500 ml						
	Alcoholic Beverages such as Sorghum Beer	Specify: Sorghum Beer	**4039**	(See Manual)						
	Nutrition Scheme Supplementation drinks (i.e. Khulani or Philani Energy drink)			7 scoops = 45 g						
	Other (Please specify)									

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/NEV
SNACKS	**Please indicate what types and amounts of snacks, puddings and sweets did you eat:**									
	Potato Crisps		**3417**	**(See Manual)**						
	Peanuts	Roasted Unsalted	**3452**							
		Roasted, Salted	**3458**							
	Cheese Curls (Nik Naks, etc.)	Average	**3267**							
		Savoury	**3418**							
	Popcorn	Plain	**3332**							
		Sugar Coated	**3359**							
	Peanuts and Raisins (mixed)	Roasted, Salted	**P0047**							
	Chocolates	Specify types and names: Assorted	**3992**							
	Candies	Sugus, gums, hard sweets (Specify)	**3986**							
	Sweets	Toffee, fudge, caramels (Specify)	**3991**							
	How many times a week did you eat snack food? _____									
CAKES, BISCUITS AND COOKIES	**Biscuits/Cookies**	Specify Type		**(See Manual)**						
	Cakes & Tarts	Specify Type								
	Pancakes/Crumpets	Specify Type								
	Rusks	Specify Types								
	Scones	White, WM	**3237**	6cm diam=35g; 8cm diam=60g						
	Muffins	Plain	**3408**							
		Bran	**3407**							
	Koeksisters		**3231**	100 x 35 = 60g						
	Savouries	Sausage Rolls	**2939**	Roll x 135mm = 165g						
		Samoosas (Meat)	**3355**	S/s = 42g						
		Biscuits e.g. Bacon Kips	**3331**	4g						
		Other								
	How many times a week did you eat cakes/cookies? _____ less than 1/week _____									

	FOOD	DESCRIPTION	CODE	QUANTITY (g/ml)	AMOUNT USUALLY EATEN (HHM)	AMOUNT USUALLY EATEN (g)	P/D	D/W	P/M	SEL/NEV
PUDDINGS	**Jelly**		**3983**	1T=35g; 1SP=75g; ½ c = 110g						
	Baked Puddings	Specify Types		Med serving = 30g 30 x 65 x 65 = 50g						
	Instant Puddings	Specify Types		1T = 45g; SP = 95g; ½ c =145g						
	Ice Cream	Commercial Regular	**3483**	Scoop = 40g; 1SP=65g; ½ c = 75g						
		Commercial Rich	**3519**							
		Soft serve	**3518**	Plain = 135g; + flake = 155g						
		Sorbet	**3491**	Scoop = 40g; 1SP=65g; ½ c = 75g						
		Ice Lollies	**3982**							
		Chocolate Coated Individual Ice Creams (E.g. Magnum)	**P0036**							
	Custard	Home Made (WM)	**2716**	T=13g; SP = 40g						
		(SM)	**2717**							
	Puddings **Specify**									
	How many times a week did you eat pudding? _____ less than 1/week _____									
SAUCES, GRAVIES, CONDIMENTS	**Tomato Sauce**		3139	1t = 6g; 1T = 25g						
	Worcester Sauce		P0037							
	Chutney	Fruit	3168	1t = 14g; 1T = 60g						
		Tomato	3114							
	Pickles		3866	1 = 10g						
	Packet Soups		3165	½ c = 125g						
	Others									

Wild birds, animals, insects or fruits and berries (hunted or collected in rural areas or on farms): Specify										
Please mention any other foods eaten by you more than once every two weeks which we have not talked about or foods eaten in other homes or places during your pregnancy.										

3. Are there any foods that you did not eat? Please list them and give reasons why you did not eat them (e.g. because of religious beliefs).		
FOODS NOT EATEN	**CODES**	**REASON**

4. EATING PATTERNS: (FREQUENCY OF EATING) Please indicate which of the following best describes the eating pattern you had during your pregnancy **(mark only one)**	
More than three meals with eating between meals	1
Three meals with eating between meals	2
Three meals with no eating between meals	3
Two meals with eating between meals	4
Two meals with no eating between meals	5
One meal with eating between meals	6
One meal with no eating between meals	7
Nibble the whole day, no specific meals	8
Others (Please specify):	9

5. Are there any foods that you ate which we haven't talked about? Please list them.							
FOODS	**DESCRIPTION**	**AMOUNT USUALLY EATEN**	**TIMES EATEN**				CODE
			Per day	Per week	Per month	Seldom/ Never	

6. Did you sometimes eat elsewhere? YES ☐ NO ☐

7. If yes, for what reason?

(1) Take food to work	(2) Buy food at work	(3) Clinic nutrition program	(4) Eating out	(5) Eat with relatives or friends	(6) Other

8. How often?

(1) > once a week	(2) weekly	(3) monthly	(4) < once a month

9. Indicate where:

(1) Family	(2) Friends	(3) Café	(4) Restaurant, Fast food	(5) Work
(6) Other (specify):				

QUESTION	**YES**	**NO**	**DON'T KNOW**	**REMARKS / OTHER**
10. Do you use iodised salt in your food?	1	2	3	
11. Are other, flavoured salts e.g. Aromat used in your food?	1	2	3	If yes, please specify:
12. Did you use any dietary supplements during your pregnancy?	1	2	3	If yes, specify:

Type	Name	Frequency	Amount
Vitamins			
Minerals			
Protein			
Energy			
Other			

Thank you for your co-operation. We appreciate your contribution.

ABBREVIATIONS:

Measures 1t = 1 rounded teaspoon 1T = 1 rounded tablespoon (15ml) 1SP = 1 rounded servingspoon (30ml) c = measuring cup (250ml) s/s = small size m/s medium L/s = large E = enriched P = plain Milk: SM = skim milk WM = whole milk BL = blend CON = condensed	Bread: Wh = white Br = brown Ww = wholewheat Meat: F = with fat FT = fat trimmed Oil/Fat B = butter HM = hard margarine Med = medium fat/light PM =- polyunsaturated SO = sunflower oil WF = white fat PB = peanut butter	BR = breakfast (Up to 09h00) IS = in-between snack L = lunch (midday (12h00-14h00) D = dinner (evening) (17h00 - 19h00) AD = after dinner Comm = commercial Home = homemade Pot = potato Cab = cabbage Carr = carrot Fill = filling Usually = at least 4x/week Other HHM = Household Measure P/D = Per day D/W = Days Per Week P/M = Per Month SEL/NEV = Seldom / Never

APPENDIX F:

Table 1: A comparison of mean (SD[#]) daily dietary intake during the first 28 days of lactation between the control and study group, as well as with the Dietary Reference Intake (DRI) (19 - 31 years) or if the latter was unavailable, the Adequate Intake (AI) for lactating women

Nutrient	Unit per day	Control Group	Study Group	p-value* (Cg vs Sg)	Mean (SD) daily intake (Cg+Sg)	DRI: Lacta-ting women (19-31 yrs)°	p-value* (between mean intake (Cg+Sg) and DRI)
Energy	kJ	9759.3 (1917.2)	9623.2 (1917.2)	0.87	9691.3 (1919.0)	11 300^	<0.01*
Total Protein	g	79.3 (16.7)	71.4 (16.7)	0.27	75.4 (16.7)	65.0^	<0.01*
Total Carbohydrate	g	305.3 (62.5)	317.3 (62.5)	0.65	311.3 (62.6)	N/A	
Total Fat	g	75.4 (17.8)	69.3 (17.8)	0.42	72.4 (17.8)	N/A	
Saturated Fat	g	22.1 (4.95)	20.8 (4.95)	0.54	21.5 (4.95)	N/A	
Poly-unsaturated Fat	g	23.5 (6.95)	21.3 (6.95)	0.45	22.4 (6.96)	N/A	
Mono-unsaturated Fat	g	22.3 (5.4)	20.6 (5.4)	0.45	21.5 (5.4)	N/A	
Total dietary Fibre	g	25.4 (6.7)	26.5 (6.7)	0.71	26.0 (6.7)	N/A	
Vitamin A (RE)[a]	µg	1054.5 (430.8)	1007.6 (430.8)	0.80	1031.1 (431.2)	1300	<0.01*
Folic acid	µg	188.7 (43.9)	194.5 (43.9)	0.76	191.6 (43.9)	500	<0.01*
Iron	mg	10.8 (2.6)	10.1 (2.6)	0.53	10.5 (2.6)	9.0	0.01*

#: Abbreviations: (SD): Standard deviation; cg= control group; sg= study group; N/A: Not available

^: AI used where no DRI was available.

°: DRI (lactating women) only available for the age group 19 – 31 years.

[a]: RE= Retinol Equivalents

*: One-sample T-test used to determine level of significance. A p-value <0.05 indicates a level of statistical significance is reached.

APPENDIX G:

Table 1: Mean (SD^) dietary intake during pregnancy (QFFQ) compared to the first 28 days of lactation (24 hour-recall) for all study participants. The control and study group were combined as no significant difference was observed in the intake of any of the studied nutrients between the two groups

Nutrient	Unit per day	Pregnancy (Qffq) (Cg +Sg)#	Lactation (24-hour recall (Cg +Sg)#)	p-value
Energy	kJ	13742.3 (4036.7)	9691.3 (1917.2)	0.00003*
Total Protein	g	96.5 (29.9) [12.3%]	75.3 (16.7) [14%]	0.001*
Total Carbohydrate	g	435.6 (130.6) [55.6%]	311.3 (62.5) [56.5%]	0.0002*
Total Fat	g	112.7 (38.8) [32.1%]	72.4 (17.8) [29.5%]	0.00001*
Saturated Fat	g	34.34 (16.4)	21.44 (4.95)	0.0003*
Poly-unsaturated Fat	g	31.62 (10.5)	22.36 (6.95)	0.0004*
Mono-unsaturated Fat	g	36.64 (13.1)	21.46 (5.4)	0.00000*
Total dietary Fibre	g	29.86 (10.1)	25.97 (6.7)	0.152
Vitamin A (RE)[a]	µg	1533.38 (1137.9)	1031.03 (430.8)	0.054*
Folic acid	µg	360.1 (156.7)	191.6 (43.9)	0.00000*
Iron	mg	14.54 (4.9)	10.44 (2.6)	0.00097*

^SD: Standard deviation; Cg: Control group; Sg: Study group

*: Mann Whitney test used for determination of p-values: p-value <0.05: Indicates a level of statistical significance

is reached.

[a]: RE: Retinol Equivalents

*: Value in [brackets] indicates percentage of total energy.

APPENDIX H

Table 1: Proposed composition of a breast milk fortifier (BMF) suited to the needs of ELBW and VLBW Xhosa premature infants, HIV-exposed or unexposed

Nutrient	Unit	Preterm breast milk composition (mean at 2-4 weeks of life) per 100 kcal	Proposed content of a BMF to be added per 100kcal EBM to meet enteral recommendations#	Enteral recommendations of the stable, growing premature ELBW and VLBW infant (pooled) (per 100 kcal) [104 (adapted*)]
Fluid	ml	156	-	104 – 173
Energy	kcal	100	-	100
Protein	g	2.50	0 – 1.3	2.5 – 3.8
Carbohydrate	g	10.31	0 – 5.2	5.4 – 15.5
Fat	g	5.31	0 – 1.2	4.1 – 6.5
Folic Acid	µg	2.50	14.5 – 42.5	17 – 45
Calcium	mg	37.16	62.8 – 182.8	100 – 220
Phosphorus	mg	22.19	37.8 – 117.8	60 -140
Magnesium	mg	5.81	2.1 – 9.2	7.9 – 15
Sodium	mg	57.34	0 – 47.7	46 – 105
Potassium	mg	87.81	0 – 18.2	52 – 106
Iron	mg	0.27	1.06 – 3.37	1.33 – 3.64
Zinc	mg	0.59	0.08 – 2.14	0.667 – 2.73
Copper	mg	0.14	0	0.08 – 0.14

#: Amounts are calculated by subtracting the EBM content from the enteral recommendation.

*: Enteral recommendations of the stable, growing ELBW and VLBW infants were pooled.

A comparison of the fortifier currently used with the proposed content of a breast milk fortifier indicates that the fortifier is theoretically able to provide in the needs of the nutrients analysed, as reflected in table 2.

Table 2: A comparison of the composition of the breast milk fortifier (BMF) currently used with the proposed fortifier

Nutrient	**Unit**	**Preterm Human Milk composition per 150ml (2 – 4 weeks postpartum)[104, 95] plus current BMF^ (per 7.5g which is added to 150ml EBM)**	**Current study: Premature milk composition per 150ml (2 - 4 weeks postpartum)**	**Proposed content of a BMF to be added per 150ml EBM***
Fluid	ml	150	150	-
Energy	kcal	126	96	30
Protein	g	3.9	2.4	1.5
Carbohydrate	g	15.98	9.9	6.08
Fat	g	5.55	5.1	0.45
Folic Acid	µg	64.65	2.4	62.25
Calcium	mg	150.45	35.67	114.78
Phosphorus	mg	89.25	21.3	67.95
Magnesium	mg	8.55	5.58	2.97
Sodium	mg	72	55.05	16.95
Potassium	mg	138	84.3	53.7
Iron	mg	2.09	0.255	1.835
Zinc	mg	1.76	0.57	1.19
Copper	mg	0.12	0.135	0

^: FM 85® from Nestlé

*: Proposed composition of fortifier (column 5) determined by subtracting column 4 from column 3.