

A 4-day test weighing study to assess volume and variations in fat and energy content of breast milk

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ABSTRACT

Background

Exclusive breastfeeding is recommended for the first six months of life. Breast milk intake and composition not only vary between populations and individuals, but also within feeds, within days and between days. Very limited information is currently available on the breast milk intake of exclusively breastfed infants, as well as on the energy and fat composition of breast milk from lactating women in South African.

Aim

The aim of this study was to assess breast milk intakes, as well as energy and fat concentration of breast milk in a convenience sample of exclusively breastfed infants and their mothers from Potchefstroom in South Africa. Furthermore, the study determined within-feed, diurnal and between-day variations in energy and fat concentration of breast milk.

Methodology

Twenty-four healthy mothers and their exclusively breastfed two to five-month old infants were recruited to stay at the metabolic unit of the North-West University in South Africa for a period of five days. The first 24 hours served as a run-in period and the remaining four days (96 hours) served as the actual test weighing period. Infants were weighed (± 1 g accuracy) before and after each feed to determine breast milk intake. A foremilk sample was collected before each feed to determine energy and fat concentration of the milk using the creamatocrit method. Additional mid-feed and hind-milk samples were collected from the first feed each day.

Results

Mean breast milk intake was low (369 ± 98 g/day), and infants consumed 52 ± 15 g of milk at each feed. Mean breastfeeding frequency was 7 ± 1 feeds/day. Mean fat and energy concentrations of sampled foremilk were 25.7 ± 7.3 g/L and 2544.5 ± 255.9 KJ/L, respectively. Mean daily fat and energy intake calculated from the measured milk intake was 14 ± 4 g and 1096 ± 302 KJ, respectively. Fat concentrations of fore- (26.8 ± 8.2 g/L), mid- (37.6 ± 7.0 g/L) and hind-feed- (50.2 ± 10.4 g/L) milk differed significantly ($P < 0.001$). Consequently, energy concentrations of fore- (2522.9 ± 323.2 KJ/L), mid- (2947.1 ± 275.3 KJ/L) and hind-feed (3463.5 ± 409.6 KJ/L) milk differed ($P < 0.001$). Milk fat concentration was significantly lower at night than the evening ($P = 0.015$). Milk energy concentration was significantly lower at night than the morning, day and the evening ($P < 0.05$). There were no differences in breast milk intake (grams) between the four days ($P = 0.371$). However, breast milk fat and energy concentration was

significantly lower at day 4 than at days 1 and 3 ($P<0.05$). Prevalence of stunting, underweight and wasting amongst the infants were 39.1%, 13.6% and 4.8%, respectively.

Conclusion

Breast milk and consequently energy intakes were low in this small sample of exclusively breastfed South African infants. This may explain the high prevalence of stunting. However, the change of environment and feeding pattern during the study could have affected milk production or intake. Furthermore, test weighing may not be a well-suited method for establishing milk intake in this population. Our results further confirm significant within-feed differences in breast milk fat and energy concentration.

Keywords Breast milk, lactation, fat, energy, test weighing

OPSOMMING

Agtergrond

Eksklusiewe borsvoeding word aanbeveel vir die eerste ses maande van die kind se lewe. Borsmelkinname en -samestelling varieer nie net tussen bevolkings en individue nie, maar ook tussen voedings, binne dae en tussen dae. Baie min inligting is tans beskikbaar oor die borsmelkinname van babas wat eksklusief op borsmelk gevoed word, sowel as oor die energie- en vetsamestelling van borsmelk van borsvoedende vroue in Suid-Afrika.

Doelstelling

Die doel van hierdie studie was om borsmelkinnames sowel as energie- en vetkonsentrasies van borsmelk in 'n gerieflikheidssteekproef van eksklusief borsvoedende babas en hulle moeders van Potchefstroom in Suid-Afrika te assesseer. Die studie wou ook bepaal wat die binne-voeding, daaglikse en tussen-dag variasies in die energie- en vetkonsentrasies van borsmelk is.

Metodologie

Vier-en-twintig gesonde moeders en hul eksklusief borsvoedende twee tot vyf maande-oue babas is gewerf om tuis te gaan by die metaboliese eenheid van die Noordwes-Universiteit in Suid-Afrika vir 'n tydperk van vyf dae. Die eerste 24 uur is gebruik as 'n aanlooptydperk en die oorblywende vier dae (96 uur) is gebruik vir die werklike toetsweegperiode. Babas is geweeg (± 1 g akkuraatheid) voor en na elke voeding om die borsmelkinname te bepaal. 'n Voormelkmonster is geneem voor elke voeding om die energie- en vetkonsentrasie van die melk te bepaal deur die 'creamatocrit' metode te gebruik. Addisionele mid-voeding en na-voeding monsters is geneem van die eerste voeding van die dag.

Resultate

Gemiddelde borsmelkinname was 369 ± 98 g/dag, en babas het ± 15 g vet ingeneem tydens elke voeding. Die gemiddelde borsvoedingsfrekwensie was 7 ± 1 voedings/dag. Gemiddelde vet- en energiekonsentrasies van monsters van voormelk was onderskeidelik 25.7 ± 7.3 g/L en 2544.5 ± 255.9 KJ/L. Gemiddelde daaglikse vet- en energie-innames, bereken op grond van die gemete melkinnames, was onderskeidelik 14 ± 4 g en 1096 ± 302 KJ. Vetkonsentrasies van voormelk (26.8 ± 8.2 g/L), mid-voeding (37.6 ± 7.0 g/L) en na-voedingsmonsters (50.2 ± 10.4 g/L) het betekenisvol verskil ($P < 0.001$). Dus het energiekonsentrasies van voormelk (2522.9 ± 323.2 KJ/L), mid-voeding (2947.1 ± 275.3 KJ/L) en na-voedingsmelk (3463.5 ± 409.6 KJ/L) verskil ($P < 0.001$). Melkvetkonsentrasies was betekenisvol laer in die nag as in die aand

($P=0.015$). Melkenergiekonsentrasie was betekenisvol laer tydens die nag as in die oggend, dag en aand ($P<0.05$). Daar was geen verskille in borsmelkinname (gram) tussen die vier dae nie ($P=0.371$), maar borsmelkvet en –energiekonsentrasies was betekenisvol laer op dag 4 as op dae 1 en 3 ($P<0.05$). Die voorkoms van ingekorte groei (“stunting”), ondergewig en wegkwyning onder die babas was onderskeidelik 39.1%, 13.6% en 4.8%.

Gevolgtrekkings

Borsmelk- en gevolglike energie-innames was laag in hierdie klein groep van eksklusief borsgevoede babas in Suid-Afrika. Dit mag die hoë voorkoms van ingekorte groei verklaar. Tog kon die verandering in omgewing en voedingspatrone deur die studietydperk ook ‘n invloed gehad het op die voedingspatrone gedurende die studie. Toetsweging mag ook nie die beste metode wees om melkinname te bepaal in hierdie bevolking nie. Ons resultate bevestig betekenisvolle tussen-voedingsverskille in borsmelkvet- en energiekonsentrasies.

Sleutelwoorde: Borsmelk, borsvoeding, vet, energie, toetsweging

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ABBREVIATIONS

AA	Arachidonic acid
ALA	α -linolenic acid
ASQ	Ages and stage questionnaire
BM	Basement membrane
BMR	Basal metabolic rate
CI	Confidence interval
CLDs	Cytoplasmic lipid droplets
DHA	Docosahexaenoic acid
ECG	Energy cost of growth
EPA	United States Environmental Protection Agency
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organisation
FDA	Flat depleted adipocytes
GJ	Gap junction
IOM	American Institute of Medicine
IQ	Intelligence quotient
JC	Junction complex
LA	Linoleic acid
LCFAs	Long chain fatty acids
LCPUFAs	Long chain polyunsaturated fatty acids
MCFAs	Medium chain fatty acids
MCPUFAs	Medium chain polyunsaturated fatty acids
ME	Myoepithelial cells
MFG	Milk fat globule
n3	Omega-3
n6	Omega-6
NCHS	National Centre for Health Statistics

NEFA	Non-esterified fatty acids
PC	Plasma cells
PUFA	Poly-unsaturated fatty acids
RER	Rough endoplasmic reticulum
TEF	Thermic effect of feeding
TEE	Total energy of expenditure
UNICEF	United Nations Children's Emergency Fund
WAZ	Weight for age Z-score
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Evidence drawn from human research has led to infant feeding being a topical issue in recent years (Fewtrell *et al.*, 2007). It has been well-established that the first 1000 days of life, the period from conception to two years of age, is the most important period in human development during which optimal nutrition is critical (Black, 2012). Breast milk has been found to provide optimal nutrition for infants (Kent *et al.*, 2006) and therefore, exclusive breastfeeding is recommended for infants from birth to six months of age (WHO, 2002). Exclusive breastfeeding as defined by the United Nations Children's Emergency Fund (UNICEF) (2015) is a process where an infant receives breast milk from his/her mother or a wet nurse, or expressed breast milk, and no other liquids or solids, apart from oral rehydration solution, drops or syrups consisting of vitamins, mineral supplements or medicines.

Unlike infant formula, the composition of breast milk varies considerably, with variations in the volume and composition of breast milk within a feed, within a day and over the duration of lactation, as well as between mothers and populations (Ballard & Morrow, 2013; Kent *et al.*, 2006; Khan *et al.*, 2013a; Quinn *et al.*, 2012). The energy in breast milk comes from fat, protein and carbohydrates (Butte *et al.*, 2002). Fat concentration of the milk is the major contributor towards energy, and it is well known that milk fat increases as the breast empties (Daly *et al.*, 1993). Variations in milk fat concentration have also been noted between breasts (Khan *et al.*, 2013b), between individuals (ranging from 28 to 57 g/L) (Khan *et al.*, 2013a) and across populations (ranging from 28 to 47 g/L) (Quinn *et al.*, 2012), resulting in marked differences in the energy concentration of breast milk. In a literature review of studies carried out in developed countries, Reilly and Wells (2005) reported that at six months of age breastfeeding probably supplies about 90% of the total energy that an infant requires. However, the fat and energy concentration of breast milk consumed by exclusively breastfed infants in the South African population is not known. Furthermore, it is not known how much breast milk exclusively breastfed infants in South Africa consume.

Kent *et al.* (2006) found that the milk production of 71 exclusively breastfeeding Australian mothers with infants aged between one and six months ranged from 478-1356 g per day (Kent *et al.*, 2006). Test weighing is a method that has been used before as a means of evaluating the milk intake of infants. Although test weighing has previously been considered as a reliable technique for assessing breast milk intake in infants (Neville *et al.*, 1988), more recently it has also been criticised by some researchers as a method that is not reliable (Nielsen *et al.*, 2011). However, some researchers have found it to be accurate if carried out according to a specific

protocol (Haase *et al.*, 2009) (outlined later in Chapter 2). Test weighing is a method that has also been criticised for being a cumbersome means of determining milk intake. However, in a developing country such as South Africa, it is likely the best method to use because it requires cheaper resources and skills that are readily available compared to isotopic methods (outlined later in Chapter 2) (Scanlon *et al.*, 2002).

1.2 Problem statement

There are limited data currently available on the nutrient composition of breast milk in South African mothers who exclusively breastfeed their infants. Minimal data exist from developing countries on breast milk composition and intake in early infancy (Agne-Djigo *et al.*, 2013; Reilly *et al.*, 2005). However, before planning large surveys with the goal to investigate breast milk micronutrient and macronutrient concentrations, as well as energy concentration of breast milk in the South African population, it is important to establish optimal sampling techniques that are suitable in the local context. Several authors have emphasized that there is a lack of sampling standardization, which may in part explain the large variability in breast milk composition reported in different studies done in different populations (Hassiotou *et al.*, 2013; Nikniaz *et al.*, 2009).

Furthermore, methods to determine 24-hour breast milk volumes consumed by infants are labour intensive and challenging to apply in field-settings. Currently, the American Institute of Medicine (IOM) proposes a mean breast milk intake for infants zero to six months of 0.78 litres per day (IOM *et al.*, 2001). However, it would be useful to establish the milk volume consumed by South African infants and have an idea of the variation in breast milk-volume consumed between infants, within day and between days in a South African population. This would help to establish whether exclusively breastfed infants in the South African population are well nourished.

This study will therefore bridge these existing gaps and will add to available knowledge of the energy adequacy breast milk for exclusively breastfed infants, as well as variations in fat and energy concentration of breast milk in a developing country.

1.3 Aims and objectives

1.3.1 Aim

The aim of this study was to assess the volume of breast milk intake and variations in fat and energy concentrations of breast milk in a purposive sample of exclusively breast-fed infants from the Potchefstroom area in South Africa participating in a four-day test weighing study.

1.3.2 Objectives

The objectives of this study were:

1. To determine the volume of breast milk intake in a convenience sample of two to five months old exclusively breastfed infants from the Potchefstroom area participating in a four-day test weighing study.
2. To measure fat and energy concentration of breast milk, and to determine within-feed, within-day and between-day variations in fat and energy concentration of breast milk consumed by two to five months old exclusively breastfed infants from the Potchefstroom area participating in a four-day test weighing study.
3. To estimate fat and energy intake of the exclusively breastfed infants from the Potchefstroom area based on determined breast milk intake and measured breast milk fat and energy concentration.

1.3.3 Assumptions and hypotheses

We assumed that test weighing over a four-day period in a clinical setting is an effective and reliable method to determine breast milk intakes in the South African population.

We therefore hypothesized that the determined mean breast milk intake in this sample of exclusively breastfed infants would be similar to that reported in other populations. We further hypothesized that fat and energy concentration of breast milk in this sample of lactating women would be similar to that reported in other populations, and that there are significant within-feed, within-day and between-day variations in fat and energy concentration of breast milk.

1.4 Structure of the dissertation

Chapter 2 is the literature review outlining the importance of breastfeeding and describing the anatomy of the breast and milk composition. It further summarizes literature on test weighing as a method of determining milk intake in breastfed infants and on variations in milk fat and energy concentration.

Chapter 3 is an article with the title “Breast milk intake, energy and fat concentration of breast milk: A 4 day test weighing study in exclusively breastfed, South African infants”. The article is formatted according to the author guidelines of the Journal of Human Lactation (JHL), however for the purpose of this dissertation, the word limit as specified in the JHL author guidelines was not adhered to, but necessary corrections will be done before the article is submitted for publication.

1.5 Contribution of the research team

Table 1.1 shows the contribution made by each member of the research team in the dissertation and writing of the article.

Table 1-1: Contribution of the research team

Team member	Role
Jeannine Baumgartner	Principal investigator of the South African arm of the iodine balance study (umbrella project) and student supervisor. Played a role in the formulation of the research problem and study design. Played an advisory role, gave guidance and assistance in the writing of this dissertation and writing of the article.
Lize Havemann-Nel	Co-supervisor; played an advisory role and gave guidance in the writing of this thesis.
Linda Siziba	PhD student; assisted with data collection and analysis of fat and energy concentration.
Susanne Dold	PhD student (ETH Zürich, Switzerland). Was involved in the design and execution of the iodine balance study (umbrella project).
Maria Andersson	Principal investigator (ETH Zürich, Switzerland) of the multi-centre iodine balance study (overall); was responsible for the conceptualization and design of the iodine balance study (umbrella project).
Sicelosethu Sihawu Siro	Involved in the formulation of the research question of this MSc project and in the execution of the iodine balance study (umbrella project). She was responsible for data collection, analysis of breast milk fat and energy concentrations, statistical analysis, and writing up of the dissertation and article.

CHAPTER 2: LITERATURE REVIEW

Breast milk is defined by Hoddinott and colleagues (2008) as “a complex living nutritional fluid that contains antibodies, enzymes and hormones, all of which have health benefits” (Hoddinott *et al.*, 2008). Furthermore Arora and colleagues (2000) stated that breast milk is a “species specific food for infants” which is absorbed easily and is rich in minerals, vitamins and protein (Arora *et al.*, 2000). In this regard breast milk has been considered the “gold standard” for infant feeding (Stam *et al.*, 2013), but it has been found that breast milk composition differs by race, region, dietary intake and stage of lactation (Shi *et al.*, 2011; Stam *et al.*, 2013). In contrast to infant formula, breast milk composition also varies within a single feed and diurnally (Chung, 2014; Khan *et al.*, 2013a).

In recent years, a lot of research has been dedicated to determining the composition of breast milk, the variations in breast milk composition, as well as factors affecting composition. A number of factors have been found to affect the composition and volume of milk. These include the age of the mother (Mello-Neto *et al.*, 2009), demands by the infant (Kent, 2007), time of day (Khan *et al.*, 2013a), the nutritional status of the mother and whether the milk is expressed or sucked (Emmett & Rogers, 1997; Lucas & Cole, 1990) among others.

2.1 Benefits of breastfeeding

Breastfeeding was shown to have a number of benefits for the infant compared to formula-feeding, both in the long and short term. These include benefits for neurological and cognitive development, as well as reduced risk of infection and onset of chronic diseases later in life (Kent *et al.*, 2006).

Rates of malnutrition are very high in developing countries and since breast milk is cheap and ready to use (Eigenmann, 2004), it is a sure means of reducing the risk of malnutrition amongst infants (Filteau, 2000; Hoddinott *et al.*, 2008). Malnutrition in infants may cause infection as it reduces the integrity of the skin and mucous membranes (protective barriers to infection) and thereby alters the immune system of the affected individual (Brown, 2003). On the other hand, nutritional status can be affected by infection because nutrient intake and absorption in the gut may be reduced by the presence of infection (Brown, 2003). Furthermore, in the presence of infection, there is generally an increase in catabolism and increased requirement of nutrients essential for growth and development (Brown, 2003). Breastfeeding is therefore a means of reducing malnutrition and thereby the risk of infection in infancy (Filteau, 2000). Furthermore, in the developing world, hygiene contributes greatly to infant mortality. Vulnerability to infection is high due to use of contaminated water, low immunisation rates and compromised immunity as a result of malnutrition, however, exclusive breastfeeding has been proven to be very effective in

optimising nutritional intake and reducing morbidity (Hoddinott *et al.*, 2008). Exclusive breastfeeding in the first six months can lead to a considerable reduction in child mortality and morbidity. A recent meta-analysis has concluded that exclusive breastfeeding had a strong protective effect with a 12% reduced risk of death in exclusively breastfed compared to non-breastfed infants (Sankar *et al.*, 2015). A pooled analysis carried out by the World Health Organisation (WHO) collaborative study team revealed that infants who were not exclusively breastfed between zero to five months had a six times and 2.5 times greater risk of dying due to diarrhoea and pneumonia, respectively, compared to exclusively breastfed infants (WHO, 2000). Researchers have also found that breastfeeding can prevent other bacterial infections, such as urinary tract infections, as well as upper and lower respiratory tract infections (Koosha *et al.*, 2008). This is attributed to the presence of immunological factors in the breast milk (Koosha *et al.*, 2008). Infants have a weak immune system but the presence of immunoglobulin, protein such as lactoferrins, lysosomes and casein, lipids, oligosaccharides and other factors were shown to help fight off infections and enhance the immune system of the infant (Egash *et al.*, 2008).

Both partial and predominant breastfeeding have benefits, but exclusive breastfeeding for six months seems to have greater benefits as shown in a study carried out by Kramer and colleagues (2008). This cluster randomised trial study in Belarus aimed to investigate whether prolonged (up to 12 months) and exclusive breastfeeding at three and six months affects the cognitive ability of a child at the age of 6.5 years compared to exclusive breastfeeding for three months (Kramer *et al.*, 2008). Mothers were randomised during pregnancy to deliver and receive post-natal care at a hospital that had implemented the WHO baby-friendly initiative to promote exclusive breastfeeding and others to hospitals and clinics that continued with the practises and policy that were already in effect at the time of the study (Kramer *et al.*, 2008). In this study, 17046 infants were recruited but only 13889 were followed up to 6.5 years of age (Kramer *et al.*, 2008). After the introduction of the baby-friendly initiative, a substantial difference was noted between the intervention group and the control group in the duration of any breastfeeding. When comparing the intervention group to the control group 72.7% compared to 60.0% were still breastfed at three months, 49.8% compared to 36.1% at six months, 36.1% compared to 24.4% at nine months and 19.7% compared to 11.4% at 12 months, respectively (Kramer *et al.*, 2008). Exclusive breastfeeding rates were higher in the intervention group both at three and six months compared to the control group at 43.3% compared to 6.4% and 7.9% compared to 0.6%, respectively (Kramer *et al.*, 2008). The Wechsler Abbreviated Scales of intelligence (four subsets of the Wechsler scales were used -vocabulary, similarities, block design and matrices) were used to establish the intelligence quotient (IQ) of children who were not yet attending school (Kramer *et al.*, 2008). Children who had been exclusively breastfed for six months or more than six months exhibited higher IQ [4.2 (95% CI, 2.8 to 5.6) points] than

those who had been exclusively breastfed for more than three months but less than six months [3.3 (95% CI, 2.7 to 4.0) points] (Kramer *et al.*, 2008). The children's teachers assessed the children who had begun school in four academic areas: reading, writing, mathematics and other subjects (Kramer *et al.*, 2008). The results indicated that children who had been exclusively breastfed for three to less than six months had a significantly higher rating (0.03 to 0.06) but no significant difference was found for exclusive breast feeding for six months or more (Kramer *et al.*, 2008).

The benefit of breastfeeding to cognitive function is further supported by McCrory and Layte (2011) who reported an enhanced neurodevelopment and cognitive function among nine-year olds who had been breastfed in infancy. McCrory and Layte (2011) performed a cross-sectional analysis using data from the Grow Up in Ireland project that recruited 8226 children aged nine years. Breastfeeding information was collected retrospectively and the authors' aim was to find out whether the breastfeeding practices correlated with standard scores in reading and mathematics (McCrory & Layte, 2011). The authors found an association between breastfeeding and scoring of the children in the tests, whereby the breastfed group scored 3.24% higher in reading ($p < 0.001$) and 2.23% higher in mathematics ($p < 0.001$) compared to the formula-fed group (McCrory & Layte, 2011). McCrory and Murray (2013) in another study also nested within the Grow Up in Ireland study determined the correlation between breastfeeding and a child's neurodevelopment using the Ages and Stages Questionnaire (ASQ) (McCrory & Murray, 2013). Breastfeeding information was gathered through interviews with the mother. The authors found that infants who at one point in their life received breast milk experienced some neurodevelopmental benefit. Focusing on age-appropriate developmental mile stones on problem solving using the ASQ at nine months, breastfed infants had a 1.2 times greater chance to achieve the milestone (McCrory & Murray, 2013). The breastfed infants also had 1.3 and 1.6 times higher odds to achieve age-appropriate fine and gross motor skills respectively (McCrory & Murray, 2013). The authors concluded that this was likely due to the presence of growth factors, hormones and dietary nucleotides that are present in breast milk but not in infant formula (McCrory & Murray, 2013).

Furthermore, breastfeeding has been associated with a reduction in the risk of childhood obesity (Armstrong & Reilly, 2002), and with a reduced risk of obesity in adulthood (Grummer-Strawn & Mei, 2004). The mechanism by which breastfeeding reduces risk of obesity in adulthood is not clear, but suggested mechanisms include that breastfed infants are able to adjust and self-regulate caloric intake compared to formula-fed infants (Birch & Fisher, 1998). Another possible mechanism would be difference in hormonal response in formula-fed and breastfed infants, with formula-feeding leading to a higher insulin response, which is likely to lead to earlier fat deposition (Lucas *et al.*, 1980; Lucas *et al.*, 1981). Another proposed

mechanism is the likelihood of a greater protein intake in formula-fed infants, which may affect glucose metabolism (Burns *et al.*, 1997).

2.2 Policies in place to improve breastfeeding rates

As much as exclusive breastfeeding has great benefits to infant growth and development, the rates of exclusive breastfeeding in the world, however, do not reflect that. At the end of the 19th century, modified cow's milk was introduced as an alternative to breast milk and this led to a decrease in breastfeeding rates (Hoddinott *et al.*, 2008). In the 20th century, because of the industrial revolution, mothers left their infants at home during the day so they could work in the cities and this is when infant formula became popular (Elgash *et al.*, 2008). Rates of breastfeeding dropped to a very low rate in the 1960s in the developing world (Hoddinott *et al.*, 2008), and by 1972, the exclusive breastfeeding rate in the first week of life had dropped to less than 30% (Elgash *et al.*, 2008). However, an increase in exclusive breastfeeding was noted in the 1990s rising from 48 to 52 % (Hoddinott *et al.*, 2008). According to UNICEF (2014), less than half of the infants in the world are exclusively breastfed for the first six months of life. Following promotion of exclusive breastfeeding in 1991 through establishing the baby friendly hospital initiative (BFHI), rates have increased both at global and most regional levels with a 38-50% increase in the least developed countries (UNICEF, 2014). Between 2009 and 2013 exclusive breastfeeding rates for the first six months in sub-Saharan Africa rates were at 36% with Eastern and southern Africa recording a rate of 51% (UNICEF, 2014).

South Africa has one of the lowest rates of exclusive breastfeeding in the world with an estimated rate of 7.4% (Shisana *et al.*, 2013). However, the South African Government has committed to setting up policies that will help encourage breastfeeding. This is evident by initiatives such as the launching of the South Africa Infant and Young Child Feeding Policy (Department of Health, 2007) adapting global policies and strategies into the South African context in order to promote healthy infant-feeding practices including promoting exclusive breastfeeding. The policy encompasses strategies, policies and regulations from international bodies and these include among others the *Innocenti Declaration*, the code of marketing of breast milk substitutes (R991) and the global strategy for Infant and Young Child Feeding (IYCF). Another such initiative was the Tshwane Declaration (2011) where the Department of Health and its stakeholders came together to pronounce their support, promotion and protection of exclusive breastfeeding for the first six months of life (Tswane Declaration, 2011).

2.3 Anatomy of the lactating breast

In order for breastfeeding to be successful, the breast should be able to synthesize, secrete and eject milk so that an infant can suck or the milk can be expressed (Geddes, 2007a). To achieve

this, the breast goes through different developmental stages: foetal, neonatal/pre-puberty and post-puberty (Geddes, 2007a). The breast begins to form during the foetal stage and at birth the breast mainly consists of rudimentary ducts with club-like tips. These ducts regress immediately after birth but begin to grow at pre-puberty stage (Geddes, 2007a). At puberty, an increase in epithelial growth occurs with each menstrual cycle and differentiation and growth of the ductile system occurs (Geddes, 2007a).

In pregnancy, the breast then goes through a lactation cycle which is divided into four stages and these include mammogenesis, lactogenesis (which consists of two stages), galactopoesis and involution (Geddes, 2007a). Figure 2-1 below shows the structure of a lactating breast.

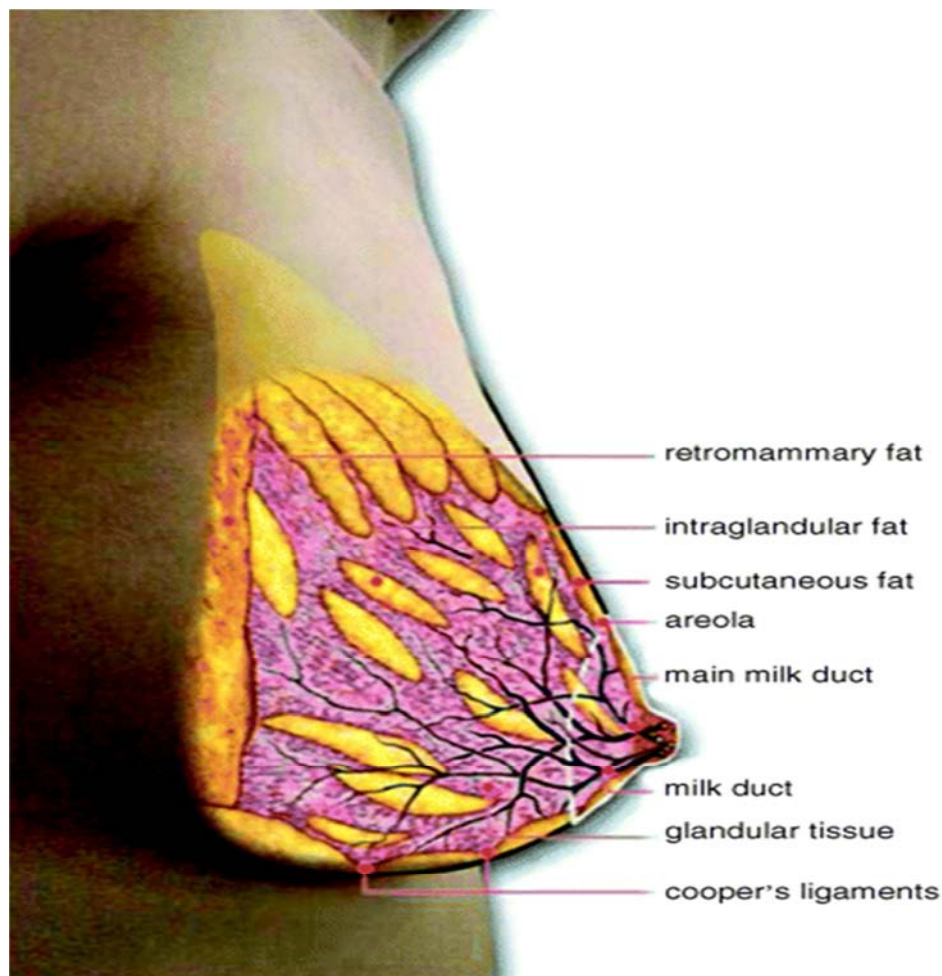


Figure 2-1: Anatomy of the lactating breast

Drawing of the gross anatomy of the lactating breast based on ultrasound observations made of the milk duct system and distribution of different tissues within the breast (Ramsay *et al.*, 2005) (Reproduced with permission from John Wiley and Sons).

Developmental differentiation: This is the first stage of breast development in readiness for lactating and occurs during the first half of pregnancy (Kent *et al.*, 2010). The breast enlarges as the ductal systems expand through further branching and the alveoli enlarge and increase in

number (Geddes, 2007a; Kent *et al.*, 2010). This process begins as early as the first month into pregnancy. Oestrogen, during this stage, stimulates proliferation and differentiation of the ductal system in the breast whilst progesterone stimulates the increase of the lobes, lobules and alveoli (Geddes, 2007a).

Lactogenesis: This is the transition from pregnancy to lactation, the cells in the mammary gland undergo differentiation to prepare them for milk production (Geddes, 2007a; Wagner, 2015). This occurs in the second half of pregnancy and colostrum begins to accumulate in the alveoli. This stage is called **lactogenesis I** and in recent years has been known as **secretory differentiation** (Kent, 2010). In this stage, there is an increase in breast size as further differentiation occurs in the alveoli and epithelial cells and they become secretory cells (Wagner, 2015). **Lactogenesis II** also known as **secretory activation** occurs post-partum, 36-96 hours after delivery (Wagner, 2015). It is characterised by an increase in milk volume, which is necessitated by the decrease in the level of progesterone in the blood (Wagner, 2015).

Galactopoies: occurs about nine days after birth (Wagner, 2015). This stage is characterised by established milk secretion which is stimulated by the continued removal of milk (Kent, 2010). Reduction of milk removal rate leads to a limitation of milk removal and hence milk production and is usually affected by the quality and quantity of infant suckling or expression of milk from the breast (Kent, 2010). The rate of milk removal has nothing to do with a mother's ability to produce milk but rather an infant's appetite (Kent, 2010), so the breast milk production is synchronised to the baby's intake (Kent, 2007a).

Involution involves the decrease of milk secretion, which occurs about 40 days after the last breastfeed (Wagner, 2015)

2.4 Physiology of breast milk synthesis

The lactating breast has a network of branching ducts and lobules containing alveoli (McManaman *et al.*, 2006). Figure 2-2 shows what the mammary alveolus looks like. Milk is secreted through the alveoli by cells called lactocytes also referred to as secretory epithelial cells (Geddes, 2007a). These secretory epithelial cells surround a central lumen in which milk is secreted into and carried to the nipple via a ductal network (McManaman *et al.*, 2006). These cells are characterised by an abundance of mitochondria, a large network of rough endoplasmic reticulum and a well-developed Golgi apparatus (RER) (McManaman & Neville, 2003). The part of the lactocytes that faces the lumen is called apical whilst the outer part is called basal region (Geddes, 2007a). In the apical region of the cell are secretory vesicles that have casein micelles and this is the area of milk secretion (McManaman & Neville, 2003; Geddes, 2007a). These epithelial cells are joined together by an apical junctional complex which is made of adherens

and tight-junctional elements, whose main function is to inhibit para-cellular interactions during lactation (McManaman & Neville, 2003). At the base, the alveolar epithelial cells are joined to the myoepithelial cells and the basement membrane (McManaman & Neville, 2003). The basement membrane serves to separate the epithelial compartment from the stroma and the vascular system (McManaman & Neville, 2003). Transfer of substance from the blood or stroma cells to the milk is regulated by apical epithelial membranes, vascular or stromal membranes, para-cellular junctional complexes, basement membrane and Golgi membranes (McManaman & Neville, 2003).

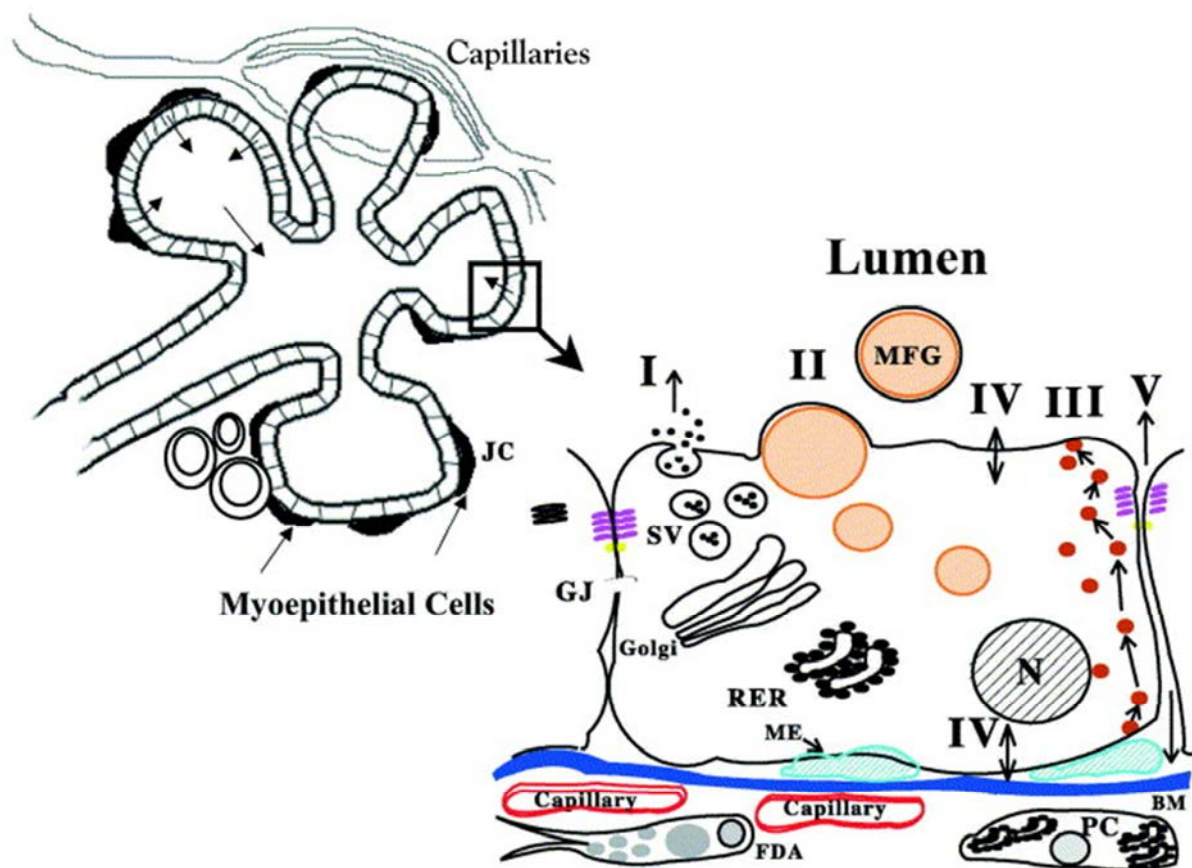


Figure 2-2: Diagram of mammary alveolus and alveolar epithelial cell showing pathways for milk secretion.

Milk is secreted by alveolar epithelial cells into the lumen (shown by arrows in the alveolus). Milk is then expressed through the ducts by contraction of myoepithelial cells that surround alveolar and ductal epithelial cells. The alveolus is surrounded by a well-developed vasculature and a stroma comprising extracellular matrix components, fibroblasts and adipocytes. The region indicated by the box is expanded to show key structural and transport properties of alveolar cells. Pathway I depicts exocytotic secretion of milk proteins, lactose, calcium and other components of the aqueous phase of milk. Pathway II depicts milk fat secretion with formation of cytoplasmic lipid droplets (CLDs) that move to the apical membrane to be secreted as a membrane bound milk fat globule (MFG). Pathway III depicts vesicular transcytosis of proteins such as immunoglobulins from the interstitial space. Pathway IV depicts transporters for the direct movement of monovalent ions, water and glucose across the apical and basal membranes of the cell. Pathway V depicts transport through the paracellular pathway for plasma components and leukocytes. Pathway V is open only during pregnancy, involution and in inflammatory states such as mastitis. Abbreviations: SV, secretory vesicle; RER, rough endoplasmic reticulum; BM, basement membrane; N, nucleus; PC, plasma cell; FDA, fat depleted adipocyte; JC, junctional complex containing the tight and adherens junctions; GJ, gap junction; ME, myoepithelial cell^{a1}.

Milk in the breast is made and stored in the alveolar cells (Neville, 1998). To improve the supply of substrate for the synthesis of milk, blood flow to the breast is increased (Neville, 1998). Water, lactose, amino-acids, minerals, vitamins, fats, immunoglobulins and other components

^{a1} Reprinted from *Advanced drug delivery reviews*, 55, Mcmanaman, J. L. & Neville, M. C. 2003, Mammary physiology and milk secretion, 629-641., (2003), with permission from Elsevier

are drawn by secretory cells from the blood and these precursor substances are converted to components of milk in the alveolar epithelial cells (Riodarn, 2005). The synthesis of breast milk is a complex process that involves five pathways and processes summarised in Figure 2-2 above. These pathways are exocytosis of milk protein, lipid synthesis and secretion, transport across the apical membrane, transcytosis of interstitial molecules and para-cellular pathway. Once breast milk is synthesised it is transported to the mammary lumen (McManaman & Neville, 2003).

Exocytosis (pathway I) is primarily the pathway for protein secretion (McManaman & Neville, 2003) and most of the components that make the aqueous phase (Neville, 1998). Protein is first synthesised in the ribosomes of the RER and then transferred to the lumen of the RER where they are enfolded into a vesicle (Neville, 1998). These are carried to the Golgi apparatus where further processing occurs with the addition of carbohydrates, calcium, citrate, phosphates and oligosaccharides (Neville, 1998). Lactose is synthesized by lactose synthetase from the precursors UDP-galactose and glucose in the Golgi apparatus (Neville, 1998; McManaman & Neville, 2003). Because the membrane of the vesicles is impermeable to lactose, water moves into the vesicle by osmosis causing the vesicles to swell (Neville, 1998; McManaman & Neville, 2003). These vesicles are then transported to the plasma membrane where they fuse with the membrane and the concentrations of the vesicle are emptied into the milk space (Neville, 1998).

The second pathway is lipid synthesis and secretion (pathway II), which is explained in detail later on in this chapter in section 2.5.2.3 where fat synthesis is explained in detail.

Transcytosis of interstitial molecules is the third pathway (pathway III) involved in the production of milk (Neville, 1998). Here intact proteins cross through the mammary epithelial from the interstitial fluid (Neville, 1998). Immunoglobulin is the most studied component of all components thought to be transferred in this way, which includes; many proteins, hormones and growth factors that are found in the milk (Neville, 1998). IgA is synthesized in the plasma of the interstitial space of the mammary gland, attaches to a receptor (polymeric immunoglobulin receptor) on the alveolar cell and the whole complex is endocytosed across the membrane. The extra cellular part of the receptor and IgA is secreted at the apical membrane (Neville, 1998).

The fourth pathway is transport across the apical membrane (pathway IV), where solute-specific transport systems of monovalent and polyvalent ions and small molecules such as glucose and amino acids are carried across from the blood into the alveoli (McManaman & Neville, 2003). This is achieved by specific transporters located at the apical and basal plasma membranes or between the basal plasma membrane and the Golgi or secretory membranes (McManaman & Neville, 2003). The transport mechanisms include i) ion transport ii) glucose transport, iii) amino acid transport and iv) transport of other agents such as drugs (McManaman & Neville, 2003).

The para-cellular pathway (pathway V) allows substances to pass in between epithelial cells where these cells are held together by structures called the tight junctions (Neville, 1998). This structure is so tight and only allows immune cells to pass through and seals tightly behind them but during pregnancy and as a result of mastitis, it becomes leaky and allows substances to move from the interstitial space into the milk (Neville, 1998). However, during lactation this pathway is closed and only pathways one to four are in use for transfer of solutes into the milk (McManaman & Neville, 2003).

2.5 Composition of breast milk

Breast milk is “not a uniform body fluid but a secretion of changing composition” (Agostoni *et al.*, 2009). Its composition varies because of a number of factors. Variation has been noted within feeds (between fore-, mid and hind-milk) diurnally (Khan *et al.*, 2013a) and the stage of lactation (Stam *et al.*, 2013).

Although breast milk composition may vary diurnally and between individuals, it still is considered the most suitable food for infant-feeding compared to infant formula, even in well-nourished populations where risk of micronutrient deficiency is minimal (Quinn *et al.*, 2012; Kent *et al.*, 2006; Ballard & Morrow, 2013). Breast milk contains protein, lipids, carbohydrates, minerals and vitamins to meet the nutritional needs of the infant for normal growth and development (Agostoni *et al.*, 2009). Breast milk also contains immune-related components, which makes it superior to infant formula. These components include sIgA, leukocytes, oligosaccharides, lysozymes, lactoferrin, interferon γ , nucleotides, cytokines and others (Agostoni *et al.*, 2009; Eglash *et al.*, 2008). They give passive immunity to the infant by protecting the gut and also the upper respiratory tract through preventing pathogens from attaching to the mucosa, hence preventing invasive infections (Agostoni *et al.*, 2009). Breast milk also contains other bioactive components such as growth factors and hormones that contribute to the growth and development of the infant (Agostoni *et al.*, 2009).

Breast milk undergoes a process of maturation from delivery to close to a month after birth (Ballard & Morrow, 2013). The first milk produced after delivery is colostrum, which is produced for the first few days of life (Ballard & Morrow, 2013). It is produced in low quantities, but is high in protein (Ballard & Morrow, 2013). These proteins mainly consist of immunoglobulins and growth factors (Ballard & Morrow, 2013). Colostrum is also low in lactose and has a higher sodium to potassium ratio compared to mature milk (Ballard & Morrow, 2013). During this period, the tight junctions in the mammary epithelium are closing, leading to secretory activation (refer to section 2.3). Secretory activation leads to the production of transition milk (Ballard & Morrow, 2013). Transition milk is produced five days to two weeks post-partum and is similar to colostrum but is in larger quantities with higher lactose levels and lower sodium to potassium

ratios (Ballard & Morrow, 2013). Thereafter, at four to six weeks post-partum, mature milk is produced (Ballard & Morrow, 2013). This is the milk that is produced throughout the period of lactation (Ballard & Morrow, 2013). Table 2-1 shows the composition of mature breast milk.

Table 2-1: Breast milk composition in the USA and the UK

Nutrient	Unit	USA (per 100g)	UK (Per 100g)
Proximates			
Water	g	87.5	87.1
Energy	kcal	70	69
Protein	g	1.03	1.3
Total lipid (fat)	g	4.38	-
Sugars, total	g	6.89	7.2
Minerals			
Calcium, Ca	mg	32	34
Iron, Fe	mg	0.03	0.07
Magnesium, Mg	mg	3	3
Phosphorus, P	mg	14	15
Potassium, K	mg	51	58
Sodium, Na	mg	17	15
Zinc, Zn	mg	0.17	0.3
Vitamins			
Vitamin C, total ascorbic acid	mg	5	4
Thiamine	mg	0.014	0.02
Riboflavin	mg	0.036	0.03
Niacin	mg	0.177	0.2
Vitamin B-6	mg	0.011	0.01
Folate, DFE	µg	5	5
Vitamin B-12	µg	0.05	Tr
Vitamin A, RAE	µg	61	58(retinol)
Carotene			24
Vitamin A, IU	IU	212	-
Vitamin E (alpha-tocopherol)	mg	0.08	0.34
Vitamin D (D2 + D3)	µg	0.1	0.04
Vitamin D	IU	3	-
Vitamin K (phyllo-quinone)	µg	0.3	-
Lipids			
Fatty acids, total saturated	g	2.009	1.8
Fatty acids, total monounsaturated	g	1.658	1.6
Fatty acids, total polyunsaturated	g	0.497	0.5
Cholesterol	mg	14	16
Other			-
Caffeine	mg	0	-

(Adapted from US Food & Drug Administration, 2012, Emmett & Rogers, 1997)

2.5.1 Energy

The energy requirement is defined as the “amount of energy acquired from the consumption of food that will meet energy expended at a body size and composition and physical activity level

that are consistent with long-term good health and will maintain physical activity that is both economically and socially desirable” (Butte, 2005). When focusing on infants, growth needs to be factored in, hence their energy requirement is the sum of total energy expenditure (TEE) and the energy cost of growth (ECG) (Butte, 2005).

The TEE can be divided further into (i) basal metabolism; (ii) thermic effect of feeding (TEF); (iii) thermoregulation and (iv) physical activity.

Basal metabolic rate (BMR) is the energy that is used in order to sustain normal cellular and tissue functions that are crucial for life and includes maintenance of body temperature, heart and respiratory functions and energy supply to the muscles at rest (Butte, 2005). The energy for basal metabolism in the infant is used mainly by the brain, heart, liver and kidneys, whereby the brain uses about 70% in the new born and between 60-65% in the first 12 months of life (Butte, 2005).

Thermic effect of feeding is defined as the energy that is spent in response to feeding (Butte, 2005). It usually accounts for about 10% of TEE (Butte, 2005). What causes the rise in energy use is transportation and conversion of absorbed nutrients into their storage forms (Butte, 2005).

Thermoregulation describes a point where temperature exceeds or is below the zone of thermo-neutrality and energy is expended to help the body maintain normal body temperature (Butte, 2005). The zone of thermo-neutrality is a point where environmental temperature at which oxygen consumption and metabolic rate is at its lowest (Butte, 2005). When temperature is lower more energy is required to maintain normal body temperature as compared to when temperatures are higher (Butte, 2005).

2.5.1.1 Energy requirements and energy intake in infancy

Taking TEE and ECG into account, according to the WHO (2002), the mean energy requirement has been set at 325-330kJ/Kg and 330-335kJ/kg body weight for boys and girls, respectively, between 4 and 6 months of age.

Fewtrell and colleagues (2003) observed that a large number of women were unable to sustain exclusive breastfeeding up to the age of six months because they perceived that infants were not being satisfied with breast milk only, especially in instances where mothers had babies with large birth weights that seemed to require more energy (Fewtrell *et al.*, 2003). This observation was further supported by Reilly and colleagues, (2005) (referred to in section 2.6.2), who in their review stated that according to their calculations, there is likely to be a 10% deficiency in energy at six months of age in exclusively breast-fed infants (Reilly *et al.*, 2005). The authors calculated

a weighted mean which amounted to 0.62kcal/g body weight, which they argue is lower than reported in WHO collaborative study on breastfeeding (0.67-0.68 kcal/g) (WHO, 1985). The difference may have a bearing on the sufficiency of energy in the six-month old exclusively breastfed infant. Lucas and colleagues (1987) found that low energy intake in breast-fed infants was more a result of low energy concentration of the breast milk than low volume. Recommendations on exclusive breastfeeding have been based on limited data about when exclusive breastfeeding ceases to be adequate for energy provision in the infant (Wells *et al.*, 2012)

2.5.1.2 Energy concentration of breast milk

The energy in breast milk comes from protein, carbohydrates and fat (Butte *et al.*, 2002). Energy concentration of breast milk, however, fluctuates during the period of lactation (Butte *et al.*, 2002). Energy concentration of milk has further been shown to vary within the day, within feeding and between breasts because of the variations in milk composition (Butte *et al.*, 2002). Fat concentration of the milk is the major contributor towards energy, contributing about 50% of total energy. However, it is well-known that milk fat concentrations vary within feeds, between feeds and diurnally as mentioned earlier, hence the variations in energy concentration. Protein and carbohydrates on the other hand have shown little variation between women though they do show variations with the stage of lactation (Butte *et al.*, 2002).

Powe and colleagues (2010) suggest that the variation that is noted in the energy concentration of breast milk could be a result of infant demand. Powe and colleagues (2010) carried out a study in 25 well-nourished exclusively breastfed infants (two to five months old) and their mothers in Massachusetts. One of the aims of the study was to establish the relationship between infant characteristics and energy requirements (Powe *et al.*, 2010). The male infants in this particular study fed more times a day (mean=9.27 times/day) than the female infants (mean=7.95 times/day) though the difference was not statistically significant. However, the milk from mothers with male infants had significantly higher energy concentrations (75.56 kcal/100ml) than that of mothers who had female infants (60.81kcal/100ml). This difference was attributed by the authors to the increased energy demand by male infants compared to their female counterparts (Powe *et al.*, 2010).

Another study carried out in Senegal had the aim of assessing the adequacy of the WHO recommendation of exclusive breastfeeding for the first six months (Agne-Djigo *et al.*, 2013). Of the 59 mother infant pairs who were enrolled, 15 were exclusively breastfeeding. The doubly labelled water method (explained in section 2.7.1.1) was used to determine milk intake and the creatinocrit method (explained in section 2.7.3) was used to determine fat and energy concentrations. The authors found that the mean energy concentration in the breast milk

amongst the exclusively breastfeeding group was 2598 ± 259 kJ/L. This mean energy concentration was within the range of values published in a study carried out by the WHO in other developing countries (WHO, 1985) and it fell within the recommended ranges given by WHO (Butte *et al.*, 2002; Agne-Djigo *et al.*, 2013).

2.5.1.2.1 Protein as a component of energy

Protein contributes about 8% to the infants energy needs (Butte *et al.*, 2002). It is not only a source of energy but also a source of essential amino acids (Butte *et al.*, 2002). Hence, it is important that an infant gets adequate amounts of protein so that both needs for tissue accretion and other metabolic functions are met as well (Butte *et al.*, 2002). Protein concentration in breast milk has been seen to decrease in concentration from 12.7 g/l in the second week of lactation in the transition milk to 8 g/L in the mature milk (at four months) then, it remains constant until the infant is weaned (Butte *et al.*, 2002).

2.5.1.2.2 Carbohydrates as a component of energy

Carbohydrates in milk consist of lactose and oligosaccharides (Czank, 2007). While lactose, which is the major component of carbohydrates, contributes close to 40% of the total energy in breast milk, oligosaccharides do not have nutritional value to the infant, but play a major role in immunity (Czank, 2007). It is a disaccharide that is made from glucose and UDP galactose by lactose synthetase in the Golgi apparatus of the lactating breast (Czank, 2007).

2.5.2 Fat concentration in breast milk

Fat is a macronutrient that plays an essential role in the normal growth and development of infants, and provides energy (Koletzko *et al.*, 2008). Fats mainly consist of triglycerides whose main constituents are fatty acids, which are further broken down to three classes by their degree of unsaturation (FAO, 2010). These classes are i) saturated fatty acids – these have no double bonds and are usually produced *de novo* by the human body ii) monounsaturated fatty acids (MUFAs)- these have one double bond and are rare compounds, the most commonly occurring being is oleic acid (FAO, 2010) iii) polyunsaturated fatty acids (PUFAs – these have two or more double bonds). PUFAs can be divided further into the omega-3 (n-3) and omega-6 (n-6) fatty acid families with linoleic acid being the parent of the n-6 and AA being the parent fatty acid for the n3 PUFAs (FAO, 2010; Ganapathy, 2009).

Breast milk has been found to contain about 40g/L of fat. Kent and colleagues (2006) (referred to in section 2.6.1) found that the mean fat concentration in breast milk of the Western Australian population was 41.1 ± 7.8 g/L ranging from 22.3 to 61.6 g/L. Khan and colleagues (2013a) (referred to in section 2.7.2) also worked with a Western Australian population with

infants (n=15) between one and six months who were exclusively breastfed. The aim of the study was to examine the relationship between macronutrient concentration over a 24-hour period of breastfeeding and breastfeeding patterns of infants between zero and six months (Khan *et al.*, 2013a). The authors found mean fat concentration in breast milk to be 43.2 ± 11.8 g/L and the concentrations ranged from 28 to 57g/L. However, fat concentration of breast milk varied within feeds, diurnally, according to dietary intake, and by phase of lactation, maternal age and parity (Khan *et al.*, 2013a)

Within-feed variation in milk was observed as early as 1473 by Meltinger, who suggested that fore-milk should be expressed first before the infant was allowed to feed since it appears runny (Hyttén, 1954). Khan and colleagues (2013a) in their study further found an overall mean difference in breast milk-fat concentration of 24 g/l between fore-milk and hind-milk (Khan *et al.*, 2013a). Statistical analysis revealed a significant difference ($P < 0.001$) between the two, whereby fore-milk had a mean fat concentration of 32 ± 12 g/L and hind-milk of 56 ± 17 g/L (Khan *et al.*, 2013a). They also found that breast milk-fat concentration was higher during the day (47.8 ± 12.3 g/L) and at night (37.9 ± 10.6 g/L) compared to the morning (40.4 ± 11.1 g/L) ($P = 0.01$ and $P = 0.02$) respectively. There was no association with the number of feeds during the day, volume of milk intake during the feed, feed duration and 24-hour milk intake from each breast (Khan *et al.*, 2013a).

The nutritional status of mothers has also been associated with the fat concentration of the milk as was established by Rocquelin and colleagues (1998) in a study that they carried out among Congolese women (Rocquelin *et al.*, 1998). Their cross-sectional nutrition survey was aimed at establishing fat concentration and fatty acid composition of the breast milk focusing on five month-old infants. Hind-milk samples were collected at two time-points, mid-morning and mid-afternoon, and were then combined for lipid extraction. The fat concentration ranged from 7.9 to 74.8g/L with a mean of 28.7 g/L, which was quite low, especially considering that these were hind milk samples. The authors further showed that underweight mothers ($BMI < 18.5$) had significantly higher breast milk fat concentrations than their normal and overweight counterparts (Rocquelin *et al.*, 1998).

As reported in the European Food Safety Authority (EFSA) report (2010), the health council of the Netherlands, recommends that infants need an adequate fat intake of 40-45% of their total energy intake and this is based on the mean fat concentration of breast milk (EFSA, 2010) . On the other hand, the IOM based their fat recommendation on observed intakes of breastfed infants and set their recommendation at 31g of fat per day (55E%) based also on an assumption that the infants requirements are being met (EFSA, 2010; IOM, 2005).

In Tabriz, Iran, a study was carried out among lactating women and their exclusively breastfed infants aged between 90 and 120 days (Nikniaz *et al.*, 2009). The aim of the study was to determine whether there was a relationship between fat concentration of breast milk and the nutritional status of the mother and the weight for age Z score (WAZ) of the infants. All infants in the study group were born full-term with normal weight at birth and had no chronic illnesses. Infants were weighed on an electronic Soehnle scale with maximum weight of 20 kg, and an accuracy of ± 10 g. WAZ was calculated according to the National Centre for Health Statistics (NCHS)/WHO (1986) recommended international reference median (Nikniaz *et al.*, 2009; WHO, 1986). Fat concentration of the milk was established using the Gerber method as described in the Encyclopedia of Dairy Science (Evers & Hughes, 2002; O'Connor & O'Brien, 2002). Mothers whose milk fat concentration was ≥ 30 g/L had infants with a mean WAZ of 0.97 which was significantly higher than their counterparts with milk fat concentration < 30 g/L (WAZ of 0.53) (Nikniaz *et al.*, 2009).

2.5.2.1 Fat composition of breast milk

In breast milk, 98-99% of the total fat is triacylglycerides (Czank *et al.*, 2007, Riordan, 2005), with palmitic (16:0) and oleic acid (18:1n9) being the major constituents (Ballard & Morrow, 2013; Czank *et al.*, 2007). Two percent is made of monacylglycerides, cholesterol (0.5%), non-esterified fatty acids (NEFA) and phospholipids (0.8%), (Czank *et al.*, 2007; Jensen, 1999). The triacylglycerides are made of three fatty acid chains attached to glycerol and there are over 200 different types of fatty acids found in breast milk and they form 85% of the triacylglycerides (Czank *et al.*, 2007). The fatty acids in breast milk can be classified into i) medium-chain fatty acids (MCFA), and ii) long-chain fatty acids (LCFA) which include the long-chain PUFAs (LCPUFAs). Short-chain fatty acids are rare in breast milk (Czank *et al.*, 2007). In mature breast milk, MCFA constitute about 15% of total fatty acids (Czank *et al.*, 2007). The major fatty acids in this group are 16:0 and 18:1n9 as mentioned earlier, which make up (in combination) up to close to half of the total fatty acids. LCPUFAs make about 12% of the total fatty acids but its concentration is influenced by maternal diet (Czank *et al.*, 2007).

Another class of fat found in breast milk are the trans-fatty acids, which are obtained from the maternal diet. Trans-fatty acid interferes with the production of LCPUFA, hence interfering with membrane function and thereby negatively affecting development of the infant (Nishimura *et al.*, 2013).

Table 2 shows the composition of fatty acids of breast milk from a population in Brazil (Nishimura *et al.*, 2013). These are the results of a study that was carried out to examine the fatty acid composition of mature breast milk among women living in Ribeirao Preto, State of Sao Paulo in Brazil who were exclusively breastfeeding their infants between five and 14 weeks old

(Nishimura *et al.*, 2013). In this study, high levels of palmitic (C16: 0) were found among the saturated fatty acids, whilst oleic acid (C18: 1n9) was high among the mono-unsaturated fatty acids, and arachidonic acid (AA, 20:4n6) was high among the LCPUFAs (Nishimura *et al.*, 2013). Essential fatty acid [α -linolenic acid (ALA) and LA] concentration was 22.42% of total fats and LCPUFAs was 0.66% of total fats (Nishimura *et al.*, 2013).

Table 2-2: Fatty acid composition (%) of mature breast milk from lactating women living in Ribeirao Preto, SP, Brazil, 2010-2011(n=47)

Fatty acids	Mean	SD
<i>Saturated</i>	42.67	5.5
C6:0 (caproic acid)	0.09	0.0
C8:0 (caprylic acid)	0.29	0.1
C10:0 (capric acid)	1.95	0.5
C11:0 (undecylenic acid)	0.012	0.0
C12:0 (lauric acid)	7.46	2.6
C13:0 (tridecanoic acid)	0.03	0.0
C14:0 (myristic acid)	6.81	2.3
C15:0 (pentadecanoic acid)	0.23	0.1
C16:0 (palmitic acid)	19.50	2.0
C17:0 (margaric acid)	0.29	0.1
C18:0 (stearic acid)	5.82	1.0
C20:0 (arachidic acid)	0.009	0.0
C21:0 (heneicosanoic acid)	0.024	0.1
C22:0 (behenic acid)	0.03	0.0
C24:0 (lignoceric acid)	0.12	0.1
<i>Monounsaturated</i>	29.15	3.7
C14:1 (myristoleic acid)	0.19	0.1
C15:1 (pentadecenoic acid)	0.02	0.0
C16:1 (palmitoleic acid)	2.11	0.7
C17:1 (10-heptadecenoic acid)	0.18	0.0
C18:1n9c (oleic acid)	26.46	2.8
C20:1n9 (gadoleic acid)	0.12	0.0
C22:1n9 (erucic acid)	0.04	0.0
C24:1n9 (lignoceric acid)	0.03	0.1
<i>Trans</i>	2.05	0.5
C18:1 trans 11 (vaccenic acid)	1.68	0.3
C18:1 trans 9 (elaidic acid)	0.28	0.1
C18:2n-6tt (linolelaidic acid)	0.09	0.1
<i>Conjugated</i>	0.49	0.1
CLA C18:2 c,t (cis, trans octadienoic acid)	0.47	0.1
CLA C18:2 t,c (trans, cis octadienoic acid)* This result represents the sum of CLA C18:2 t,c with CLA C18:2 7t9c	0.02	0.0
<i>n-3 Polyunsaturated</i>	2.11	0.4
C18:3 n-3 (α -linolenic acid)	1.54	0.4
C20:3 n-3 (eicosatrienoic acid)	0.40	0.1
C20:5 n-3 (eicosapentaenoic acid)	0.08	0.1
C22:6 n-3 (docosahexaenoic acid)	0.09	0.1
<i>n-6 Polyunsaturated</i>	21.87	4.6
C18:2 n-6 (linoleic acid)	20.96	4.4
C18:3 n-6 (γ -linoleic acid)	0.09	0.1
C20:3 n-6 (dihomo- γ -linoleic acid)	0.34	0.1
C20:4 n-6 (arachidonic acid)	0.48	0.1

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2.5.2.2 Role of fat in breast milk

Fat in breast milk provides about half the amount of energy and essential nutrients that play an important role in the development of the central nervous system (Lauritzen *et al.*, 2001), particularly the fatty acids docosahexaenoic acid (DHA, 22:6n3) and AA (Lauritzen *et al.*, 2001). DHA is an n-3 LCPUFA that in the human body is made from ALA but in small amounts, thus, should be provided by the diet to meet the body's needs (Huang *et al.*, 2013). In the exclusively breastfed infant, the source of DHA is breast milk and is found in varying quantities in the breast milk because it is greatly influenced by maternal diet (Huang *et al.*, 2013). DHA is critical for infants as it is crucial for the structure and function of the central nervous system, the retina of the eye and the immune system (Huang *et al.*, 2013). AA, in turn, is synthesized from linoleic acid (LA) and is a precursor in the synthesis of prostaglandins and leukotrienes, which form part of the immune system (Ganapathy, 2009; Koletzko *et al.*, 2008). It is also of structural importance in the membranes of cells throughout the body (Koletzko *et al.*, 2008). In the breast milk, AA has been found to be more constant across populations with an average concentration of 0.45% of total fatty acid concentration while DHA (ranging from 0.1 to 3.8% of total fatty acids) varies a lot and is affected by diet (Ganapathy, 2009). Fat in breast milk is also a source of fat-soluble vitamins (Koletzko *et al.*, 2001; German & Dillard, 2006) and plays an important role in the absorption of the same since they are not soluble in the aqueous phase of the cells (German & Dillard, 2006).

During the first four to six months, the infant accrues about 1.4 to 1.7 kg of fat (Koletzko *et al.*, 1988), and spares protein for the growth of the lean muscles (Innis, 2007a). The accrued fat serves as a thermic insulator and plays a structural role in the tissue.

2.5.2.3 Fat synthesis in the breast

Fat in the breast milk comes from two sources – *de novo* synthesis in the cytoplasm of mammary epithelial cells or from lipids in the maternal blood (Czank *et al.*, 2007). Lipids in the maternal blood, in turn, come from three different sources that are tissue synthesis, adipose tissue and the diet (Czank *et al.*, 2007).

De novo synthesis accounts for about 20% of the total fatty acids found in the breast milk and are mainly medium-chain fatty acids. They are made from acetyl-CoA that is a derivative of glucose and reducing equivalents that are products of the pentose phosphate pathway (Czank *et al.*, 2007). Acetyl-CoA is converted to malonyl-CoA by the addition of a C₂ unit through the action of acetyl-CoA carboxylase (Czank *et al.*, 2007; Innis, 2007b). The fatty acid synthase complex undergoes a series of reactions whereby C₂ is continually added to the chain. A cytosolic medium-chain acyl thioesterase called thioesterase 11 stops the elongation of the

chain at eight to 14 C by hydrolysing the thioester bond that holds the fatty acid and enzyme complex together (Czank *et al.*, 2007; Innis, 2007b).

The long-chain fatty acids found in the breast milk come from the blood. These long-chain fatty acids are transported by chylomicrons or very low-density lipoproteins or from non-esterified fatty acids bound to albumin circulating in the blood (Czank *et al.*, 2007). However, the pathway of transporting this particular group of fatty acids to the alveolar cell basal membrane is not clear (Czank *et al.*, 2007). Once the fatty acids get to the alveolar cell basal membrane, they move into the cell either by diffusion or by means of a membrane transport, which involves fatty acid binding protein (FABP) that are found on the membrane (Barber *et al.*, 1997). Inside the cell, the fatty acid binds either with acetyl-CoA binding protein or with FABP in the cytosol. These fatty acids will be ready for assimilation into triacylglycerides (Czank *et al.*, 2007).

Hachey and colleagues (1987) carried out a study to determine mechanisms that affect transport of fatty acids from the diet into the breast milk, and whether there are different transport mechanisms for specific fatty acids (Hachey *et al.*, 1987). They also aimed to describe the process of triacylglyceride synthesis in the breast and to establish how much of the dietary fatty acids are incorporated into the milk triacylglycerides. The authors focused on three fatty acids only in their study including 16:0 (palmitic acid), 18:1 n 9 (oleic acid) and 18:2 n 6 (linoleic acid). They used the stable isotope tracer method to trace these fatty acids so that they could determine quantitatively how much of the fatty acids ended up in the milk. They found that about 10 to 12% of the 16:0 (palmitic acid), 18:1 n 9 (oleic acid) and 18:2 n 6 (linoleic acid) found in milk originated from the diet and are transported as chylomicrons (Hachey *et al.*, 1987).

Triacylglyceride synthesis occurs in the smooth endoplasmic reticulum of the epithelial cells of the mammary gland (McManaman & Neville, 2003; Neville, 1998). Triacylglyceride is a product of glycerol-3-phosphate and fatty acyl-CoA. Glycerol-3-phosphate undergoes acylation of the free hydroxyl groups with two fatty acyl groups producing phosphatic acid. The phosphatic acid undergoes dephosphorylation, resulting in diacylglycerol. The addition of a fatty acyl group leads to the formation of a triacylglyceride.

Some fatty acids are synthesized *de novo* by adipose tissue, however, not so much during lactation (Czank *et al.*, 2007). The *de novo* synthesis during lactation is hindered by the action of pyruvate dehydrogenase and acetyl-CoA, hence, most of the fatty acids in breast milk that are from the tissue come from the liver. The liver synthesises some fatty acids through *de novo* synthesis and absorbs some that are released into the blood from the adipose tissue as triacylglyceride in the form of very low-density lipoproteins.

2.5.2.4 Secretion of fat into the milk

Triacylglyceride is synthesised and released as small droplets of different sizes called milk fat or milk lipid globules (Heid & Keenan, 2005). These globules are covered by a milk fat globule membrane (Heid & Keenan, 2005). After coalescence, the cytoplasmic lipid droplets are transported to the apex of the cell (Neville, 1998; Heid & Keenan, 2005). These large droplets push against the membrane, are enfolded into the membrane and then break free as milk fat globules (Neville, 1998; Heid & Keenan, 2005). The membrane of the milk fat globule is maintained and helps to prevent further coalition of the fat globules, which could render them too big for excretion and further provide cholesterol and phospholipids for the breast-fed infant (Neville, 1998). The milk fat globules are presented as triacylglyceride core, covered by a milk fat globule membrane consisting of phospholipids, protein, cholesterol and enzymes. These globules range in size from 1 micron to 12 microns with a majority being about 4 microns.

2.6 Variation in breastfeeding frequency and breast milk volume intake

2.6.1 Feeding frequency

It is recommended that infants should breastfeed on demand; as a result, variation has been noted in the feeding patterns of infants. These patterns include unpaired feeding, which is having a complete meal from one breast only; paired feeding whereby an infant breastfeeds from both breasts within 30 minutes; and cluster feeding whereby an infant breastfeeds from one and same breast within 30 minutes of feeding from the other breast (Kent, 2007).

Breastfeeding patterns vary within populations. Kent and colleagues (2006) (referred to in section 2.5.2) carried out a cross-sectional study in Western Australian infants to determine the volume and pattern of milk intake amongst exclusively breastfed infants who were one to six months old (Kent *et al.*, 2006). The frequency of feeding in this study population ranged from six to 18 feeds per day with an average feeding frequency of 11 feeds per day (Kent *et al.*, 2006). During the day, frequency of feeds ranged from four to 13 with an average of eight feeds per day (Kent *et al.*, 2006). Time lapses between two feeding sessions ranged from one hour 50 minutes to six hours (Kent *et al.*, 2006).

As breastfeeding patterns vary between individuals, they also vary between populations. Hörnell and colleagues (1999) carried out a descriptive study in Sweden, Uppsala, to investigate three aspects of breast feeding patterns: length of feed, time lapse between feeds and frequency of feeds (Hörnell *et al.*, 1999). Infants were recruited at birth until the mother's second menstruation post-partum or new pregnancy that led to a mean age of 8.6 ± 3.4 months. This study showed that infants in this population were breastfed more frequently during the day than the night (2.9 to 10.8 feeds compared to 1.0 to 5.1 feeds). They also reported longer feeds

during the day compared to the night time (20mins to 4h 35mins compared to 0 to 2h 8 minutes) (Hörnell *et al.*, 1999).

The occurrence of more frequent feeds during the day rather than the night was reported by Konner and Worthman (1980), who carried out a study among the !Kung hunters in Namibia and Botswana. One of their aims was to establish the feeding patterns of the !Kung hunters (Konner & Worthman, 1980). The authors observed mother-infant pairs from dawn to dusk and interviewed mothers to get information about night feeds. The infants observed were aged between 12 to 139 weeks, and the author established that the infants were fed very frequently throughout the day, but were fed only once or twice throughout the night. The mean number of feeds per hour was about four with a mean length of 1.92 ± 0.18 minutes per bout, which resulted in a mean of 7.83 ± 1.27 minutes per hour. The mean length of time between the feeds was 13.19 ± 1.28 minutes with a mean maximum length of 55.6 ± 3.79 minutes. Konner and Worthman (1980) further reported that there was no relation between the length of feeds and the age of the infants.

2.6.2 Breast milk volume intake

Breast milk production is affected by the mother's nutritional status - the quality may be good but the quantity reduced in mothers with severe malnutrition (UNICEF, 2014). Thus, the composition of breast milk from malnourished mothers may not be significantly different in quality to that of well-nourished mothers but the volume might be significantly less (Agostoni *et al.*, 2009). However, breast milk volumes have been found to vary insignificantly between mothers from developed and undeveloped countries with the average milk volume being 750ml per day for up to six months of exclusive breastfeeding (Kent, 2007).

In the past, breast milk volume intakes for exclusively breastfed infants was set at 850ml by both the Food and Agriculture Organisation (FAO) and the WHO (FAO, 1950). With further investigation, some researchers have found lower values. Jelliffe and Jelliffe (1978) conducted a literature review of studies performed not later than 1978 to determine breast milk volume and composition. They found that in the first six months of infancy, milk intakes ranged from 600ml to 700ml a day among well-nourished mothers and 500 to 700ml in poorly nourished mothers (Jelliffe & Jelliffe, 1978). However, it is not stated whether these studies were carried out on exclusively breastfed infants. On the other hand, according to IOM, the average intake of milk for exclusively breastfed infants is 780ml per day (IOM *et al.*, 2001).

A systematic review by Reilly *et al.* (2005) (referred to in section 2.5.1.1) investigating the daily breast milk volume transferred from mother to infant reported the mean milk volume to be 779 g/d at two to four months, 827 g/d at 5 months and 894 g/d at six months. The review focused

on breast milk volume intake, and whether breast milk intake increased with the energy demands of the infants and how much metabolisable energy was present in milk. Metabolisable energy is calculated by subtracting heat of urine combustion from digestible energy (Butte, 2005). All the studies they included in their literature search (n=44) were from developed countries and included healthy infants between three and six months where 1041 were between three and four months and 72 infants were six months old.

Haisma and colleagues (2003), in Pelotas Brazil, aimed to determine how much of breast milk intake was replaced by water, tea, juices and other complementary feeds by using the dose to mother deuterium-oxide turnover method. The study included exclusively breastfed, predominantly breastfed, and breastfed infants also given formula or cow milk and breastfed infants who were complementarily fed. They found that exclusively breastfed infants at the age of four months consumed an average of 815 g of breast milk per day (Haisma *et al.*, 2003).

Breast milk intakes vary, and recommended breast milk intakes for exclusively breastfed infants have been arrived at using data from different studies. A review carried out by the United States Environmental Protection Agency (EPA) (2011) on breast milk intake in seven studies recommend breast milk intakes of 510 ml/ day for infants under one month, 690 and 770 ml/day for infants aged one to less than three months and three to less than six months, respectively (Moya *et al.*, 2011). However, this systematic review only included studies performed in the USA, with the exception of one study that included data from Finland and Sweden.

A few studies have been conducted in developing countries on the breast milk intake of exclusively breastfed infants hence limited data are available on the intakes in developing countries. Moreover, large discrepancies can be observed in the data that has been collected on the breast milk intake of exclusively breastfed infants.

2.6.2.1 Breastfeeding techniques and milk intake

Effective breastfeeding leads to adequate nourishment for the infant (Mulder, 2006). However effective breastfeeding can be affected by poor positioning, latching, sucking and transfer of milk to the infant (Mulder, 2006). Both mother and infant should be comfortable so that the infant is able to latch on to the breast correctly, and therefore can effectively suck and remove milk from the breast (Mulder, 2006). Poor position may lead to, among other problems, poor let-down of milk, poor milk supply and ineffective feeding (Mulder, 2006).

It is important that an infant correctly latches on to the breast covering both the nipple and the areola with its mouth, hence forming a seal between the breast and its mouth (Mulder, 2006). Correct latching leads to effective feeding, and to achieve a good latch, the positioning of the infant should be good too (Mulder, 2006). According to Cadwell (2007), incorrect latching can

lead to poor milk intake. Caloric malnutrition and dehydration may cause a baby to “behave well” or be “sleepy” all the time, hence should never be a measure of a satisfied baby but rather an investigation should be done to establish whether milk intake is adequate (Cadwell, 2007).

In a study among 92 healthy mother-infant pairs in Sweden, it was established that among the mothers who had breastfeeding problems, incorrect sucking technique was the main problem (94%) (Righard, 1998). It was observed that the infants engaged in superficial nipple sucking. The mother-infant pairs in this study were stratified into two groups; those who had breastfeeding problems (N=52) and those who had no problems (N=40) (Righard, 1998). The infants were aged from one week to 17 weeks in the problem group. Their breastfeeding techniques were observed for poor positioning and attachment (latching) (Righard, 1998). The authors indicated that superficial sucking was likely a result of the use of pacifiers and bottle feeding. Thus, the use of pacifiers and bottle feeding may lead to ineffective sucking that fails to stimulate milk letdown (Righard, 1998). Finally milk transfer is to occur from the nipple into the infant’s mouth (Mulder, 2006). However, for milk transfer to occur there should be milk letdown which stimulates correct and effective suckling and can be achieved by correct position and latching (Mulder, 2006).

Most of the studies that have explored the effect of breastfeeding techniques focus on the duration of breastfeeding. However, the effects of breastfeeding techniques on milk intake have not been fully explored.

2.6.2.2 Milk ejection reflex and milk intake

Milk ejection is stimulated by oxytocin (Prime *et al.*, 2007). Oxytocin is a hormone that is produced in the pituitary gland in the brain (Prime *et al.*, 2007). The oxytocin is released into the blood and when it gets to the breast, attaches on receptors found in the myoepithelial cell and causes contraction of the alveoli, which leads to the release of milk from the alveoli into the milk duct (Prime *et al.*, 2007). The release of oxytocin is stimulated by either the cry of an infant or suckling on the breast (Prime *et al.*, 2007). For lactation to be successful, it is important that the let-down reflex or milk ejection reflex works perfectly well (Prime *et al.*, 2007). However, this let-down reflex can be affected by psychological stress and may in the long run affect milk yield and consequently milk intake (Prime *et al.*, 2007).

It has been established that stressors such as verbal arithmetic problems, noise, moderate electric shock and pain or placing feet in ice cold water may lead to temporary inhibition of oxytocin (Newton & Newton, 1948; Ueda *et al.*, 1994). A decrease in milk yield was observed in a woman who was exposed to different distractions. Exposure to distractions led to a decreased milk yield of 99 mml compared to 168 ml when she was not distracted (Newton & Newton,

1948). The mother was exposed to one of three different distractions at every morning feed during the test day which could be placing her feet in ice cold water for 10s after every 30s, verbal mathematical problems coupled with a mild electrical shock if she gave an incorrect response or delayed responding, and the third was pulling of the big toes to cause pain (Newton & Newton, 1948). On these test days (12 days in total), prior to feeding, saline or Pitocin (oxytocin) was injected into her blood (Newton & Newton, 1948). On the days saline was injected the milk intake was notably lower (99g) compared to when Pitocin was used (153g) (Newton & Newton, 1948). The milk intake when Pitocin was used prior to feeding was similar to the control day (eight days in total). These findings reflect the effect that the distractions can have on the release of oxytocin, considering that milk intake was normal after administration of oxytocin (Newton & Newton, 1948).

The inhibition of oxytocin was further established by Ueda *et al.* (1994). The authors recruited 22 mothers five days post-partum with normally shaped nipples, good milk production, and who had given birth vaginally (Ueda *et al.*, 1994). The women were randomly allocated to three groups; the control group, the group exposed to mental calculation and the group exposed to noise (building construction at mean of 70 DB). The women in the two latter groups were exposed to the stressor during a 20-minute breastfeeding episode. The mean basal oxytocin levels in all three groups before feeding were similar. However, significantly lower levels of oxytocin were observed during breastfeeding in both groups exposed to the stressors, and there was a decrease in the pulsatile release of oxytocin in the mental calculations group (1.28 ± 0.76 pulses) and the noise exposed group (1.14 ± 0.38 pulses) compared to the control group (2.25 ± 0.71 pulses per 20 minutes) ($P < 0.05$ and $P < 0.01$ respectively) (Ueda *et al.*, 1994). When the milk ejection reflex is impaired, then breast emptying is affected with some milk remaining in the breast, hence leading to the down regulation of milk synthesis (Dewey, 2001). The above studies indicate how maternal stress can impair the milk let-down reflex (Dewey, 2001).

Subtle stress in lactating mothers usually goes unheeded but has an effect on milk ejection, therefore it is important to support and encourage lactating mothers (Geddes, 2007b). Nielsen *et al.* (2011), in their study found that infants of mothers that had support during lactation had a high intake of breast milk. However, in their study, they did not mention who gave support and there was no group to compare to. Most studies that have been carried out to establish the effect of support to lactating mothers only relate to effects of breastfeeding duration and there are limited data on the effects of support on breast-milk intake.

2.7 Assessing breast milk intake and composition

2.7.1 Assessing breast milk intake

Different methods have been used to measure breast milk intake of infants and different studies have come up with different mean values.

2.7.1.1 Doubly labelled water

In the use of doubly labelled water, infants are given small doses of water labelled with non-radioactive isotopes of deuterium and oxygen¹⁸ (Scanlon *et al.*, 2002). The initial step in the process is the collection of saliva and urine samples, which provide baseline data. Further samples are then collected over a five to 15-day period. Total energy expenditure is then calculated from the differential disappearance rate of the isotopes (corrected for change in body composition), and breast milk intake is calculated using the energy balance equation (Scanlon *et al.*, 2002).

Neilsen and colleagues (2011) found that breast milk intake in infants between 15 and 25 weeks old from Glasgow, Scotland, was higher than values that are reported in the literature with a value of 923 ± 122 g per day among boys at three to four months compared to 779 g per day that was reported by Reilly and colleagues (2005). At six months of age, recorded milk intake volume was 999 ± 146 g per day compared to 894 g per day as reported by Reilly and colleagues (2005). The study had a longitudinal design and infants were followed from 15 weeks to 25 weeks of age to determine volume of milk intake and energy intake in exclusively breastfed infants. The authors used the doubly labelled water technique to measure milk intake, energy intake and milk energy concentration. Validation of the method was done against indirect calorimetry in hospitalised preterm infants and post-surgical term infants. The doubly labelled water that was used was a mixed sterilised >99.9% atom % ²H₂O and 10.40 atom % H₂O¹⁸ and infants were given a 5 ml oral dose of the doubly labelled water. Pre-dose measurement of saliva was done a day before dosing, and on the day of dosing. Post-dosing measurement of urine were done a day after dosing. Analysis was done using isotope ratio mass spectrometry. The isotope elimination rates and carbon dioxide production were calculated using the plateau method on a pre-coded sheet. Milk intake was established by using the elimination rate of deuterium and correction for insensible water influx was also calculated. Milk energy was calculated as total energy intake divided by milk intake (KJ/g) (Neilsen *et al.* 2011).

Advantages of using doubly labelled water are that there is no need for observation or equipment to carry out measurements during feeding, hence the feeding schedules of the infant are not interfered with (Scanlon *et al.*, 2002). It allows for observation in field setup. However,

the limitations of using doubly labelled water are that both expertise and analytical equipment are not widely available in developing countries and that it is expensive (Shetty, 2002; Prentice, 1999, Scanlon *et al.*, 2002). When using the doubly labelled water technique, should the infant consume any unmeasured quantity of supplementary food, then the validity of the standard of the technique is threatened (Scanlon *et al.*, 2002). As a means of preventing this from occurring, the mother, instead of the infant, is dosed (Scanlon *et al.*, 2002). However added expense on doubly labelled water when observing exclusively breastfed infants is that the doubly labelled water has to be given to the mother and both the mother and infant samples need to be analysed (Scanlon *et al.*, 2002). The doubly labelled water is given to the mother in order to validate exclusive breast feeding practice. If the infant is given any other food or liquid that is not breast milk from its mother, the researcher is able to pick it up.

2.7.1.2 Test weighing

The test weighing method is a technique where an infant is weighed before and after a feed without a change of clothes or diapers, and the difference in weight between the second measured and the first measurement is considered the amount of milk consumed (Haase *et al.*, 2009; Scanlon *et al.*, 2002). The test weighing method has been widely used by researchers because it is more affordable compared to isotopic methods and uses less specialised equipment and expertise. The test weighing method has been validated in the past (Neville *et al.*, 1988). Validation of the method was carried out among hospitalised infants where bottled milk was weighed before and after the feed and test weighing the infants, thereafter, the results were compared and an “acceptable small errors” of approximately 3% found (Neville *et al.*, 1988). Neville and colleagues (1988) carried out a two phase study where mothers initially test weighed their infants before and after every feed and in the second phase hourly-pumped breast milk volumes were measured, thereafter compared the results. The mothers carried out test weighing over 48 hours and then were admitted to hospital where milk was pumped on an hourly basis for eight hours (10 minutes or until milk stopped flowing a drop of synthetic oxytocin was administered intra-nasally and pumping would resume for five minutes) (Neville *et al.*, 1988). Collection vials for the milk collection were weighed before and after the collection. They found a random error of $\pm 3\%$ between test weighing of the infants and weights of the formula hence reflecting that test weighing has “acceptable small errors” (Neville *et al.*, 1988).

Many researchers, however, have criticised the test weighing method reporting that it is either inaccurate or imprecise (Nielsen *et al.*, 2011; Reilly *et al.*, 2005; Savenije & Brand, 2006). However, Haase and colleagues (2006) have attributed these shortcomings to the scales or technique used in the test weighing. The authors argued that many researchers, as they carry out test weighing, have not considered possible confounders in the test weighing such as infant

clothing, hanging blankets, and infant movements, hence they developed an improved test weighing method for infants who are under constant monitoring.

Savenije and Brand (2006) assessed the accuracy and precision of the test weighing method in a clinical setting. They studied 100 infants who were either bottle-fed, cup-fed or fed by nasogastric tubes at an infant ward in a hospital in the Netherlands (Savenije & Brand, 2006). Triple measurements were carried out pre-, post- and 15 minutes post-feed. These measurements were done using two identical Avery Berkel Pesa ERR330 electronic balances (Avery Berkel, Breda, the Netherlands) that were calibrated and maintained according to the Dutch Weights and Measure Act (Savenije & Brand, 2006). These digital scales gave single gram readings with no decimals. Measurements were taken with infants fully dressed and with their nappies on only. Those fed with nasogastric tubes were weighed with the tubes. Both the feeding staff and the person weighing the infants were blinded to the amount of feed given to the infants (Savenije & Brand, 2006). Milk was given to the infants through a 20ml or 50ml syringe and intake was read off from the syringe after the feed. In some cases, milk containers were weighed before and after the feed to establish milk intake. Milk intakes were recorded on a separate sheet from the weights and all spillages recorded and all those weighing the infants were blinded to the milk intakes measurements (Savenije & Brand, 2006). The median amount of formula or breast milk given to the infants was 37.5 (24.5 to 45) ml. There was a non-significant, weak correlation between the volume established through test weighing and the actual volume of milk given to the infant ($r = -0.18$, $P = 0.09$) (Savenije & Brand, 2006). Evaporative water loss was 1 (-1 to 2) g (Savenije & Brand, 2006). The authors' conclusion was that the test weighing method was accurate but imprecise because it over- or under-estimated the amount of milk intake by up to 15ml per feed and 40% of the milk intake per feed (Savenije & Brand, 2006). Regurgitation and vomiting episodes occurred in 14 (15%) infants and spillage in 20 (21%) infants. Part of the imprecision was due to spills and vomiting, but excluding such cases still led to an imprecision in 95% of the test weighing up to 14ml per feed (Savenije & Brand, 2006). Another contributor to the imprecision was that the scales were not sensitive enough to detect small changes (Savenije & Brand, 2006).

In an effort to develop an accurate and precise test weighing technique, Haase and colleagues (2006) carried out a study among pre-term and high-risk hospitalised infants to establish confounding factors that influence the test weighing method (Haase *et al.*, 2009). They included a convenience sample of 19 infants who were in the intensive care unit of a hospital in South Carolina. In the first phase, the authors observed variations in the test weighing technique and circumstances that influenced accuracy. Some of the variables that seemed to affect the weighing outcomes were: 1) The type of scale used: one scale (Scaletronix paediatric scale) gave a variation of 10g per measurement or more depending on the position of the infant on the

scale; 2) Infant movement such as flailing of arms and legs; 3) clothes and blankets draping over the scale. Therefore, the authors recommended that a reliable scale needs to be used and should always be placed correctly and in the same position pre- and post-feed in order to obtain accurate results. They also recommended swaddling of the infant in order to minimise infant movements and to avoid blankets and clothing hanging over the scales. Furthermore, they recommended that all clothing articles that the infant was weighed in during the pre-feed should be the same during the post-feed weighing. After implementing these recommendations, the authors found a correlation close to one between the amount milk intake and weight gain by test weighing were $r^2 = 0.998$ for infants without leads (intravenous lines and monitor wires) and $r^2 = 0.997$ for infants with leads.

Nielsen and colleagues (2011) argued that the test weighing method is not as accurate in measuring of breast milk intake and energy compared to the use of double-labelled water (Nielsen *et al.*, 2011). This is further supported by a review carried out by Reilly and colleagues (2005) who found a difference of 66 g per day (95% CI: 11-123 g per day; $P=0.02$) intake of breast milk between the test weighing method and isotopic techniques (Reilly *et al.*, 2005). In the 38 studies that used test weighing, the mean estimated milk intake was 799 ± 47 g per day compared to 864 ± 63 g per day in the three studies that used isotopic methods (Reilly *et al.*, 2005). They further concluded that test weighing was a cumbersome technique on the mothers who carry out the test weighing, especially in the case of frequent feeders and involved a higher risk of under-reporting as compared to using isotopic methods (Reilly *et al.*, 2005).

Most studies that have performed test weighing have ranged from a few hours to 24-Hrs, however, carrying out the study over a longer period of time is likely to reduce imprecision as mentioned by Neville and colleagues (1988).

2.7.2 Sampling and analysis of breast milk for fat and energy concentration determination

There is a lot of discrepancy in breast milk sampling between different studies, and often sampling procedures are not described in detail. For example, in a previous study carried out by Lin *et al.* (2011) to find the equation that can show the relation between the creatinocrit value and calories in Chinese population breast milk sampling was done by expressing fore and hind-milk. What is not clear, however, is whether they combined the fore and the hind for analysis or whether they analysed them separately (Lin *et al.*, 2011). On the other hand, Kociszewska-Najman and colleagues (2012) in their research to determine the changes in creatinocrit, energy and fat concentration of colostrum, transitional and mature milk in both pre-term and term infants collected milk over the first two weeks of lactation. They collected two samples of breast milk per participant per day: one in the morning (0600-0900) and one at night (0000-

0300) for seven days (Kociszewska-Najman *et al.*, 2012). One milk sample was collected at each of the time points but it is not mentioned whether the sample were of fore, mid or hind-milk, however they established that there were variations in creatinocrit, energy and fat with the stage of lactation (Kociszewska-Najman *et al.*, 2012).

Hassiotou and colleagues (2013) aimed to establish changes in breast milk fat concentration, protein and cellular concentration with degree of breast fullness. Samples were collected before and immediately after the feed, as well as 30 minutes, one hour, 1.5 hours, 2.5 hours and 3 hours after the feed. Participants were not supposed to express or breastfeed for three hours before the feed so that the breast would be full or close to full at the next feed. The authors found that there was a higher fat concentration post feed compared to before feed, which increased even further 30 minutes post feed (Hassiotou *et al.*, 2013).

Khan and colleagues (2013a) (referred to in section 2.5.2) found significant differences in fat between the pre- and post-feed samples whereby fat levels were higher post-feed compared to pre-feed (Khan *et al.*, 2013a).

Of interest is the study that was carried out to determine the effects of freezing of breast milk on creatinocrit concentrations (Vázquez-Román *et al.*, 2014). They found that freezing of breast milk at -20 C can lead to a decrease in both measured fat and energy concentrations of the milk of which a significant difference was already found in the first three months of storage (Vázquez-Román *et al.*, 2014). This is attributed to the action of lipase which leads to the hydrolysis of triglycerides and increases the free fatty acids in the milk (Vázquez-Román *et al.*, 2014). Hassiotou *et al.* (2013) also found that the sampling method (automatic versus manual expression) could have an impact on the variations in breast milk fat. They found that there was significantly less fat in the hand-expressed breast milk than the pumped milk suggesting that the standardised vacuum applied when pumping results in a more homogenous sample with regards to fat distribution (Hassiotou *et al.*, 2013). However, it is not well established which of the sampling methods provides a representative sample of natural suckled breast milk.

These studies highlight the importance of breast milk sampling when determining breast milk fat concentration and eventually concentrations of other breast milk constituents. Since it has been established that there are variations in fat and therefore energy concentrations between fore-, mid- and hind-feed milk, sampling of the three would be best to achieve a more accurate measure of fat and energy. It would also be ideal to collect samples throughout the day for comparison considering that differences have been found between day and night feeds. A sampling strategy should be followed for more accurate results in research studies and should be described in detail in the publications in order to allow comparison between studies. To prevent alteration within the sample during storage, temperatures lower than -70°C need to be

utilised. Lipoprotein lipase and bile salts lipase have been found to be active even when milk is frozen at -20C, but have been found to be inactive at -70C (Berkow *et al.*, 1984).

2.7.3 Analysis of fat concentration by creamatocrit

The creamatocrit method is a simple means of determining fat and energy concentration of milk samples. Milk is placed into a capillary tube and then centrifuged (Jensen, 1999). In the past, the length of fat column is then determined and calculated as a percentage of the total milk column. The value obtained is then converted to % fat using standard curves of other method of determining fat especially solvent extraction method (Jensen, 1999).

A new centrifuge has since been introduced and has been validated by Meier and colleagues (2002). In their study, they sought to establish and validate the reliability and accuracy of the creamatocrit plus for performing creamatocrit of breast milk (Meier *et al.*, 2002). They compared their results to those of a standard centrifuge with a haematocrit reader and the standard centrifuge with callipers using a total of 36 milk specimens. The authors found no significant differences in the intra- and interrater reliability tests amongst the three techniques and the absolute creamatocrit values were comparable among the three. They therefore, concluded that the creamocrit plus is a reliable means of determining lipid and energy concentration in breast milk (Meier *et al.*, 2002)

The new centrifuge has an in-built formula to calculate fat and energy from the creamatocrit reading. Below are the formulae used:

$\text{Creamatocrit} = (\text{length of cream layer} / \text{total length of the milk column}) \times 100;$

$\text{Fat} = 3.968 + (5.917 \times \text{creamatocrit});$

$\text{Energy} = 385.422 + (55.656 \times \text{creamatocrit}).$

2.8 Conclusion

Exclusive breastfeeding is recommended for the first six months of life, as it is associated with lower infant morbidity and mortality. It is imperative that information on breast milk intake and nutrient supply in exclusively breastfed infants be obtained because it helps define adequate nutrient intake during infancy (Grote *et al.*, 2016). However, limited data are available from developing countries, and in particular from the South African population. Hence, this study will

provide data on the breast milk intake of exclusively breastfed infants and the energy and fat composition of the breast milk.

Methods used to determine breast milk intake in populations are the doubly labelled water technique and test weighing. The test weighing method has been criticized by some researchers as an inaccurate and imprecise method; however, if used in a clinical setting it is expected to be reliable. Considering that it is cheaper and does not require specialised skills and equipment to perform, it is well-suited in resource restricted settings and hence will be used in this study.

CHAPTER 3: ARTICLE

3.1 Article

Student research: Original research

Title: Breast milk intake and variations in breast milk energy and fat concentration: A 4-day test weighing study in exclusively breast-fed South African infants

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Key Messages

- *It is well known that fat and consequently energy concentrations in breast milk vary within feed, between feed and diurnally but the variations do not affect milk intake.*
- *However limited data are available on milk intakes and composition amongst exclusively breastfed infants in South Africa.*
- *We used the test weighing method to establish breast milk intakes in exclusively breastfed infants and the results of our study indicate low milk intake levels and consequently low estimated fat and energy intakes*

Abstract

Background: Breast milk intake varies between populations and composition varies within feed, diurnally and between mothers. Very limited information is available on the breast milk intake of exclusively breast fed infants, including breast milk energy and fat concentrations, in South Africa.

Research Aim: To assess breast milk intakes and variations in fat and energy concentrations of breast milk in exclusively breast fed South African infants and their mothers participating in a 4-day test weighing study.

Methods: Twenty-four healthy mothers and their exclusively breast-fed 2-5 month-old infants stayed at a metabolic unit for five days. Over 96 hours, infants were weighed before and after each feed to determine breast milk intake. At each feed, a fore-milk sample was collected to determine fat and energy concentrations using the creamatocrit method. Additional mid-feed and hind-feed samples were collected from the first feed each day.

Results: Mean breast milk intake was 369 ± 98 g per day. Mean fat and energy concentrations of fore-milk were 26 ± 7 g/L and 2545 ± 256 kJ/L, respectively. Calculated mean fat and energy intake per kilogram (kg) body weight per day were 2 ± 1 g and 192 ± 60 kJ, respectively. Mean fat and energy concentrations of fore- (27 ± 8 g/L; 2545 ± 256 kJ/L), mid- (38 ± 7 g/L; 2947 ± 275 kJ/L) and hind-feed (50 ± 10 g/L; 3464 ± 410 kJ/L) milk differed significantly ($P < 0.001$).

Conclusion: Based on this small sample and using a test weighing method, exclusively breastfed South African infants may have low breast milk intakes, hence

are likely to be energy deficient. Further studies are needed to confirm these findings in a larger sample and with different methods.

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Keywords: Breastfeeding, milk energy, milk fat, milk intake, test weighing

Background

Exclusive breastfeeding is recommended for the first six months of life¹, and is associated with lower infant morbidity and mortality particularly in developing countries.²

Different populations have varying feeding patterns and breast milk intake levels.³⁻⁵ In infants from developed countries, mean breast milk intakes of 779 g/d, 827 g/d and 894 g/d were established for the ages 2-4 months, 5 months and 6 months, respectively, through a review of 33 studies that assessed daily breast milk volumes passed on from mother to infant.⁶ The United States Environmental Protection Agency, on the other hand, recommends that infants less than one month of age require an average amount of 510 mL per day, whilst 690 mL and 770 mL per day are needed for infants one to three months and three to six months, respectively.⁷ These recommendations were arrived at through reviewing six studies that used test weighing to establish milk intake, and one review that aimed to establish breast milk intakes in the United States. All six studies included were performed in America, with the exception of one study that included European data as well.⁷ Therefore, the current reference values for breast milk intake in infants below six months of age have mostly been established using data from developed countries. It is, however, unclear whether breast milk intakes in developing countries are comparable to those of developed countries, considering that malnutrition is more prevalent in low- and middle-income countries and breast milk production may differ between well-nourished and under-nourished mothers.⁸

Variations in breast milk fat and energy concentrations within feed and between days have been documented in the past, and both infant and maternal characteristics

were found to influence these variations.^{5,9,10} The energy concentration of breast milk is known to be influenced by the variation in fat concentration, whereby fat constitutes about 50% of total energy in breast milk.¹¹ Variations in breast milk fat concentrations between individuals and between populations have also been documented, with variations between individuals ranging from 28 to 57 g/L¹², and between populations ranging from 28 to 47 g/L.¹³ It is also well-known that there is a significant variation in fat concentrations between fore-, mid- and hind-milk.¹² However, to our knowledge, the fat concentration of breast milk has not been assessed in the South African population.

To establish whether exclusively breastfed infants in a population are getting enough energy from milk to ensure optimal growth, it may be useful to measure breast milk intake and energy concentration. Breast milk intake can be assessed using either isotopic methods or the test weighing method.¹⁴ Test weighing involves weighing infants before and after feeding to establish the quantity of milk consumed.¹⁵⁻¹⁷ Test weighing has been criticized by some as a method that is inaccurate and imprecise^{6,18,19}, whilst others have demonstrated it to be an effective means of establishing intake.¹⁵ In addition, it is cheap and does not require specialised skills and equipment to perform, and is well suited in resource restricted settings.

Forming part of an iodine balance study with the primary aim to determine the iodine requirements in early infancy, this study aimed to determine breast milk intake using the test weighing method and to assess within feeds, within day and between day variations in breast milk energy and fat concentration in exclusively breast-fed South African infants and their mothers residing at a metabolic unit for five days. We also critically evaluated whether test weighing in a controlled environment is a feasible

method to establish breast milk intake in a South African population. To the best of our knowledge, this is the first study that has carried out test weighing to determine breast milk intake in a South African population.

Methods

Design and setting

This test weighing study was nested within an iodine balance study that was designed to estimate the dietary amount of iodine needed to achieve iodine balance in early infancy (0-6 months), and to define the median urinary iodine concentration cut-off that can be used to define adequate iodine status in infants (0-6 months). The study was conducted at the metabolic unit of the Centre of Excellence for Nutrition at the North-West University (NWU) in Potchefstroom, South Africa from June 2015 to August 2015. Ethical approval for this study was obtained from the North-West University Health Research Ethics Committee (NWU-00090-14-A1).

Sample

A purposive sample of 24 mothers with a two to five month old exclusively breast fed infant from any ethnical and socio-economic background was recruited from the Potchefstroom area in South Africa. The inclusion and exclusion criteria of this study were based on the iodine balance study this study was nested in. Inclusion criteria for the infants were: 1) healthy; 2) currently being exclusively breast fed (determined by use of an infant feeding practice questionnaire); 3) born at full-term (in week 38 to 42); 4) normal birth weight (≥ 2500 g); 5) and no known history of thyroid disease. Exclusion criteria for the infants were: 1) receiving infant formula or solid food; 2)

receiving iodine-containing supplements; 3) moderate anaemia (haemoglobin [Hb] <9 g/dL).

The inclusion criteria for the lactating mothers were 1) apparently healthy; 2) no known history of thyroid disease; 3) singleton birth; 4) currently exclusively breastfeeding (both at breast and/or expressed). Exclusion criteria for lactating mothers were: 1) use of X-ray / CT contrast agent or of iodine containing medication within the last year; 2) moderate anaemia (Hb <11.5 g/dL); 3) UIC at pre-screening <100 µg/L; 4) currently smoking; 5) tested HIV positive during pregnancy (information obtained from Road to Health booklet).

Recruitment of study participants was done through advertisements in local newspapers, flyers and posters and by word of mouth. Interested mothers and their spouses/life partners were invited to the metabolic unit at NWU (Potchefstroom Campus) for an information session and pre-screening. After receiving all the necessary information and having a chance to ask questions, interested mothers gave provisional written informed consent for the pre-screening. The pre-screening included finger pricking of the mother and heel pricking of the infant to obtain a capillary blood spot to measure the haemoglobin concentration. In addition, information from the Road to Health booklet (maternal HIV status, gestational age and birth weight) was obtained. A spot urine sample (5 ml) from the mother and the infant using an adhesive urine collection bag (U-Bag paediatric or infant size (Hollister Inc, Libertyville, IL) was collected for the analysis of UIC.

Mother-infant pairs that met the inclusion criteria were invited to participate in the study. They were given an information sheet that they took home, read, and discussed with other members of the family. Appointments were scheduled for the

stay at the metabolic unit for data collection. All mothers who participated gave further written informed consent upon admission to the metabolic ward. All mothers who participated were given a token of appreciation which included ZAR 900, a gift hamper for the infant, and ZAR 100 airtime for their use during their stay at the metabolic unit.

Data collection and analysis

Mother-infant pairs were admitted to the metabolic ward of the Centre of Excellence for Nutrition at NWU for five nights (Monday 08:00 a.m. to Saturday 08:00 a.m.). On admission, a standardised culture-sensitive breakfast (each mother participating in the study received the same menu in the same order) was served and the study protocol was explained once more in the language preferred by the mother. Mothers gave written informed consent to participate in the test weighing study. The first day at the metabolic ward served as the run-in phase and the following four days (96 hours) as the test weighing period. Trained study assistants completed a case report form for each of the mother-infant pairs. The mothers were asked questions regarding breastfeeding practices and socio-economic status and habitual dietary intake.

On the run-in day, anthropometric measurements were taken. The height of each mother was measured using a Leicester height measure Mk11 stadiometer and weight was measured in kg using a Seca 813 robusta high-capacity electronic scale. Body mass index (BMI) was calculated for each of the mothers using the following formula: $BMI = \text{mass (kg)} / (\text{height [m]})^2$. Infant length (without clothing) was measured supine on a ShorrBoard infant/child portable height-length measuring board. Infant weights (in g) were measured using a SECA 728 infant scale with an

accuracy of ± 1 g. Weight-for-age z-scores (WAZ) and length-for-age z-scores (LAZ) of the infants were calculated using the WHO 2006 references (WHO AnthroPlus, version 1.0.2 software).

Mothers were given three culture-sensitive meals and three snacks a day. The infants were exclusively breastfed during the entire study duration. At the first feed of each day (starting at 8:00 a.m.), a sample of breast milk from the fore-, mid-feed and hind-milk was collected (5 ml each) with the assistance of trained research nurses or nursing assistants for the purpose of analysing energy and fat concentration and to establish the extent of within-feed variations in breast milk fat and energy concentrations. Expression was done manually after cleaning the breast with a wet cloth. The fore-milk sample was collected from the breast the mother would intend to use for feeding. The infant was then put to the breast to suckle for 2 to 2.5 min after let-down, judged as the time-point when the infant began to swallow actively. Subsequently another 5 ml of breast milk was collected to obtain a mid-feed sample. The infant then suckled on the breast until fully satisfied, after which a third 5 ml sample, the hind-milk sample, was collected. At each of the other feeding sessions, the mother was requested to express 5 ml of fore-milk and to feed the infant only from one breast at each feed and to alternate breasts between feeds. Each sample of fore-milk was analysed for fat and energy concentration to establish whether there were any within-day variations in the milk. The nurses at the metabolic unit recorded the time of each feed.

Fat (in g/L) and estimated energy concentrations (in kJ/L) in breast milk samples were determined in fresh samples by analysis of creatocrit using the Creatocrit Plus System (Separation technology, Inc). Creatocrit is well-recognised within

clinical settings as an accurate method to measure fat and energy concentration of breast milk and is validated by Meier *et al.* (2002). Its performance is comparable to the standard centrifuge using a haematocrit reader or digital callipers and has been found to provide accurate results on fat concentration and energy concentration of breast milk.²⁰ The following equations are used by the device:

$$\text{Creatocrit (unit)} = (\text{length of cream layer} / \text{total length of the milk column}) \times 100$$

$$\text{Fat (unit)} = 3.968 + (5.917 \times \text{creamocrit}); \text{Energy (unit)} = 385.422 + (55.656 \times \text{creamocrit})$$

Creamatocrit, fat and energy concentrations were recorded for each sample.

Breast milk intake was quantified through test weighing the infant. Trained research nurses weighed the infant using a SECA 728 infant scale to the nearest gram before and after each feeding session. The infants were weighed with the clothing articles and bibs that they wore to feed and were weighed after feeding dressed in the same items. The daily consumption of breast milk was calculated by adding up the weights of recorded intakes for each day and presented in grams (g). Mean consumption (for 4 days) was calculated by adding up daily consumption for the four days and dividing by four. The mean energy and fat concentrations were obtained by summing up energy and fat concentration of the fore-, mid-, and hind-milk sample and dividing by three. The fat and energy intakes (in g and kJ respectively) of the exclusively breastfed infants (daily and mean across 4 days) were calculated as a product of breast milk intake (in g) and fat and energy concentrations (g/L and kJ/L

respectively) measured in breast milk, respectively. To analyse milk intakes, and fat and energy concentrations with respect to time of day, the 24-hour period was divided into a morning (04:01-10:00), day (10:01-16:00), evening (16:01-22:00) and night (22:01-04:00) phase.

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics (version 23; IBM Co.). Shapiro-Wilk test and Q-Q plots were used to determine normality of continuous variables. We performed independent T- test to establish whether there were any differences between the male and female infants. Pearson's correlations were performed to establish correlations between normally distributed continuous variables. Spearman's correlations were performed to establish whether there were any associations between non-normally continuous variables. To establish whether there were any differences in fat or energy concentrations between fore-, mid-feed, and hind milk samples, as well as within day and between days, repeated-measures ANOVAs were performed. *P*-values <0.05 were considered significant.

Results

A total of 33 mother-infant pairs were recruited, and 24 were enrolled and completed the study. Nine mothers were excluded because they failed to produce enough milk for their infants during the run-in day (the first 24 hours of the study). Further interviews revealed that they had not been exclusively breastfeeding before entry into the study (n=8), even though they had declared doing so at recruitment. One mother was referred to the local health clinic after she reported that she had been struggling to breastfeed her infant prior to study enrolment.

Characteristics of the study group are shown in Table 1. The infants had a mean age of 13.5 ± 4.7 weeks, mean length of 57 ± 5 cm and weight of 5896 ± 1061 g. There was no significant difference in age, length and weight between the male and female infants ($P > 0.05$). The mothers had a mean age of 26 ± 6 years and a mean BMI of 28.1 ± 6.6 kg/m². All the mothers reported to have taken folic acid, vitamin C and ferrous sulphate during pregnancy except for one who had used multivitamins. However, at the time of the study, none of the mothers were taking any nutrient supplements.

Table 1. Frequency distribution of characteristics of mother-infant pairs participating in 4-day test weighing study (N=24)

Parameters	Frequency [N (%)]
Infant	
Gender	
Female	10 (42)
Male	14 (58)
Length for age Z-Score [n (%)]	
Severely stunted (<-3SD)	6 (25)
Stunted (<-2SD)	3 (13)
Normal (>-2SD but < 3SD)	15 (62)
Mother	
BMI (N=23)	
Normal weight	9 (37.5)
Overweight	4 (16.7)
Obese	10 (41.7)
Ethnicity	
Black	23 (96)
Coloured	1 (4)
Level of education	
Primary	1 (4)
Secondary	21 (88)
Tertiary	2 (8)
Marital status	
Married	2 (8)
Single	22 (92)
Employment	
Employed	3 (13)
Not employed	21 (87)

Test weighing as performed for each feed. Table 2 provides a summary of milk intake over the 4-day test weighing study. The male infants' mean milk intake per day was 381 ± 83 g and the female infants' intake was 353 ± 118 g, but there was no significant difference ($P = 0.277$). There was no significant variation in milk intake between the four days ($P > 0.05$).

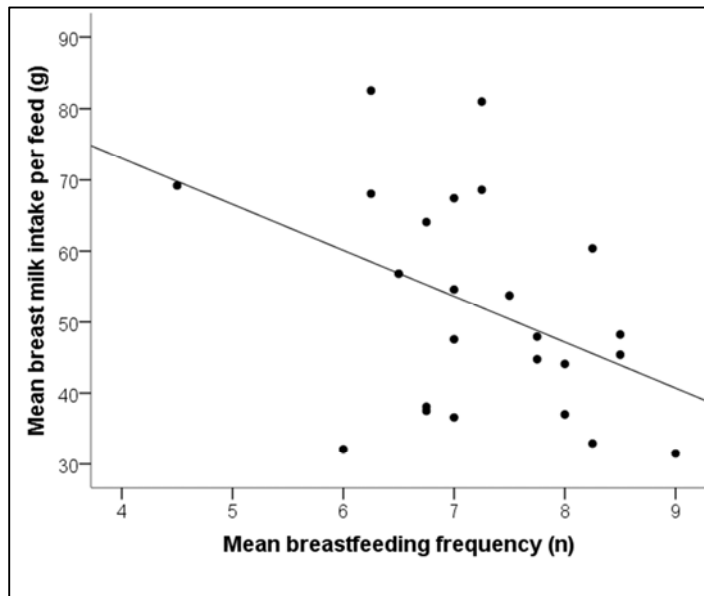
Table 2. Milk intake over the 4 day test weighing study (N=24)

Milk intake	Day 1	Day 2	Day 3	Day 4	Mean day 1-4	<i>p-value</i>
	(n=24)	(n=24)	(n=24)	(n=24)	(n=24)	
	M(SD)	M(SD)	M(SD)	M(SD)	M(SD)	
Per day (g)	351(123)	370(118)	363(120)	392(120)	369(98)	0.356
Per feed (g)	50(21)	53(18)	51(17)	54(17)	52(15)	0.401
Per day (g/kg BW)	61(23)	64(21)	64(24)	70(26)	65(20)	0.246
Feeding frequency	7(1)	7(1)	7(1)	7(2)	7(1)	0.567

Note: Mean milk intake per each study day. Per day intake is the mean intake obtained by totalling of all milk intakes measured over the course of the day and establishing the mean for the group. Per feed (g) intakes were established by dividing total mean intake per day by frequency of feeds per day. Milk intakes did not differ significantly between the days ($P>0.05$).

The males' breastfeeding frequency was 8 ± 1 whilst the females' was 7 ± 1 , but did not differ significantly ($P = 0.104$). There was an inverse correlation between mean daily breastfeeding frequency and milk intake per feed ($r_p = -0.446$, $P = 0.029$) as shown in Figure 1, but there was no correlation with mean estimated daily fat and energy intake from breast milk ($P > 0.05$). The mean milk intakes per feed for the female infants was 53 ± 18 g and that of the male infants was 51 ± 14 g and there was no significant difference between the two ($P=0.824$).

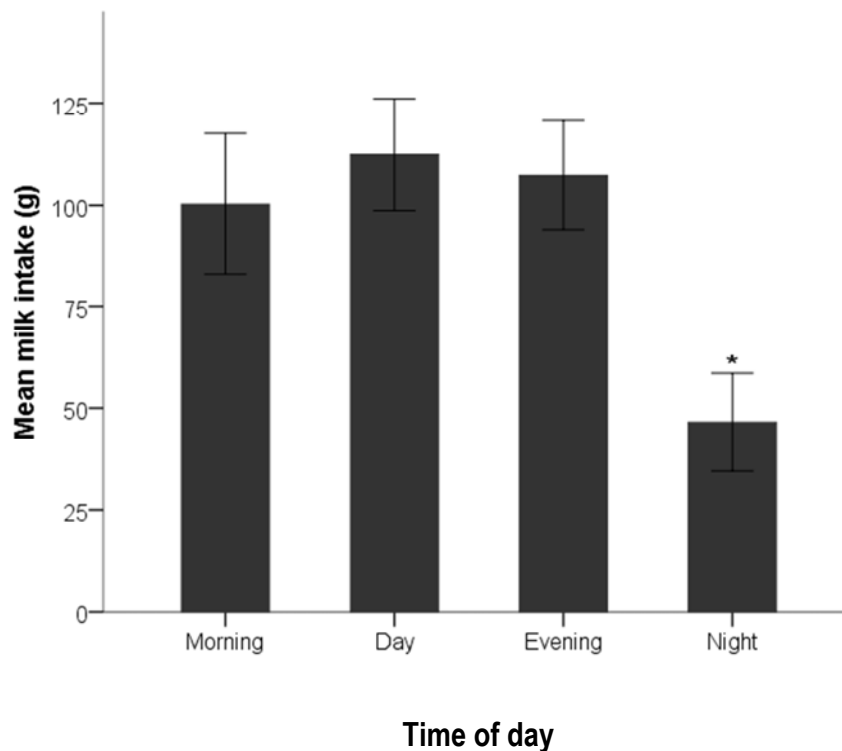
Figure 1. Correlation of breastfeeding frequency and mean milk intake per feed for the four days of the study (N=24)



Note: Mean milk intake per feed and mean feeding frequency are for each individual infant over the four days. We did a Pearson's correlation and obtained an $r_p = -0.446$ and $P = 0.029$.

Using repeated measures ANOVAs, we found a significant difference in milk intake between the different times of the day ($P < 0.001$) (Figure 2). Mean milk intake was significantly lower during the night (47 ± 29 g) than during the morning (100 ± 41 g; $P < 0.001$), day (112 ± 32 g; $P < 0.001$) and night (107 ± 32 g; $P < 0.001$).

Figure 2. Mean milk intake at each time of the day (N=24)



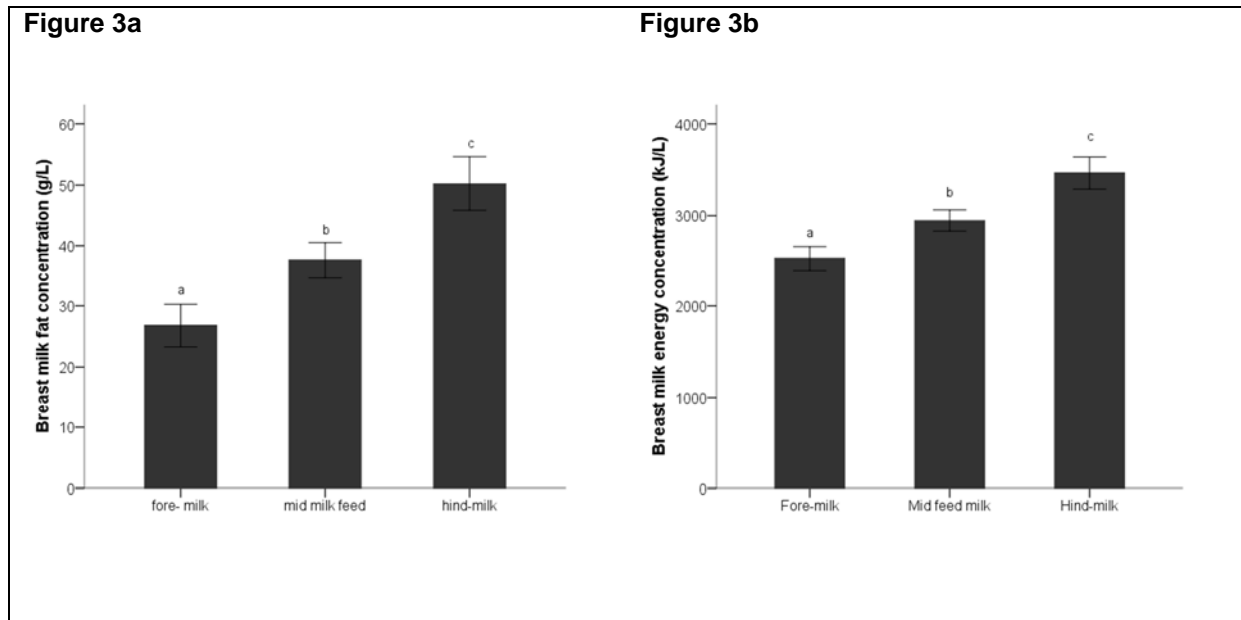
Note: Mean milk intakes are the average milk intakes over the four days of the test weighing study. Morning was from 04:01 to 10:00, day 10:01 to 16:00, evening 16:01 to 22:00 and night 22:01 to 04:00.

*Mean milk intake at night was significantly lower than during the day ($P < 0.001$).

Mean fat concentration of fore-milk across the four days was 26 ± 7 g/L. Milk fat concentration of milk consumed by male infants (41 ± 5 g) was significantly higher than from milk consumed by female infants (35 ± 6 g) ($P = 0.014$). Mean fat concentrations of fore- (27 ± 8 g/L), mid-feed (38 ± 7 g/L) and hind- (50 ± 10 g/L) milk differed significantly ($P < 0.001$) as shown in Figure 3a. Fat concentration in the fore-milk samples varied between days; milk at day one had higher fat concentration (31 ± 9 g/L) than at day two (25 ± 5 g/L; $P = 0.013$) and four (22 ± 5 g/L; $P < 0.001$). Fat concentration of breast milk in day three was also higher (28 ± 8 g/L) compared to day four ($P < 0.001$). However, no significant difference was observed in the mean

fat concentration of the first sample of the day that comprised fore-, mid-feed and hind-milk ($P < 0.05$) across the four days.

Figure 3. Within feed variations of breast milk in fat and energy concentration (N=24)



Note: Figure 3a: Within feed variation in fat concentration in the first sample of breast milk in the day.

Figure 3b: Within feed variation in energy concentration in the first sample of breast milk in the day.

Data for the first sample of the day to establish within feed variations.

^{a,b,c} shows a significant difference in fat and energy concentration between fore- mid- and hind-milk ($P < 0.05$).

The fore-, mid-feed and hind-milk energy concentrations in this sample were 2545 ± 256 kJ/L, 2947 ± 275 kJ/L and 3464 ± 410 kJ/L, respectively and differed significantly ($P < 0.001$) as shown in figure 3b. Mean energy concentration of milk consumed by male and female infants was 2970 ± 273 kJ/L and 2989 ± 231 kJ/L, respectively, with no significant difference ($P = 0.859$).

Breast milk energy concentration was higher on day one (2684 ± 316 kJ/L, $P < 0.001$) and day three (2609 ± 426 kJ/L; $P = 0.006$) compared to day four (2329 ± 187 kJ/L). There was a positive correlation between infant age and mean breast milk energy concentration ($r_s = 0.427$, $P = 0.037$) as shown in Figure 4a. Infant age was positively correlated with mean energy concentrations of both mid-feed ($r_s = 0.427$, $P = 0.037$)

(Figure 4b) and hind-milk ($r_s=0.556$, $P=0.005$) (Figure 4c), but not of fore-milk ($r_s=0.222$ $P=0.297$).

Figure 4. Correlation of energy concentration of breast milk and infant age (N=24)

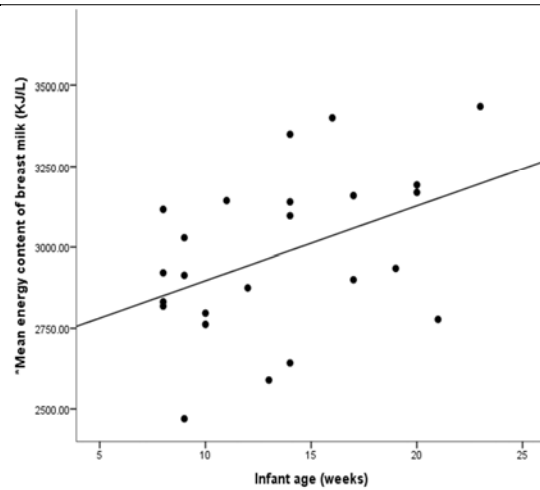


Fig 4a

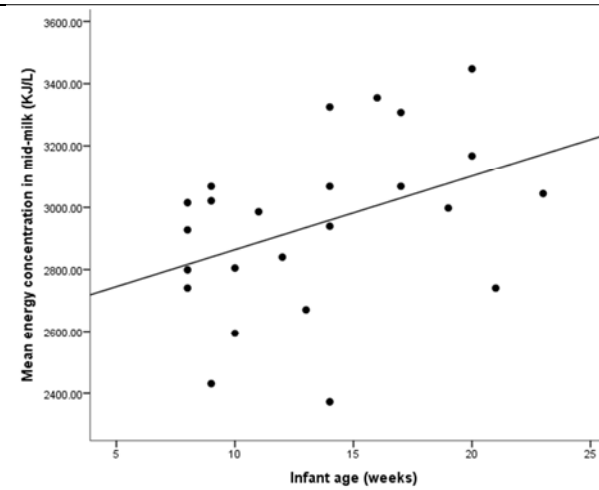


Fig 4b

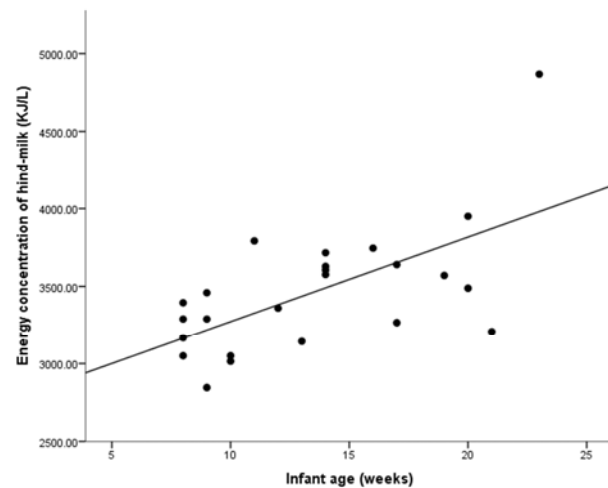


Fig 4c

Note: We used Spearman's correlation

Figure 4a: Correlation of mean energy concentration of the first sample of the day (fore-, mid- and hind-milk) of breast milk and infant age $r_s=0.427(P=0.037)$.

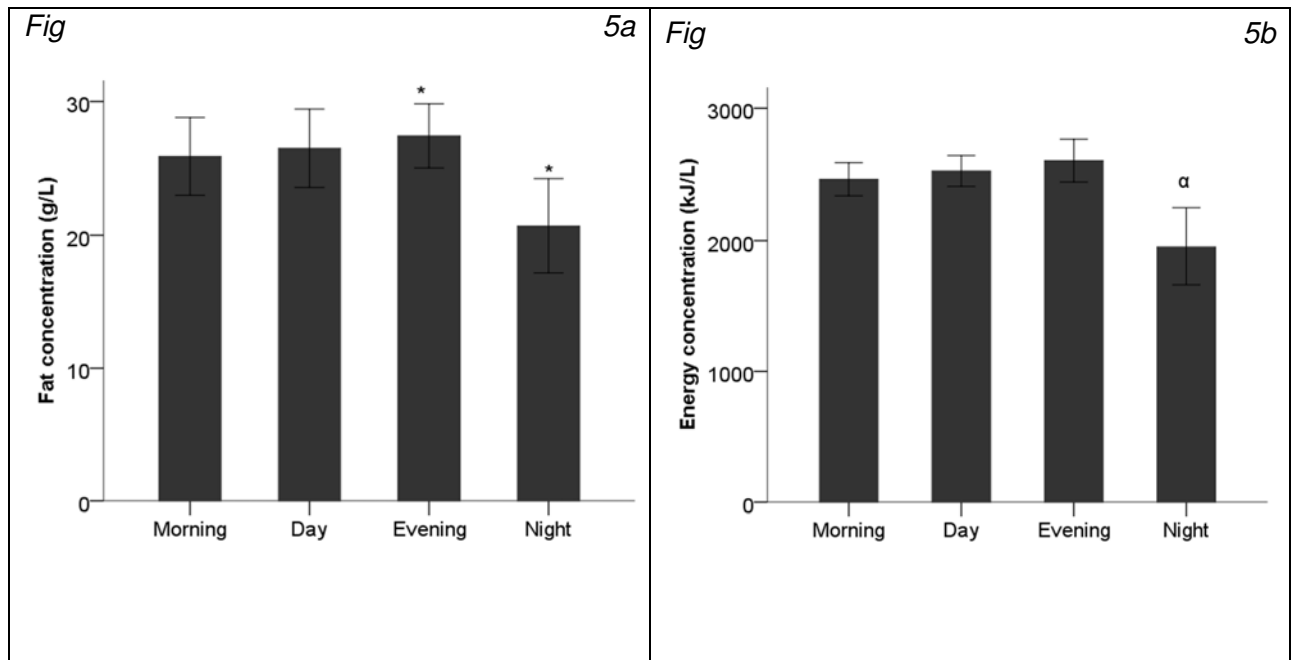
Figure 4b: Correlation of mean energy concentration of mid-milk and infant age. $r_s=0.427 (P=0.037)$.

Figure 4c: Correlation of mean energy concentration of hind- milk and infant age. $r_s=0.556 (P=0.005)$.

The mean energy concentration obtained by summing up energy concentration of fore- mid- and hind-milk sample and dividing by three.

There was a significant variation in the fat concentration of the milk between time of day ($P=0.004$) (Figure 5). Breast milk-fat concentration was significantly lower during the night (20.7 ± 8.2 g/L) than during the evening (27.4 ± 5.5 g/L) ($P=0.015$).

Figure 5. Mean fat and energy concentrations in fore-milk at different times of the day (N=24)



Note: Data for the all fore-milk samples collected throughout the day. Figure 5a: Mean fat concentration in fore-milk consumed at each time of the day. Figure 5b: Mean energy concentration in fore-milk consumed at each time of day. Morning was from 04:01 to 10:00, day 10:01 to 16:00, evening 16:01 to 22:00 and night 22:01 to 04:00.

*Milk fat concentration was significantly lower at night than during the evening ($P=0.015$).

^aMilk energy concentration was significantly lower at night than the morning ($P=0.021$), day ($P=0.006$) and the evening ($P=0.002$).

Consequently breast milk energy concentrations also differed between time of day ($P<0.001$) (Figure 5b). During the night (1953.1 ± 374.9 kJ/L), breast milk energy concentrations were significantly lower than during the morning (2463.4 ± 289 kJ/L) ($P = 0.021$), day (2526.2 ± 271 kJ/L) ($P = 0.006$) and evening (2604.4 ± 373.9 kJ/L) ($P=0.002$). Breast milk energy concentrations did not differ between morning, day, and evening.

Table 3 shows the estimated fat and energy intakes based on measured breast milk intake and estimated mean fat and energy concentrations of the first sample of the day. The estimated mean daily fat intake for the male infants was 16 ± 4 g/day and for the females 12 ± 5 g/day, but no significant differences by sex were found ($P = 0.085$). There was no significant difference between female (2 ± 1 g/kg/day) and male (3 ± 1 g/kg/day) infants ($P = 0.797$).

Table 3. Estimated fat and energy intake of infants over the course of 4 days in the test weighing study (N=24)

Variable	Day 1	Day 2	Day 3	Day 4	Mean day 1-4	<i>p-value</i>
	(n=24)	(n=24)	(n=24)	(n=24)	(n=24)	
	M(SD)	M(SD)	M(SD)	M(SD)	M(SD)	
Fat intake						
Per feed (g)	2(1)	2(1)	2(1)	2 (1)	2(1)	0.774
Per day (g)	14(8)	13(6)	14(6)	15(6)	14(4)	0.802
Per day (g/kg BW)	2(1)	2(1)	2(1)	3(1)	2(1)	0.653
Energy intake						
Per feed (kJ)	153(72)	149 (49)	151(46)	163(60)	154(45)	0.405
Per day (kJ)	1065(462)	1048(315)	1093(407)	1178(389)	1096(302)	0.602
Per day (kJ/kg BW)	182(69)	182(56)	195(88)	209(82)	192(60)	0.245

Note: Fat and energy intakes are a product of mean fat and energy concentration of the first sample of the day (mean of the fore- mid- and hind-mid milk) and the mean milk intakes of each day. Using repeated-measures ANOVAs, we found no difference in both fat and energy intakes between the days ($p>0.05$).

There was no significant difference between the mean energy intakes per day of male (1139.6 ± 294.7 kJ) and female (1035.4 ± 316.5 kJ) infants ($P=0.416$).

Discussion

To our knowledge, the test weighing method has not been used to measure breast milk intakes amongst exclusively breastfed infants in South Africa and little is known about

breast milk composition of South African lactating mothers. In this four-day test weighing study we found that breast milk intakes of the infants participating in this study were low, ranging from a mean intake of 193 g to 538 g per day. This translated to low estimated fat and energy intakes. However, fat and energy concentrations of breast milk were comparable to other studies³ and varied within feed, between feeds and diurnally as expected.¹²

Measured breast milk intakes in this small sample of reportedly exclusively breastfed infants were lower than intakes reported in other studies.^{4,21-23} In a study carried out by Nielsen and colleagues (2011) in infants between 15 and 25 weeks old (n=50) from Glasgow, Scotland, using a doubly labelled water technique, milk intakes were found to be 923 ± 122 g per day and 999 ± 146 g per day at weeks 15 and 25, respectively. They found that when mothers are supported to exclusively breastfeed during the first six months of life, the breast milk intakes of the infants are high and breast milk intakes also increase during lactation to meet the energy requirements of the infant.¹⁸ However, the study does not mention the source of support. In our study population, mothers were mostly single and unemployed, hence, it is likely that the mothers in this study may not have enough support (emotional, financial, sufficient dietary intake) which could likely have impacted on the milk production, leading to low intakes in this population. Interventions to support and promote exclusive breastfeeding such as peer counselling, visitation and counselling by community health care-workers have been implemented^{24,25}; however, not in all the provinces in South Africa²⁶. The prevalence of stunting in this group of infants is a cause for concern. There was no association between the length of the infants and measured breast milk intakes. However, it cannot be disputed that these infants' energy consumption was below the required daily intake.²⁷ Estimated energy intake per kg body weight was about half the recommended energy intake. In a

study amongst Senegalese infants, a mean milk intake of 993 ± 135 g per day and energy intake of 2598 ± 259 kJ/L were observed amongst exclusively breastfed infants at six months of age³, showing that exclusive breastfeeding can be sufficient as a means of infant feeding. However, the question that arises is whether there is a possibility that mothers do not breastfeed correctly.

Milk intake per feed correlated inversely with breastfeeding frequency, but there was no significant association between mean daily milk intake and feeding frequency. This finding is in agreement with a study done by Kent *et al.* (2005) in Western Australian infants, and hence confirms that infants have either frequent small feeds or fewer but larger feeds.

In contrast to our study, Kent *et al.* (2005) found that the male infants in their group had a higher milk intake per feed compared to the girls (155 ± 55 g and 130 ± 29 g, respectively).⁴ In our study there was no difference between milk per feed of the male and female infants. We found no relationship between the infant's sex and milk intake, fat, and energy concentrations. This is in contrast to the findings of Powe *et al.* (2009) who found that the mothers of male infants produced milk with a higher energy concentration compared to their female counterparts (75.56 kcal/100ml and 60.811 kcal/100ml $P=0.049$).⁵ Furthermore, they found that the male infants consumed more breast milk than the females which is also contrary to our results. In our study, although non-significant, breast milk from mothers with male infants had a higher fat concentration compared to their female counterparts. The male infants in our study were younger than their female counterparts but were larger in terms of length and weight though these differences (in age, weight and length) were not significant. This, however, may explain why there was no relationship between the sex and energy concentration of the milk and milk volume intake. After controlling, Powe and colleagues

(2010) allude to the fact that faster-growing infants demand more milk from their mothers. As infants grow, demand for milk increases, consequently milk production increases, and this probably then leads to the mother producing higher caloric milk to meet the infant's needs.⁵

In this study, fat concentration of breast milk was significantly higher during the evening compared to the night. Similarly, Kent and colleagues (2006) demonstrated that fat concentration was higher in the evening and day compared to the night and morning ⁴. Khan *et al.* (2013) on the other hand showed that fat concentration was higher during the day compared to morning, and at night, fat concentration was lower than in the morning. Milk fat concentration is known to be affected by degree of breast fullness where less full breasts have higher fat concentrations compared to fuller breasts.^{11,28} The high fat concentration in our study may therefore be explained by the higher feeding frequency during the day which leads to less full breasts at evening time. Fat concentration was also higher on day one compared to the other days. Fat concentration was also higher in day four than day three. It is likely that the fluctuations in the fat concentrations between days can be explained by maternal diet that varied in fat concentration, as it has been previously established that a high fat diet can increase fat concentration in breast milk.^{29,30,31}

Test weighing is a cheap method that does not require specialized expertise in performing. It has been used in different populations to determine milk intake amongst breastfeeding infants. However, Savenije and Brand (2006) found test weighing to be an imprecise method in measuring breast milk intake largely because of scales that were incapable of picking up even the slightest change in weight.¹⁹ Nonetheless, the SECA 728 infant scale used in this study has a proprietary damping system which factors out the discrepancy in measurements caused by movement of the infant. The

scale also has a fine graduation, which makes it sensitive even to a small change in weight. Furthermore, Neville and colleagues (1988) validated the test weighing method and found that if test weighing is carried out by motivated and educated participants it yields results with minimal errors.²² In our study, trained research nurses and nursing assistants carried out the test weighing in an attempt to reduce error and ensure high accuracy. We did not factor in insensible water loss, however, as its consideration would not increase the breast milk intake to expected levels.

We faced a few challenges as we performed the study: First, during the run-in day of this study, we observed that mothers kept their infants on the breast for an indefinite period and were using the breast as a dummy. Thus, in order to ensure accurate determination of milk intake during test weighing, we had to encourage mothers to remove the infants from the breast when they sensed the infant had stopped feeding and only allow the infant back on the breast when it was showing signs of being hungry. We cannot rule out that this interference with the feeding routine of the mother-infant pairs may have had an impact on breast milk intakes. However, infants were never denied feeding on cue.

Second, self-reporting on exclusive breastfeeding may have been inaccurate. South Africa has one of the lowest exclusive breastfeeding rates (<8%)³², and it has been reported that in the past, mothers have not been honest about exclusive breastfeeding practices, leading to over-estimation of the prevalence of exclusive breastfeeding according to the WHO recommendation.³³ In this study, eight mothers dropped out because they were not exclusively breastfeeding. The question is whether we can trust that the mothers we included in the study were all exclusively breastfeeding their infants prior to enrolment?

Third, the change of environment from home to the metabolic unit and change of diet may have also affected milk production, hence leading to low milk intakes. Anxiety and stress affect the release of oxytocin hence leading to a reduced milk let-down reflex.^{34,35} However, the metabolic unit was set in a homely way and the food served was culturally sensitive and standardized across the mothers (each mother participating in the study received the same menu in the same order). However, mothers were allowed to request a different meal if it was not to their liking. Furthermore, the sampling process together with test weighing could also have interfered with the milk let-down reflex.

Nonetheless, if the data of our study are a true reflection of the intakes of the infants, then our study shows that there is likely to be an energy deficiency experienced by exclusively breastfed infants in the Potchefstroom area. However, we are unable to generalize these results considering that this study was carried out in a small purposive sample. Furthermore, our study was inadequately powered to determine differences within sub-groups (infant age categories, mothers' weight category, height-for-age z score categories). We did not monitor growth as we carried out the study; this would have helped us validate the low intakes we established as we carried out the study. However the strength of our study was that it was performed in a controlled environment where a sensitive, calibrated scale was used, and test weighing was carried out by trained research nurses and nursing assistants. Consequently it is likely that all data points were accurately collected. Furthermore, test weighing was performed over four days which increased the precision of the test weighing method.

Further studies need to be done in a more representative sample to establish breast milk and energy intake of exclusively breastfed infants in South Africa. The test weighing method can be a cumbersome way of collecting breast milk intake but with the aid of research staff, all data points can be captured. However, in a population where

mothers use the breast as a dummy, test weighing is not the best method to use for determining breast milk intake and isotopic methods may be the better method to use in order to avoid interference with the breastfeeding routine. This will further help establish whether mothers are truly exclusively breastfeeding, should the study be carried out from their homes. Using isotopic methods, a study with a duration of 10-14 days will further help to establish whether a change of environment affects breastfeeding outcomes and also how long it takes for adaptation.

The low intakes and the high prevalence of stunting we observed in our study highlight the importance of growth monitoring and breast milk intake in exclusively breastfed infants so that there is a possibility of timely intervention to prevent or address growth faltering. Lack of equipment, resources, shortages and lack of understanding of growth monitoring indices in most South African public health institutions affect growth monitoring.³⁶ Furthermore growth monitoring involves measuring of weight and length, but rarely is length measured.³⁷⁻³⁹ The results of this study suggest a need to monitor for stunting even earlier than six months of age.

Conclusion

In conclusion, although the fat and energy concentrations of breast milk were comparable to other populations, breast milk and consequently estimated fat and energy intakes were low in this small sample of exclusively breastfed South African infants. The change of environment and feeding pattern during the study could have affected milk production or intake. Furthermore, test weighing may not be a well-suited method for establishing milk intake in this population.

Declaration of conflict of interests

No conflict of interest declared

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CHAPTER FOUR: CONCLUSIONS AND RECOMMENDATIONS

4.1 Introduction

The main aim of this MSc project was to assess the volume of breast milk intake and variations in fat and energy concentration of breast milk in a purposive sample of exclusively breastfed infants from the Potchefstroom area in South Africa. We used the test weighing method to establish breast milk intakes amongst 24 exclusively breastfed infants over a 96-hour period. Mother-infant pairs were recruited to stay at the metabolic unit of the Centre of Excellence for Nutrition at the North-West University, where breast milk intake was measured using the test weighing method by trained research nurses. A fore-milk sample was collected before every feed, and additional mid- and hind-samples were collected at the first feed of the day. Fat and energy concentrations in the breast milk samples were measured using the creatatocrit plus system. Daily fat and energy intakes were estimated as a product of breast milk intake with estimated mean daily fat and energy concentrations measured in the first feed per day (mean fore-, mid-feed, hind-milk samples).

4.2 Main findings

In this test weighing study, we found low mean daily milk intakes (193 g to 538 g per day) in this small sample of exclusively breastfed infants. In comparison to other studies and recommended intakes, intakes in this study were low. The low milk intake consequently led to low fat and energy intakes.

Most of the mothers in this sample group were single (92%) and unemployed (87%). There were also high levels of stunting among the infants in this group (n=9, 38%) with six infants (25%) being severely stunted. There were, however, no correlations of milk, energy or fat intake with any of the assessed infant characteristics.

We found that fat and energy concentrations varied within feed, within day and between day as expected. In agreement with previous studies, fat concentrations were higher in hind-milk than in both mid and fore-milk. The within-feed differences in fat concentrations consequently led to significant differences in breast milk energy concentrations.

4.3 Conclusion

Fat and energy concentrations in this study sample were similar to those recorded in other studies. Both fat and energy concentrations also varied within feeds and between days and diurnally as expected. However, milk intakes in this population were very low, hence

affecting both fat and energy intake amongst these exclusively breastfed infants. These results may suggest energy deficiency among exclusively breastfeeding infants in Potchefstroom. However, due to the experienced challenges and potential sources of error when performing such a four-day test weighing study, further studies need to be carried out using isotopic methods to confirm or repudiate the above findings. Isotopic techniques will help to establish whether mothers are exclusively breastfeeding in the first place and will be more applicable in this setting as it has no interference with the breastfeeding routine. Furthermore isotopic techniques allow for the study to be performed in a field setting so that mothers are able to go through the study while they are in their usual environment.

4.4 Recommendations

Exclusive breastfeeding is recommended for the first six months of life, and in other settings was proven to provide adequate fat and energy for optimal growth and development. Nonetheless, it is important that monitoring of intakes and infant growth be done to ensure that infants receive adequate nourishment. Mothers also need to be trained in effective feeding techniques such as correct positioning of infants and latching techniques so as to ensure that their infants are adequately fed. If the data of this research study are a true reflection of milk, fat and energy intakes of this study population, efforts need to be made to help mothers to adequately breastfeed their infants.

4.4.1 Support systems to encourage exclusive breastfeeding

Most mothers in this study were single and unemployed. For breastfeeding to be successful, a support system is necessary. Breastfeeding support groups can be created in the community for single mothers so that they can support and encourage each other, especially during the phase of exclusive breastfeeding. Support during breastfeeding can increase the amount of milk intake in infants (Nielsen *et al.*, 2011). Community peer counselling has been found to work well in developing countries to increase compliance with exclusive breastfeeding for the first six months of life (Nankunda *et al.*, 2006; Tylleskär *et al.*, 2011) and has been practised in other parts of South Africa (Tylleskär *et al.*, 2011). Hence, introduction of the concept throughout the country is likely to help, not only to increase compliance with exclusive breastfeeding, but it is likely to help increase milk production in the mothers and hence intake of the infants. The role of peer counsellors in the PROMISE-EBF study was to share breastfeeding information and to support and encourage lactating mothers, as well as to do referrals in cases when women had breastfeeding problems (Tylleskär *et al.*, 2011). It has been established that psychological stress experienced by lactating mothers can affect the release of oxytocin, hence affecting the amount of milk an

infant gets from its mother (Ueda *et al.*, 1994). It is likely that peer counselling can reduce psychological stress, which in turn may have an effect on milk ejection. However, it is important that research is carried out to establish effects of peer counselling on milk intakes.

4.4.2 Child growth monitoring

Mothers are encouraged to take their children for monthly monitoring. However, weight is the main indicator that is used to monitor growth. The Road to Health booklet has made provision for monitoring length or height. It is, however, not mandatory to monitor length or height as the IYCF policy emphasizes monitoring of weight only (Department of Health, 2007). Stunting is associated with a number of adverse outcomes that may be irreversible. Some of these adverse outcomes include cognitive deficits, poor schooling outcomes and poor productivity (Measelle *et al.*, 2016; Walker *et al.*, 2007; Ivanovic *et al.*, 2004; Victora *et al.*, 2008). However, should catch-up growth occur, there is likely to be no long-term cognitive deficit (Crookston *et al.*, 2010). In cases of severe stunting, catch-up growth may not be achievable (Crookston *et al.*, 2010). This therefore makes growth-monitoring imperative at an earlier stage, in order to intervene with an appropriate intervention, which will promote growth and development.

4.4.3 Monitoring for effective breastfeeding techniques

Mothers need to be taught correct positioning, latching and sucking of the infant on to the breast so that there is effective milk transfer to the infant. It is well established that improper breastfeeding techniques can affect the duration of breastfeeding especially exclusive breastfeeding (Cadwell, 2007; Mulder, 2006; Righard, 1998). However, it is not very clear to what extent it affects milk intake. Mothers, especially primiparous mothers, are likely taught effective breastfeeding techniques post-delivery in the maternity wards as it is a requirement of the mother-baby friendly initiative. It is, however, important that a follow-up be done to ensure that the mother breastfeeds her infant correctly. This may be incorporated into the growth monitoring plan, so that when mothers do their monthly visits the health-care workers will observe how they breastfeed so that they may correct any incorrect feeding techniques. Studies observing breastfeeding techniques and determining milk intake of infants need to be conducted to establish whether there is a relationship between the two.

As much as efforts can be made to assist mothers with increasing their milk production, it is important that further research be done in this field.

4.4.4 Further research

This study was done in a small purposive sample of exclusively breastfeeding mothers and their infants, hence the results of this study cannot be generalised to the wider population. It is imperative that a larger study be carried out using isotopic methods so that a true picture can be attained concerning breast milk intakes in exclusively breastfed infants in South Africa. Also if the sample is large enough, then associations of milk intake with maternal and infant characteristics can also be assessed. Isotopic methods will interfere less with the feeding routine and are more appropriate for field study. This will therefore allow for breast milk intakes to be assessed while mothers are in their respective homes, as a result minimizing the possible effects of anxiety and stress of being away from home. A further advantage would be that the researcher would not have to rely on self-reported exclusive breastfeeding but will be able to establish this from their analysis. Furthermore, it will be important that in future research energy intakes of the mothers be assessed, in order to establish whether the intakes of the infants are related to the energy intakes of the mothers.

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3 October 2015

Dr J Baumgartner
Nutrition

Dear Dr Baumgartner

HREC APPROVAL OF YOUR APPLICATION

Ethics number: NWU-00090-14-A1 Establishing the Iodine Requirement in Infancy: A Multi-Centre Metabolic Balance Study

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the Health Research Ethics Committee (HREC) secretariat.

Project title: Test weighing study to assess the breast milk volume and variation in energy and micronutrient content of breast milk over a 4 day period

Project leader/supervisor: Dr J Baumgartner

Student: SS Siro

Application type: Sub-study

Risk level descriptor: Minimal

You are kindly informed that at the meeting held on 10/09/2015 of the HREC, Faculty of Health Sciences, the aforementioned was approved.

The period of approval for this project is from 03/10/2015 to 30/11/2016.

After ethical review:

Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).

The HREC requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the protocol or other associated documentation must be submitted to the HREC prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form.

A progress report should be submitted within one year of approval of this study and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC must be notified if the study is temporarily suspended

or terminated. The progress report template is obtainable from Carolien van Zyl at Carolien.VanZyl@nwu.ac.za. Annually a number of projects may be randomly selected for an external audit.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.

Please note that for any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

The HREC complies with the South African National Health Act 61 (2003), the regulations on Research with Human Participants of 2014 of the Department of Health and Principles, the Declaration of Helsinki, 2013, the Belmont Report and the Ethics in Health Research: Principles, Structures and Processes (SANS document).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Ethics Office at Carolien.VanZyl@nwu.ac.za or 018 299 2089.

Yours sincerely



Prof Minrie Greeff
HREC Chairperson

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Applications\2015 Applications\Applications 08 - 10 September 2015\NWU-00090-14-A1 (J Baumgartner-SS Siro)\NWU-00090-14-A1 (J Baumgartner-SS Siro) - AL\NWU-00090-14-A1 (J Baumgartner-SS Siro) - AL.docm
03 October 2015

File reference: 9.1.5.3

ANNEXURE B-INFORMATION LEAFLET AND CONSENT FORM



PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

Establishing the iodine requirement in infancy: A multi-center metabolic balance study

REFERENCE NUMBERS: EK 2013-N-21 and NWU 00090-14-S1

PRINCIPAL INVESTIGATOR: Prof. Marius Smuts

CO-PRINCIPAL INVESTIGATOR: Dr. Jeannine Baumgartner

ADDRESS:

Centre of Excellence for Nutrition
North-West University
Faculty of Health Sciences
Private Bag X6001
2520 Potchefstroom

CONTACT NUMBER:

018 299 2086

You are being invited to take part in a research project with the goal to find out the right amount of the nutrient iodine required for optimal health of infants.

*Please take some time to read the information presented here, which will explain the details of this project. Please ask the researcher any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are free to withdraw from the study at any point, even if you do agree to take part.*

This study has been approved by the Ethics Committee of the Swiss Federal Institute of Technology in Zurich, Switzerland (EK 2013-N-21) and by the Health Research Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU 00090-14-S1) and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki and the ethical guidelines of the National Health Research Ethics Council. It might be necessary for the research ethics committee members or relevant authorities to inspect the research records.

What is this research study all about?

Iodine is a nutrient that is very important for proper growth and development of babies. As babies are growing quickly, they need much more iodine in relation to their body weight than adults. Therefore, it is important that young babies get the right amount of iodine with breast milk or formula milk. However, breast milk and infant formula on the market contain varying amounts of iodine and not enough research has been done on how much iodine a baby actually needs per day.

Therefore, the goal of this study is to find out the right amount of iodine that healthy babies need for optimal development during their first 6 months of life. In order to find out, we will study how much iodine babies receive from breast milk and how much of this amount is excreted again via stool, urine and saliva. We will then determine how much iodine the body of a healthy baby keeps back and this will be the amount needed by the baby's body.

Who can participate?

You can participate in this study if:

- You are healthy
- You have no known history of thyroid disease
- You have given birth to only one baby at a time (no twins)
- You are currently exclusively breastfeeding your baby (only feeding breast milk)

You cannot participate if:

- You received X-ray or CT contrast agent or iodine containing medication within the last year
- You are moderately anaemic (tested during pre-screening)
- You are currently smoking

- You have been tested HIV positive during pregnancy (information will be obtained from Road to Health booklet)

Your baby can participate in this study if:

- He or she is 2-5 months old (during the study)
- He or she is healthy
- He or she is currently being exclusively breast fed (only receiving breast milk)
- He or she was born at full-term (in week 38 to 42)
- He or she had normal birth weight (above 2500 g)
- He or she has no known history of thyroid disease

Your baby cannot participate in this study if:

- He or she is receiving infant formula or solid food
- He or she is receiving iodine-containing supplements
- He or she is moderately anaemic (tested during pre-screening)

What will your responsibilities be?

The aim of this study is to measure all iodine that your baby receives from your breast milk and all iodine that your baby excretes in his/her urine, stool and saliva. To do so, we will need you and your baby to come and stay at the research clinic of the Centre of Excellence for Nutrition at the North-West University for 5 days (5 nights) from Monday Morning to Saturday Morning.

What will be your responsibility during the pre-screening (done at information event):

In order to make sure that you and your baby are fulfilling the inclusion criteria, we will ask permission to obtain data from your baby's Road to Health Chart (including data on your HIV status during pregnancy) during the information meeting at NWU. We will also ask you that a qualified nurse does a finger prick to obtain a blood spot from you and a heel prick to obtain a blood spot from your baby to test whether you or your baby is anemic or not. We will ask separate permission from you to collect a blood spot from you and your baby, and to get information from your baby's Road to Health booklet (pre-screening). By signing the permission to participate in the pre-screening you will not be forced to participate in the main study, and you will be given time to think about it before we will contact you to make an appointment for the main study.

What will be your responsibility during the study at the research clinic?

During the first day at the research clinic we will:

- Ask you questions regarding you and your baby's health, feeding habits, and socio-economic status
- Measure your and your baby's body temperature
- Collect a urine sample from you
- Collect a urine sample from your baby using a special urine collection pad
- Collect a 5 ml blood sample from you and a 2 ml (ca. 1 teaspoon) blood sample from the arm of your baby
- Measure your height and weight
- Measure your baby's length and weight
- Collect a breast milk sample (5 ml of fore milk) each time you are feeding your child (1 time per day, we will also collect a mid-feed and hind milk sample)
- Weight your baby before and after each feeding session

We will collect a blood sample from you and your baby to monitor to check whether your and your baby's thyroid (organ that produces important hormones) is functioning properly. We will also measure other nutrients in your blood including but not limited to iron, vitamin A and essential fatty acids. In blood, we will also measure whether you or your baby have any infection at the time of the study (e.g. from a flue).

Furthermore, we will ask you to bring along a 5 g salt sample from your household, so that we can determine how much iodine is in the salt that you consume.

During the second to last day at the metabolic clinic (Tuesday to Saturday morning) we will:

- Collect 24h urine from you (all your urine)
- Measure your and your baby's body temperature
- Collect a breast milk sample (5 ml of fore milk) each time you are feeding your child (1 time per day, we will also collect a mid-feed and hind milk sample)
- Weight your baby before and after each feeding session
- Collect all the soiled diapers and cleaning tissues from your baby
- Collect a saliva sample (1 ml, ca. 1/2 teaspoon) once a day from your baby

We will collect the urine, the soiled diapers and cleaning tissues as well as the breast milk and saliva samples and measure their content of iodine at our iodine laboratory at the North-West University and at a laboratory at ETH Zürich, Switzerland. If you give permission, we will also measure other nutrients in your breast milk including but not limited to iron, vitamin A and essential fatty acids.

Only professional nurses and nursing assistants will work with your baby during your stay. At least one professional nurse and one nursing assistant will be with you at the metabolic clinic at any time during 24 hours.

Will you benefit from taking part in this research?

By participating in this study you will find out more about your and your baby's nutritional health, as we will not only determine your and your baby's iodine, iron, essential fatty acid, and vitamin A, and thyroid hormone status. You will also find out more about the energy content of your breast milk.

The bigger benefit will be to the research community in gaining a better understanding on the iodine requirements of infants, which is important for the monitoring of iodine nutrition in infants in South Africa and the rest of the world.

Are there risk in you taking part in this research?

Collecting a blood sample from the arm of you and your baby (only once during screening day) can sometimes lead to bruising. In order to minimize this risk and discomfort for you and your baby, the blood will only be collected by nurses with adequate training and experience.

In total, 4 mother-baby pairs will be staying at the research clinic. Therefore, there is an increased risk for cross-infection should a baby or mother become sick during the course of the study. To minimize this risks, we will make sure that the research clinic is cleaned as frequently as possible and that the clinic will be properly aired. The nurses will furthermore monitor the health of all participating mothers and their babies by measuring body temperature daily. Should you or your baby become sick during the study, we will have to exclude you from the study (without loss of any benefits) and will refer you to your health clinic/house doctor.

We will also make sure that your privacy is not affected during this study by giving you your own private room (equipped with baby cot) in the clinic. Furthermore, all the measurements will be performed in private rooms and all the personnel/researchers working with you or your baby will be female.

Who will have access to the data?

We will also handle all your information as confidential as possible by allocating a study code to you and your baby. All samples will be labelled with this code and only the principal investigator and co-principal investigator will have access to the records containing your name. Only the researchers will work with your data. Data will be kept safe and secure by locking hard copies in locked cupboards in the researcher's office and for electronic data it will be password protected. Reporting of findings will be anonymous.

What will happen in the unlikely event of some form of discomfort occurring as a result of you taking part in this research study?

Please let us know if you experience any physical or emotional discomfort during or after participating in the study and we will make appropriate arrangements for you to talk to a medical doctor or psychologist.

Will you be paid to take part in this study and are there any costs involved?

You will receive a reimbursement to the value of ZAR 900 for the time you spend at the research clinic, during which you are not able to work. Furthermore, you will receive a gift hamper with goods for you and your baby (toys, clothes, receiving blanket, toiletries) as a token of appreciation and an airtime voucher to a value of ZAR 100 to communicate with family members and friends during your stay at the research clinic at the start of the baseline study.

Furthermore, we will arrange transport for you and your baby to come to the research clinic on the first study day, as well as to go home at the final day of the study.

We will provide you with meals and snacks, as well as diapers, toiletries and towels that you and your baby will need during the period of your stay in the research clinic. Furthermore, entertainment like television, magazines and newspapers will be available to you to make your stay more pleasant. You will be allowed to have visitors (family

members) on specific days and times. Thus, there will be no costs involved for you, if you take part.

Is there anything else that you should know or do?

- You can contact Prof. Marius Smuts at 018 299 2086 or Dr. Jeannine Baumgartner at 018 299 4018 if you have any further queries or encounter any problems.
- You can contact the Health Research Ethics Committee via Mrs Carolien van Zyl at 018 299 2094; carolien.vanzyl@nwu.ac.za if you have any concerns or complaints that have not been adequately addressed by the researcher.
- You will receive a copy of this information and consent form for your own records.

How will you know about the findings?

We will give you immediate feedback of results that we can determine during the study, namely whether you and your baby are anaemic or not. We will also be able to give you information regarding the energy content of your breast milk during the course of the study.

However, be aware that it will take time to perform the other analyses and that the results will only be available after several months. Once the study is completed and all the results are available, we will invite you to another information event at the North-West University at which we will present to you the study findings and in single sessions provide you with your individual results. Should we find an abnormal value during our analyses that needs medical attention, we will inform you immediately and refer you to your health clinic or house doctor.

Declaration by participant

By signing below, I agree to take part in a **pre-screening** for a research study entitled: *Establishing the iodine requirement in infancy: A multi-center metabolic balance study*

I declare that:

- I have read this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions to both the person obtaining consent, as well as the researcher and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the researcher feels it is in my best interest, or if I do not follow the study plan, as agreed to.

Signed at (*place*) on (*date*) 20....

.....
Signature of participant

.....
Signature of witness

Declaration by person obtaining consent

I (*name*) declare that:

- I explained the information in this document to
- I encouraged her to ask questions and took adequate time to answer them.
- I am satisfied that she adequately understands all aspects of the research, as discussed above
- I did not use an interpreter.

Signed at (*place*) on (*date*) 20....

.....
Signature of person obtaining consent

.....
Signature of witness

Declaration by researcher

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did not use an interpreter.

Signed at (*place*) on (*date*) 20....

.....
Signature of researcher

.....
Signature of witness

Declaration by participant

By signing below, I agree to take part in a research study entitled: *Establishing the iodine requirement in infancy: A multi-center metabolic balance study*

I declare that:

- I have read this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions to both the person obtaining consent, as well as the researcher and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the researcher feels it is in my best interest, or if I do not follow the study plan, as agreed to.

Signed at (place) on (date) 20....

.....

Signature of participant

.....

Signature of witness

Declaration by person obtaining consent

I (name) declare that:

- I explained the information in this document to
- I encouraged her to ask questions and took adequate time to answer them.
- I am satisfied that she adequately understands all aspects of the research, as discussed above
- I did not use an interpreter.

Signed at (place) on (date) 20....

.....

.....

Signature of person obtaining consent

Signature of witness

Declaration by researcher

I (*name*) declare that:

- I explained the information in this document to
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did not use an interpreter.

Signed at (*place*) on (*date*) 20....

.....
Signature of researcher

.....
Signature of witness



Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich
Institute of Food, Nutrition and Health
Laboratory of Human Nutrition

CASE REPORT FORM

“A balance study in breast fed infants to establish the iodine requirement in infancy”

Date (DD/MM/YY): / /

Subject Code:

INFANT		<input type="checkbox"/> female <input type="checkbox"/> male	MOTHER	
Date of birth:	<input type="text"/> / <input type="text"/> / <input type="text"/>		Date of birth:	<input type="text"/> / <input type="text"/> / <input type="text"/>
Age (in weeks):	<input type="text"/>		Age (in years):	<input type="text"/>
Pregnancy week of delivery:	<input type="text"/>		Nationality:	<input type="text"/>
Delivery between pregnancy week 38 and 42*:	<input type="checkbox"/> Yes* <input type="checkbox"/> No		Ethnicity:	<input type="checkbox"/> Black <input type="checkbox"/> Colored <input type="checkbox"/> Indian of Asian <input type="checkbox"/> White
Birth weight:	<input type="text"/> g		Residing in since:	
Birth weight \geq 2500 g*:	<input type="checkbox"/> Yes* <input type="checkbox"/> No		at least 1 year <u>before</u> delivery	<input type="checkbox"/> Yes <input type="checkbox"/> No
Birth length:	<input type="text"/> cm		since delivery (together with infant)	<input type="checkbox"/> Yes <input type="checkbox"/> No
Delivery:	<input type="checkbox"/> vaginal <input type="checkbox"/> cesarian		History of Thyroid Disease*:	<input type="checkbox"/> Yes <input type="checkbox"/> No*
History of Thyroid Disease*:	<input type="checkbox"/> Yes <input type="checkbox"/> No*		If YES, please specify:

Date (DD/MM/YY): |_|_|/|_|_|/|_|_|

Subject Code:

INFANT	
Good health*:	<input type="checkbox"/> Yes* <input type="checkbox"/> No
If NO, please specify:
Is your baby on any medication?	<input type="checkbox"/> Yes <input type="checkbox"/> No
If YES, please specify:	Name of medication:
Does your baby have any disability (including birth defects)?	<input type="checkbox"/> Yes <input type="checkbox"/> No
If YES, please specify:
Does your baby have any illness or disease?	<input type="checkbox"/> Yes <input type="checkbox"/> No
If YES, please specify:
Average number of regurgitation episodes per day:	_ _
Average number of colics per day:	_ _
Average number of stools per day:	_ _
Usual consistency of stools:	<input type="checkbox"/> Liquid <input type="checkbox"/> Semi-liquid <input type="checkbox"/> Solid

MOTHER	
Good health:	<input type="checkbox"/> Yes* <input type="checkbox"/> No
If NO, please specify
Are you on any medication:	<input type="checkbox"/> Yes <input type="checkbox"/> No
If YES, please specify:	Name of medication:
Information from pregnancy road to health card:*	
HIV Test Result:	<input type="checkbox"/> Tested HIV positive <input type="checkbox"/> Tested HIV negative* <input type="checkbox"/> Not tested
Do you smoke at present?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Did you smoke before pregnancy?	<input type="checkbox"/> Yes <input type="checkbox"/> No
Within the last year: Use of ... ?	
X-ray / CT contrast agent*	<input type="checkbox"/> Yes <input type="checkbox"/> No*
Iodine containing medication (e.g. Amiodaron as antiarrhythmic agent)*	<input type="checkbox"/> Yes <input type="checkbox"/> No*
Are you currently employed? <input type="checkbox"/> Yes <input type="checkbox"/> No	
Marital status?	<input type="checkbox"/> Married <input type="checkbox"/> Single <input type="checkbox"/> Living together <input type="checkbox"/> Divorced / separated <input type="checkbox"/> Widowed
Educational level?	<input type="checkbox"/> Primary (grade 1-7) <input type="checkbox"/> Secondary (grade 8-12) <input type="checkbox"/> Tertiary (college/university) <input type="checkbox"/> None <input type="checkbox"/> Other:

Date (DD/MM/YY): I _ I _ / I _ I _ / I _ I _

Subject Code:

MOTHER NUTRITION	
Use of supplements during pregnancy:	<input type="checkbox"/> Yes <input type="checkbox"/> No
If <u>YES</u> , please specify:	Name of supplement: How often did you take the supplement? <input type="checkbox"/> Daily <input type="checkbox"/> I _ I times per week
Use of supplements at the moment:	<input type="checkbox"/> Yes <input type="checkbox"/> No
If <u>YES</u> , please specify:	Name of supplement: How often did you take the supplement? <input type="checkbox"/> Daily <input type="checkbox"/> I _ I times per week
Where do you buy your salt?	<input type="checkbox"/> Supermarket <input type="checkbox"/> Spaza shop <input type="checkbox"/> Other:

INFANT NUTRITION		
Breast Milk		
Is the infant currently exclusively fed with breast milk?*	<input type="checkbox"/> Yes*	I _ I feedings per day
	<input type="checkbox"/> Yes, except for*	<input type="checkbox"/> Water <input type="checkbox"/> Tea <input type="checkbox"/> Juices <input type="checkbox"/> Other:
	<input type="checkbox"/> No	
If YES or YES EXCEPT FOR: Since when is the infant fully breast fed?		Since I _ I _ / I _ I _ M Y
Infant Formula		
Has the infant <u>ever</u> received infant formula?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
If YES: Does the infant <u>currently</u> receive infant formula?	<input type="checkbox"/> Yes	Received infant formula <u>only</u> before: I _ I _ / I _ I _ M Y
	<input type="checkbox"/> No*	
Semisolid food, solid food, fluids with nutritional value (except tea and juices), supplements		
Is the infant <u>currently</u> receiving semisolid food, solid food and/or fluids with nutritional value (e.g. porridge, pap, cereal) (in addition to / instead of breast milk)?*	<input type="checkbox"/> Yes <input type="checkbox"/> No*	
If <u>YES</u> , please specify:		
Is the infant <u>currently</u> receiving iodine containing supplements?*	<input type="checkbox"/> Yes <input type="checkbox"/> No*	
If <u>YES</u> , please specify:		

INFANT DIAPERS	
Which diaper do you currently use?	Diaper brand name: Diaper size:

Date (DD/MM/YY): / /

Subject Code:

COMMENTS
.....
.....
.....

INCLUSION CRITERIA (see *) FULLFILLED:	<input type="checkbox"/> Yes	<input type="checkbox"/> No
WRITTEN INFORMED CONSENT SIGNED:	<input type="checkbox"/> Yes	<input type="checkbox"/> No

Name investigator	Signature investigator	Place, Date (DD/MM/YY)
.....

ANNEXURE D-FEEDING DIARY

Study day X	[Date]	[Infant]
--------------------	---------------	-----------------

Feeding 1		(sample label)
Time feeding (HH:MM, 24h format):	_ _ : _ _	
Foremilk sample collected? (Desired: 4 ml)	<input type="checkbox"/> Yes: ca. _ _ ml <input type="checkbox"/> No	
Weight infant before feeding:	_ _ _ _ g	
Weight infant after feeding:	_ _ _ _ g	
Breast milk consumption during feeding:	_ _ _ g	
Comments:		
Name investigator/nurse:		

Feeding 2		(sample label)
Time feeding (HH:MM, 24h format):	_ _ : _ _	
Foremilk sample collected? (Desired: 4 ml)	<input type="checkbox"/> Yes: ca. _ _ ml <input type="checkbox"/> No	
Weight infant before feeding:	_ _ _ _ g	
Weight infant after feeding:	_ _ _ _ g	
Breast milk consumption during feeding:	_ _ _ g	
Comments:		
Name investigator/nurse:		

Feeding 3		(sample label)
Time feeding (HH:MM, 24h format):	_ _ : _ _	
Foremilk sample collected? (Desired: 4 ml)	<input type="checkbox"/> Yes: ca. _ _ ml <input type="checkbox"/> No	
Weight infant before feeding:	_ _ _ _ g	
Weight infant after feeding:	_ _ _ _ g	
Breast milk consumption during feeding:	_ _ _ g	
Comments:		
Name investigator/nurse:		

ANNEXURE E-CREAMATOCRIT RECORDING FORM

Study day: Date: / / Infant code:

Feed 1 (sample label)			
	Creamatocrit	Fat (g/L)	Energy (KJ)
Fore-milk sample	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
Mid-feed sample	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
Hind-feed sample collected?	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
Comments:			
Name investigator/nurse:			

Fore-milk samples				
Feed number	Sample label	Creamatocrit	Fat (g/L)	Energy (KJ)
2		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
3		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
4		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
5		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
6		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
7		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
8		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
9		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
10		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
11		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
12		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
Pooled sample		<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> %	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/>
Comments:				
Name investigator/nurse:				

ANNEXURE F-FOOD DIARY

Study day: I _ I _ I

Participant code: I _ I _ I

Person responsible for recording _____

Please record every food item eaten per day and the quantities eaten. Please also include water and fluid (juices and drinks) taken.

Time	Food item	Qty

