Subclinical mastitis and HIV-1 in South African women

Juana Francisca Willumsen

University College London

PhD

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Abstract

HIV is transmitted from mother-to-child through breastmilk and without antiretroviral prophylaxis can account for up to half of the vertical transmission observed. Virus may be present in the milk, but not result in infection in the breastfed infant and little is known about which factors influence viral shedding.

Two studies were conducted in Durban. South Africa, an area of high HIV seroprevalence, to test the hypothesis that during subclinical mastitis (as indicated by raised breastmilk sodium:potassium ratio (Na/K)) viral shedding into breastmilk increases and high levels of breastmilk inflammatory cytokines cause damage to the infant intestinal mucosa (thereby allowing virus to enter the infant circulation.

In a cross-sectional study the prevalence of subclinical mastitis among 321 lactating mothers of unknown HIV status was 25.7% (only in one breast in 18.5% of all mothers). Breastmilk Na/K correlated with the inflammatory cytokine IL-8. Mothers who had exclusively breastfed their infant in the previous 24 hours had lower Na/K ratios than those who had supplemented breastmilk with formula. Interestingly, mothers who had given complementary foods in addition to breastmilk had the lowest Na/K ratios.

A cohort study of 145 HIV-infected women and their infants during the first 3 months of lactation breastmilk Na/K ratio is strongly associated with viral load and there is an interaction between feeding mode and Na/K. Exclusive breastfeeding during early lactation resulted in significantly lower breastmilk viral load than mixed feeding, but this effect was reduced at later time points. There was no association between breastmilk IL-8 and infant intestinal permeability. It is important to note that breastmilk Na/K only predicted between 11 and 26% of viral load and any intervention to reduce the prevalence of subclinical mastitis would be expected to have a small impact on the overall rate of mother-to-child transmission of HIV.

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1: Background

1.1 South Africa and study site

1.1.2 Geography and population

South Africa has a land mass of 1,219,000 square km which since 1994 has been divided in to 9 provinces (see figure 1.1.1). It is considered to be a country "in transition" from developing to developed nation, with rapid economic development, good infrastructure and telecommunications networks and a very wealthy elite minority. However, for many South Africans life is still a hard struggle, based on subsistence agriculture in poorly serviced rural areas. In addition, the legacy of apartheid has meant that generations have been denied education and unemployment rates are high. There is still a strong traditional cultural base and life for many Black Africans is little different from that in neighbouring, developing countries. This situation means that South Africa is a unique location, combining First World facilities with Third World health and social problems. In addition, the rapid spread of HIV/AIDS in recent years has raised awareness of the inequity in health and social provision to large sectors of the population and poses South Africa with an enormous challenge over the next few decades. How the country responds to this crisis and lessons that can be learnt from this experience will hopefully serve many other African countries and could shape policy on a larger scale.

Figure 1.1.1: Map of South Africa indicating provincial boundaries after democratic elections in 1994

Taken from Globetrotter Guide to South Africa, by Peter Joyce, 1999.

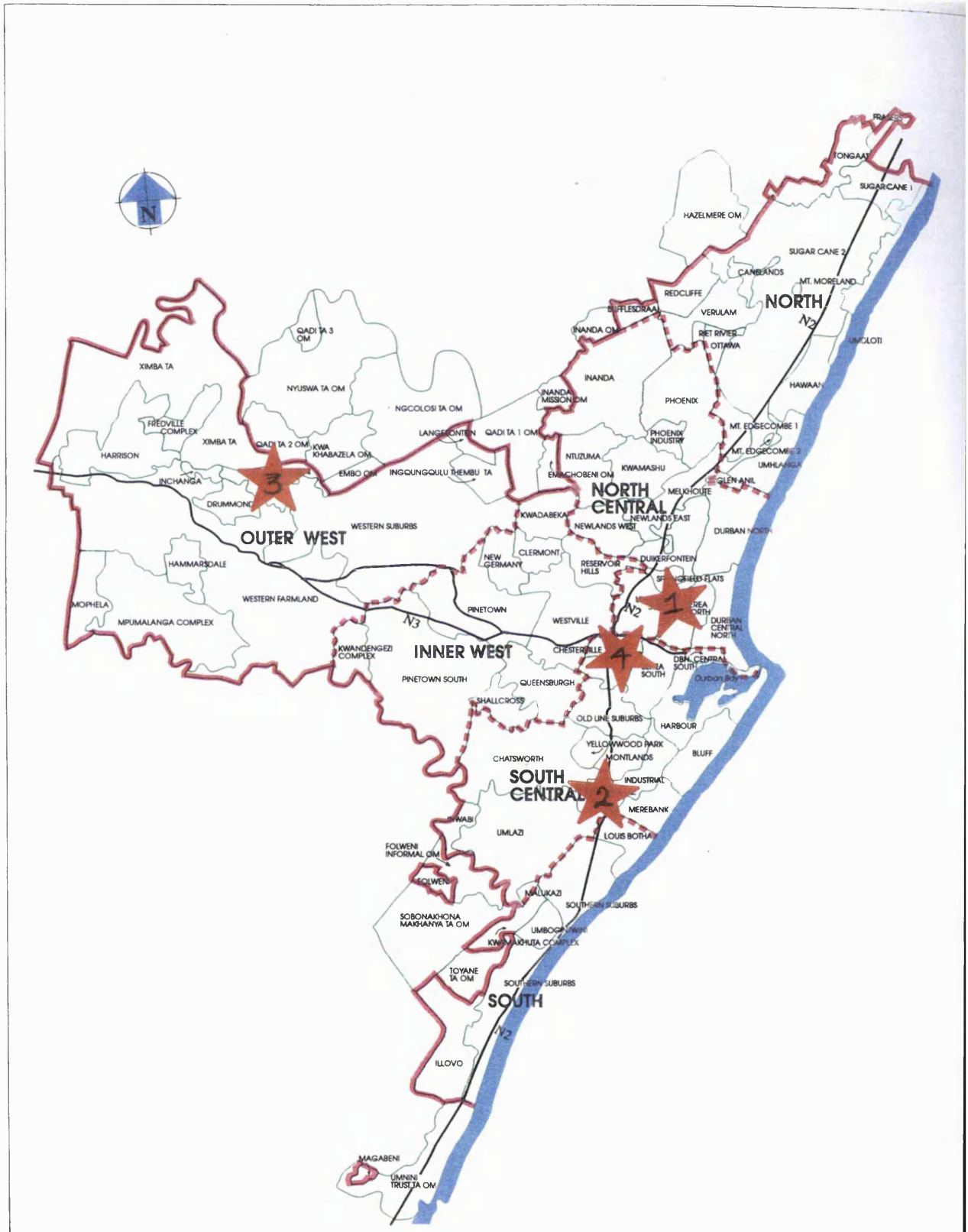
According to the census of October 1996 (1), Kwa-Zulu Natal (KZN) occupies 7.6% of the land mass, yet is home to 8.4 million people, or 20.7% of the country's population of approximately 40.5 million. In KZN nearly 82% of the population are Black African and 9% Indian, and over 56% live in rural areas. The majority language spoken is iZulu (the mother tongue of 80% of the population) and only 1% of those living in KZN were born outside South Africa.

Nearly 23% of the adult population has received no schooling, and of these over 61% are women. 39% of those aged 15-65 are unemployed, and 27% of those in employment earn less than R500 (about £50) per month, whereas GNP (gross national product) per capita is estimated at US$3160 (2). KZN has the third highest percentage of the population with no schooling or unemployed in the country. 32% of the population live in traditional dwellings and an additional 11% in informal settlements. Almost 30% of households use wood for cooking, over 60% do not have an inside tap and 15% have no toilet facilities (1). KZN has the third highest percentage of population using wood for cooking and without toilet facilities in the country, behind Northern Province and the Eastern Cape. National infant mortality is estimated to be 50/1000 (1996), maternal mortality 230/10,000 births and life expectancy 65 years (2).

Durban is the third largest city in South Africa and the largest in KZN, with a population estimated at between 2.1 and 2.8 million (3;4). It is a major port and holiday destination, with a large industrial sector, including oil refinery. There are two large (black) townships to the north and south of the city and a number of informal settlements. A map of the Durban Metropolitan area is shown in figure 1.1.2. Some of the informal settlements are now being developed, houses are being built and facilities provided, but the rate at which new homes are erected does not meet demand and many households remain without basic facilities. Figure 1.1.2 below indicates the extent of the Durban Metropolitan area and the location of the study sites involved in this project (McCord's Hospital, Lamontville Clinic, Valley Trust Primary Health Care Centre and Cato Manor Clinic) are shown.

Figure 1.1.2: Durban Metropolitan Area settlement areas 1996

1 = McCord Hospital; 2 = Lamontville Clinic; 3 = Valley Trust PHC; 4 = Cato Manor Clinic



SETTLEMENT AREAS & POPULATION ESTIMATE PROJECT 1995

Metropolitan Boundary (January 1996)
Metropolitan Transitional Substructure Boundary (January 1996)
Cluster Boundary
Roads
OM Indicates if an area lies outside the Metropolitan area

Urban Strategy Department • Corporate Services • City of Durban
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Reference to this material must be fully acknowledged

DURBAN METROPOLITAN AREA
Spatial Clustering

Map 2

1.1.3 Political history

In the late 1940s and early 1950 the white South Africa government introduced the policy of racial segregation, known as apartheid. The basic principles of this were to keep the Black, Indian, Coloured and White population separate, to protect employment opportunities for whites and restrict blacks to live in designated "homelands" or townships outside the urban and economically active areas. The need for low-cost black manual labour especially in the mining industry led to an increase in the use of migrant workers, who were housed in single men's hostels far from their homes. This system of migrant labour led to family instability and men only returned to their families and homes during vacations (5). Many men from the former homeland of Zululand worked in the mining industry or as migrant workers in Durban, where a number of men's hostels are still standing today. All facilities, including health services, were segregated and it was considered that the education provided to black school pupils should only be in line with the likely requirements of the labour market and very basic.

The first multiracial, democratically elected government came to power in 1994 and instituted a number of changes aimed at redressing the imbalance of the past. However, the poor economic state of the country at that time meant that plans for housing, water and sanitation development, job creation have not yet yielded the promised results. Children under 6 years of age and pregnant women are eligible for free health care, schools are no longer segregated on racial lines and affirmative action and racial equality targets which aim to empower the black population have been put into action. South Africa remains a divided and polar society, with enormous economic disparity, which is now not solely along racial lines, but remains predominantly so.

1.2 The HIV epidemic in South Africa and KwaZulu-Natal

1.2.1 History of the HIV epidemic

In the 1980s HIV was not regarded as a problem for South Africa; the epidemic was considered to be restricted to the homosexual community and there was widespread denial of the issue. The first group to test positive for HIV were migrant mine workers. Since 1990 sentinel surveillance of HIV seroprevalence at government antenatal clinics (ANC) has been carried out in each province on an annual basis. In 1990 the national average seroprevalence of HIV was 0.76%, but by 1994 this had risen to 7.5% in antenatal surveys. After the change of government in 1994 the four provinces and the homelands were merged and divided into 9 provinces (see figure 1.1.1). The geographic boundaries of KwaZulu-Natal were not significantly changed, and so data are available for KZN from 1990. However, for the other provinces data is only available since 1994 (6).

In 1991 the prevalences of HIV infection in Uganda, Zambia and Zimbabwe were 27.6%, 24.5% and 17.1% respectively, whereas in South Africa the national average was 1.35%. It is estimated that South Africa will reach 27% seroprevalence in 1999, which is eight years later than Uganda, seven years after Zambia and five years after Zimbabwe, suggesting a southward movement of the epidemic. In South Africa the epidemic is essentially clade C restricted and heterosexually and vertically transmitted.

In 1994 14% of the women surveyed at antenatal clinicsin KZN were infected with HIV, compared with the national seroprevalence at antenatal clinics of 7.57%. KZN has consistently had a much higher seroprevalence than the rest of the country. There are a number of reasons that may account for this:

- The high population density in the province, with 46% of the population under 20 years of age
- High unemployment and poor socio-economic conditions
- The migration of large numbers of workers from their homes in KZN to the gold mines of Gauteng province, which has been occurring since the time of apartheid and continues today
- KZN has two very busy sea ports (Durban and Richard's Bay) and many important transport routes to northern countries (6).

1.2.2 Current HIV prevalence estimates

The most recent results available are from the 1998 antenatal clinic (ANC) surveillance exercise carried out in November of that year, when all women booking for antenatal care at selected clinics in that month are anonymously tested for HIV. This indicates that 22.8% of women nationally and 32.5% of the black women in KZN attending antenatal clinics are infected with HIV (6). Previous estimates indicate that the seroprevalence is higher in urban areas than rural areas (2), which would suggest that the seroprevalence in Durban could be higher than the 32.5% provincial average. In KZN, about 20% of the women who were infected with HIV were in the 20-29 age category. Many factors contribute to the high prevalence in younger women, including a preference for male partners older than themselves, who have greater economic power and a preference by older males for young female partners, who are thought to be "free of disease". The seroprevalence is higher than the provincial average in the townships around Durban, which are areas of extreme poverty (44% for KwaMashu to the north and 36.6% for Umlazi to the south of the city in 1999). In addition, at the informal settlement of Cato Manor seroprevalence is estimated to be 41.4%, as indicated in early results of the ANC surveillance study in 1999 (6).

In Durban state primary health care is provided by City Health Department clinics serving the local population. There are currently 24 purpose-built clinics and a further 18 operating from church halls and other public spaces on a weekly or fortnightly basis (City Health Department personal communication, 2000). Women are required to book for antenatal care at their local City Health Department clinic and will be referred to a tertiary centre if needed. Most women

deliver in one of the hospitals, although there are plans to open some City Health Department clinics 24 hours a day with on-site facilities for normal deliveries. The main centre for tertiary care is the King Edward VIII teaching hospital at the University of Natal Medical School, with another 6 tertiary hospitals serving the city and surrounding townships (4).

The current South African Ministry of Health policy does not include the provision of antiretroviral drugs for the reduction of mother-to-child-transmission of HIV. In addition, there has been recent controversy, with the president expressing concerns as to the safety of zidovudine. The recent results from Uganda of a trial of intrapartum nevirapine plus a dose to the infant (7) at very low cost were greeted with much excitement in South Africa and it is hoped that as soon as results from a similar trial being carried out in South Africa are available that this drug regime will be adopted as national policy by the Minister of Health.

1.3 Collaborative links and selection of study location

Collaborative links between the Centre for International Child Health and the Department of Paediatrics and Child Health at the University of Natal Medical School were first established in 1996. As a result of this a number of research projects have been jointly designed and carried out. Further details of how the current project was established are provided in Chapter 3. This academic link has also provided training in laboratory skills to personnel in Durban and visits to both sites by researchers to further develop studies and collaborate on statistical analysis.

Durban is the largest urban centre in KZN, which is the most densely populated, relatively socio-economically disadvantaged province with the highest HIV seroprevalence in South Africa. The sector of the population utilising the free mother and child health services provided by City Health Department clinics is generally poor, unemployed, with little education, poor housing conditions and very high seroprevalence of HIV compared with other sectors of society. Many people have migrated to the city in search of work, yet still retain their links with their rural homes and hold traditional cultural beliefs. For many of these HIV-infected pregnant women, infant feeding options are limited due to financial and social constraints; formula alone will cost R150 (£15) per month to purchase and exclusive formula feeding may be seen as disclosure of

HIV status. Given these conditions, Durban serves as an ideal location in which to investigate factors associated with MTCT among HIV-infected women who chose to breastfeed with the hope of making recommendations on ways to improve breastfeeding practices among HIV-infected women in order to reduce the risk of breastmilk transmission of HIV. Further details of the study sites involved in this project and how these were established as research sites are provided in Chapter 3.

2: Introduction

2.1 Mother-to-child-transmission of HIV

The global spread of HIV through heterosexual contact has lead to a high prevalence of infection among sexually active women of reproductive age (2). The overwhelming majority of paediatric HIV infections are a result of mother-to-child transmission (MTCT) of the virus, either during pregnancy (8), delivery (9) or the postnatal period (10-12). With the increase in numbers of women of child-bearing age infected with HIV the number of paediatric HIV infections will also rise, with a substantial impact on infant and child mortality, especially in developing countries where the vast majority of HIV-infected women live.

In the absence of therapeutic interventions an estimated 15-20% of HIV-infected mothers in Europe and North America and 25-35% in Africa, India and Thailand will transmit the virus to their infant (13). UNAIDS estimates that MTCT accounted for over half of new infections in 1998, of which 90% were in Africa (14). The relative importance of *in utero*, intrapartum and postnatal transmission can only be estimated, but there is a consensus that a substantial proportion of transmission occurs in late pregnancy or during delivery (13;15;16) with MTCT also associated with breastfeeding (17). Vertical transmission is a multifactorial event, which is influenced by a number of variables. In order to develop targeted interventions to reduce the risk of transmission it is important to identify the maternal and infant factors associated with the risk of transmission. As the main objective of this thesis relates to MTCT of HIV through breastfeeding, only a brief overview of the risk factors for transmission during pregnancy and delivery will be included.

2.1.1. Risk factors for perinatal transmission of HIV

Most studies of the association of maternal characteristics and risk of perinatal transmission have been carried out in developed countries where few HIV-infected women breastfeed their infant. Risk factors which have been shown to be important in some studies include age (18), genetic determinants of immune responses (19), level of HIV-induced immunodeficiency (20) and viral characteristics (21). Co-infection with sexually transmitted diseases, chorioamnionitis (22) and placental inflammation (23-25) may increase the risk of transmission through increased viral load in the cervical tract or disruption of the placental or amniotic barrier and exposure of the fetus to HIV.

A number of studies have reported increased risk of transmission with high maternal viral load (26-30), although only one study has proposed a threshold below which transmission is unlikely to occur (31). Maternal viral load increases as disease progresses and in one study when adjusted for maternal CD4+ count near the time of delivery, women with detectable HIV RNA load were shown to be almost six times as likely to transmit HIV as women with undetectable viral load (32). Mofenson *et al.* reported that among 480 women who all received zidovudine before or from early pregnancy, the only independent risk factor for transmission was maternal baseline (third trimester) HIV RNA levels, with a 2.4 increase in odds ratio per log increase in copy number. None of the 84 women with undetectable levels of HIV RNA at baseline or the 107 with undetectable levels at delivery transmitted to their infant (15). Even when a reduced period of zidovudine therapy is used, as in the short-course regimen from Thailand in which therapy was initiated at 36 weeks gestation, the reduction in maternal HIV viral load between 36 weeks and delivery explained about 80% of the 50% reduction mother-to-child transmission *in utero* and intrapartum (mothers did not breastfeed) (33). Such a short course of antiretroviral therapy has also been shown to be effective in reducing MTCT even among women who breastfeed, with a 37% reduction in Cote d'Ivoire at 3 months (34) and 38% reduction in a multicentre trial in West Africa at 6 months (35) and 30% at 15 months (36).

The relationship between maternal antibodies to HIV and the risk of MTCT remains unclear (37;38). Potential risk factors amenable to practical interventions that could reduce the risk of perinatal transmission include ascending infections and maternal viral load, although to reduce the latter would require antiretroviral therapy, which is not available to most people in developing countries.

However, maternal characteristics and viral load are not the only factors important for transmission. It is estimated that between two thirds to three-quarters of perinatal MTCT occurs during delivery (16;39;40) in a non-breastfeeding population. Intrapartum events such as long duration of labour, extended time between rupture of membranes and delivery (41), prematurity (20;26) and events during delivery which expose the infant to mother's blood (42) have all been associated with increased risk of MTCT (13). Studies of twins have indicated that exposure to virus in cervical secretions during delivery is an important risk factor, as first born twins are more likely to be infected, especially after vaginal delivery (43;44).

Elective caesarean section has long been proposed as a means of reducing fetal exposure to HIV-laden maternal secretions or blood and a possible intervention to reduce MTCT. Some retrospective studies of mode of delivery indicate that perinatal MTCT could be reduced to half by caesarean section, even after adjusting for maternal factors (45;46) including viral load (47), whereas other studies do not (18). A randomised trial of mode of delivery for HIV-infected women carried out in Europe confirms the findings of observational studies that elective caesarean sections reduced the rate of transmission from 10.5% in women randomised to vaginal delivery to 1.8% in those randomised to caesarean section (48). Reports from a meta-analysis of 15 prospective cohort studies indicate that the risk of transmission is reduced by 87% if elective caesarean section is carried out in women who also received antiretroviral therapy during pregnancy (46) and there may be an interaction between zidovudine therapy and caesarean section (41;49). If antiretroviral drugs are not given during pregnancy, mode of delivery may not be as important a risk factor for transmission as cervicovaginal infection, duration of rupture of membranes and maternal p24 antigenemia (41).

The geographical differences in transmission rates may represent differences in relative importance of associated risk factors in different settings. Most studies of perinatal MTCT risk factors have been carried out in Europe and North America and have concentrated on maternal viral and immunological characteristics that could be amenable to antiretroviral or immuno-modulatory interventions. Interventions at the time of delivery to reduce infant exposure to maternal secretions containing virus, such as caesarean section, pose little added health risk to the mother in developed countries. All these potential interventions also assume that infants will not be exposed to HIV after delivery. However, in developing countries antiretrovirals are often not affordable or available, operative delivery carries a great risk of subsequent infection (50), which in the immuno-compromised HIV-infected mother may be fatal and increases obstetric risks in subsequent pregnancies and many infants will continue to be exposed to HIV through breastfeeding. In addition, the majority of HIV-infected women living in developing countries are unaware of their status, do not have access to voluntary counselling and testing nor advice on infant feeding options. In these situations infants will continue to be exposed to HIV after delivery and interventions need to be found which might reduce the risk of postnatal transmission, but which are not detrimental to the health of infants of uninfected mothers.

2.1.2 Postnatal transmission through breastfeeding

Early in the history of the HIV epidemic, clinical case histories suggested that postnatal transmission was an important route of infection and breast milk was implicated as a bodily fluid possibly responsible for this. A 1985 report of vertical transmission from a mother (who had acquired the infection through a postpartum blood transfusion) to her breastfed infant (51) indicated that in the absence of *in utero* or intrapartum transmission possibilities, breastmilk was likely to be the route of infection.

A review of the available epidemiological studies of vertical transmission from mothers who had acquired the infection postpartum indicated that the risk of transmission through breastmilk was 29% (95% CI 16-42%) (17). However, reported postnatal transmission rates vary greatly, ranging from 16% in Zambia (52) to 27% in Australia (53) and 40% in Rwanda (54). The study by Hira in Zambia recruited 1954 women who were seronegative at delivery, and determined subsequent HIV status in 634 women and their infants who returned for testing a year later. Sixteen of these women

seroconverted during the 12 months after delivery and three of their 19 children acquired HIV, presumably through breastfeeding; the risk of transmission is much higher during primary infection in the mother (52). The high vertical transmission rate from the study in Australia represents three breastfed children whose mothers were infected with HIV-1 through postpartum blood transfusions (10 women) or intravenous drug use (one woman) (53). The study in Rwanda reports vertical transmission rates from a prospective cohort of 212 women known to be seronegative at delivery who were tested for HIV-1 antibodies at three monthly intervals for approx. 17 months postpartum. Of this cohort 16 women became seropositive and nine of their infants acquired the infection. In four of these infants born to the ten women who seroconverted between four and 21 months postpartum, HIV-1 infection is thought to have been acquired through breastmilk; the remaining six women seroconverted in the first three months, thereby not excluding *in utero* or intrapartum transmission (54). These high vertical transmission rates may be partially explained by variation in HIV-1 virulence during seroconversion, concurrent infections and breastfeeding rates.

As the majority of HIV-infected women are already infected before pregnancy, it is important to estimate the MTCT rate due to breastfeeding by women with established HIV infection. However, it is difficult to identify the timing of infection and separate *in utero*, intrapartum and early breastfeeding transmission. In the early years of research in this field RNA PCR was not widely used and early kits were not as reliable as those available today. Therefore results must be viewed with caution and difficulties of determining the exact timing of infection taken into account (55). Dunn *et al.* combined the results from six studies of infection rates in breast and bottle-fed infants in Europe, USA, Australia and Zaire and found that when the maternal infection is acquired antepartum the additional risk of transmission through breastfeeding is 14% (95% CI 7-22%) (17). The higher transmission rates seen in studies of women who acquired HIV-1 postpartum may be due to high viral load during the primary stage of infection, whereas infants born to mothers who had acquired the HIV infection before delivery may benefit from lower breastmilk viral load, the presence of antibodies to HIV-1 in breastmilk and transplacental exposure to neutralising antibodies. Further discussion of risk factors for postnatal transmission of HIV will concentrate on women with established infection at the time of delivery.

2.1.3 Risk factors for breastmilk transmission

2.1.3.1 Breastmilk risk factors

Given that even in HIV-infected populations which practise breastfeeding the majority of children are not infected (56), there has been considerable interest in factors which may account for this. HIV is found in breastmilk as both cell-associated (DNA) and cell-free (RNA) virus (57). Some studies have examined the quantity and characteristics of the virus in breastmilk to investigate if there are viral factors that determine which mothers do transmit the virus and which do not. In addition the presence of antibodies to HIV and other conditions in the breast or infant may increase the risk of HIV infection in the breastfeeding infant.

The origin of HIV in breastmilk remains unclear, although HIV particles and infectivity have been detected both in the liquid phase of breastmilk and in association with breastmilk cells. There is evidence that mammary epithelial cells (MEC) can be infected by the virus (58) and that hormones normally associated with lactation (triiodothyronine (T3), β-estradiol and prolactin) may stimulate virus replication. Although *in vitro* HIV replicates in MEC to a lesser extent than in lymphoid cells, MEC-derived HIV might have selective advantages for the infection of mucosal epithelial cells and be trophic for infant oral or gastrointestinal epithelial cells (58).

HIV may be transmitted by cell-to-cell contact, which implicates milk leukocytes, possibly in combination with defective breastmilk anti-infective substances such as antibodies. A study of 215 lactating HIV-infected women in Rwanda showed that the greatest risk of vertical transmission occurred in women with HIV DNA detectable by PCR in their breastmilk at 15 days postpartum, especially if breastmilk IgM antibodies were not present, when likelihood of infection was 47%, compared with 18% if breastmilk did not contain HIV-1 infected cells (57). Secretory IgA antibodies are the main component of humoral immunity in breastmilk (59) and they probably protect against viral infection by coating and blocking attachment. However, HIV-1 specific IgA was rarely detected in breastmilk samples from women in Rwanda, although this may be compensated for by increased production of non-specific IgM in the mammary gland (57) which is involved in the lysis of HIV-1 infected cells.

In a study of breastmilk samples from 201 seropositive Ugandan women, Guay *et al.* attempted to detect p24 antigen and DNA in a single sample collected at about six weeks postpartum (60). p24 antigen was not detected in any breastmilk sample, even after Immune Complex Dissociation (ICD). Breastmilk cell pellets were only available from 20 of the 47 women who had transmitted HIV to their infant, and HIV-1 DNA could be detected in 16 of these samples (80%). HIV-1 DNA was detected in 75 samples from 104 women (72%) whose infants were classified as uninfected, according to HIV-1 DNA PCR at >6 months of age. When six infants who had evidence of having been infected *in utero* or intrapartum were excluded, there was no significant difference in transmission rates from mothers with (11/86, 13%) or without (2/30, 7%) detectable HIV-1 DNA PCR in their breastmilk at 6 weeks, although the number of subjects is very small. There was no difference in the median duration of breastfeeding between infants who acquired HIV and those who did not (15.8 vs. 14.4 months); the rate of transmission did not differ according to the duration of breastfeeding, although data on total breastfeeding duration was only reported on the larger cohort of 345 women, in which 91 infants were HIV-infected. There was a slight (non-significant) difference in vertical transmission rate between the cohort of women from whom breastmilk samples were analysed (23.4%) and the entire cohort (26.4%). This study has various limitations, not least the fact that only a single breastmilk sample per woman was analysed and is not necessarily representative of the infant's HIV exposure throughout breastfeeding. In addition, no indication is given whether the sample size obtained would have been sufficient to detect a difference in transmission rates from women with and without detectable HIV-1 DNA.

In a longitudinal study of only 47 seropositive Haitian women, Ruff *et al.* (61) found that the percentage of breastmilk samples with detectable HIV-1 DNA varied between 70% (33/47) at 0-4 days, 51% (n=35) at 3 months, 50% (n=30) at 6 months, 35% (n=24) at 9 months and 53% (n=15)at 12 months. This may indicate that HIV is shed intermittently into the breastmilk, as in other viral infections (11). However, it should be noted that there was considerable loss to follow-up in this study, with only 24 women providing breastmilk samples at 9 months and 15 at 12 months. Representative patterns of HIV shedding in breastmilk in women in this study show that some women shed virus throughout lactation, whereas others never have detectable viral load in breastmilk. Another group of women only shed virus in breastmilk at certain time points, although no information was given on the number of women falling into each of these categories (61). This variability in detection may account for the differing results found in a study of 208 breastfeeding

Rwandan women, where HIV-1 DNA was only detected in 47% (60/129) of samples taken at 15 days postpartum and 21% at 6 months (n=96) (57). The concentration of milk lymphocytes falls throughout lactation from 10^6 to 10^3 cells/ml (62), which may account for some decrease in levels of cell-associated virus. In addition the PCR kits available at that time had lower sensitivity than those available today.

It has also been suggested that as colostrum and early breastmilk contain a much higher concentration of breastmilk leukocytes than mature milk (63), that this may increase the risk of transmission. Nduati *et al.* (64) analysed 212 breastmilk samples from 107 HIV-infected women and found that PCR could detect HIV DNA in only 58% of all the breastmilk cell pellet samples. Interestingly, 77/212 samples were collected during the first week postpartum, when colostrum is produced (63) and in only 51% of these first week samples could HIV DNA be detected, whereas 64% of the 8-90 day samples had detectable HIV DNA. The prevalence of HIV DNA was significantly higher in mature milk than in colostrum, or in late milk over 9 months postpartum (p=0.02 and 0.01 respectively) and the volume of milk consumed in later stages of lactation much greater.

In summary HIV is present in breastmilk as cell-free and cell-associated virus and may be blocked from adhering to the infant mucosa by breastmilk antibodies. Levels of HIV in breastmilk vary throughout lactation and in each individual woman, making it difficult to quantify infant exposure. This would suggest that postnatal transmission is a multifactorial event.

2.1.3.2 Other risk factors

As many breastfed infants of HIV-infected mothers are exposed to cell-associated and cell-free virus through breastmilk, why do only some of them become infected? One factor that has been proposed is the duration of exposure through breastfeeding. A multicentre pooled analysis of late postnatal transmission of HIV considered late breastfeeding transmission to have occurred if an infant was HIV PCR negative at 2.5 months of age, but became positive on subsequent testing. The cut-off of 2.5 months to account for any intrapartum infection is due to the latent period between infection and sufficient replication of the HIV to permit detection by PCR. It is difficult to distinguish between intrapartum and postpartum acquisition of infection in the first

2.5-3 months of life. As breastfeeding was only common in studies from developing countries the analysis concentrated on 902 children of whom 49 were deemed to have acquired HIV through breastfeeding (overall late postnatal transmission 5%). Of these, 20 children had details of timing of transmission and from these it was estimated that if breastfeeding had stopped at 4 months transmission would have occurred in no infants and in 3/20 if breastfeeding had stopped at 6 months (65).

In a study conducted in Dar es Salaam Karlsson *et al.* reported that 8/139 (5.7%) of infants known to be HIV uninfected by PCR or p24 antigen test at 6 months of age became infected after 11 months of age (66). It has been speculated that late postnatal transmission may occur when infants develop teeth and cause injury to their mother's nipples, but in this study no mother who transmitted HIV after 6 months reported any breastfeeding problems, including cracked nipples or mastitis.

Miotti *et al.* (67) have recently reported breastfeeding transmission incidence rates in a cohort of 672 children from Malawi. Time of seroconversion was estimated as the midpoint between the last negative PCR and the first positive results and duration of breastfeeding was as reported by mothers at each follow-up. The HIV infection incidence was 0.7% per month between 1.5 to 5 months, 0.6% from 6-11 months and 0.3% between 12 and 17 months. Cumulative transmission rate was 10.3% after 23 months in infants who were breastfed beyond 1 month of age. In this study mothers were asked about breastfeeding problems, cracked nipples and mastitis from 6 months onwards; there was no significant difference in transmission among the 73/427 (17%) who reported breast problems compared with those who did not.

Mastitis is an inflammatory process, during which the paracellular pathways between mammary epithelial cells open up. This is characterised by an increase in breastmilk sodium concentration and also inflammatory cells (63;68) and is a local response usually observed in one breast only. This may result in increased breastmilk viral load. A study by Semba *et al.* (69) using stored breastmilk samples collected at 6 weeks of age only, as part of a vitamin A supplementation trial in Malawi, found that breastmilk sodium >12mmol/L, which the authors speculate is indicative of subclinical mastitis, was found in 28.4% of mothers of 88 HIV –infected infants, but only 12.5% of mothers of 240 uninfected infants at 6 weeks of age and was associated with infection status

of the infant at 6 weeks. Breastmilk viral load was determined for a subsample of 134 women; median breastmilk viral load was 700 copies/ml in mothers of infected, versus undetectable in mothers of uninfected infants and 920 copies/ml in women with raised breastmilk sodium versus undetectable in those with normal sodium levels. Mothers who had raised breastmilk sodium levels also had lower CD4 and higher plasma viral load and breastmilk lactoferrin concentration. Breastmilk sodium, lactoferrin at 6 weeks and plasma viral load during pregnancy were all associated with increased risk of MTCT at 6 weeks and 12 months. However, the risk of MTCT at 12 months was not assessed for infants uninfected at 6 weeks and therefore it is difficult to draw conclusions regarding the increased risk of transmission during that period for uninfected infants exposed to breastmilk with high levels of Na. It should also be noted that this study used archive breastmilk samples which had only been collected at one point (6 weeks postpartum) during lactation. As both breastmilk sodium (70) and viral load (57;61) vary throughout lactation in each individual woman, it may be difficult to attribute breastmilk transmission of HIV to one episode of subclinical mastitis or high viral load, especially when other factors known to influence the risk of transmission, such as maternal CD4 are also associated with the observed outcome. It is difficult to attribute causality and determine whether high breastmilk sodium in one breastmilk sample at one time point is independently associated with breastmilk transmission or infant infection status at that time point, or simply a marker of poor maternal immune status and systemic inflammation.

Acquisition of HIV infection from HIV-contaminated breastmilk is likely to be influenced by a number of factors. Not least of these is the hypothesised portal of entry into the infant circulation. It has been suggested that MTCT occurs primarily through contact between HIV virus and infant mucosal surfaces (71). During breastfeeding the infant oral, tonsillar and intestinal mucosa come into close contact with virus. HIV may enter through breaches in the mucosal barrier, or through infection of mucosal associated lymphoid tissue. If cell-free or cell-associated virus is able to infect lymphocytes in the submucosa by passing through breaches in the intestinal mucosa, conditions that result in intestinal epithelial damage may increase the risk of transmission. It has been suggested that early weaning or the introduction of formula may damage the infant gut (72) and it has recently been observed that infant feeding practices are associated with different MTCT risk. Data from a randomised controlled trial of breastfeeding versus formula feeding in Nairobi, Kenya showed that in the group assigned to breastfeeding

MTCT rose from 19.9% at 6 weeks (which would account for *in utero* and intrapartum transmission, plus some breastmilk transmission) to 28% at about 6 months. However, it is interesting to note that in the formula feeding group transmission also increased from 9.7% at 6 weeks to 15.9% at about 6 months probably due to mothers randomised to formula feeding also giving occasional breastfeeds; compliance with randomisation to formula feeding was only 70%, although no data is given as to the timing of non-compliance (73). By 24 months of age the cumulative probability of HIV infection was 36.7% (95% confidence interval (CI) 29.4-44.0%) in the breastfeeding arm and 20.5% (95% CI 14.0-27.0%) in the formula feeding arm. However, even though entry criteria into the study included access to municipally treated water and formula was provided free of charge, the 24 month mortality rate in the formula feeding arm was similar to that in the breastfeeding arm (20.0% (95% CI 14.4-25.6% vs. 24.4% (95% CI 18.2-30.7%) and HIV-free survival was lower in the formula feeding arm (74).

It may be that the quality of breastfeeding influences the risk of transmission. In a prospective study of 549 mother-infant pairs in South Africa Coutsoudis *et al.* reported that, even after controlling for potential confounders, exclusive breastfeeding to 3 months had a significantly lower transmission risk than mixed feeding (hazard ratio 0.52, CI 0.28-0.98), and a similar risk to no breastfeeding (0.85, CI 0.51-1.42) (75). This study only reported transmission risk at 3 months, but it would indicate that the actual mode of feeding is important, especially early in life, possibly because of the beneficial effects of exclusive breastfeeding on infant gut maturity and integrity.

2.1.4 Summary of mother-to-child transmission

There are a number of factors that influence mother-to-child transmission of HIV which may interact and have different relative importance in different settings. Many potentially important determinants of MTCT are still unknown, such as infant immunological susceptibility and antibody levels. Factors of major importance about which there is some evidence include:

In utero

- maternal viral load and viral strain
- maternal clinical and immune status (CD4)
- chorioamnionitis and ascending co-infections

Intrapartum

- prematurity
- obstetric procedures and mode of delivery
- duration of labour and rupture of membranes

Postpartum

- breastmilk viral load and non-specific antibody levels
- breast health (cracked nipples and mastitis)
- duration of breastfeeding
- feeding mode
- infant intestinal integrity

2.2 Subclinical breast inflammation

2.2.1 Breastmilk composition

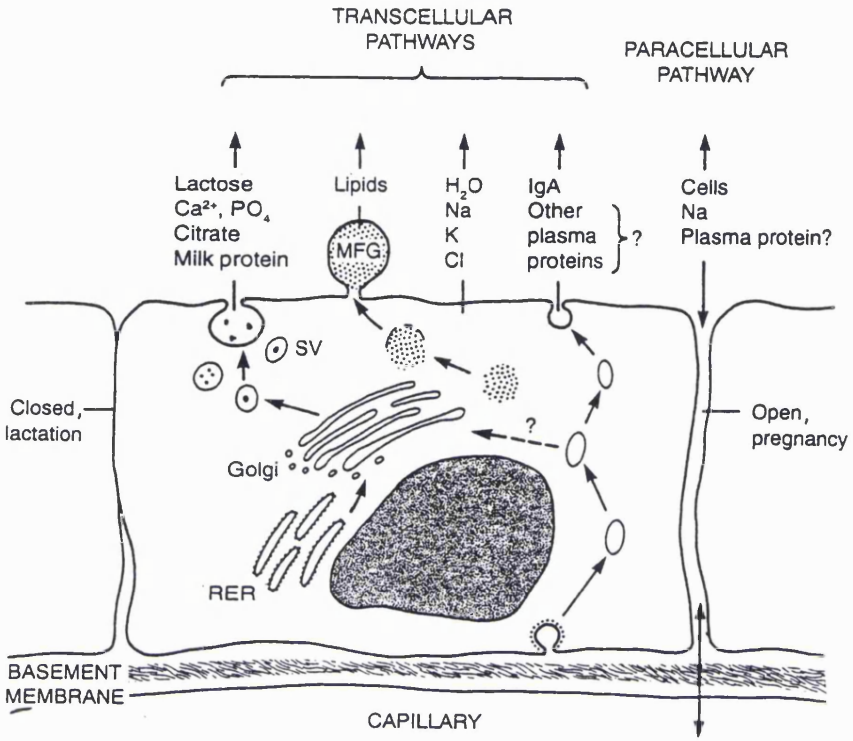
Several studies have reported levels of breastmilk components, including electrolytes in healthy, normal lactating women. These are usually from breastmilk samples donated by small numbers of "keen" breastfeeding women in developed countries, in whom infections are rare and rapidly treated. Electrolytes are found predominantly in the aqueous phase of milk and can be measured by direct flame photometry, with prior acid digestion making no significant difference to the results obtained (76).

2.2.1.1 Breastmilk composition during stages of lactation

Breastmilk composition varies greatly in the first weeks of lactation, during the transition from colostrum (1-5 days postpartum) to mature milk. Thereafter, the concentration of most components remains stable until weaning, when mammary gland involution disrupts the mammary epithelium and milk synthesis is affected (63).

Human breastmilk is iso-osmotic with plasma, but contains lower levels of ions, the main osmotic component being lactose. As shown in figure 2.2.1, lactose is synthesised in the Golgi apparatus of the alveolar epithelial cells and as lactose concentrations increase in the alveolus an osmotic gradient develops, drawing water in. Electrolytes follow their concentration gradients and direct leakage of electrolytes is prevented by the tight junctions of the alveolar epithelial cells (63;77).

Figure 2.2.1: Normal breastmilk production



A. Lactogenesis

Lactogenesis in humans is defined as the period from delivery to approximately day 5, by which time milk production is established and copious (78). Lactogenesis is initiated by the fall in progesterone levels at delivery, in the presence of maintained prolactin concentration.

Two physiological mechanisms are hypothesised to be involved in lactogenesis:

a) the closure of junctional complexes between mammary alveolar cells, which prevents extracellular fluid components from entering the alveolar space (which would result in dramatic changes in sodium, chloride and lactose concentrations) (79).

b) the increase in mammary alveolar cell milk synthesis, resulting in increased milk secretion and thereby raised lactose concentration in the alveolar space. This provides an osmotic gradient and results in changes in electrolyte concentrations (80).

During the first 3 days postpartum sodium concentrations fall dramatically from approximately 40 mmol/L to 13 mmol/L. Potassium, in contrast, rises from 13 mmol/L to 18 mmol/L in that time, in line with the rise in lactose concentration from 100 mmol/L to 160 mmol/L (78). The rise in lactose concentration is most likely a result of the change in cellular metabolism and milk secretion, at the same time as the closure of the tight junctions between mammary epithelial cells. The increase in lactose concentration corresponds most closely to the decrease in sodium, either as a result of the changes in osmotic gradient or the closure of tight junctions, or a combination of the two. Neville *et al.* provide evidence that the closure of the tight junctions precedes the onset of copious milk secretion, as there is a delay between the ionic composition changes and major increase in milk volume (78).

There are known differences between the milk secreted by mothers of preterm and term infants. Breastmilk sodium concentrations of mothers of preterm infants are consistently higher, even after the dramatic decline four days postpartum, when term breastmilk sodium levels reach a steady state and remain stable throughout the day (81).

B. Mature milk

Studies of breastmilk composition over the first six months of life have indicated that once lactation is established (between one and six months) the concentrations of sodium and potassium vary by ≤25% over time (82), with significant differences between individual women, suggesting that the secretory pattern is set in early lactation and maintained through the first six months in healthy women from developed countries. Other studies have found that sodium and potassium concentrations were stable over the first four months of lactation, after an initial decline (83), with greatest variability seen in sodium levels even in healthy women. There is a strong inverse relationship between electrolyte and lactose concentration.

Potassium is thought to permeate across the apical membrane of the alveolar cell, such that its concentration in mature milk represents equilibrium across the membrane (78), and thus remains constant in the aqueous phase even during episodes of mastitis. For this reason sodium is expressed as a ratio with potassium, to control for the fat content of a given milk sample. The Na/K ratio in one of the only large studies (n=269) of breast milk electrolyte levels, decreases from 1 during the first week of lactation to 0.5 at five weeks (84).

C. Weaning

During weaning breastmilk volume decreases and the mammary gland milk synthesis declines. Studies of electrolyte concentrations during weaning have shown that sodium levels increase dramatically, up to 220% of baseline (85) when daily breastmilk volume fell below 400 ml (78) or 300 ml/day (86). This increase in sodium concentration is due to increased leakage of plasma sodium between the tight junctions, over the normal transcellular transport, as the mammary gland undergoes involution.

2.2.1.2 Effects of breastmilk sampling

Results from studies by Neville *et al.* (70) indicate that it is possible to obtain a representative sample for the determination of components of the aqueous phase, without disrupting the breastfeeding dyad. They found that the composition of the aqueous phase did not vary significantly between feeds and that a small mid-feed sample gave the same results as the pooled, pumped contents of one breast, so that a "spot" sample is adequate for the determination of aqueous phase components in population studies. Koo and Gupta (81) also found no significant difference in sodium concentration if breastmilk samples were collected before or after a feeding episode in 45 women breastfeeding term infants and 22 with preterm infants. In contrast, other groups (87;88) have observed differences in electrolyte concentration between fore- and hind-milk, with both sodium and potassium approximately 5% higher in hind-milk (76), although this was established in a very small group of women (n=14).

During the first 45 days of lactation the composition of breastmilk from both breasts seems to remain remarkably similar for each woman and the greatest variation observed between women rather than between feeds, although this has been reported on only six women (70). There were, however, occasionally considerable and inconsistent differences observed between breasts of individual women and it has been suggested that elevations in sodium concentration due to mastitis (one subject complained of tenderness and swelling, but not fever during one such episode) may contribute to these (70). For this reason Neville recommends that breastmilk samples be collected from both breasts separately and suggests that sodium levels be measured to account for breast inflammation.

When the diurnal variation in breastmilk electrolytes was investigated, it was found that there was a reciprocal relationship between sodium and potassium when samples were collected every four hours, over a 24 hour period at 3.5 to 32 weeks postpartum (n=28) (76). The diurnal change in sodium concentration ranged from 22 to 80%, with a more consistent pattern in subjects after eight weeks postpartum. Lowest mean sodium concentrations were recorded from samples taken between 10:00 and 12:00 hrs., but large inter-individual differences made the pattern difficult to discern.

It has been hypothesised that some of the diurnal variation in breastmilk sodium levels could be due to maternal dietary sodium intake. As plasma sodium concentration is regulated by hormonal and osmotic mechanisms, it is possible that any effect of maternal diet on breastmilk electrolyte concentrations would be transitory. However, Ereman *et al.* (89) found no significant postprandial variation in breastmilk sodium or potassium levels of eight exclusively breastfeeding mothers, even after a high (2175 mg) or low (130 mg) sodium lunch; Keenan *et al.* (76) also found that a low sodium and potassium (10.8 and 60-100 mmol/d respectively) diet did not affect breastmilk electrolyte concentration in 28 healthy lactating women in Texas. Diet was also found not to influence breastmilk sodium in teenage mothers (90) when 24-hour dietary recall data was analysed.

Concern has been raised that the method of breastmilk collection may also contribute to differences observed in composition. A partial cross-over trial of hand *vs.* pump expression of breastmilk revealed a significantly higher sodium content in hand expressed milk (14.2 vs. 8.3 mmol/L at 12 days postpartum) (91). It is suggested that this may be due to the compression of lactiferous ducts (causing intercellular leakage or cellular damage) to obtain milk by hand expression, as opposed to the intermittent suction applied by mechanical pumps. However, this study was carried out on a small number of British mothers (16 manual, 11 pump) of premature infants requiring neonatal intensive care and may not be representative of normal breastmilk sodium levels.

2.2.1.3 Normal breastmilk composition

Most studies of breastmilk composition and electrolyte levels have been carried out on small and highly selected groups of women, generally keen and dedicated breastfeeding women from university towns. Few studies have collected breastmilk from both breasts and analysed them separately, although condition such as mastitis and breast inflammation, which may alter breastmilk composition are often subclinical and unilateral (70). Given that it is known that the inter-individual variation in breastmilk composition is very high (70), it is therefore difficult to extrapolate these data to the normal levels of breastmilk sodium and potassium in a population of breastfeeding women who are not particularly highly educated, well nourished or healthy, but more truly represent the majority of breastfeeding women in the world. Table 2.2.1.1 summarises the data on Na/K ratio at different infant ages and by method of expression. Only during very early lactation (84) and weaning is Na/K ratio greater than 1, and apart from the study by Dewey (92), all ratios are less than 0.6.

Table 2.2.1.1: Comparison of breastmilk sodium/potassium levels by infant age and characteristics

reference (expression method)	age /stage of lactation	n =	sodium (mmol/L)	potassium (mmol/L)	Na/K ratio
Koo 1982 (hand)	1 d term	17	64.8		
	3 d term	18	21.4		
	5 d term	19	16.2		
	15-28 d term	48	6.9		
	1 d prem	5	70.9		
	3 d prem	8	21.8		
	5 d prem	5	15.6		
	15-28 d prem	52	10.6		
Hazebroek 1983 (not specified)	1-3 d	35	19.8		1.00
	5 weeks	213	8.5		0.50
Allen 1991 (hand)	21 d	13	9.2	16.2	0.56
	45 d	13	7.2	15.1	0.48
	90 d	13	6.3	13.9	0.45
	180 d	13	6.0	12.4	0.48
Wack 1997 (hand and pump)	0-60 d	27	7.9	15.0	0.52
	61-120 d	20	5.6	12.5	0.45
	121-180 d	25	5.9	12.4	0.47
Dewey 1983 (hand)	1 m	13	9.9	13.5	0.73
	2 m	16	11.5	12.2	0.94
	3 m	18	8.0	12.0	0.66
	4 m	16	7.6	11.9	0.64
	5 m	14	7.2	11.8	0.61
	6 m	15	5.8	11.0	0.53
Keenan 1982 (pump)	3.5-6 w	14	7.9	15.2	0.52
	8.5-18 w	14	4.7	13.8	0.34
	20-32 w	12	5.4	13.3	0.41
Dewey 1984 (hand/pump)	>500 ml/d	11	4.2	10.0	0.42
	300-500 ml/d	6	7.34	10.6	0.69
	<300 ml/d	6	22.8	9.6	2.38

Note: hand = hand expression; pump = expression using manual or electric breast pump

Cytokines are small, soluble glycoproteins with established autocrine, paracrine and endocrine functions within the immune system. Given the immuno-modulatory potential of breastmilk, there has been considerable interest in cytokine concentration and function in breastmilk. There are substantial technical difficulties in measuring cytokines in breastmilk, due to proteolytic and binding functions of some breastmilk components. Therefore the cytokines discussed below are not an exhaustive list, but those which may be important for protection against mastitis, which is the focus of the present work.

Many cytokines can be detected in breastmilk and there is evidence that interleukin (IL)-6, -8 (93;94) and TNF (94) are produced by mammary gland epithelial cells. IL-6 has been detected in colostrum and mature milk samples from 10 women and found to correlate with mononuclear cell number and to be involved in IgA production by milk mononuclear cells after stimulation with *Staphylococcus aureus in vitro* (95). However, other groups have not been able to detect IL-6 in colostrum, transitional and mature milk from mothers of preterm and term infants, although maternal breastmilk cells did express mRNA for IL-6 in 6/12 samples (96).

Srivastava *et al.* (96) report that cytokines are only present in the whey fraction of breastmilk and some require activation by gastric acid. Data on individual cytokine levels are presented, but given the very small sample size, ranging from 2 to 27 healthy American women, and the observation that there is considerable variability between individual women, it is difficult to extrapolate these data into other populations.

Interleukin-8 is the cytokine involved in mastitis of which there is most information in humans and for which assays in milk are most reliable (97). It has been detected in breastmilk and mRNA for IL-8 has been found in both breastmilk cells and mammary gland epithelial cells (93;94;96) indicating that it is produced in both. This cytokine is of particular importance as it is a pro-inflammatory cytokine that is chemoattractant to neutrophils (98) (99). The accumulation of leukocytes, in particular neutrophils may be an important part of the mammary gland's defences to infection.

In spite of large amounts of information, it is difficult to establish normal levels of IL-8 in human breastmilk, since wide variation is seen within studies (96;97). In a large community-based study in Bangladesh, Filteau *et al.* reported IL-8 levels from 212 women enrolled in a vitamin A supplementation trial. These were considerably lower than the values reported by Srivastava. At two weeks postpartum IL-8 concentration in breastmilk samples from Bangladesh was 35.8 pg/ml (95% CI 29.0-44.1) and 29.7 pg/ml (95% CI 25.9-34.9) at 3 months (97). Data from a study of red palm oil and sunflower oil supplementation in Tanzania indicated that IL-8 concentration ranged from 35 pg/ml to approximately 90 pg/ml during the first 3 months of lactation, although geometric means are not reported and it is not clear exactly how many women gave pooled breastmilk samples for this analysis (100).

2.2.2 Breast inflammation

Mastitis and mammary gland inflammation have long been recognised by the dairy industry as having a dramatic effect on the quantity and quality of milk produced, as well as sometimes devastating effects on dairy herd health (101). A considerable amount of research into the effects of mastitis on cow, sheep and goat mammary gland epithelial integrity, lymphocyte recruitment to the gland and mammary gland cytokine production has yielded much data which can be extrapolated to the effects of similar inflammatory responses during human lactation.

Experimental time course studies in dairy animlas have suggested a role in mastitis for the cytokines IL-1, 6, 8 and TNF and the complement component C5A (99;101-103). During episodes of endotoxin-induced mastitis in cows, udder swelling preceded increased concentration of inflammatory cytokines such as IL-1β (102) IL-6, TNF-α (101) and IL-8 (104), which are produced locally; inflammation was restricted to the infected gland. During *Staphylococcus aureus* induced mastitis in sheep IL-8 and TNF-α concentrations in milk increase in parallel with increase in leukocyte concentrations. IL-8 has been shown to be chemotactic for sheep neutrophils and increased in concentrations before the increase in leukocyte recruitment to the affected udder (105). A similar pattern is observed during endotoxin-induced mastitis in sheep (106). Interestingly, IL-8 has been found to be responsible for neutrophil chemotactic activity during episodes of mastitis, but not in non-mastitic mammary glands in cows. Results from studies in cows and goats have indicated that milk from mastitic

and non-mastitic bovine (99) and caprine (107) udders display chemotactic potential for neutrophils. However, when anti-IL-8 antibodies are added to the milk in culture, chemotactic activity of mastitic milk, but not that of non-mastitic milk is blocked (99); physiochemical characteristics of the neutrophil chemoattractant in mastitic goat milk would also suggest that it is IL-8 (107). During mastitis IL-8 may be produced locally in mammary gland epithelial cells, and milk cells. The indication that IL-8 is the cytokine involved in neutrophil chemotaxis only during mastitis suggests that increased IL-8 concentration is a specific response to local inflammation.

The 2.9% incidence of puerperal mastitis among 966 women in the US in the first 7 weeks of lactation indicates that problems of breast health affect women in developed as well as developing countries (108). A study from Australia has estimated the incidence of mastitis as 5% and prevalence of 6% among 16,351 deliveries (103). Both these studies have relied on hospital records and in the latter the denominator used was 50% of all recorded deliveries, using the assumption that 50% of women would breastfeed, and estimated of incidence amy not be reliable. A prospective cohort using questionnaires and telephone follow-up in Australia estimated the self-reported mastitis incidence rate in the first 6 months of lactation to be 20% (219/1075) (109). In this study 25% of the episodes of mastitis occurred within the first 14 days and 50% by 21 days of lactation. As the majority of women (76%) with suspected mastitis seek health advice from their general practitioner and only 6% present at the hospital emergency room (109) it is important to have prospective community-based data on the incidence and prevalence of mastitis.

Breast inflammation was found to be a sizeable problem in Gambian women, who may be lactating for most of their reproductive years. Prentice *et al.* (110) found a monthly incidence of mastitis of 2.6% among an average of 159 women seen every month at the local health clinic. In this study women who presented at the local clinic with mastitis (defined as breast abscesses, breast pain with local heat with or without pyrexia or breast pain without signs of engorgement) expressed breastmilk, which was then analysed for serum-derived immunoproteins, lactose, sodium and transferrin levels. Sodium levels were considerably raised during the mastitis episode and returned to normal levels upon recovery. Lactose concentrations decreased to 41% of normal during mastitis and returned to normal upon recovery (110). This pattern of

changes can be explained by the appearance of the paracellular pathway between the tight junction of the mammary epithelial cells. This permits serum-derived components to enter the milk and lactose to leave. The opening of the paracellular pathway may be an active response to breast inflammation, rather than reflecting partial loss of structural integrity at the site of inflammation, as the whole breast mammary tissue would seem to be involved. The paracellular pathway is short-lived and sodium and lactose levels returned to normal within one week.

Raised sodium levels were also reported in one woman from the USA who developed mastitis in the first two weeks postpartum. Upon analysis it was found that the milk from the affected breast had a sodium concentration of 103 mmol/L, compared with the unaffected breast sodium concentration of 3 mmol/L and control level of 6 mmol/L (111). The author speculated that chronic infection had resulted in continually elevated levels of sodium, which were high enough to alter the taste and acceptability of the breastmilk to the infant. This may explain why in another study infants who consumed milk with high sodium levels (>16 mmol/L) had slightly reduced weight gain; weight gain from birth to one month was 818g (n=60), compared with 994g (n=65) (68). It is speculated that infants who consume breastmilk with persistently high levels of sodium may dislike the taste (111) and consequently consume less breastmilk than they require for optimal growth; or that small infants do not suckle effectively, resulting in milk stasis and disruption of the mammary gland epithelium and raised breastmilk sodium.

There have been very few reports of electrolyte levels during episodes of breast inflammation. A community-based study conducted in Bangladesh investigated Na/K ratio as an indicator of breast inflammation in 212 women at two weeks and three months postpartum. Using a cut-off of a Na/K ratio >0.6 to indicate subclinical breast inflammation, they reported a prevalence of 24% at 2 weeks and 12% at 3 months postpartum. In this study Na/K ratio correlated very strongly with breastmilk IL-8 concentration (97). Using the same cut-off for Na/K, Filteau *et al.* also reported that 13% (11/83) and 10% (9/87) of women had subclinical mastitis at 1 and 3 months of lactation respectively in a community-based study in Tanzania (100).

Risk factors for mastitis remain unclear, but cracked nipples (112), attachment difficulties (113) and milk stasis (114) may be associated with increased risk. Infectious agents, such as *Staphylococcus aureus* (112) and *Candida* (115) are known to cause some, but not all cases of mastitis. During infective and non-infective mastitis, effective emptying of the breast has been found to significantly reduce symptoms and improve outcome (116). Lactation counselling has been effective in reducing milk sodium concentration of women presenting with problems of lactation (68) and would presumably also reduce the high levels of IL-8 associated with raised Na/K (97;117). This may be of benefit to the infant, as high ingestion of IL-8, which is relatively resistant to digestive processes (118), is speculated to be associated with inflammation and damage to the infant gut epithelia. In addition mastitis has been implicated in postnatal transmission of HIV from mother-to-child (117). This study is detailed further in section 2.1.3.2.

2.2.3 Summary of subclinical breast inflammation

- There is very little data on the prevalence of subclinical mastitis and to date no study that has collected samples from both breasts

- During mammary gland inflammation breakdown of the mammary epithelium tight junctions results in increased leakage of sodium from plasma into milk secretions.

- Mammary inflammation results in increased neutrophil recruitment, mediated by IL-8

- During episodes of mastitis and subclinical mastitis in humans elevated breastmilk Na/K and IL-8 levels have been noted

- Subclinical mastitis (raised Na levels without symptoms of mastitis) is common and has been associated with HIV infection in the infant

2.3 Intestinal permeability

Absorption across the healthy intestinal mucosa is highly selective and intestinal transport systems have affinity for certain nutrients, which are absorbed by active transport (119). The absorptive surface and integrity, and therefore permeability of the intestinal mucosa is influenced by a number of factors. Lactulose and mannitol are two sugars that reflect transcellular and paracellular uptake respectively. The recovery of these sugars in urine samples collected over 5 hours after an intravenous dose is high (around 97% for lactulose and 70% for mannitol) (120) and this differential permeability test provides useful information regarding small-intestine pathology. The lactulose/mannitol (L:M) ratio is not influenced by factors such as gastric emptying, peristalsis or glomerular filtration rate, which might affect the urinary elimination of each individual sugar probe (72). Mucosal damage results in malabsorption and reduced energy intake and has been associated with growth faltering in children (121).

2.3.1 Normal infant intestinal permeability

In young infants the intestinal mucosa is still developing and it is known that trophic factors in breastmilk assist in the maturation of the gut epithelium. Studies of intestinal permeability in newborns have shown that during the first 6 days of life, the L:M ratio fell in breastfed infants (n=11), compared with no decline in formula-fed infants (n=9). The difference in the slopes of these changes in intestinal permeability between the two feeding groups was significant ($p < 0.01$), although the mean values are not given (122). Catassi *et al.* found that in 36 breastfed infants L:M ratio was significantly lower at 7 days compared with 36 formula-fed infants at the same age (0.22 ±0.25 vs. 0.47 ±0.41, $p = 0.002$), but that the L:M ratio fell over the first week of life in both groups (72). A study of infant feeding practices in preterm infants has also shown that by 28 days postpartum human milk feeding (n =38) was associated with a decrease in L:M ratio when compared with formula feeding (n =25, $p = 0.02$), and that L:M ratio also fell with increasing age (123).

There have been few reports of intestinal permeability in healthy, older infants in developing countries, most studies having concentrated in altered mucosal integrity during episodes of, or recovery from, diarrhoea. A community-based study of intestinal permeability in Guatemala successfully completed L:M tests on 158 infants who had been free of diarrhoea for at least 1 week, and showed that 30% of children aged less than 1 year had a L:M ratio above the cut-off of 0.07, which the authors defined as normal, based on some early work by Ford *et al.* (124;125). Interestingly, the L:M ratio was positively associated with age and in infants less than 6 months of age, with non-breastfeeding. Exclusively or predominantly breastfed infants (n = 76) had significantly lower L:M ratios than mixed (n =59) or exclusively formula-fed (n =23) infants (p =0.004). L:M ratio was negatively correlated with the age at termination of breastfeeding, but not the duration of time since termination of breastfeeding, indicating that early cessation may have an effect on intestinal permeability (125).

2.3.2 Factors which may alter intestinal mucosal integrity

Human milk is known to contain many trophic factors which influence infant gut development, adaptation and maturation (126-128). However, in addition to these positive factors, breastmilk also contains cytokines which are able to trigger inflammatory responses (94;96;129). Cytokines can mediate local and systemic effects of intestinal inflammation and its consequences, such as diarrhoea, cellular activation and mucosal permeability. Cytokines produced by activated macrophages such as IL-1, 6 and 8 have been shown to be increased in inflamed intestinal tissue (130).

2.3.3 Summary of intestinal permeability

- The neonatal intestine is not fully mature and develops during the postpartum period, under the influence of breastmilk trophic factors

- The lactulose:mannitol test has been accepted as a measure of intestinal mucosa permeability, reflecting tight junction integrity and absorptive surface area respectively

- Breastfed infants have been found to had a lower L:M ratio than formula-fed infants, and exclusively breastfed infants had a lower L:M ratio than those not exclusively breastfed in one community-based study of healthy children

- Cytokines have a negative effect on intestinal permeability and are associated with inflammation

2.4 Hypothesis and objectives of research

Breastmilk is a known route of MTCT of HIV-1, yet the majority of children breastfed by their HIV-infected mother do not become infected during the period of breastfeeding. Breastmilk viral load is thought to be an important determinant of postnatal MTCT, but it remains unclear what are determines breastmilk viral load.

Breast inflammation and subclinical mastitis (as measured by breastmilk Na/K) results in disruption of the mammary gland epithelia and may be more prevalent than previously thought. Milk stasis and mammary gland involution due to reduced breastmilk drainage and production are also known to result in increased Na/K levels and it is likely that infant feeding practices could be associated with subclinical mastitis. Increased mammary gland permeability and inflammation may cause increased recruitment of HIV-infected cells or cell-free virus into the breastmilk.

Subclinical mastitis is also associated with increased levels of inflammatory cytokines in breastmilk, which if they pass into the infant intestine intact may cause inflammation and increased intestinal permeability. It is speculated that these conditions (high breastmilk viral load and increased infant intestinal permeability) may favor the acquisition of breastmilk HIV by the infant.

In order to test the hypothesis that subclinical mastitis is associated with breastmilk viral load and infant intestinal permeability, both potential risk factors for postnatal MTCT, two studies were planned:

1. A cross-sectional study of the prevalence of subclinical mastitis among healthy lactating women attending Well Baby clinics in a population with a high HIV seroprevalence

2. A study of:

    a) the prevalence of subclinical mastitis during the first 3 months of lactation among a cohort of HIV-infected women who had chosen to breastfeed

    b) the association between subclinical mastitis, breastmilk viral load and infant intestinal permeability and how these relationships are affected by infant feeding practices among a cohort of HIV-infected women who had chosen to breastfeed.

3: Methods

3.1 Sample size

3.1.1 Prevalence Study

Sample size was calculated on the basis of the only community-based data on the prevalence of subclinical breast inflammation available at the time from a study in Bangladesh (97). In that study 25% of women at 1 week and 12 % at 3 months postpartum had Na/K ratio >0.6, taken to be indicative of subclinical breast inflammation. In order to detect a similar prevalence (within 5% of the true value) in South Africa, it was estimated that 100 women would need to be recruited at 1 week, 75 at 6 weeks and 100 at 3 months postpartum.

3.1.2 Cohort Study

Sample size calculations were conducted for 5% significance and 80% power. An outline of mother-infant pairs available at each time point is shown below, showing assumptions of the percentage of mothers still breastfeeding at each time point.

About 45% of women would be expected to have the high milk viral loads associated with increased risk of HIV transmission (131). Assuming this broke down into 30% among women with normal milk Na/K ratio and 60% among women with high Na/K, we would need 50 women in each of the two groups. As no data were available on the prevalence of subclinical breast inflammation at different stages of lactation among HIV-infected women, it was assumed that this would be at least as high as the prevalence of raised Na/K in the study in Bangladesh at 1 week postpartum and remain constant. However, during the cohort study, results from a study of the prevalence of subclinical breast inflammation among women in Durban who were breastfeeding an infant less than 1 year of age, became available. Sample size requirements for the cohort study were reassessed assuming a prevalence of raised Na/K of approximately 35%, (see section 4.5), and breastmilk viral load assumptions as above, and it was estimated that 200 women would need to be recruited.

Based on gut permeability differences between breast- (L:M ratio 0.22 ±0.25)and bottle-fed (L:M ratio 0.47 ±0.41) infants (72), 30 infants per group would be required to determine differences in intestinal permeability between infants of women with and without high Na/K ratios at the same sampling time.

			Assumptions
1 week	200 breast feeding		100% breast feeding
		130 normal Na/K 70 high N/K	35% high Na/K
6 weeks	160 breast feeding		80% breast feeding
		104 normal Na/K 56 high Na/K	35% high Na/K
14 weeks	130 breast feeding		65% breast feeding
		85 normal Na/K 45 high Na/K	35% high Na/K

3.2 Study population and recruitment

Durban was chosen as the site for this study for a number of reasons:

1. The prevalence of HIV was very high in KwaZulu-Natal (26.9% in 1997) (6). Although HIV was not considered in the study of the prevalence of subclinical breast inflammation, this study was planned to provide valuable background information for a longitudinal cohort study of lactating HIV-infected mothers (see 3.2.2)

2. The urban population of Durban was served by a number of stable clinics from which it would be possible to carry out this work.

3. Collaborative projects between the Department of Paediatrics and the Centre for International Child Health were under way. These included an already established project investigating gut permeability in infants of HIV-infected women and it was planned that the cohort study be allied to this project (see 3.2.2).

For these reasons it was decided to locate the two phases of this study in Durban and collaborate with the Department of Paediatrics at the University of Natal Medical School. For further information on Durban and the HIV epidemic in South Africa, please refer to Chapter 1.

3.2.1 Prevalence Study

Study sites were not randomly selected, but instead chosen because of previous links with collaborators on this project, the Department of Paediatrics and Child Health, University of Natal Medical School, Durban. The map of the Durban Metropolitan Area shown in Chapter 1 (Figure 1.1.2) indicates the location of the following study sites. Ethical permission to conduct this study was obtained from the relevant committees at the University of Natal and the Institute of Child Health and Great Ormond Street Hospital.

3.2.1.1 Study sites

3.2.1.1.1: McCord's Hospital

A mission hospital in Durban City, serving a mostly black (about 5% Asian, <1% white) population from Mayville, Clare Estate, Sydenham and the informal settlement at Cato Manor (see figure1.1.2).

This was the site of an ongoing mother and infant cohort in whom a number of studies were being conducted (75;132) (Filteau *et al.*, submitted 2000). As contact had already been established with the hospital administration and a study clinic was running at the site 5 days a week, it was decided to approach the hospital medical superintendent for permission to conduct this cross-sectional study within the routine post-natal clinic. At this time, McCord's Hospital received a subsidy from the Provincial Department of Health to provide free mother and child (to age 6) services to people living within the hospital catchment area, including the informal settlements of Cato Manor.

Women who had delivered at the hospital were asked to return with their infant on Friday morning, one week after discharge and when the infant was 6 weeks old. At each visit weight was recorded and the mother and baby seen by a nurse. At the second visit first Diptheria Pertussis and Tetanus (DPT) immunisation was administered and the mother referred to her local City Health Department clinic for continuing immunisation and childcare. At the time of this study, approximately 80 infants were seen each Friday.

The purpose of the study was explained to the Sister-in-Charge and the clinic staff agreed to make a screened cubicle available for interviews with mothers. Mothers arrived early at the clinic (7-7.30am), had their baby weighed and then sat in a queue until seen in turn by a nurse.

Routine Voluntary Counselling and Testing for HIV was offered at the McCord's antenatal clinic. At the time of this study approximately 180 women were booking for antenatal care at the clinic each month, of which 22% were HIV-infected (1997, personal communication, Dr E Spooner, SAVITA study.). However, in the confines of this cross-sectional study women were not asked about HIV status.

The nursing staff at McCord's ante- and postnatal clinic were very motivated and had recently attended a breastfeeding course. There were several "home-made" posters promoting breastfeeding and at each visit mothers were asked if they were breastfeeding and if they had any problems. Staff reported that 90% of the mothers attending McCord's postnatal clinic were breastfeeding. Staff did not formally record this figure, but a selection of check-ups that I witnessed (approximately 40, over two sessions) would support this claim.

The cohort of HIV-infected mothers and infants recruited for a placebo-controlled trial of vitamin A supplementation during pregnancy (75) also attended McCord's Hospital. These women were seen by a dedicated team in a separate area of the ante- and postnatal clinic. For details of this study, please see section 3.2.2.1.1. Permission was obtained from the study co-ordinator to approach women in this study to participate in the cross-sectional study.

3.2.1.1.2: Lamontville City Health Department Clinic

A City Health Department Clinic located in Lamontville Township, to the south of Durban City, serving a black population.

Contact with this clinic was made through a clinician working for the Durban City Health Department. Lamontville clinic was chosen from a list of possible City Health Department Clinics, as it was situated near the large township of Umlazi, to the south of Durban city centre. Permission to conduct this study was granted by the Durban City Health Department. The clinic was open 3 days a week and staffed by nurses and assistants, with a clinician one session per week.

The purpose of the study was explained to all the clinic staff and a screened off area in an infrequently used waiting area was made available for interviews. Mothers and infants of all ages attended this clinic, for Mother and Child Health services and primary health care. The majority of women attending the Lamontville clinic had delivered their baby at the King Edward VIII Hospital in Durban. Routine antenatal HIV testing was not offered by Durban City Health Department clinics. There was no evidence of breastfeeding promotion at the clinic and due to some staff unwillingness it was not possible to sit-in on a routine check-up visit. Mothers arrived at the clinic throughout the morning, had their notes put into a queue and were then called up to have the baby weighed before being sent through to the immunisation room, or referred to the doctor.

3.2.1.1.3: Valley Trust Primary Health Care Clinic

A peri-urban clinic some 50km west from central Durban, serving the rural black communities of the Valley of a Thousand Hills.

This site was chosen for its strong academic and teaching links with the Department of Paediatrics at the University of Natal Medical School. The clinic was a 7-days a week facility with one full-time clinician and 6 professional nurses. Permission was given by the medical superintendent and the paediatric services sister to recruit women attending the child health clinic. Mothers attending the clinic were first seen by nurse assistants who weighed the child, administered immunisations and then referred patients on to the sister if needed. Valley Trust had for many years had a particular interest in nutrition and there were many posters promoting "healthy eating". However, no special emphasis was given to breastfeeding. Routine HIV-testing was not offered at Valley Trust. There were 24-hour delivery facilities, but many women still delivered at home.

Permission to carry out the study in each of the sites was obtained from the medical superintendent (McCord's and Valley Trust) and the Durban City Health Department. The purpose of the study was explained to all the nursing staff and efforts were made not to disrupt the normal functioning of the clinics involved.

At all sites every child had a Road to Health card with details of birth weight, gestational age (not always completed) and immunisations.

3.2.1.2 Recruitment

The study was carried out between 21st November 1997 and 30th March 1998.

A Zulu-speaking research assistant approached breastfeeding women in turn as they waited in line to be seen by the clinic staff. She explained the purpose of the study, the type of questions that would be asked, that all answers would be confidential and that we would like to collect a small (3-5 ml) sample of breastmilk from each breast by manual expression.

Given the stigma attached to HIV-infected status and the difficulties in broaching this subject within the confines of a rapid, cross-sectional study, it was decided not to enquire about maternal HIV status.

Inclusion criteria: Verbal, informed consent.
Breastfeeding an infant between 3 days and 12 months old.
Willing to provide a breastmilk sample from each breast.

Exclusion criteria: none

Women who gave informed verbal consent were then taken to a private screened area and asked to answer questions about breast health, infant birth weight and gestational age (taken from Road to Health cards), and a brief 24-hour recall of infant feeding practice. Mothers were asked what their infant had consumed "since this time yesterday". This data was not collected from the 26 mothers in the HIV study at McCord's Hospital for logistical reasons. This was an open question, which the research assistant recorded on a list of options, or as other, with details, if the answer given was not listed. The options listed on the questionnaire were developed with the help of local clinic staff to incorporate most of the common foods given to infants less than 1 year of age. This data was collected in order to compare the prevalence of subclinical breast inflammation in mothers who were exclusively breastfeeding, compared with those who were supplementing breastmilk with either formula or complementary foods. The mother was asked to manually express 3-5 ml of breast milk from each breast into a sterile polypropylene jar.

The Zulu-speaking research assistant, who recorded all answers in English on the form provided, administered the questionnaire. The time required for completion of the questionnaire and collection of breastmilk samples ranged from about 4 minutes to 10 minutes, depending on how easily the woman was able to manually express about 3 ml of breastmilk from each breast. No mother was made to feel obliged to provide a breastmilk sample and if she was encountering difficulties the research assistant assured her that this was not a problem and thanked her for her participation. Any woman who reported problems breastfeeding (n=2, both at the McCord's site) was referred to a qualified nurse with lactation support experience. The questionnaire used is included in Annex 1.

3.2.2 Cohort Study

3.2.2.1 Study sites

3.2.2.1.1 McCord's Hospital

HIV-infected breastfeeding women, who were already enrolled in a placebo-controlled trial of vitamin A supplementation during pregnancy (132) and effects on infant intestinal permeability (Filteau *et al.*, submitted 2000) were eligible for inclusion in this study. Women taking part in the vitamin A supplementation trial were from the McCord's Hospital population as described in 3.2.1.1.1 above. Recruitment commenced in December 1997 and finished in September 1998.

The vitamin A trial commenced in July 1995. Pregnant women booking at the antenatal clinic at McCord's Hospital were offered Voluntary Counselling and HIV Testing (VCT) by 2 qualified HIV counsellors. Women who consented to be tested had blood samples taken which were analysed for HIV-1 antibodies by ELISA at McCord's Hospital laboratories. 22% of women consenting to HIV testing were found to be HIV-infected in 1997 (personal communication, Dr E Spooner, SAVITA study). Women who tested positive, were less than 28 weeks gestation and gave informed, written consent were eligible for inclusion in the study.

Women received either capsules containing 15 mg vitamin A and 30 mg β–carotene or an identical placebo. Women were asked to take the supplement every day and were given a 2-4 week supply, depending on the date of their next antenatal appointment. At each antenatal visit women were seen by the study clinician, who assessed their health status, asked specifically about adverse effects of the vitamin A supplement and counselled women on infant feeding choices. Maternal baseline blood samples were taken at recruitment for CD4 and CD8 estimation by FACScan. Several studies have reported that maternal CD4 and CD8 counts do not decline more rapidly as a result of pregnancy (133) and percentages remain stable (134).

At delivery a cord blood sample was collected and women were visited by the study clinician or HIV-counsellor on the postnatal ward before discharge. Mothers were given a capsule containing 60 mg vitamin A or placebo within one week of delivery. Mother-infant pairs were given a follow-up appointment when the infant was about 1 week of age. A study of infant intestinal permeability and HIV transmission was nested within the Vitamin A supplementation trial and commenced in May 1997. HIV-infected women were recruited into this trial either during antenatal visits, or after delivery for a small group of mothers who booked too late in gestation to be included in the Vitamin A supplementation trial. Information about delivery was recorded from women's clinical notes while they were still on the post-delivery ward. Gestational age was estimated routinely from date of last menstrual period, fundal height or by ultrasound during antenatal visits. All women booking before the third trimester had a routine ultrasound scan in the second trimester. No formal assessment was made of gestational age of the infant at delivery.

At each follow-up visit infants were weighed to the nearest 10g, length and head circumference measured to the nearest 0.1cm. At the 1, 6 and 14 week visits a paediatric urine collection bag was fitted and the infant was given 2ml/kg body weight of sterile lactulose:mannitol solution (prepared by the Pharmacy Dept., King Edward VIII Hospital, Durban). Urine was collected for the following 5 hours. During this time, mothers were asked to provide a 7-10ml sample of breastmilk from each breast into a clean polypropylene jar by manual expression (see section 3.3.1.2.1 for details of breastmilk collection and processing methodology). 3ml of blood was taken from the infants into an EDTA tube for retrospective viral load analysis. The study clinician examined mother and infant and a detailed history of recent morbidity (since last visit) and infant

diet was taken. In particular mothers were asked about breastfeeding (date started, date stopped), formula feeding (assessment made of whether feeds were being mixed correctly and education provided as appropriate) and any additional drinks or foods given. During the 5-hour wait mothers were given tea, biscuits and lunch. An air-conditioned waiting area with a TV was provided for the sole use of mothers involved in these studies. In addition, mothers were given R40 to cover transport and alternative childcare expenses incurred by the visit and 5 hour urine collection.

When the infant was 6, 9 and 15 months of age, mother-infant pairs were asked to return for follow-up to assess health status and record dietary history of the infant. Infants were weighed to the nearest 10g, length and head circumference measured to the nearest 0.1cm. At 9 months a 3ml sample of blood was taken from each infant and this was analysed for HIV-1 antibodies by ELISA at the Virology Laboratories of King Edward VIII Hospital. If the ELISA was positive, IgG3 or HIV antigen was determined. In this setting IgG3 has been shown to be a reliable marker of infant HIV infection from 6 months of age onwards (135). If the ELISA was positive, but IgG3 negative, the child was deemed to be negative, with maternal antibodies to HIV still in the circulation. At 15 months the infant blood sample was analysed for HIV-1 antibodies by ELISA only.

Mothers who failed to attend any scheduled visit were contacted by telephone (if available) by the Zulu-speaking counsellor and asked to attend the following week. Written remainders were not sent. Copies of questionnaires used are included in Annex 1.

At the time of establishment of this study McCord's Hospital was receiving a Provincial Health Department subsidy to provide free mother-and-child health services to the hospital catchment area. Antenatal care, delivery, postnatal check-ups and medication were provided free. In July 1998 the Provincial Health Department withdrew its subsidy to McCord's Hospital as part of a cost-cutting exercise. From July 1998 onwards all women booking for antenatal care at McCord's were required to pay R300 to cover routine antenatal care and pay R800 (£80, the cost of a normal vaginal delivery) by the 36th week of gestation. A Caesarean section would cost R2000 (£200). Due to the introduction of user-fees the population profile of the hospital changed considerably and the number of women booking for antenatal care dropped from 45 to 25 per

week. Antenatal nursing staff became uncomfortable with suggesting that "fee-paying" clients see the HIV counsellor and VCT rates dropped in the first few months. In addition, the higher socio-economic status of those women booking for antenatal care resulted in a lower prevalence of HIV among the women consenting to HIV testing. The mothers and infants already enrolled in the study continued to be followed-up, and the research team were committed to providing free care for these infants. This resulted in the Vitamin A Study taking on the cost of medication and treatment for infants and mothers, which increased the cost of running the study considerably during the last six months at this site. For these reasons it was decided to investigate an alternative site for the current breastmilk study, in order to achieve the required sample size.

3.2.2.1.2 Cato Manor Clinic

The Cato Manor Clinic was identified as a possible alternative site for the current study through discussions with the clinician working with the Durban City Health Department. This clinic was located in a large informal settlement in Durban and served a population of up to 80,000 people (information from estimates carried out by the Cato Manor Development Association) in the surrounding areas. When the study commenced, the clinic was open only on Tuesday, Wednesday and Friday for general medical care and Thursday morning for antenatal care. During the study the clinic opened 5 days a week, but a Durban City Health clinician was only available on Tuesdays, Wednesdays and Fridays. It was staffed by nurses and family planning sisters.

When the Durban City Health Department was approached, HIV counselling and testing was available at Cato Manor on a very limited basis. Although some of the nurses had been trained in HIV counselling, the work load was such that only clients who specifically requested a test, or in whom there were clinical reasons for suggesting HIV testing, were counselled and tested. Blood samples were collected by City Health and taken to King Edward VIII Hospital for HIV antibody detection by ELISA. Results were transmitted back to the clinic via the City Health Department and could take over 2 weeks to return to the clinic.

In discussion with the staff at the clinic it was decided to establish voluntary HIV counselling and testing at the antenatal clinic on Thursday mornings. As part of the research project 2 trained HIV counsellors were made available to the antenatal clinic and VCT was introduced as part of the normal routine service for women booking for antenatal care. I transported blood samples back to King Edward VIII Hospital and collected results directly, thereby ensuring that these were available the following week.

The study protocol was submitted to the Durban City Health Department for approval, which was finally obtained in October 1998. Antenatal HIV counselling and testing was started at the end of October 1998. Due to limited nursing staff, only the first 20 of the women who presented to the clinic each week for their first antenatal visit were booked. The number turned away each week varied from 0-10. Women were counselled in groups of three and given the opportunity to give written consent to the test individually in private. 78.5% (907/1156 in Nov 98-October 99) of women counselled consented to testing, the remainder stating that they were either "not ready" or did not wish to know their results. Blood samples for HIV testing were taken if consent was given at the same time as blood sampling for syphilis testing. Depending on gestational age at booking visit women were given a clinic appointment 4 or 2 weeks later. During the period of the study the seroprevalence of HIV in women consenting to HIV testing at this antenatal clinic was 39%.

Women who were pregnant, had tested positive for HIV during antenatal care and planned to remain in the clinic catchment area for 9 months after delivery were eligible for inclusion in the study. The purpose of the study was explained and women who gave informed, written consent were recruited. The Subject Information leaflet in English and Zulu is included in Annex 1. At recruitment and each antenatal visit the study clinician counselled women on infant feeding choices, explaining the advantages and disadvantages of formula feeding, including the cost (about R150/month) of infant formula. Early results from the Vitamin A trial at McCord's Hospital became available just as recruitment started at Cato Manor. These indicated that there was no additional risk of transmission to 3 months of age with exclusive breastfeeding, compared with formula feeding, but that mixed feeding carried a much greater risk of MTCT (75). For this reason, and given the poor sanitary conditions in the informal settlement, women who did not have stable employment or financial support from family or partner and so chose to breastfeed,

were advised to exclusively breastfeed in the first few months of life. The study clinician supported every woman in her individual decision and provided advice and help in achieving that. Infant feeding advice for HIV-infected women was discussed with the clinic staff and a common message agreed upon, to ensure that women would receive similar advice from all the healthcare workers they came into contact with at the Cato Manor Clinic. However, the majority of women from Cato Manor deliver at King Edward VIII Hospital, where on occasion women were given conflicting advice regarding infant feeding choices.

All women enrolled in the study and at more than 28 weeks gestation were given capsules containing 15 mg vitamin A and 30mg β-carotene or multivitamins, to be taken daily as a nutritional supplement. This dose was the same as that given in the vitamin A arm of the Vitamin A supplementation trial at McCord's Hospital (see 3.2.2.1.1). Multivitamins, which contained 0.8 mg vitamin A and 0.2 mg β-carotene, were provided to the last 17 women (who delivered after the end of July 1999), when supplies of vitamin A and β-carotene had run out. Maternal baseline blood samples were taken at recruitment for CD4 and CD8 estimation by FACScan. In addition, women were asked to return to the clinic on the first Tuesday after their infant was born. At this visit mothers were given a capsule containing 60 mg of vitamin A. This was taken to be the 1 week follow-up visit. Women were asked about mode of delivery and duration of rupture of membranes. Gestational age was estimated routinely from date of last menstrual period, fundal height or ultrasound during antenatal visits. Most women booking at Cato Manor had one ultrasound scan during the second or third trimester. No formal assessment was made of gestational age on the infant at delivery.

At each visit at 1, 6 and 14 weeks of age, infants were weighed, given a 2ml/kg dose of lactulose:mannitol solution and urine collected for the next 5 hours in a paediatric urine collection bag, as described in 3.3.1.2.2. Breastmilk samples were collected from breastfeeding mothers as per protocol detailed in 3.3.1.2.1. Mothers were provided with a dedicated room with a TV in which to wait, and given tea, biscuits and lunch and R20 to cover transport and others costs incurred by the 5-hour urine collection.

When the infant was 6 and 9 months of age, mother-infant pairs were asked to return for follow-up to assess health status and record dietary history of the infant. Infants were weighed to the nearest 10g, length and head circumference measured to the nearest 0.1cm. At 9 months a 3ml sample of blood was taken from each infant and this was analysed for HIV-1 antibodies by ELISA at the Virology Laboratories of King Edward VIII Hospital. If the ELISA was positive, IgG3 or HIV antigen was determined (as per 3.2.2.1.1). Infants who were no longer breastfeeding and had HIV status determined were discharged. Infants who were still receiving breastmilk, or in whom HIV status could not be determined were given a further follow-up date 3 months later. After 12 months the infant blood sample was analysed for HIV-1 antibodies by ELISA only.

Mothers who failed to attend any scheduled visit were visited at home by the community health worker who was employed as a research assistant on the study and asked to attend the following week. Written remainders were not sent. Copies of the questionnaires used are included in Annex 1.

3.2.2.3 Summary of study procedure

Antenatal:	HIV voluntary counselling and testing; if HIV-infected invited to participate; counselled re infant feeding options and given vitamin supplements (after 28 weeks gestation). CD4/CD8 measured. Socio-economic details recorded.
1, 6 and 14 weeks:	Infant anthropometry recorded, L:M test and blood samples taken. Mother asked to give breastmilk samples and body temperature measured. Infant feeding practices recorded.
12 months:	Infant HIV status determined by ELISA and IgG3

3.3 Sample collection and laboratory analysis

3.3.1 Sample collection, processing and storage

3.3.1.1 Prevalence Study

3.3.1.1.2 Breastmilk samples

Mothers were asked to manually express 3-5 ml of breast milk from each breast into a separately labelled 50ml sterile polypropylene jar with screw-top lid. No special cleaning of the breast was required and mothers were not asked to restrict breastfeeding prior to collection, nor to empty the whole breast. All samples were collected between 7.30 am and 12.30 am and kept in a coolbox until transported to the Medical school (within 4 hours), where they were gently swirled to mix and aliquotted into two 1.5ml eppendorf tubes and stored at -20°C until analysed for Na/K and IL-8 as described in 3.3.2.1 and 3.3.2.2 respectively.

Samples to be analysed for Na/K were transported to London by air, packed in dry ice, as suitable analytical facilities were not available in Durban.

3.3.1.2 Cohort Study

3.3.1.2.1 Breastmilk samples

Mothers were asked to manually express 7-10ml of breastmilk into separately labelled 50ml sterile polypropylene jars with screw-top lids. No special cleaning of the breast was required and mothers were not asked to restrict breastfeeding prior to collection, nor to empty the whole breast. Breastmilk samples were collected between 9.00 am and 12.00 p.m. and stored in a cool room and transported to the Medical School in within 2 hours of collection.

Breastmilk samples were gently swirled to mix and two 1ml aliquots of whole milk stored in cryovials. One aliquot was used for Na/K analysis and one for IL-8 analysis.

3ml of whole milk were placed in a lidded plastic test tube and centrifuged at approximately 1000g for 10 minutes. The fat layer was removed from the top using cotton wool buds. Two 1ml aliquots of supernatant were stored in cryovials. In addition, the cell pellet was re-suspended in a small volume of supernatant and also stored in a cryovial. One aliquot of supernatant was used for cell-free HIV viral load quantification by PCR.

All breastmilk samples were initially stored in liquid nitrogen and periodically transferred to –70°C freezer in the Department of Paediatrics for long term storage. Samples to be analysed for Na/K were transported to London by air, packed in dry ice.

3.3.1.2.2 Intestinal permeability test

Intestinal permeability was measured by the dual sugar absorption test, which has been validated (136) and accepted as an indirect technique for monitoring intestinal mucosal changes in children (121;124). At each visit at 1, 6 and 14 weeks of age, infants were weighed, given a 2ml/kg dose of a solution containing 200mg/ml lactulose and 50mg/ml mannitol. Urine was collected for the next 5 hours using a paediatric urine collection bag, which was changed 5-8 times, depending on urine production. Difficulties were encountered in urine collection from female infants and the research assistants responsible for urine collection devised a system to ensure as complete a collection as possible. Urine bags for female infants had two cotton wool balls placed inside, to absorb urine as soon as it was produced and limit the leakage out of the bag. In addition, the disposable nappy was used inside-out, lined with a layer of clingfilm and a nappy liner, with an additional two cotton wool balls placed between the nappy liner and the infant. This was to reduce faecal contamination of the urine collection. The photograph in figure 3.3.1.2.2 shows the research assistant collecting samples from an infant on the study.

Figure 3.3.1.2.2: Urine collection from young infant

a) Administering the lactulose:mannitol solution

b) Changing urine collection bag

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Urine collection bags were emptied into a large screw-top plastic container, which had been washed out with disinfectant solution immediately prior to use. These jars were tightly capped and urine stored in a cool room and transported to the Medical School within 1 hour of completion of the collection period.

Urine samples were gently swirled to mix. Two 1ml aliquots were stored in cryovials at –20°C. Samples were transported to London by air, packed in dry ice, as analysis could not be carried out in Durban.

3.3.2 Laboratory analysis

3.3.2.1 Breastmilk Sodium/Potassium

Flame photometry analysis was used to quantify Na/K in breastmilk. This was carried out at the Chemical Pathology Laboratory of Great Ormond Street Hospital for Sick Children in London, as suitable facilities were not available in Durban.

The IL943 is a digital flame photometer used to determine sodium and potassium in urine, plasma and other body fluids. Percentage recovery of Na/K in spiked breastmilk samples using this technique was 104% ±1%, and was not influenced by milk fat content (Woodfin and Filteau, 1999, unpublished) and coefficient of variation was 0.94% for Na and 1.18% for K (n=58).

3.3.2.1.1 Principle of flame photometry

The alkaline metals (Group I of the periodic table) absorb energy when heated. This energy absorption converts the atoms into their excited state. As the atoms cool, the energy is re-emitted as radiation, some of which is in the visible part of the spectrum, at wavelengths specific for individual elements. In dilute solutions, the amount of emitted light is proportional to the concentration of the element.

In the flame photometer, a small amount of the sample is passed through a propane gas flame, which causes the respective colours of the constituent elements to be emitted. These are detected within the machine through a set of filters. Due to the instability of a flame - any disturbance can greatly affect the colours - an internal standard is used. In most cases, a disturbance or "noise" can be compensated by the machine by comparing back to this standard. In the IL943, the internal standard used is a 1.5 mmol/L Caesium solution. In some cases, the viscosity of the sample will affect the accuracy of the result obtained. For analysis of breastmilk, the urine setting was used, as this has a wider range than the serum setting. Analysis was carried out according to the instrument instructions.

3.3.2.2 Breastmilk Interleukin-8

The method used was ELISA, utilising the PeliKine Compact™ Human IL-8 ELISA kit from CLB (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, catalogue number M1918). Adaptations for use with human breastmilk had been developed by Dr. Suzanne Filteau at the Centre for International Child Health, Institute of Child Health in London (97).

Percentage recovery of IL-8 in spiked breastmilk samples using this technique was 141% ±46% (97) ; intraplate and interplate coefficient of variation for quality control samples included in each assay run in Durban were 4.88% (n=10) and 19.57% (n= 72) respectively. ELSIA techniques are subject to greater variation than many other laboratory techniques. The high recovery of IL-8 after spiking needs to be investigated further.

3.3.2.2.1 Principles of ELISA

Enzyme-linked immunosorbant assays (ELISA) are based on the principle of antibody binding. In a sandwich ELISA, such as that used to quantify IL-8, microtire plates suitable for ELISA are coated with a specific antibody to the protein to be measured. When the sample or standard is added, the specific proteins bind to the antibody during the incubation period, to reach a point of equilibrium. Binding is such that proteins bound to antibody are not washed off during washing steps, but excess protein is effectively removed. The addition of a detergent, such as Tween20

to the washing buffer reduces non-specific binding and helps in removal of unbound antibodies during the washing process. Another specific antibody to the protein in question, conjugated to a marker, such as biotin, is then added, and this binds in the same way. By adding horse-raddish peroxidase (HRP) conjugated strepavidin, which binds to the biotin conjugated antibody, it is possible to produce a colour reaction on the addition of a substrate. After washing to remove excess conjugate a substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide is added this will form a colour complex. The reaction can be stopped by the addition of 2M sulphuric acid and the optical density of the colour complex in each well can be measured by an ELISA reader. This is used to extrapolate IL-8 concentration from a standard curve.

3.3.2.3 Human immunodeficiency viral load

Quantification of breastmilk cell-free HIV ribonucleic acid (RNA) was carried out using the Amplicor HIV-1 Monitor™, version1.5 kit from Roche Diagnostic Systems, Inc. by polymerase chain reaction (PCR), which has been shown to be sensitive for HIV clades present in KwaZulu-Natal (personal communication, Dr Dennis York, University of Natal Medical School, 1999)

3.3.2.3.1 Principles of PCR

The polymerase chain reaction (PCR) can be used to generate copies of a piece of DNA, which can then be bound to a probe, allowing colorimetric determination. For the quantification of cell-free HIV RNA a 155 base target sequence of HIV-1 RNA first undergoes reverse transcription to generate cDNA. This target sequence is in a highly conserved region of the gag gene. By using specific thermostable enzyme (*Thermus thermophilus* DNA Polymerase) under suitable conditions, reverse transcription and cDNA amplification using specific primers occur in the same mixture during a number of temperature cycles. The amplified DNA is then denatured to form single-strand DNA that can be bound to specific probes on a microwell plate by hybridization. The addition of avidin-horseradish peroxidase conjugate results in specific binding to biotin-labelled DNA amplicon captured by the plate-bound probes. After washing to remove excess conjugate a substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide is added this will form a colour complex. The reaction can be stopped by the addition

of weak acid and the optical density of the colour complex in each well determined on a microplate reader. The use of an internal Quantitation Standard of known copy number controls for the amplification process and compensates for effects of inhibition, to allow the accurate calculation of the number of copies of target RNA by comparison.

3.3.2.4 Intestinal permeability test

Lactulose and mannitol concentrations in urine are determined by enzymatic methods using a CobasFara centrifugal analyzer (Roche) and using methods adapted by Willumsen *et al.* (137). Percentage recovery of 0.125 mg/ml lactulose and mannitol in spiked urine samples using this technique was 100.80% and 99.98% respectively (Willumsen, MSc dissertation, unpublished 1994) and coefficient of variation for assays run as part of this study was 20.83% for lactulose and 4.93% for mannitol (n=37).

3.3.2.4.1 Principles of lactulose:mannitol test

This test uses two sugars of different molecular size, which are not normally digested in the gut, but excreted intact in the urine. The ratio of lactulose to mannitol (L:M) excreted in the urine after a standard dose and 5 hour urine collection time, gives an indication of intestinal mucosal permeability; as permeability increases, more of the larger, paracellularly-transported sugar (lactulose) is present in the urine. If absorptive suface area is reduced, less of the smaller, transcellularly-transported sugar (mannitol) is present in urine. Both these situations result in a high L:M ratio.

The mannitol assay is based on the oxidation of mannitol to fructose by NAD-specific *D*-mannitol dehydrogenase from *Leuconostoc mesenteroides* (Biocatalysts Ltd, UK). The change in absorbance at 340 nm is measured to indicate the rate of reduction of NAD to NADH. Hexokinase is added to the coenzyme mixture to abolish the inhibitory effect of increasing fructose concentration (138)

The lactulose assay uses a quadruple enzyme mixture to produce NADPH from lactulose, which will also act on the glucose, fructose and lactose also present in the urine sample. For this reason, it is necessary to incubate the urine sample with and without β-galactosidase, in order to determine the amount of NADPH produced by the free monosaccharides and those then derived from lactulose. The method used (137) had been adapted from that of Northrop *et al.* (139).

3.4 Statistical analysis

Data was entered and double-checked using EpiInfo version 6 and SPSS 8.0. Statistical analyses were carried out using SPSS 8.0. Variables that were not normally distributed were natural log (Na/K and IL-8) or log base 10 (viral load) transformed and geometric means and 95% Confidence Intervals (95%CI) have been reported. Gestational age, maternal age and CD4 count were categorised for inclusion in some analyses. Analyses were considered significant at the $p < 0.05$ level.

Statistical methods used include paired and independent t-tests for comparison of groups and interbreast differences, Analysis of Variance (ANOVA) to investigate the association between feeding mode and Na/K, with post-hoc analysis by Student-Newman-Keuls where appropriate, Chi Square and bivariate correlation between breastmilk Na/K, IL-8 and viral load and mean breastmilk Na/K and IL-8 and L:M. Univariate and multivariate analyses were carried out by linear regression for continuous dependent variables and logistic regression for binary dependent variables to investigate factors important in determining breastmilk Na/K and viral load levels. Further details are provided in chapters 4 and 5 when describing statistical analyses.

3.5 The researcher's role

As this work was carried out as part of a collaborative project between the Centre for International Child Health in London and the Department of Paediatrics and Child Health in Durban, and part was nested within on-going studies, it is appropriate to clarify my role in the research.

The prevalence study was designed in collaboration with Dr Suzanne Filteau in London. I designed the questionnaires, recruited, trained and supervised the research assistant and sought permission from the study sites with help from Dr Anna Coutsoudis in Durban. I was responsible for sample collection and processing and data entry.

The cohort study was initially nested within an on-going study of gut permeability of infants born to HIV-infected mothers who were part of a placebo-controlled study of Vitamin A supplementation during pregnancy. The Vitamin A supplementation trial was co-ordinated by Dr Anna Coutsoudis and the gut permeability study by Dr Nigel Rollins. The study site at McCord's Hospital had been established and mothers and infants were under the clinical care of the study clinicians. Blood samples were taken by the research phlebotomist/nurse and processed by a research assistant at the Department of Paediatrics. Urine collection was carried out during the follow-up visits by the research assistant, supervised by the research nurse and myself. I added the breast health questions to the morbidity questionnaire, developed and co-ordinated the collection of breastmilk and processing of the samples.

I was responsible for the establishment of the Cato Manor clinic study site. I was involved in securing funding for the study from UNICEF – South Africa. I approached the City Health Department clinician at Cato Manor, requested permission from the Durban City Health Department to conduct a study at the clinic, met with clinic staff to discuss establishing antenatal HIV screening and explain the purpose of the study, recruited HIV counsellors and assisted in training them in aspects of antenatal HIV counselling and the study and equipped the clinic for the purposes of the study. The research clinician and phlebotomist/nurse from the McCord's Hospital study site also worked at Cato Manor. A new research assistant from within the Cato Manor community was appointed, following mathematics test and interviews with the

clinic community health workers. In addition, regular update meeting were held with the clinic staff to feed back progress with HIV screening and the study, and allow issues concerning them to be dealt with.

Breastmilk Na/K and urinary lactulose:mannitol analysis was carried out by Steve Bilotta, Richard Beesley and Abigail Woodfin, under the supervision of Frances Taylor of the Chemical Pathology Laboratory and Dr. Suzanne Filteau of the Centre for International Child Health.

I carried out breastmilk IL-8 analysis at the Department of Paediatrics laboratory of the University of Natal Medical School in Durban.

HIV PCR was carried out by Subitha Dwarika , under supervision of Dr. Denis York at the Department of Virology laboratory of the University of Natal Medical School in Durban.

I was responsible for the planning of and carried out all the statistical analyses present in this thesis.

4: Prevalence of subclinical mastitis among lactating women in Durban

4.1 Objective

To determine the prevalence of subclinical mastitis among lactating women in and around Durban, KwaZulu-Natal, South Africa.

4.2 Study design

To investigate the prevalence of subclinical mastitis among lactating women in urban and peri-urban Durban, a cross-sectional study was carried out of women attending routine vaccination clinics with their breastfed infants. Breastfeeding mothers from 3 sites in and around Durban, including some women from an on-going study of HIV-infected mothers at one of the sites, were interviewed, asked about infant feeding practices in the previous 24 hours and infant birthweight and gestational age were recorded from Road-to-Health cards. Women were asked to provide a small (3-5 ml) sample of breastmilk from each breast by manual expression. For further details, please refer to 3.2.1.

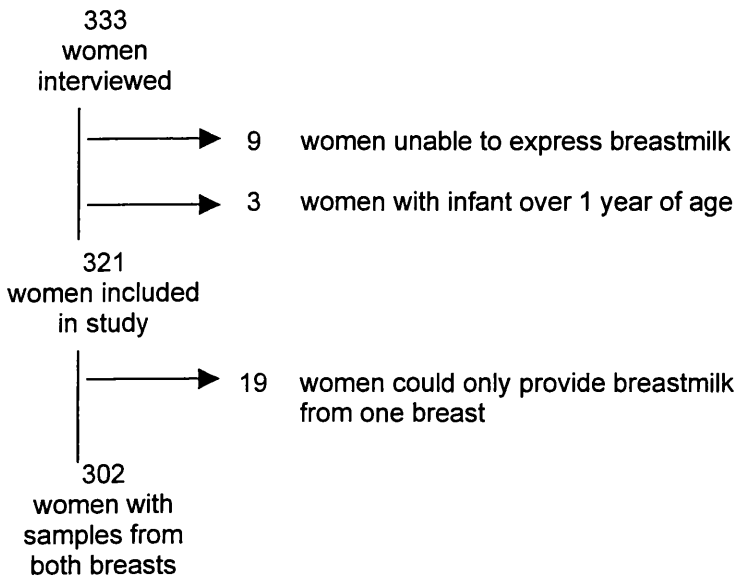
4.3 Subject characteristics

Although no data was collected on the number of women refusing to participate in this study, or their reasons for not wishing to participate, anecdotal information indicates that the reasons most commonly given for not taking part were that they would lose their place in the queue and what would they gain by participating (payment?). In the light of this it was decided to offer all women who agreed to participate in this study a carton of juice (for them, not their baby) in appreciation of their time. This was introduced after one session at McCord's Hospital and resulted in an improvement in recruitment.

A total of 333 women were interviewed between November 1997 and March 1998. Of these, 9 were unable to provide a breastmilk sample and 3 had an infant over one year of age and these 12 have been excluded from the analysis.

Data from the remaining 321 women have been included in the current study. Of these, 19 were able to provide a milk sample from only one breast, leaving 302 women with a breastmilk samples from both right and left breast. In the case of twins, infant data from the first-born twin only has been used. An outline of the recruitment is shown in figure 4.3.1.

Figure 4.3.1 Outline of recruitment and inclusion into the study



Subject characteristics are shown in table 4.3.2. Maternal age was not recorded for all women and birthweight and gestational age were not always recorded on Road-to-Health cards. In addition, it is possible that birthweight was not recorded accurately on some cards, as for example it is rare for a baby born at only 850g to survive in this setting. No gestational age had been recorded for this child. The national policy in South African public hospitals is to not ventilate a neonate of less than 1,000g, but provide them with full supportive care, as chances of survival are small and there is a chronic shortage of ventilators and staff to care for these babies. King Edward VIII University Hospital, which is the tertiary referral hospital for the whole

of KwaZulu-Natal, follows this policy, but supportive care is of a very high standard and some neonates of 750g have survived (personal communication Prof. M Adhikari, Department of Paediatrics, King Edward VIII Hospital).

Overall, 15.8% (46/292) of mothers were teenagers (≤ 19 years), 4.8% (12/249) of infants were preterm (<37 completed weeks of gestation) and 10.2% (31/304) of low birth weight (≤2,500g at birth). There were significantly more young mothers, premature and low birthweight infants at the Valley Trust (14/37, 37.8%, 1/11, 9.1%, 6/31, 19.4% respectively) than Lamontville (16/104, 15.4%, 1/91, 1.1%, 7/97, 7.2%respectively) or McCord's (16/150, 10.7%, 10/146, 6.8%, 14/150, 9.3% respectively).

Table 4.3.2: Subject characteristics at each site and for the study as a whole

Mean ± SD	McCord's n = 153	Lamontville n = 104	Valley Trust n = 38	HIV study cohort at McCord's n = 26	All n = 321
Maternal age, yrs (range) n =	27.7 ± 6.0 (15-43) 150	25.3 ± 5.6 (15-42) 104	23.1 ± 6.5 (16-41) 37		26.2 ± 6.1 (15-43) 291
Infant age, wks (range) n =	4.0 ± 5.1 (0.4-43.1) 153	14.0 ± 9.0 (0.3-46.0) 104	19.9 ± 12.4 (2.0-47.0) 38	11.9 ± 4.0 (5.3-19.1) 26	9.8 ± 9.6 (0.3-47.0) 321
Gest age, wks (range) n =	39.3 ± 1.8 (32-42) 146	40.0 ± 1.0 (34-42) 91	38.8 ± 4.3 (26-41) 11		39.5 ± 1.8 (26-42) 248
Birthweight, g (range) n =	3114 ± 474 (1450-4200) 150	3217 ± 561 (850-5700) 97	2997 ± 632 (1450-4500) 31	3066 ± 553 (1540-3940) 26	3131 ± 529 (850-5700) 304

According to the original sample size calculations 100, 75 and 100 infants were required aged 1, 6 and 14 weeks respectively. As attendance at immunisation clinics was not at exactly these ages, infants have been grouped into the age groups shown in table 4.3.3 below. These include a young age group ≤2 weeks and then age groups around the time of DPT immunisations, followed by an age group to include all other infants. Due to the different clinic criteria, (see 3.2.1.1) there was not an even spread of infant age groups across the different study sites, as indicated by mean infant age in table 4.3.2.

Table 4.3.3: Infant age group distribution at each study site

Age group (weeks)	McCord's (%)	Lamontville (%)	Valley Trust (%)	HIV study cohort (%)	All (%)
0-2	79 (51.6)	5 (4.8)	1 (2.6)		85 (26.5)
2.1-6	35 (22.9)	15 (14.4)	4 (10.5)	3 (11.5)	57 (17.8)
6.1-14	34 (22.2)	33 (31.7)	10 (26.3)	16 (61.5)	93 (29.0)
14.1-20	3 (2.0)	35 (33.7)	6 (15.8)	7 (26.9)	51 (15.9)
20.1-52	2 (1.3)	16 (15.4)	17 (44.7)		35 (10.9)
total	153	104	38	26	321

Percentages presented for each site and may not add to 100 due to rounding

As milk stasis during weaning results in high Na/K ratio due to involution of the mammary epithelium (78), the effect of breastmilk displacement by other energy sources on breastmilk Na/K ratio was of interest. Data elating to infant feeding mode were therefore split into three categories: breastmilk only (no water, no juice etc.), breastmilk and infant formula (volume not specified) or breastmilk and water (there were only 3 infants who had received water, but not formula) and breastmilk and other foods (including commercial baby foods and family foods). Some infants in the latter category had also been offered infant formula, but were categorised as breastmilk and other foods. Infant diet varied by age group and study site, as can be seen below in table 4.3.4. Infant feeding data was not collected from the HIV study cohort.

Table 4.3.4: Infant feeding by age and at each study site

Age group (wks)	Dietary recall	McCord's n = 152	Lamontville n = 104	Valley Trust n = 38	All (%) n = 294	
0-2	BM only	72	4	1	77	(90.6)
	BM + formula	5			5	(5.9)
	BM + foods	2	1		3	(3.5)
2.1-6	BM only	26	11	3	40	(74.1)
	BM + formula	7	3	1	11	(20.4)
	BM + foods	2	1		3	(11.1)
6.1-14	BM only	22	10	4	36	(47.4)
	BM + formula	8	15	2	25	(32.9)
	BM + foods	3	8	4	15	(19.7)
14.1-20	BM only	2	2	2	6	(13.6)
	BM + formula		10		10	(22.7)
	BM + foods	1	23	4	28	(63.6)
20.1-52	BM only					
	BM + formula		1		1	(2.9)
	BM + foods	2	15	17	34	(97.1)

BM = breastmilk. Percentages presented for each age group and may not add to 100 due to rounding

The WHO/UNICEF guidelines in use at the time of this study recommended that infants be exclusively breastfed (EBF) for 4-6 months (140). Taking a median age from these recommendations of 5 months (20 weeks) and using a definition of EBF as no water, juice, tea, infant formula or other foods in the previous 24 hours (140), 159/294 (54.1%) of infants aged ≤20 weeks were exclusively breastfed in the preceding 24 hours. As can be seen from table 4.3.4, 57.9% (150/259) of the infants ≤20 weeks of age were recruited from McCord's, 34.0% (88) from Lamontville and only 8.1% (21) from Valley Trust. For the infants for whom dietary recall data was available, table 4.3.5 indicates that there was a difference in the number of infants ≤20 weeks of age who were exclusively breastfed at each study site.

Table 4.3.5: Infants ≤20 weeks of age who were exclusively breastfed at each study site

Feeding	McCord's (%)		Lamontville (%)		Valley Trust (%)		All (%)	
EBF	122	(81.3)	27	(30.7)	10	(47.6)	159	(61.2)
Mixed feeding	28		61		11		100	
total	150		88		21		259	

EBF = exclusive breastfeeding.

4.4 Breastmilk biochemistry

Breastmilk sodium/potassium ratio (Na/K) and IL-8 were not normally distributed and were thus natural log (ln) transformed before statistical analysis. Geometric means, with 95% confidence intervals are presented throughout.

Breastmilk samples were available from 321 women. 302 women gave breastmilk samples from both breasts and for 19 women a breastmilk sample was available from one breast only. In some cases the breastmilk expressed was insufficient for analysis of both Na/K and IL-8. Only women who were unable to provide a breastmilk sample from either breast were excluded.

Geometric mean Na/K ratio was 0.57 (95%CI 0.52-0.62, n=311) in the left breast, 0.62 (0.56-0.69, n=312) in the right and 0.59 (0.55-0.63) in the total of 623 breastmilk samples. Na/K ≤0.6 is considered to be normal (see table 2.2.1.1). Geometric mean Na/K did not significantly differ between sites (ANOVA p=0.60) and in further analysis sites have been combined.

Geometric mean IL-8 concentrations were 179.5 pg/ml (95%CI 146.9-219.2, n=304) and 244.7 pg/ml (194.9-307.2, n=309) in the left and right breasts respectively, and 208.5 pg/ml (194.7-223.2) in the sample as a whole. No cut-off was available for normal IL-8 concentrations in breastmilk. Geometric mean IL-8 did not significantly differ between sites (ANOVA p= 0.37) and in further analysis sites have been combined.

The mean values for Na/K and IL-8 in left and right breasts overlap considerably and in future analysis no consideration will be made as to whether a breastmilk sample originated from the left or right breast. However, differences between breasts were important, as indicated by the significant difference between individual pairs of breastmilk samples. A Paired t-test of Na/K showed a significant difference in Na/K (p=0.032) and IL-8 (p=0.01) between breasts, indicating that interbreasts differences are important within individuals. Very few women reported breast pain or tenderness (9/293, 3.1%) or sore or cracked nipples (19/293, 6.5%) when specifically asked about such problems. There was no significant difference in Na/K ratio in samples from women who reported breast or nipple problems compared with those who did not (0.60, 95%CI 0.41-0.90, n=16 samples vs. 0.59, 0.55-0.63, n=556 samples, p=0.901 and 0.62, 0.48-0.81, n=35 samples vs. 0.58, 0.54-0.63, n=542 samples, p=0.667 respectively) and this has not been analysed further. It should be noted that 2/9 women with breast tenderness and 3/19 women with sore or cracked nipples were unable to give a breastmilk sample from the affected breast.

Cut-off points for Na/K ratio were set as follows: normal Na/K ≤0.6, slightly raised >0.6 ≤ 1.0, very raised >1.0. The Na/K cut-off points used were the same as those used in the study by Filteau *et al.* in Bangladesh (97) and are derived from published Na levels in milk of healthy women (see section 2.2.1.3). Na/K >1 is equivalent to about 18 mmol/L sodium, which after the first few days of lactation is indicative of mammary gland inflammation, or involution due to milk stasis (78). Histograms of Na/K and IL-8 are shown in Annex 2, figure 4.4.1. The percentage of breastmilk samples with each level of Na/K ratio are shown below in table 4.4.1.

Table 4.4.1: Proportion of breastmilk samples with low, mildly and severely raised Na/K

	n= 302 (%)
bilaterally normal	149 (49.3)
unilaterally mildly raised	49 (16.2)
bilaterally mildly raised	24 (7.9)
Bilaterally ≤1.0	**222 (73.5)**
unilaterally severely raised	24 (7.9)
one mildly, one severely raised	32 (10.6)
Unilaterally >1.0	**56 (18.5)**
bilaterally severely raised	24 (7.9)
Bilaterally >1.0	**24 (7.9)**

Percentages may not add to 100 due to rounding. Not all women were able to provide sufficient sample from both breasts for all laboratory analysis. The women included here gave sufficient breastmilk from both breasts for Na/K analysis. Normal Na/K ≤0.6, mildly raised >0.6 ≤1.0, severely raised >1.0.

As severely raised Na/K would be expected to have greatest impact on breast health indicators and possibly infant health, further analysis will concentrate on Na/K >1.0. More than a quarter of women (80/302, 26.5%) had Na/K ratio >1 in at least one breast. Of the 302 women for whom two breastmilk samples were available, 56 (18.5%) had Na/K >1 in only one breast and 24 (7.9%) had very raised Na/K in both breasts. Na/K correlated with levels of IL-8 in the breastmilk (correlation coefficient= 0.64, p<0.001). Partial correlations, controlling for infant age in days, were significant in women where Na/K was low in both breasts and in women with unilaterally raised Na/K in both the severely raised and in the normal breast. However, in women with bilaterally severely raised Na/K, breastmilk IL-8 and Na/K did not correlate, as shown in table 4.4.2.

Table 4.4.2: Partial correlation between breastmilk Na/K and IL-8, controlling for days postpartum

	partial correlation	p =	no. of women
unilaterally severely raised breast	0.27	0.045	}
unilaterally normal breast	0.37	0.006	} 55
bilaterally severely raised breasts	0.18	0.233	22
bilaterally normal breasts	0.34	<0.001	
			218

Total number of women with breastmilk Na/K and IL-8 data available from both breasts =295. Normal Na/K ≤1.0, severely raised >1.0.

4.5 Subclinical mastitis

4.5.1 Subclinical mastitis and infant age

Both unilaterally and bilaterally raised Na/K was more common in mothers of younger infants, as shown in table 4.5.1.1.

Table 4.5.1.1: Number of women with mildly and severely raised breastmilk Na/K ratio in each infant age group

Infant age	0-2 wks n= 78 (%)	2.1-6 wks n= 56 (%)	6.1-14 wks n= 88 (%)	14.1-20 wks n= 46 (%)	20.1-52 wks n= 34 (%)	All n= 302 (%)
bilaterally normal	33 (42.3)	24 (42.9)	44 (50.0)	24 (52.2)	24 (70.6)	149 (49.3)
unilaterally mild	12 (15.4)	10 (17.9)	13 (14.8)	9 (19.6)	5 (14.7)	49 (16.2)
bilaterally mild	6 (7.7)	4 (7.1)	8 (9.1)	5 (10.9)	1 (2.9)	24 (7.9)
Bilaterally ≤1.0		**38 (67.9)**	**65 (73.9)**	**38 (82.6)**	**30 (88.2)**	**222 (73.5)**
	51 (65.8)					
unilaterally severe	4 (5.1)	7 (12.5)	7 (7.9)	4 (8.7)	2 (5.9)	24 (7.9)
one mild, one severe	13 (16.7)	7 (12.5)	9 (10.2)	2 (4.3)	1 (2.9)	32 (10.6)
Unilaterally >1.0		**14 (25.0)**	**16 (18.2)**	**6 (13.0)**	**3 (8.8)**	**56 (18.5)**
	17 (21.8)					
bilaterally severe	10 (12.8)	4 (7.1)	7 (7.9)	2 (4.3)	1 (2.9)	24 (7.9)
Bilaterally >1.0	**10 (12.8)**	**4 (7.1)**	**7 (7.9)**	**2 (4.3)**	**1 (2.9)**	**24 (7.9)**

Percentages presented for each age group and may not add to 100 due to rounding. Note: breastmilk samples from both breasts only available from 302 women. Unilaterally raised = Na/K ratio >0.6 in one breast only; bilaterally raised = Na/K >0.6 in both breasts; bilaterally normal = Na/K ≤0.6 in both breasts.

Unilateral Na/K >1.0 was present in 17/78 (21.8%) mothers of infants ≤2 weeks of age. Interestingly, when the lower cut-off of >0.6 was used in this age group 20.7% (6/29) of cases of bilaterally raised Na/K had only marginally raised Na/K (between 0.6 and 1) in both breasts, whereas 44.8% (13/29) had mildly raised in one breast and severely raised in the other. Mildly bilaterally raised Na/K (>0.6 ≤1.0) may be due to the mammary epithelium tight junctions still being open during early lactation. Mean infant age according to type of subclinical mastitis is shown in table 4.5.1.2 below.

Table 4.5.1.2: Mean infant age (days) by type of subclinical mastitis

	Mean infant age (days) ± sd	n =
bilaterally normal ≤0.6	80.1 ± 75.0	149
unilaterally mildly raised	66.0 ± 61.7	74
bilaterally mildly raised	49.7 ± 51.7	79
bilaterally normal ≤1	75.6 ± 71.5	222
unilaterally severely raised	50.5 ± 52.0	56
bilaterally severely raised	47.2 ± 45.8	24

Normal Na/K ≤0.6, mildly raised >0.6 ≤1.0, severely raised >1.0.

It can be seen that women with bilaterally mildly raised Na/K >0.6 tended to have the youngest infants (49 days vs. 66 or 80 days in unilaterally raised and bilaterally low Na/K ratio groups), but the standard deviation in each group was very large. This trend was confirmed by ANOVA of infant age according to type of subclinical mastitis, which was significant when using both the 0.6 and 1.0 cut-off for Na/K (p=0.005 and 0.011 respectively). Post-hoc analysis, using the Student-Newmans-Keuls test for homogenous subsets, indicated that there was a trend for the infants of women with bilateral Na/K >0.6 to be younger than those with bilateral Na/K ≤0.6, but this trend did not reach significance at the 1.0 cut-off.

As bilaterally raised Na/K may be more common in women who are still producing colostrum, analysis was restricted to the 0-2 week age group. However, although there was trend for mothers with bilaterally raised Na/K >0.6 or >1 in this group to have the youngest infants, this did not reach significance by ANOVA (p=0.13 and 0.17 respectively), possibly due to the small number of cases in this sub-group.

4.5.2 Subclinical mastitis and infant feeding

Fifty-four percent (159/294) of all infants for whom dietary recall data was available had been exclusively breastfed, 17.7% (52/294) had received formula and the remainder (28.2%, n=83) had received complementary foods in addition to breastmilk in the previous 24 hours. Analysis of variance indicated that infant feeding in the previous 24 hours influenced breastmilk Na/K levels (p<0.001) and all three groups were significantly different. Mothers of the 52 infants who

had received formula in addition to breastmilk had higher Na/K (geometric mean 0.95, 95%CI 0.74-1.22) than the 159 mothers of infants who had been exclusively breastfed (0.58, 0.53-0.62) and those who had used complementary foods had the lowest Na/K (0.43, 0.38-0.47, p<0.001) and each feeding group was significantly different from the other two.

Of the 279 women for whom breastmilk from both breasts and infant dietary recall data was available, 54.1% (151/279) reported exclusive breastfeeding in the previous 24 hours. The number of women with bilaterally normal, severely raised or unilaterally severely raised Na/K >1.0 according to infant dietary recall is presented in table 4.5.2.1.

Table 4.5.2.1: Type of subclinical mastitis by 24-hour infant dietary recall

Dietary recall	EBF n= 151 (%)	BM + formula n= 48 (%)	BM + foods n= 80 (%)
bilaterally normal	109 (72.2)	27 (56.3)	69 (86.3)
unilaterally severely raised	32 (21.2)	9 (18.8)	9 (11.3)
bilaterally severely raised	10 (6.6)	12 (25.0)	2 (2.5)

Percentages presented for each feeding group and may not add to 100 due to rounding. Breastmilk samples from both breasts and infant dietary recall only available from 279 women. Unilaterally severely raised = Na/K ratio >1 in one breast only; bilaterally severely raised = Na/K >1 in both breasts; bilaterally normal = Na/K ≤1 in both breasts.

As can be seen from this table 4.5.2.1, among women with severely raised Na/K, more women who reported exclusive breastfeeding (32/42), or using complementary foods (9/11) in the last 24 hours had unilateral than bilateral Na/K >1. However, women who had given formula (12/21) were more likely to have bilateral Na/K >1 than unilateral.

21.2% (32/151) of the women reporting exclusive breastfeeding in the previous 24 hours had unilaterally raised Na/K >1. Interestingly, when the lower cut-off of 0.6 was used, this rises to 39/151 (25.8%), indicating that the majority (32/39) of cases of unilaterally raised Na/K were >1.0 and not in the marginal 0.6-1.0 range. A Chi square analysis of type of subclinical mastitis and feeding in the last 24 hours showed that there was a significant difference between groups, using the 0.6 or 1.0 cut-off for Na/K ratio (p<0.001). More women with unilaterally raised Na/K reported exclusive breastfeeding than using formula or complementary foods in addition to breastmilk.

4.6 Discussion

This prevalence study was conducted at three different sites in and around Durban. The sample population was breastfeeding women attending free Mother and Child Health services with an infant of less than one year of age. Due to the different clinic criteria there was not an even spread of infant ages across the three sites. In addition, there were differences in indicators of infant and mother health and well-being, as suggested by the higher proportion of young mothers, preterm and low birthweight infants at the rural site (Valley Trust). This may be due to the different population profile of the clinic catchment areas. However, these indicators of socio-economic and health status do not seem to influence breastmilk biochemistry and during all further analysis of breastmilk components study sites were combined.

Exclusive breastfeeding rates were higher than have been previously reported for sub-Saharan Africa (no figures were available for South Africa) as a whole (141). This maybe due to the large number of mothers of young infants recruited from the McCord's study site. McCord's Hospital had a proactive and very supportive attitude amongst nursing staff towards breastfeeding, which was not noted at the other study sites. The very low EBF rates reported at Lamontville clinic could be said to be more typical of an urban population in a country in transition, such as South Africa, where formula feeding is considered "modern" and is accessible, if not always affordable. A previous study of constraints to EBF among 58 women at Valley Trust indicated that 42% of infants were EBF at 6 weeks and only 4% at 3 months (142). In that study the main reasons given for supplementation were "insufficient milk" or that breastfeeding was "for the poor". In the present study 73.5% (25/32) of infants 6 weeks of age were EBF. The study by Carter (142) used a lengthy structured questionnaire administered to women with infants less than 6 months of age attending the Valley Trust clinic and asked about infant feeding practices since birth, including age of introduction of other formula and other foods. In contrast, this study asked specifically about infant feeding in the preceding 24 hours, which is the recommended definition of EBF (140) and less likely to be biased by a lengthy recall period. Given the very proactive attitude towards breastfeeding at McCord's it is possible that some women felt obliged to report exclusive breastfeeding when interviewed at the hospital. This was very difficult to double-check in the context of a cross-sectional study, but might be considered in future work relating to exclusive breastfeeding and the risk of subclinical mastitis.

This study has analysed samples taken from both breasts and some interesting differences were found in the possible aetiology of subclinical mastitis depending on whether one or both breasts were involved. When Na/K was raised in one breast only (unilaterally severely raised Na/K) it was found to correlate with breastmilk levels of the inflammatory cytokine IL-8. However, when Na/K was raised in both breasts (bilaterally raised Na/K), breastmilk IL-8 did not correlate with Na/K. This finding would suggest that unilaterally raised Na/K resulted from a local inflammatory response as IL-8 is known to be produced locally by the mammary epithelium (93). This local inflammation may be due to infective agents or mechanical damage due to poor infant positioning during suckling.

In contrast, in cases of bilaterally raised Na/K, the lack of correlation between Na/K and IL-8 suggests either a systemic inflammatory response or milk stasis and mammary gland involution. The former would result in the mammary epithelium becoming increasingly permeable to Na ions and/or systemically produced IL-8 entering the breastmilk due to a systemic inflammatory response. The latter would result in an increase in Na concentration due to mammary gland involution and disruption of the mammary epithelium tight junctions, due to gradual weaning, fall in breastmilk production and milk stasis.

The finding that bilaterally raised Na/K was more common in mothers of younger infants raises the possibility that this was due to sampling from women in whom the tight junctions of the mammary epithelium had not yet closed. Although in the study sample as a whole there was a trend for women with bilaterally raised Na/K to have the youngest infants (by ANOVA), this was probably not due to a greater number of these women being in early lactation; an ANOVA of type of subclinical mastitis and infant age in the 0-2 week age group only was not statistically significant, indicating that raised Na/K was not just a physiological phenomena as found in colostrum. It is possible that women with younger infants were experiencing difficulties in positioning the infant at the breast. This might result in damage to the mammary epithelium and inflammation. However, very few women reported breast pain or cracked nipples when specifically asked about these problems.

Milk stasis due to weaning or the displacement of breastmilk by formula might be another cause of bilaterally raised Na/K. The results indicate that a higher proportion of the women reporting that their infant had consumed formula in the previous 24 hours had bilaterally raised Na/K (25.0%) compared with those who had breastfed exclusively (6.6%). Mothers of infants who had received formula had a higher geometric mean Na/K ratio than those who had exclusively breastfed, and mothers who had given complementary foods had the lowest mean Na/K (0.95 vs. 0.58 vs. 0.43 respectively, $p<0.001$). It is possible that infant formula displaces breastmilk, resulting in sub-optimal emptying of the breast, milk stasis and mammary gland involution. The finding that mothers who reported having given some complementary foods had lower mean Na/K than those exclusively breastfeeding is more difficult to explain. Complementary foods are introduced in small quantities very early in life. In the confines of this cross-sectional study no attempt was made to ask mothers to estimate portion size or volume of feeds. However, if very small quantities of complementary feeds are given which do not displace breastmilk, or if the infant is given additional foods because he is very hungry, breast emptying would be expected to be optimal. This would avoid the problems of milk stasis, reduce the chances of infections and disruption of the mammary epithelium tight junctions.

4.6.1 Summary of main findings

- Raised Na/K ratio indicative of subclinical mastitis was common, occurring in 26.5% of healthy women attending free mother-and-child health services in and around Durban ***(table 4.4.1)***.

- Raised Na/K ratio >1 in one breast only occurred in 18.5% of the study population. Unilaterally raised Na/K, correlated with breastmilk IL-8 and was suggestive of a local inflammatory response ***(table 4.4.2)***. This may be due to local infection or mechanical damage due to poor infant positioning during suckling.

- Bilaterally raised Na/K was found in 7.9% of women and did not correlate with breastmilk IL-8 levels ***(table 4.4.2)***. Bilaterally raised Na/K may be due to non-closure of the mammary epithelium tight junctions during early lactation, or disruption during mammary gland involution when breastmilk production falls. Bilaterally raised Na/K was associated with infant age ANOVA ***(table 4.5.1.2)***. However, in the 0-2 week age group there was no association between infant age and bilaterally raised Na/K, indicating that this finding was not simply due to non-closure of the tight junctions during very early lactation.

- Bilaterally raised Na/K was associated with supplementation of breastmilk with infant formula ***(table 4.5.2.1)***. As formula may displace breastmilk feeds, this could result in milk stasis, disruption of the mammary epithelium tight junctions and an increase in breastmilk Na/K, but not IL-8 levels in both breasts.

- Women who exclusively breastfeed had significantly lower breastmilk Na/K than those using formula. However, 21.2% of women breastfeeding exclusively had unilaterally raised Na/K, which correlates with breastmilk IL-8 levels and was indicative of subclinical mastitis. The causes for this may be infective or mechanical (poor positioning and latch-on). ***See section 4.5.2.***

- Women who gave complementary feeds in addition to breastmilk had the lowest mean Na/K. This may be because small quantities of complementary foods are introduced very early in life and do not significantly displace breastmilk, or additional foods are given to very hungry infants. In both these cases, breast emptying could be expected to remain adequate to prevent milk stasis and gland involution. ***See section 4.5.2.***

5: Determinants of breastmilk viral load in HIV-infected women and intestinal permeability in their breastfed infants

5.1 Objectives

Although HIV is known to be present in breastmilk, it remains unclear why most breastfeeding mothers do not transmit the virus to their infant. One possible explanation for transmission may be that the presence of subclinical mastitis in a minority of women could be associated with increased viral load and increased levels of inflammatory cytokines in breastmilk (such as IL-8), which in turn may increase the risk of MTCT through increased infant intestinal permeability. Conversely, lack of subclinical mastitis could be one of several explanations for the absence of breastmilk transmission of HIV in most cases.

The objective of this study is to clarify the association between subclinical mastitis, breastmilk viral load and infant gut permeability, as a first step in understanding the role of breast health as a potential risk factor for postnatal mother-to-child transmission of HIV.

5.2 Study design

To investigate the determinants of and association between indicators of breast health, breastmilk viral load and infant intestinal permeability, a prospective study of lactating HIV-infected women and their infants was carried out. This study was based at a hospital (McCord's, receiving provincial subsidy to provide free mother and child health care) and a primary health care clinic (Cato Manor, serving an informal settlement) in Durban and HIV-infected mothers and their breastfed infants were enrolled. Mothers and infants were followed-up for the first 3 months of life and invited to attend at 1, 6 and 14 weeks of age. At each visit the infant underwent a test of intestinal permeability, using the lactulose:mannitol dual sugar absorption test, anthropometry was recorded and a detailed infant feeding history obtained from the mother. If the mother was still breastfeeding she was asked to donate a small sample of breastmilk from each breast by manual expression. Infant urine samples were analysed for

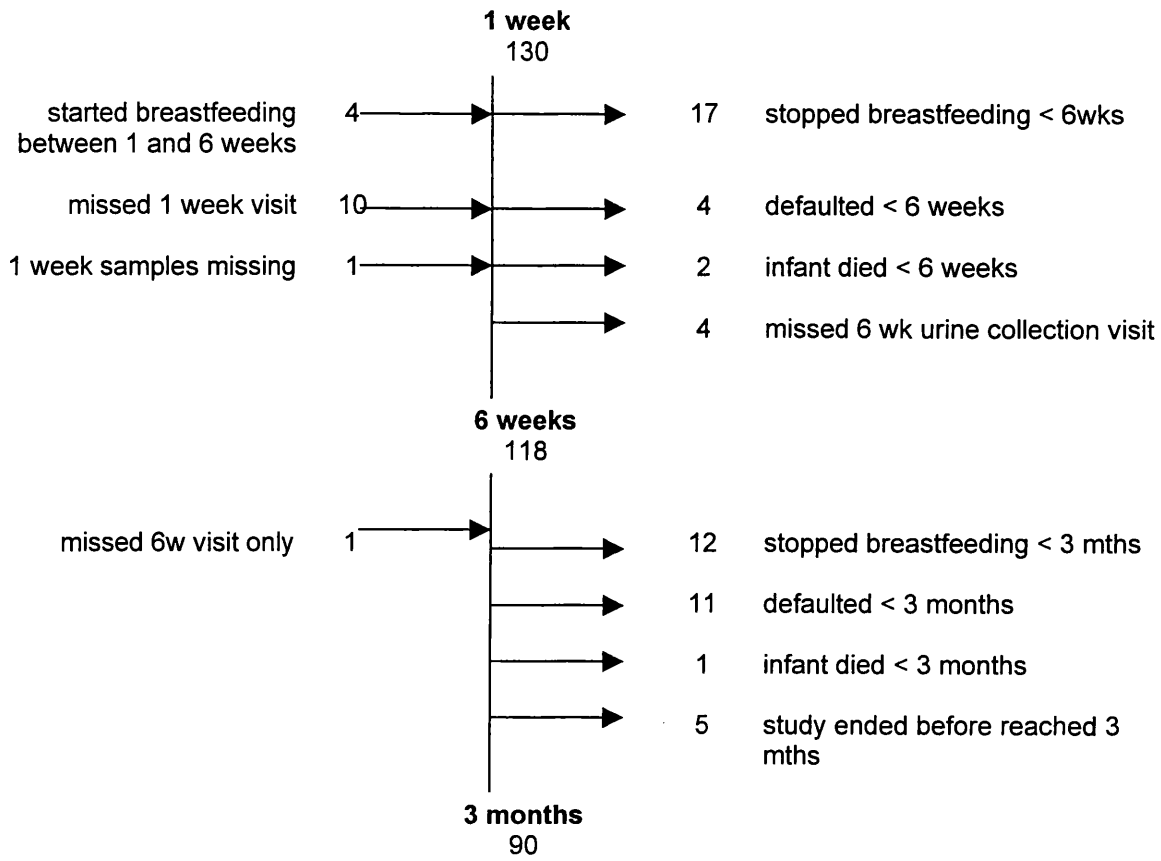
lactulose:mannitol ratio (L:M); breastmilk samples were analysed for sodium:potassium ratio (Na/K) and interleukin-8 (IL-8) as indicators of subclinical mastitis, and cell-free HIV RNA viral load. For further details, please refer to section 3.2.2. and 3.3.

5.3 Subject characteristics

Recruitment commenced in November 1997 and final samples were collected in mid December 1999. During this period a total of 145 lactating, HIV-infected women and their infants were recruited into the study: 106 women and 110 infants (4 sets of twins) were enrolled from an on-going study of intestinal permeability taking place at the McCord's Hospital site and a further 39 women and their 39 infants were enrolled at the Cato Manor clinic site, which was established after McCord's became a fee-paying hospital. Further details of the population served by these health-care facilities are available in section 3.2.2.1. In the case of twins, only the first-born twin is considered in this analysis as the primary outcomes are breastmilk factors.

At the McCord's site 187 women were recruited for the intestinal permeability study over that period. 81 (43%) of these either chose not to breastfeed or declined to participate in this study. At the Cato Manor site 55 women recruited during pregnancy returned for a 1-week follow-up visit with their infant. Of these 16 (29%) either chose not to breastfeed or refused to participate in this study. At both sites women may have missed scheduled visits, stopped breastfeeding or dropped out of the study between delivery and 3 months. In addition, some women attended the clinic late in the day and were given an alternative date to attend for the 5 hour urine collection. In this case dietary and morbidity data was collected by the clinician at the first visit, but breastmilk and urine at the subsequent visit up to a week later. Some women attended the clinical follow-up session, but failed to attend for the urine and breastmilk collection session. The figure below (figure 5.3.1) shows the number of lactating women sampled at each time point and explains the flow of women in and out of the study.

Figure 5.3.1 Cohort profile



A total of 68 women gave breastmilk samples at each of the three visits and in future will be referred to as the core group. In only 55 of these an infant urine sample for L:M analysis is also available at the same time. 130 women and infants gave samples at 1 week, 118 at 6 weeks and 90 at 3 months of age.

Subject characteristics are shown in table 5.3.1. The characteristics of the mothers enrolled at McCord's reflect those of the catchment area of the hospital. Although there were some slight differences in birthweight and gestational age, delivery by caesarean section and prematurity (<37 completed weeks gestation) between the two sites, these were not statistically significant. In summary, these data indicates that women attending the Cato Manor clinic were generally of lower socio-economic status, from poorer housing conditions and less well educated than mothers attending McCord's Hospital. There were however, no statistically significant differences in maternal CD4 count, infant birthweight or gestational age. As there were no major differences in the demographic profile of the study participants from the two sites which were

likely to influence the main outcome variables, the two study sites were combined for further analysis. It is interesting to note the very high caesarean section rates at both sites. At the Cato Manor site most women delivered at King Edward VIII Hospital, which is the tertiary teaching hospital in Durban. Personal communication with a member of the Obstetrics and Gynaecology department revealed that almost no instrumental vaginal deliveries are carried out and if any complications develop during labour an emergency caesarean section is performed immediately.

There were no differences in mean birthweight, gestational age, maternal age, baseline CD4 or infant weight in the group of 68 women who provided breastmilk samples at each visit (core group) compared with those who either stopped breastfeeding, missed a visit or defaulted before 3 months. There were also no significant differences in any subject characteristics between mothers who were exclusively breastfeeding (EBF), mixed feeding (breastmilk plus water, formula or complementary foods) or had decided to stop breastfeeding at any time point, except that more unemployed women (59/82) were still EBF at 3 months ($p=0.003$), more women without electricity (51/67) were EBF at 6 weeks ($p=0.025$) and more women who did not cohabit with the father of the baby (49/76) had decided to stop breastfeeding by 6 weeks ($p=0.078$).

Table 5.3.1 Subject characteristics at each site and for the cohort as a whole

	McCord's	n=	Cato Manor	n=	All	n=
Maternal age, yrs (SD)	25.8 (±4.6)	104	24.6 (±4.9)	38	25.5 (±4.7)	142
Maternal education ≤9 yrs (%)	62/98 (63.3%)		29/39 (74.4%)		91/137 (66.4%)	
Maternal unemployment (%)	70/100 (70.0%)		32/38 (84.2%)		102/138 (73.9%)	
Maternal baseline CD4 (SD)	450 (±233)	80	481 (±260)	35	459 (±241)	115
Gestational age at recruitment (SD)	29 (±4)	93	33 (±5)	39	30 (±5)	132
Urban informal housing (%)	38/101 (37.6%)		39/39 (100.0%)		77/140 (55.0%)	
No electricity (%)	39/99 (39.4%)		33/39 (84.6%)		72/138 (52.2%)	
No inside water tap (%)	58/99 (58.6%)		25/39 (64.1%)		83/138 (60.1%)	
Primigravida (%)	33/105 (31.4%)		15/39 (38.5%)		48/144 (33.3%)	
Caesarean section delivery (%)	27/104 (26.0%)		12/39 (30.8%)		39/102 (27.3%)	
Duration of rupture of membranes, hrs (SD)	4.8 (±10.1)	91	4.7 (±7.4)	35	4.8 (±9.4)	126
Infant female:male ratio (% female)	59:47 (55.7%)		23:16 (59.0%)		82:63 (56.5%)	
Infant birth weight, kg (SD)	3.16 (±0.48)	106	3.15 (±0.40)	39	3.16 (±0.46)	145
Infant gestational age, wks (SD)	39.5 (±1.4)	91	39.3 (±2.2)	37	39.4 (±1.7)	128
Low birthweight, ≤2.5kg (%)	11/106 (10.4%)		3/39 (7.7%)		14/145 (9.7%)	
Prematurity, <37 wks (%)	5/91 (5.5%)		5/37 (13.5%)		10/128 (7.8%)	

There were no statistically significant differences in maternal age, baseline CD4 count, infant birthweight, gestational age, proportion low birthweight or premature between the two sites. Caesarean section rates were very high at both sites. The Cato Manor clinic served an informal settlement, but a proportion of the women attending McCord's Hospital also lived in an informal settlement. The only significant difference between the sites was in the proportion of women living without electricity (Chi Square $p<0.001$).

5.4 Laboratory analysis

Breastmilk Na/K, IL-8 and viral load are not normally distributed. In accordance with standard practice Na/K and IL-8 were natural log (ln) transformed and viral load was log transformed using log base 10 (log) for statistical analysis (histograms of these variables are shown in Annex 2, figure 5.4.1). Geometric mean and 95% confidence (95%CI) intervals are presented throughout for variables that were ln and log transformed.

Lactulose:Mannitol (L:M) ratio was ln transformed and had a bimodal distribution. This was due to approximately 10% of samples having undetectable lactulose concentration, which had been set to the limit of detection (0.0001). This may be due to very dilute urine sample or very low levels of paracellular absorption of lactulose by a healthy, intact intestinal epithelium. In these cases the limit of detection for L:M ratio was therefore set to 0.01 and L:M was natural log transformed for further analysis.

Women and their infants may have attended for scheduled visits earlier or later than invited to do so. Mean age at the 1 week visit was 12 days (SD ±5 days, range from 4-30 days postpartum), at 6 weeks mean age was 46 days (±6, range 35-66) and at 3 months 95 days (±10, range 73-138).

Geometric mean Na/K ratio, IL-8 and viral load and percentage of samples with undetectable (<200 copies/ml) viral load at each time point are shown in table 5.4.1. Geometric mean values for right and left breasts separately are shown in table 5.3.2 in Annex 2.

Table 5.4.1 Geometric mean breastmilk Na/K, IL-8 and viral load, median and detectable viral load at each time point

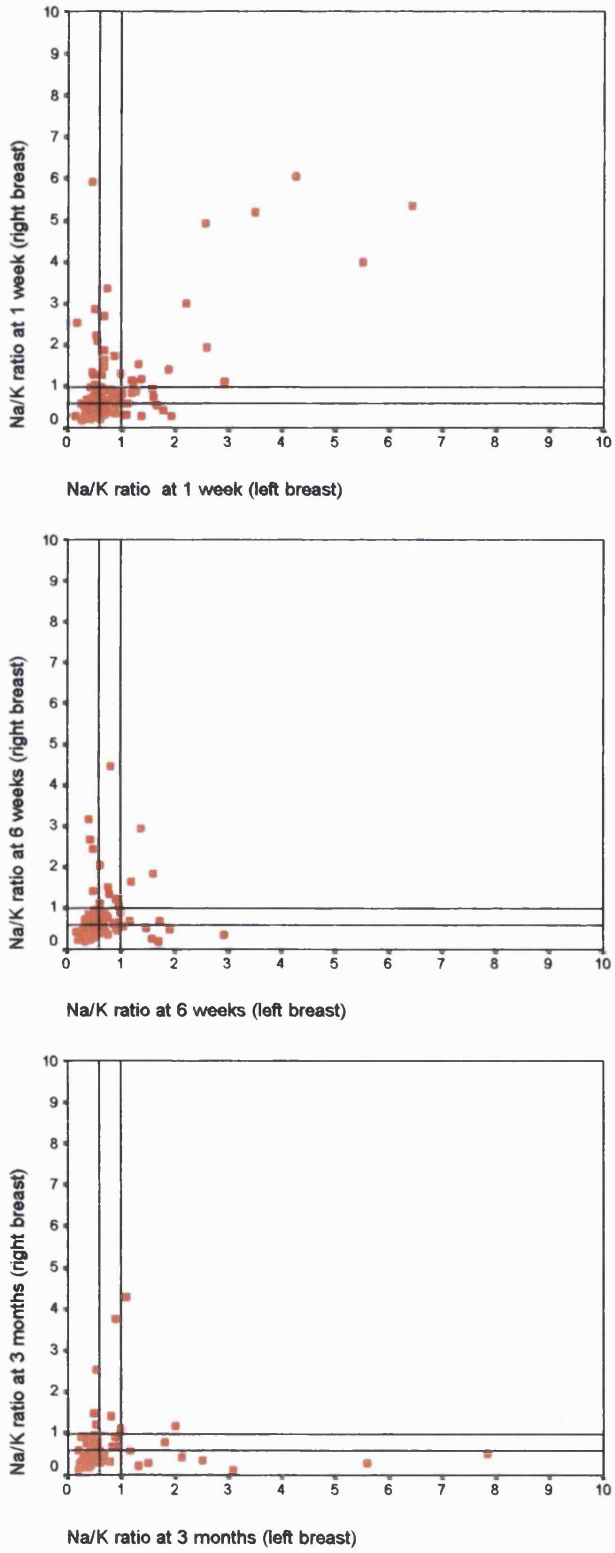
	(n samples)	geometric mean (95%CI, or %)
Na/K ratio	1 wk (255)	0.67 (0.62-0.73)
	6 wks (233)	0.57 (0.52-0.63)
	3 mths (166)	0.56 (0.49-0.64)
IL-8 (pg/ml)	1 wk (258)	292 (235-362)
	6 wks (234)	192 (160-232)
	3 mths (172)	233 (190-287)
Viral load (copies/ml)	1 wk (185)	1094 (829-1443)
	6 wks (193)	1039 (790-1366)
	3 mths (160)	914 (690-1210)
Median viral load (range)	1 wk (185)	597 (200-1,258,925)
	6 wks (193)	500 (200-1,148,153)
	3 mths (160)	408 (200-208,929)
Viral load undetectable (≤200 copies/ml)	1 wk (185)	63/185 (34.1%)
	6 wks (193)	73/193 (37.8%)
	3 mths (160)	68/160 (42.5%)

Note: not every woman was able to provide sufficient sample from both breasts for all laboratory analysis at each time point. Na/K ≤0.6 is considered to be normal. No data is available on normal IL-8 concentrations in breastmilk. Breastmilk viral load is known to be lower than plasma viral load and an increasing proportion of breastmilk samples had undetectable viral load as lactation progressed.

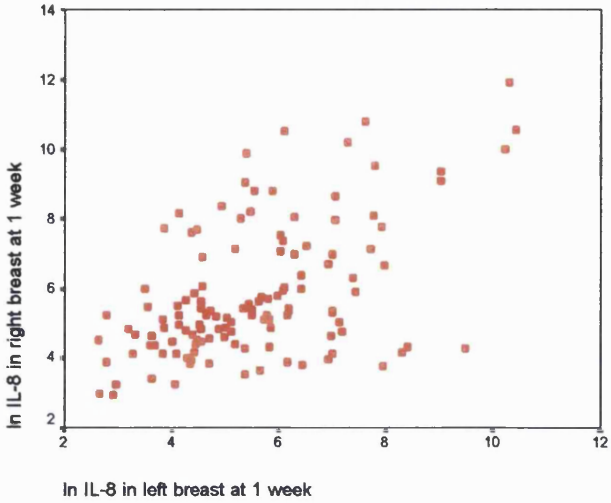
Although there was considerable overlap in values between right and left breast, there were occasional significant differences between the two breasts of an individual woman at any given time point. Scattergraphs in figure 5.4.1 show the relationship between Na/K, IL-8 and viral load in right and left breasts of individual women. As can be seen from the graphs of Na/K, there are very few cases of bilaterally raised Na/K >1 at 6 weeks and 3 months, but still a proportion of unilaterally raised Na/K at each time point. There are also a number of discordant breastmilk samples with respect to IL-8 at each time point. Breastmilk viral load would appear to be correlated in left and right breast at 1 and 6 weeks, but by 3 months of age there was a random scatter. It is interesting to note that in many women there is unilaterally undetectable viral load and often considerable virus present in the sample from the other breast.

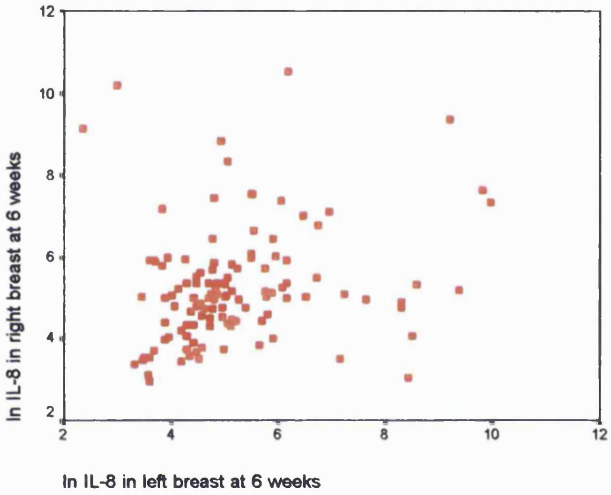
Figure 5.4.1: Scattergraphs of (a) individual Na/K (with cut-off values of 0.6 and 1.0 shown), (b) ln IL-8 and (c) log viral load (with limit of detection ≤200 copies/ml shown) in breastmilk from right and left breasts at each time point

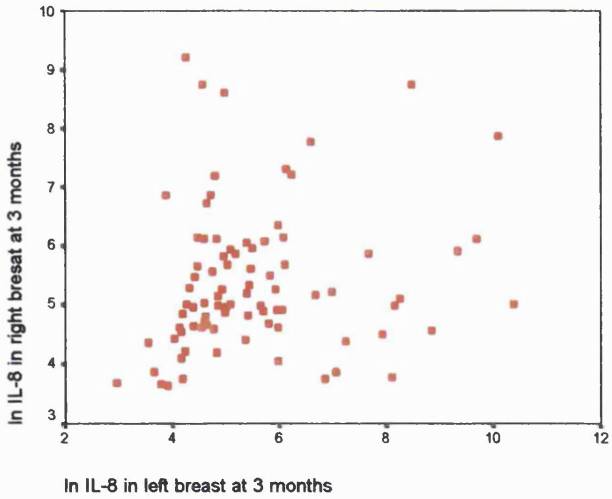
(a)



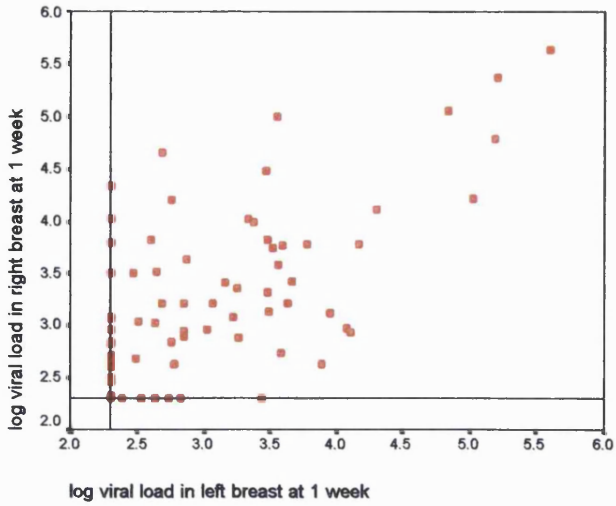
(b)

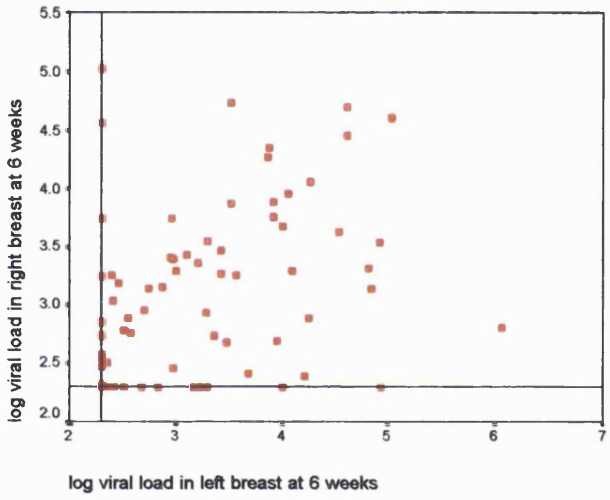

ln IL-8 in right breast at 1 week
ln IL-8 in left breast at 1 week

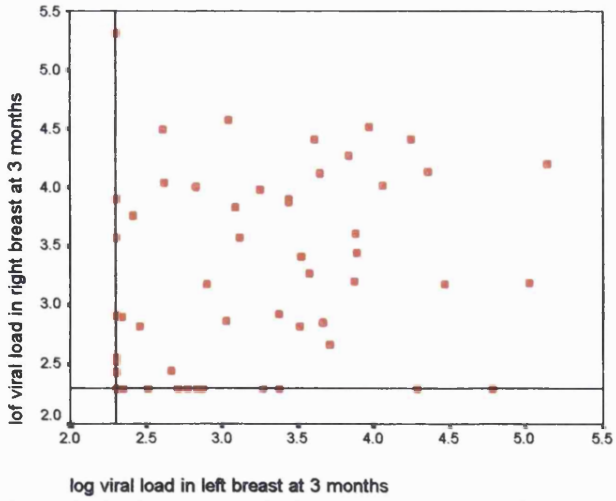

ln IL-8 in right breast at 6 weeks
ln IL-8 in left breast at 6 weeks


ln IL-8 in right bresat at 3 months
ln IL-8 in left breast at 3 months

(c)


log viral load in right breast at 1 week
log viral load in left breast at 1 week


log viral load in right breast at 6 weeks
log viral load in left breast at 6 weeks


lof viral load in right breast at 3 months
log viral load in left breast at 3 months

Paired T-tests indicated a statistically significant difference between breasts for IL-8 (p=0.033) and viral load (p=0.008) at 1 week, but not at any other time. There were no statistically significant interbreast differences in Na/K ratio. There was a statistically significant difference in Na/K at 1 week and IL-8 at 1 week and 3 month, between the core group of 68 women who provided breastmilk samples at each of the 3 visits and those who either stopped breastfeeding, missed a visit or defaulted (non-regular) before 3 months (as shown in table 5.4.2.) There was no significant difference between these groups in viral load at any time point.

Table 5.4.2 Geometric mean breastmilk Na/K and IL-8 in women who gave breastmilk samples at each visit (core group) and those who did not (non-regular)

		core group (95%CI) [n samples]	non-regular (95%CI) [n samples]	p =
Na/K ratio	1 wk	0.61 (0.50-0.75) [136]	0.75 (0.64-0.87) [119]	**0.028**
	6 wks	0.56 (0.51-0.62) [136]	0.60 (0.50-0.71) [97]	0.491
	3 mths	0.53 (0.47-0.61) [165]	0.69 (0.47-1.02) [30]	0.128
IL-8 (pg/ml)	1 wk	236 (180-311) [135]	367 (263-514) [123]	**0.045**
	6 wks	119 (149-239) [136]	198 (146-268) [98]	0.811
	3 mths	197 (159-243) [134]	428 (253-726) [38]	**0.002**

Note: not every woman was able to provide sufficient sample from both breasts for all laboratory analysis at each time point.

Using recommended cut-off values for Na/K to define subclinical mastitis the prevalence of subclinical mastitis at 1 and 6 weeks and 3 months respectively is shown in table 5.4.3 below.

Table 5.4.3 Prevalence of subclinical mastitis at each time point

	1 week (%) n=126	6 weeks (%) n=116	3 months (%) n= 83
Bilaterally normal	46 (36.5)	57 (49.1)	45 (54.2)
Unilaterally mildly raised	23 (18.3)	22 (19.0)	14 (16.9)
Bilaterally mildly raised	14 (11.1)	11 (9.5)	3 (3.6)
Bilaterally ≤1.0	**83 (65.9)**	**90 (77.6)**	**62 (74.7)**
Unilaterally severely raised	16 (12.7)	15 (12.9)	15 (18.1)
One mildly and one severely raised	14 (11.1)	7 (6.0)	4 (4.8)
Unilaterally >1.0	**30 (23.8)**	**22 (19.0)**	**19 (22.9)**
Bilaterally severely raised	13 (10.3)	4 (3.4)	2 (2.4)
Bilaterally >1.0	**13 (10.3)**	**4 (3.4)**	**2 (2.4)**

Note: Percentages may not add to 100 due to rounding. Not all women were able to provide sufficient sample from both breasts for all laboratory analysis. The women included here gave sufficient breastmilk from both breasts for Na/K analysis. Normal Na/K ≤0.6, mildly raised >0.6 ≤1.0, severely raised >1.0.

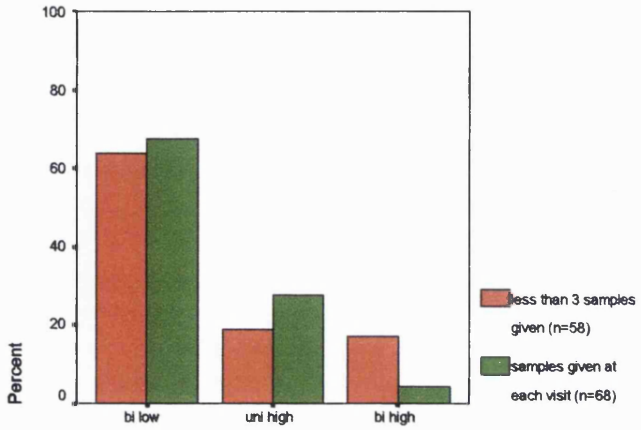
As can be seen from the table the proportion of women with normal Na/K increases a little over time and the proportion with bilaterally severely raised Na/K falls sharply after the first week. However, the proportion with unilaterally severely raised Na/K remains fairly constant over the three time points. This could indicate that bilaterally severely raised Na/K might be a physiological phenomenon associated primarily with early lactation, whereas unilaterally raised Na/K can occur throughout lactation and could be the result of mechanical damage, unilateral involution or inflammation due to poor feeding method. As can be seen from table 5.4.3, 34.9% (43/126), 22.4% (26/116) and 25.3% (21/83) of women have Na/K >1.0 in at least one breast (unilaterally severely raised plus bilaterally severely raised at each time point) at 1 and 6 weeks and 3 months respectively. It is interesting to note however, that Na/K is >1.0 in one breast only and ≤0.6 in the other in the majority of cases. As severely raised Na/K can be expected to have the greatest impact on other breastmilk and infant parameters, it was decided for the purpose of this analysis to concentrate only on the severely raised Na/K (>1.0) and its association with other risk factors for postnatal transmission, defining any Na/K ratio ≤1.0 as low. Na/K ratio of 1.0 is equivalent to about 18 mmol/L Na^+ (78), which is 2-3 times greater than the Na/K ratio found in breastmilk of normal, healthy women (see table 2.2.1.1 for details).

It is possible that women who were breastfeeding at every visit may have different risk factors for subclinical mastitis than those who stopped breastfeeding, missed a visit or defaulted before 3 months. Figure 5.4.2 below compares subclinical mastitis at each time point in women who gave breastmilk samples at each visit (core group) compared with those who did not (non-regular). Table 5.3.3 in Annex 2 shows the breakdown of categories of subclinical mastitis in these women.

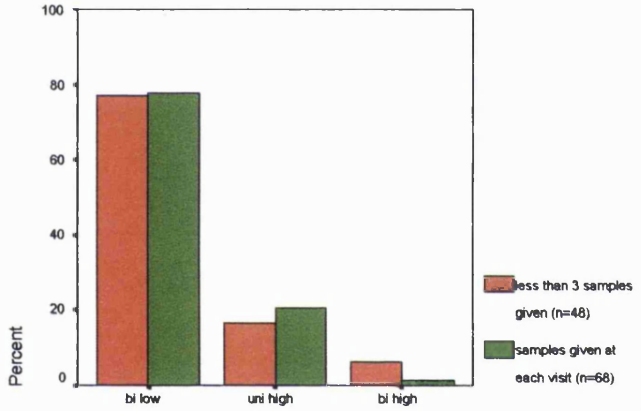
As can be seen from figure 5.4.2 a higher proportion of women who did not breastfeed to 3 months or missed visits during the study had bilaterally raised Na/K at each time point. However, the proportion of women with unilaterally raised or bilaterally low Na/K was similar whether they were part of the core group that provided samples at each time point or not. Therefore, the women who attended every visit, breastfed from birth to at least 3 months and provided a breastmilk sample at each visit were less likely to have severely raised Na/K in both breasts. There was a trend for slightly more women in the core group than those who did not

provide breastmilk at each visit to have unilaterally severely raised Na/K. However this difference was small and would not suggest that these 68 women were a completely separate group with a very different subclinical mastitis history throughout lactation from those who defaulted or stopped breastfeeding. However, the slightly increased prevalence of bilaterally severely raised Na/K may be one factor in women deciding to stop breastfeeding before 3 months postpartum. Of the 10 women who were non-regular attenders with bilaterally high Na/K at 1 week, 4 defaulted from the 6 week visit, 3 were breastfeeding exclusively at 1 week and one of these was using breastmilk and water at 6 weeks, 3 who were using mixed feeding at 1 week and 2 had stopped breastfeeding by 6 weeks.

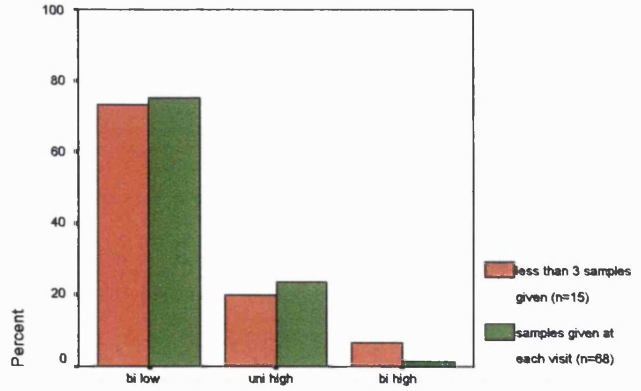
Figure 5.4.2: Percentage of women in the core group or those who did not attend regularly with bilaterally low (both breasts ≤1.0), unilaterally high (one breast >1.0 only) or bilaterally high (both breasts >1.0) Na/K at each time point



type of subclinical mastitis at 1 week



type of subclinical mastitis at 6 weeks



type of subclinical mastitis at 3 months

5.5 Relationship between subclinical mastitis and breastmilk viral load

In order to investigate whether Na/K is associated with breastmilk IL-8 concentration and viral load, bivariate correlations were carried out. Na/K was found to correlate strongly with IL-8 and with breastmilk viral load at each time point. Univariate regression was carried out to determine the strength of the association between breastmilk Na/K, IL-8 and viral load. The results are summarised in table 5.5.1.

Table 5.5.1: Association between breastmilk Na/K, IL-8 and viral load at each time point

		1 week	6 weeks	3 months
Na/K & IL-8	correlation coefficient	0.593	0.581	0.607
	univariate coefficient	1.455	1.165	0.971
	p =	<0.001	<0.001	<0.001
	# samples =	253	232	164
Na/K & viral load	correlation coefficient	0.377	0.259	0.282
	univariate coefficient	0.465	0.309	0.258
	p =	<0.001	<0.001	<0.001
	# samples =	183	191	152
IL-8 & viral load	correlation coefficient	0.333	0.255	0.431
	univariate coefficient	0.163	0.155	0.244
	p =	<0.001	<0.001	<0.001
	# samples =	182	192	158

As can be seen from table 5.5.1 there is a strong correlation between Na/K and IL-8 at each time point and one unit increase in ln Na/K was associated with between 1.5 and 1.0 unit increase in ln IL-8. The association between Na/K and viral load was strongest at 1 week, and the unit increase in log viral load when ln Na/K increased by 1 unit was greatest at 1 week (0.465) and declined as lactation progressed (to 0.258). Although IL-8 and viral load were correlated, the size of this effect was marginal.

Multivariate regression requires that all observations contributing to the model be independent. However, in this study breastmilk samples have been collected from each breast of each woman and analysed independently for breastmilk factors, which are of primary concern in this study. As has been shown above, breastmilk Na/K, IL-8 and viral load are often raised in one breast only. Great consideration was given to the best way of analysing this data, which would reflect what happens in each breast independently, but also consider the limitation imposed by multivariate modelling.

The explanatory variables which influence breastmilk Na/K and the relationship between Na/K and viral load were investigated using the mean value for Na/K and viral load from both breasts of each woman and also by using one breast only (arbitrarily chosen as the right breast). However, this poses some difficulties:

a) Taking the mean value of breastmilk Na/K and viral load will eliminate many of the extreme values for Na/K and viral load. As can be seen from the scattergraphs in figure 5.4.1 and from table 5.4.3, bilaterally high Na/K is uncommon, especially after the first week of life. The prevalence of unilaterally high Na/K however, remains almost constant throughout lactation and in the majority of cases breastmilk Na/K is >1 in one breast and ≤0.6 in the other. In these cases mean Na/K value would tend to be low and comparable with mean Na/K from women with bilaterally low Na/K.

b) The use of one breast only also reduces the power of the study. Having noted that breastmilk factors are often unilaterally raised it becomes increasingly obvious that samples must be collected from both breasts in order to establish a clearer picture of what is happening in breastmilk at any given time. The use of samples from a randomly picked breast may mean that high breastmilk Na/K, IL-8 or viral load in the other breast is missed and not taken into account.

Additional analyses were carried out to explore the differences resulting from using either one breast only or the mean value of both breasts and the models did not change substantially. For these reasons, it was decided to include information relating to both breasts from each woman in the following models, although it must be stated that these may not be fully independent. Possible maternal and infant explanatory characteristics have thus been included twice, once for each breast and the relationship between the explanatory variable and the dependant variable includes the effect of confounding variables relating to the women or infant for each breast as if they were independent.

5.5.1 Determinants of breastmilk Na/K

As Na/K was hypothesised to be the primary explanatory variable for breastmilk viral load, and given the changing prevalence of subclinical mastitis over time, univariate regression was carried out to determine the factors influencing breastmilk Na/K at each time point, as these might differ. Na/K was used as a continuous variable rather than subclinical mastitis category in order to make full use of the data (there were very few women with bilaterally high Na/K after the first week of lactation) and explain the relationship between variables over the range of Na./K values. Potential explanatory variables for Na/K included:

- factors indicative of socio-economic status (housing, availability of electricity and piped water in the home, maternal education and employment)
- maternal age (categorised as ≤25 years and >25 years as there was an indication that age was a confounder, but few mothers were younger than 20 years)
- maternal well-being (CD4, CD8 count during pregnancy, maternal temperature at visit, mode of delivery)
- infant factors that might influence effectiveness of breastfeeding (birthweight, gestational age, sex, current weight or percentage weight increase from last visit, age at visit)
- feeding mode at time of visit (but not restricted to a 24-hour recall of infant feeding and defined as "EBF" according to WHO/UNICEF definitions, or "mixed" if any water, formula, other liquids or foods had also been given)
- whether the mother breastfed to at least 3 months and also attended every visit (core group)

Where there was a significant effect the potential explanatory variables were then entered into a multivariate model to predict breastmilk Na/K, keeping variables considered to be important *a priori*. Although explored, no interaction terms were found to be statistically significant and none was included in the multivariate analysis.

The final models were chosen after inclusion of potential confounding variables and variables considered to be important *a priori* (such as infant age and weight). The effect of each of these variables was considered in the light of the other variables in the model and those that subsequently had little impact on Na/K and were not considered important were excluded.

At 1 week the following model predicted 6.9% of the Na/K (adjusted R^2=0.069, p=0.011). The variables included in the model are detailed in table 5.5.1.1. Only sex and feeding mode were significant : female infants were consuming breastmilk with higher Na/K than male infants and women who were exclusively breastfeeding had lower Na/K than those who were mixed feeding.

Table 5.5.1.1: Determinants of Na/K ratio at 1 week

variable		n*	univariate coefficient for variable (95%CI)	multivariate coefficient # in model (95%CI)	p value
sex	Female	97	0.334 (0.162-0.506)	0.358 (0.127-0.589)	0.003
	Male	58	0	0	
maternal age (yrs)	≤25	96	0.105 (-0.076--0.286)	0.154 (-0.074-0.383)	0.183
	>25	59	0	0	
feeding	EBF	115	-0.088 (-0.288-0.113)	-0.205 (-0.501-0.0005)	0.050
	mixed feeding	40	0	0	
attendance	regular	94	-0.195 (-0.369--0.021)	-0.125 (-0.360-0.110)	0.297
	non-regular	61	0	0	
% weight inc. 1-6w		155	-0.191 (-0.471--0.089)	-0.147 (-0.512-0.218)	0.428
maternal temperature	>38°C	8	0.366 (-0.042-0.774)	0.154 (-0.074-0.383)	0.183
	≤38°C	147	0	0	

*n= number of breastmilk samples in each group/variable included in the final model. # multivariate coefficient in the model, allowing for the effect of other variables included in the model. EBF = exclusive breastfeeding. Regular attendance = core group who provided samples at all three time points; non-regular = stopped breastfeeding, missed visits of defaulted from study.

At 6 weeks the model which predicted 5.7% of the Na/K included sex, maternal age and percentage weight increase 6 weeks-3 months (adjusted R^2=0.057, p=0.006). The variables included in the model are detailed in table 5.5.1.2. It is interesting to note that at 6 weeks it is difficult to predict breastmilk Na/K. This may be because there are many factors which change at about this time: the infant may be going through a growth spurt (which is why future weight trajectory is an explanatory variable) or breastfeeding has become established and has not yet been influenced by weaning (as opposed to the situation in early lactation at 1 week and the time of possible introduction of other foods at 3 months) and therefore Na/K is less influenced by external factors. Infants who grew faster between 6 weeks ad 3 months were drinking breastmilk with considerably lower Na/K at 6 weeks. It may be that these infants were efficient breastfeeders who effectively emptied the breast and thus avoided milk stasis developing and breastmilk Na/K increasing and also obtained sufficient nutrition for rapid growth. However, there was no significant difference in mean percentage weight increase from 6 weeks to 3 months in the different mastitis categories or by exclusive vs. mixed feeding at 6 weeks by ANOVA (p=0.681 and 0.553 respectively).

Table 5.5.1.2: Determinants of Na/K ratio at 6 weeks

variable		n*	univariate coefficient for variable (95%CI)	multivariate coefficient # in model (95%CI)	p value
sex	Female	103	0.258 (0.070-0.446)	0.152 (-0.045--0.350)	0.129
	Male	78	0	0	
maternal age (yrs)	≤25	96	0.223 (0.031-0.414)	0.120 (-0.075-0.314)	0.227
	>25	85	0	0	
birthweight		181	-0.235 (-0.448--0.022)	-0.270 (-0.503--0.037)	0.023
% weight inc. 6w-3m		181	-0.473 (-0.913--0.034)	-0.646 (-1.098--0.193)	0.005

*n= number of breastmilk samples in each group/variable included in the final model. # multivariate coefficient in the model, allowing for the effect of other variables included in the model.

At 3 months univariate analysis indicated that only weight and percentage previous weight increase (6 weeks-3 month) were likely to be important explanatory variables. The final model included weight gain and whether women had breastfed and attended follow-up visits throughout the study (adjusted R^2=0.070, p=0.002). This model explained 7% of the Na/K and the variables included in the model are detailed in table 5.5.1.3. It is interesting to note that infants who had grown well since the 6 weeks visit were consuming breastmilk with considerably lower Na/K. As at 6 weeks, this may be an indicator of effective breastfeeding and infants who empty the breast and obtain sufficient nutrition for rapid growth. Exclusively breastfed infants (n=66) had a significant higher percentage weight increase between 6 weeks and 3 months than those who were mixed fed (n=17) by ANOVA (p=0.011). It is worth noting that at 3 months 68 women who had attended all appointments and were still breastfeeding provided samples, whereas only 15 who had not attended regularly provided samples. Infants whose mother had bilaterally severely raised Na/K at 3 months had poorer growth between 6 weeks and 3 months than those whose mothers had either unilaterally raised (n=17) or bilaterally normal (n=57) Na/K by ANOVA (p=0.021). However, it is important to note that there were only 2 infants in the bilaterally severely raised group at 3 months of age.

Table 5.5.1.3: Determinants of Na/K ratio at 3 months

variable		n*	univariate coefficient for variable (95%CI)	multivariate coefficient # in model (95%CI)	p value
% weight inc (6w-3m)		152	-0.691 (-1.312--0.069)	-0.696 (-1.310--0.083)	0.026
attendance	regular	128	-0.262 (-0.599-0.075)	-0.391 (-0.735--0.048)	0.026
	non-regular	24	0	0	

*n= number of breastmilk samples in each group/variable included in the final model. # multivariate coefficient in the model, allowing for the effect of other variables included in the model. Regular attender = core group who provided samples at all three time points; non-regular = stopped breastfeeding, missed visits or defaulted from study.

As the explanatory variables for Na/K may differ according to the type of subclinical mastitis present (bilaterally low, unilaterally high and bilaterally high Na/K), an attempt was made to investigate potential explanatory variables for breastmilk samples from women in each category of subclinical mastitis. However, there were very few women in each category, once those with missing values for some variables had been excluded, so this analysis could not be pursued.

5.5.2 Determinants of the relationship between Na/K and viral load

As IL-8 correlates with Na/K and there is colinearity between Na/K and IL-8 it would be inappropriate to use both in any model to predict viral load. In the following analysis Na/K will be used as the explanatory variable. The relationship of interest is that between Na/K and viral load. In order to determine the factors influencing this relationship univariate analysis was carried out including relevant variables. Given that the prevalence of raised Na/K and the explanatory variables for Na/K change during the course of lactation, indicating that varying processes could be important at different stages, it would be expected that the relationship between Na/K and viral load would be influenced by different factors at each point in lactation. Therefore the relationship between Na/K and viral load was investigated at each time point separately.

Potential confounding variables for the relationship between Na/K and viral load included

- factors indicative of socio-economic status (housing, availability of electricity and piped water in the home, maternal education and employment)
- maternal age (categorised as ≤25 years and >25 years as there was an indication that age was a confounder, but few mothers were younger than 20 years)
- maternal well-being (CD4, CD8 count during pregnancy, maternal temperature at visit, mode of delivery)
- infant factors that might influence effectiveness of breastfeeding (birthweight, gestational age, sex, current weight or weight gain from last visit, age at visit, feeding mode)
- whether the mother breastfed to at least 3 months and also attended every visit

Where there was a significant effect, or the variable altered the coefficient of Na/K, it was then entered into a multivariate model to predict the relationship between breastmilk Na/K and viral load, keeping variables considered to be important *a priori*. Potential confounding variables included infant age and weight and maternal baseline CD4 at all time points, feeding mode, maternal body temperature at 1 and 6 weeks, mode of delivery, low birthweight, maternal age at 1 week only, prematurity at 6 weeks only and weight gain at 3 months only.

Interaction between variables and Na/K were also tested and those that were significant or seemed to alter the relationship between Na/K and viral load were included in a multivariate model. Sex, feeding mode and mode of delivery were found to interact with Na/K at 1 week, 6

weeks and 3 months. However, given the sample size the use of more than one interaction term would render the number in each cell very small. Therefore, it was decided to only use one interaction term and as it would appear that feeding mode has an effect on the relationship between Na/K and viral load by its action through Na/K, this interaction term was chosen; sex and mode of delivery were included as potential confounders.

The final models were chosen after inclusion of potential confounding variables, variables considered to be important *a priori* (such as infant age and weight) and interaction terms. The effect of each of these variables was considered in the light of the other variables in the model and those that subsequently had little impact on the relationship between Na/K and viral load and were not considered important were excluded.

The final model chosen that best predicts the relationship between Na/K and viral load at 1 week takes into account infant sex, CD4 group (<200, 200-500, >500 cells/ml), maternal age category (≤25 or >25 years) and the interaction term between feeding mode and Na/K (Adjusted $R^2 = 0.263$, $p<0.001$). This model predicts 26.3% of the breastmilk viral load at 1 week. The variables included in the final model are detailed below in table 5.5.2.1.

Table 5.5.2.1: Determinants of viral load at 1 week

variable		n*	univariate coefficient for variable (95%CI)		multivariate coefficient# feeding in model (95%CI)	p
ln Na/K		107	0.465 (0.297-0.633)	Mixed †	0.967 (0.671-1.317)	<0.001
				EBF†	-0.070 (-0.284-0.144)	0.591
sex	Female	67	0.157 (-0.073-0.387)		0.145 (-0.078-0.367)	0.201
	Male	78	0		0	
CD4 (cell/ml)	<200	29	0.508 (0.168-0.847)		0.475 (0.160-0.790)	0.003
	200-500	73	0.214 (-0.057-0.485)		0.293 (0.043-0.544)	0.022
	>500	43	0		0	
maternal age (yrs)	≤25	95	0.200 (-0.036-0.437)		0.315 (0.092-0.539)	0.006
	>25	50	0		0	
feeding	EBF	113	-0.198 (-0.464-0.067)		-0.739 (-1.074--0.403)	<0.001
	mixed feeding	32	0		0	

*n= number of breastmilk samples in each group/variable included in the final model. # multivariate coefficient in the model, allowing for the effect of other variables included in the model. This model includes the interaction term between feeding mode and Na/K. † when this category used as the reference value. EBF = exclusive breastfeeding.

Taking mixed feeding as the reference group in the interaction term, women who were giving their infant other foods or liquids in addition to breastmilk (mixed feeding) had a 0.967 unit increase in log viral load for a one unit increase in ln Na/K; when exclusive breastfeeding was taken as the reference group in the interaction term, mothers who were exclusively breastfeeding their infant had a (non-significant) 0.070 unit decrease in log viral load for a one unit increase in ln Na/K, when taking into account the confounding variables also included in the

model. It is interesting to note that the other variables included in the model have a small effect, apart from CD4 count during pregnancy. In the group of women with CD4 counts below 200 cells/ml there is a statistically significant increase in viral load compared with women with CD4 counts >200 cells/ml. Mothers aged less than 25 had a significantly higher viral load than those aged over 25 years. Female infants consumed breastmilk with a (non-significantly) higher viral load than male infants.

This indicates that although the model is only able to predict about 26% of the breastmilk viral load, there was a strong linear relationship between Na/K and viral load when taking into account the other confounding variables and that this relationship differed according to feeding mode. A substantially greater increase in viral load accompanies an increase in breastmilk Na/K in women who are not exclusively breastfeeding compared with those who are. As breastmilk viral load is presumed to be an important risk factor for breastmilk transmission of HIV, this data suggests that exclusive breastfeeding during early lactation may be associated with a reduced amount of virus present in milk. However, it is important to remember that this model is not a strong predictor of breastmilk viral load overall and many other factors are obviously also involved, the most likely of which is maternal plasma viral load (which was not measured in this study).

At 6 weeks the final model chosen that best predicts the relationship between Na/K and viral load at 6 weeks takes into account infant sex, CD4 group (<200, 200-500, >500 cells/ml), and the interaction term between feeding mode and Na/K (Adjusted R^2 = 0.112, p=0.001). This model predicts 11.2% of the breastmilk viral load at 6 weeks. The variables included in the final model are detailed below in table 5.5.2.2.

Table 5.5.2.2: Determinants of viral load at 6 weeks

variable		n*	univariate coefficient for variable (95%CI)		multivariate coefficient in model (95%CI)	p
ln Na/K		153	0.465 (0.297-0.633)	Mixed †	0.331 (0.026-0.636)	0.033
				EBF †	0.390 (0.165-0.615)	0.001
sex	Female	80	-0.015 (-0.256-0.226		-0.101 (-0.372-0.170)	0.462
	Male	73	0		0	
CD4 (cells/ml)	<200	26	0.592 (0.213-0.971)		0.585 (0.202-0.968)	0.003
	200-500	71	0.150 (-0.139-0.440)		0.128 (-0.181-0.437)	0.415
	>500	56	0		0	
feeding	EBF	123	0.143 (-0.153-0.439)		0.172 (-0.218-0.562)	0.385
	mixed feeding	30	0		0	

*n= number of breastmilk samples in each group/variable included in the final model. # multivariate coefficient in the model, allowing for the effect of other variables included in the model. This model includes the interaction term between feeding mode and Na/K. † when this category used as the reference value. EBF = exclusive breastfeeding

Taking mixed feeding as the reference group in the interaction term, women who were giving their infant other foods or liquids in addition to breastmilk (mixed feeding) had a 0.331 unit increase in log viral load for a one unit increase in ln Na/K; when exclusive breastfeeding was taken as the reference group, mothers who were exclusively breastfeeding their infant had a 0.390 unit increase in log viral load for a one unit increase in ln Na/K when taking into account the confounding variables also included in the model. It is interesting to note that there was not a great difference in the coefficient for mixed feeding compared with exclusive breastfeeding at this age, indicating that the relationship between Na/K and viral load at this stage in lactation is

similar for mixed and exclusive breastfeeding. In addition, CD4 <200 cells/ml during pregnancy was the only covariant with a statistically significant coefficient. As expected, women with CD4 <200 cells/ml again had a higher breastmilk viral load than those with CD4 >200. Although this model is only able to predict about 11% of the breastmilk viral load, there is a strong linear relationship between Na/K and viral load when taking into account the other confounding variables, which was not as strongly influenced by feeding mode as at 1 week.

At 3 months the final model chosen that best predicts the relationship between Na/K and viral load at 3 months takes into account infant sex, CD4 category (<200, 200-500, >500 cells/ml), maternal age category (≤25, >25 years), percentage weight increase between 6 weeks and 3 months and the interaction between feeding mode and Na/K (Adjusted R^2 = 0.184, p<0.001). This model predicts 18.4% of the breastmilk viral load at 3 months. The variables included in the final model are detailed below in table 5.5.2.3.

Table 5.5.2.3: Determinants of viral load at 3 months.

variable		n*	univariate coefficient for variable (95%CI)		multivariate coefficient # in model (95%CI)	p
ln Na/K		109	0.258 (0.116-0.400)	Mixed †	0.049 (-0.258-0.356)	0.752
				EBF †	0.310 (0.096-0.524)	0.005
sex	Female	52	0.235 (-0.012-0.482)		0.212 (-0.075-0.500)	0.146
	Male	57	0		0	
CD4 (cells/ml)	<200	20	0.288 (-0.097-0.672)		0.396 (-0.030-0.794)	0.052
	200-500	49	0.048 (-0.255-0.352)		-0.041 (-0.359-0.278)	0.801
	>500	40	0		0	
maternal age	≤25	59	0.167 (-0.082-0.416)		0.248 (-0.034-0.530)	0.084
	>25	50	0		0	
% weight inc 6w-3m		109	-0.556 (-1.191-0.078)		-0.920 (-1.559--0.280)	0.005
feeding	EBF	87	0.397 (0.094-0.699)		0.549 (0.169-0.929)	0.005
	mixed feeding	22	0		0	

*n= number of breastmilk samples in each group/variable included in the final model. # multivariate coefficient in the model, allowing for the effect of other variables included in the model. This model includes the interaction term between feeding mode and Na/K. † when this category used as the reference value. EBF = exclusive breastfeeding

Taking mixed feeding as the reference group in the interaction term, women who were giving their infant other foods or liquids in addition to breastmilk (mixed feeding) had a (non-significant) 0.049 unit increase in log viral load for a one unit increase in ln Na/K; when exclusive breastfeeding was taken as the reference group in the interaction term, mothers who were exclusively breastfeeding their infant had a 0.310 unit increase in log viral load for a one unit increase in ln Na/K, when taking into account the confounding variables also included in the model. As at 1 week women with CD4 <200 cells/ml during pregnancy and women aged less than 25 years had a borderline significantly higher breastmilk viral load than those with CD4 >200 cells/ml or > 25 years of age. Infants who had gained more weight as a percentage of their weight at 6 weeks consumed milk with a significantly lower viral load. No other confounding variables were statistically significant.

This indicates that although the model is only able to predict about 18% of the breastmilk viral load, there is a linear relationship between Na/K and viral load when taking into account the other confounding variables, and differed according to feeding mode. However, the effect was the inverse of that seen at earlier time points, and at 3 months of age those mothers who were exclusively breastfeeding had a greater increase in log viral load for every one unit increase in ln Na/K than those practising mixed feeding, although the latter was non-significant.

In order to determine if the women who were regular breastfeeding attenders were different from those who did not attend regularly with respect to the relationship between Na/K and viral load, the best models at each time point were run including a variable coding for regular breastfeeding attendance as a confounder. This analysis showed that the relationship between Na/K and viral load was not statistically significantly influenced by attendance and breastfeeding for at least 3 months and that the size of the effect of the various coefficients did not substantially change at any time point. The relationship between Na/K and viral load was also not significantly influenced by the type of subclinical mastitis present (bilaterally low, unilaterally high or bilaterally high Na/K).

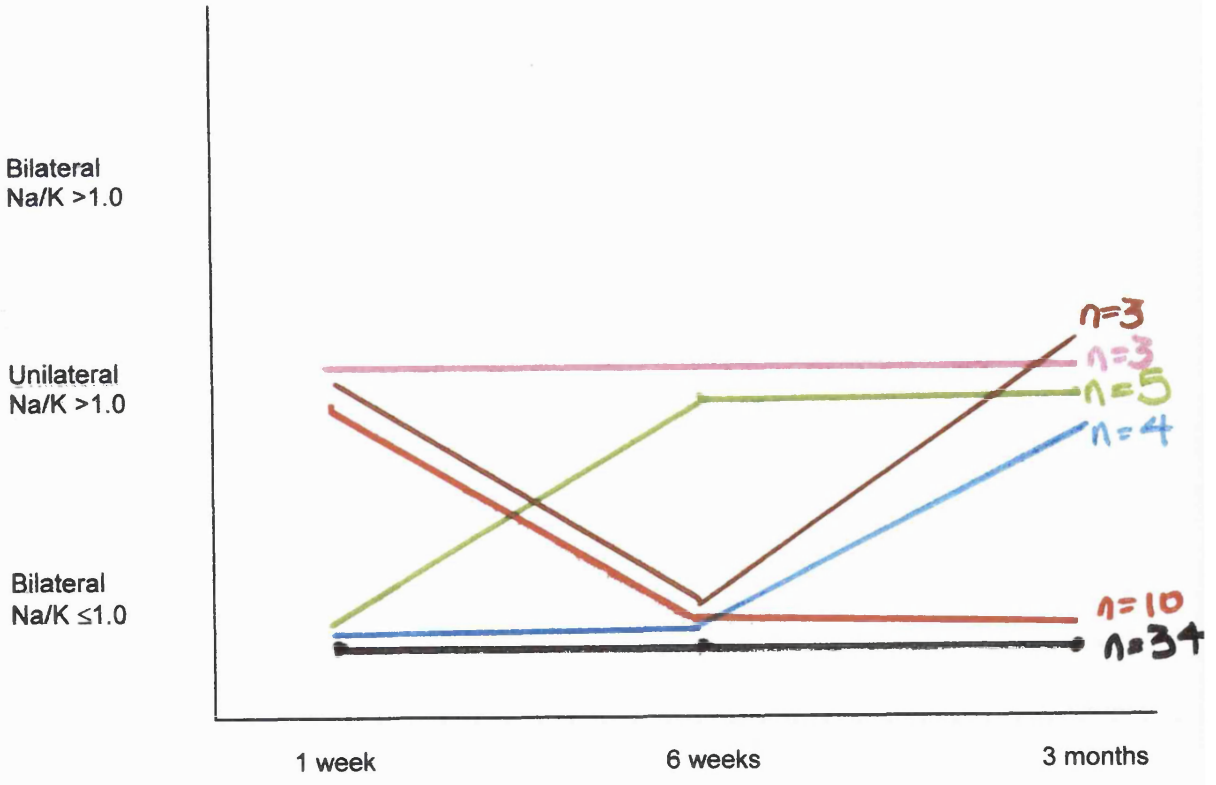
Breastmilk viral load was below the limit of detection of the HIV RNA PCR assay (≤200 copies/ml) in 34.1% (63/185), 37.8% (73/193) and 42.5% (68/160) samples at 1, 6 weeks and 3 months respectively. In the analyses above breastmilk samples with undetectable viral load have been assigned the value of 200 copies/ml, which is the assay-specific limit of detection. To explore which factors are associated with detectable breastmilk viral load at each time point. logistic regression was carried out with viral load detectability as the binary dependent variable. Univariate regression at each time point indicated a similar trend as seen in linear regression above using viral load as a continuous variable. For example, Na/K was strongly associated with viral load detectability, although at 1 week the odds ratio (OR) for ln Na/K was 1.19 (95%CI 0.74-1.89, p=0.472) but not significant. At 6 weeks the OR was 1.93 (1.12-3.33, p=0.018) and 3 months the OR was 1.64 (1.03-2.60, p=0.037). This indicates that for a 1 unit increase in ln Na/K there was between a 19 and 93% increase chance of viral load being detectable in that sample. However, due to the relatively low number of samples in each cell it was not possible to pursue multivariate modelling further.

5.5.3 Longitudinal patterns of subclinicai mastitis and viral load

The group of 68 women who breastfed from birth to at least 3 months of age and attended every follow-up visit provide a unique opportunity to investigate the longitudinal patterns of subclinical mastitis and viral shedding in breastmilk. Earlier analysis indicated that there was no significant difference between women in this core group and those who stopped breastfeeding, missed a visit or defaulted before 3 months of age with respect to the proportions with each type of subclinical mastitis. In addition attendance at each visit was not included in multivariate models to predict breastmilk viral load as it was not found to be a confounder or to significantly alter the relationship between Na/K and viral load. For these reasons, it would be reasonable to speculate that patterns of Na/K and viral load seen in these women would likely be replicated in women who continued to breastfeed, but were not seen at each visit and HIV-infected breastfeeding women in the first 3 months of lactation in general.

Subclinical mastitis category (bilaterally normal, unilaterally high and bilaterally high Na/K) at each time point were compared. As can be seen from figure 5.5.3.1, 34 of the 68 women (50%) had bilaterally normal Na/K at each time point. 10/68 (14.7%) had unilaterally high Na/K at 1 week and bilaterally normal at 6 weeks and 3 months. The remaining 24 women had various combinations of possible subclinical mastitis categories, with only one women having bilaterally high Na/K at 3 months (having previously had unilaterally high Na/K at 1 and 6 weeks). In women with more than 1 episode of unilaterally high Na/K (n= 14) the affected breast at each time point was noted. In 9 (64.3%) Na/K >1 occurred in the same breast during each unilateral subclinical mastitis episode. However, in 5 (35.7%) women different breasts were affected during each episode. This suggests that unilateral subclinical mastitis is not simply the results of a "defective" breast which is always responsible for raised breastmilk Na/K, but more likely to be the result of poor feeding practice or mechanical damage to a single breast at any time.

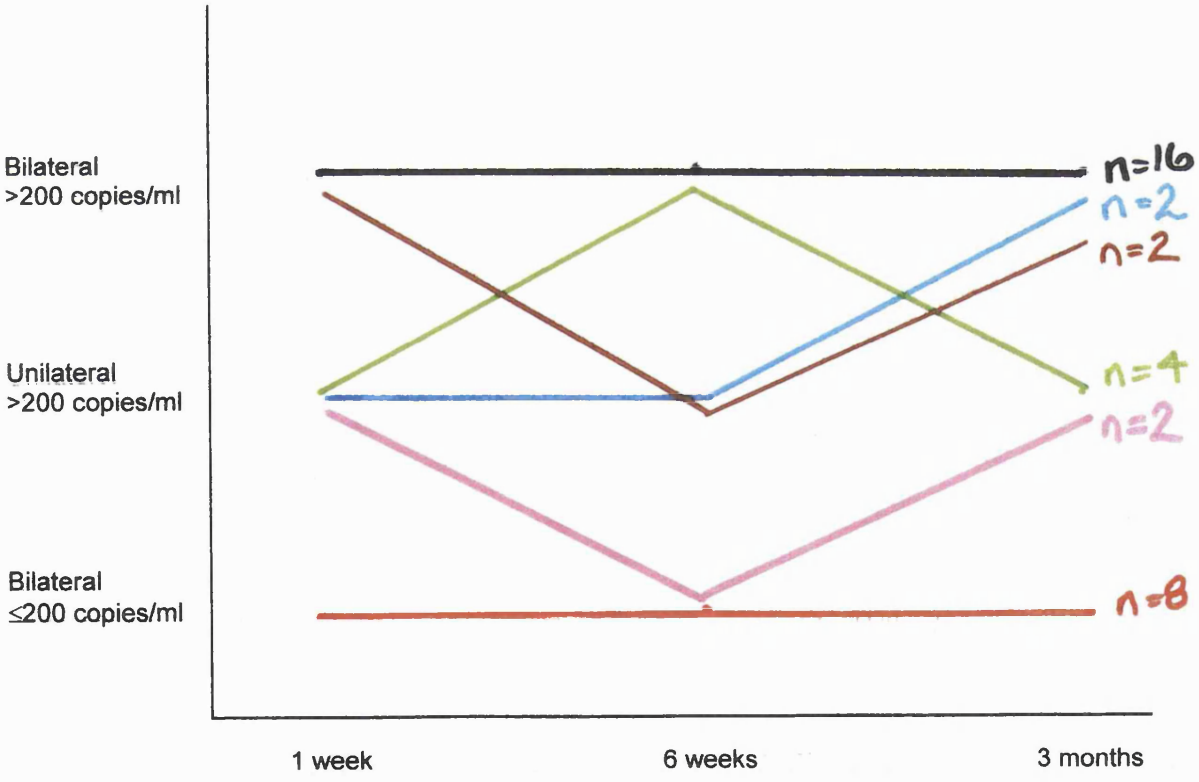
Figure 5.5.3.1: Longitudinal patterns of breastmilk Na/K among women in the core group



Note: only the 6 most common patterns are shown

Breastmilk viral load was often undetectable (<200 copies/ml) in one breast, but detectable in the other, as can be seen from figure 5.4.1. Breastmilk viral load was only available at all three time points from 47 of the 68 women who breastfed and attended each follow-up visit. As can be seen from figure 5.5.3.2, among these women 16 (34.0%) had bilaterally detectable breastmilk viral load and 8 (17.0%) had bilaterally undetectable viral load at each time point. The remaining 23 women had various combinations of bilaterally undetectable, unilaterally detectable or bilaterally detectable viral load at each time point. In women with more than one unilaterally detectable viral load episode (n=10), 3 had detectable viral load in the same breast during each episode and 7 had detectable viral load in different breasts during each episode.

Figure 5.5.3.2: Longitudinal patterns of breastmilk viral shedding among women in the core group



Note: only the 6 most common patterns are shown

5.5.4 Relationship between IL-8 and intestinal permeability

Another possible risk factor for breastmilk transmission of HIV is infant intestinal permeability, as it is hypothesised that increased breastmilk viral load in conjunction with increased intestinal permeability may result in transfer of the HIV into the infant circulation. The relationship between breastmilk Na/K and IL-8 and intestinal permeability was investigated, to determine if breastmilk factors were involved in increased intestinal permeability as well as increased breastmilk viral load and whether subclinical mastitis was a common factor in both situations. Geometric mean L:M at each time point is shown in table 5.5.4.1

Table 5.5.4.1 Geometric mean urinary L:M at each time point

	(n samples)	geometric mean (95%CI)
L:M ratio	1 wk (126)	0.171 (0.132-0.221)
	6 wks (115)	0.140 (0.111-0.177)
	3 mths (82)	0.108 (0.077-0.151)

It is important to note that the intestinal permeability of infants in this study is within the normal range at all time points. Healthy infants in the UK have been found to have L:M of 0.12 (± 0.09) (121). One possible mechanism for increased intestinal permeability is through damage or inflammation caused by high levels of breastmilk cytokines, such as IL-8. However, the infant gut is exposed to milk from both breasts, and as has been shown above there are often dramatic differences between breasts (as shown in table 5.4.3 and figure 5.4.1). There was no correlation between the mean breastmilk IL-8 in both breasts and infant intestinal permeability at any time point. There was a significant correlation between mean Na/K and L:M at 3 months only. Univariate regression was carried out to determine the relationship between mean breastmilk Na/K, IL-8 and L:M at each time point. The results are summarised in table 5.5.4.2. Given the very small range of L:M values in this study, it would be difficult to determine a relationship with any breastmilk factors or feeding mode. In addition, all infants were at least partially breastfed, and the majority were exclusively breastfed at the time of intestinal permeability testing, which could explain the very low L:M values noted.

Table 5.5.4.2: Association between mean breastmilk Na/K and IL-8 from both breasts and L:M at each time point

		1 week	6 weeks	3 months
Na/K	correlation coefficient	0.023	0.184	0.253*
&	univariate coefficient	0.054	0.387	0.676
L:M	p =	0.806	0.054	0.025
	# samples =	120	111	78
IL-8	correlation coefficient	0.038	0.035	0.007
&	univariate coefficient	0.035	0.040	0.009
L:M	p =	0.680	0.712	0.951
	# samples =	122	111	81

* Correlation is significant at the 0.05 level (2-tailed)

L:M would appear to be more closely related to Na/K than to IL-8, although this relationship was only borderline significant at 6 weeks and significant ($p<0.05$) at 3 months. This may be because IL-8 does not pass into the infant intestine intact, due to denaturing in the stomach. The univariate coefficient for L:M and Na/K at 6 weeks and 3 months was considerable. However, this might be due to an association between Na/K and L:M at the low end of the scale, as extreme L:M values are not found in this study. The data indicate that although there may be a weak association between breastmilk Na/K and intestinal permeability, there are many other factors that contribute to permeability which need to be considered. Breastmilk Na/K as a measure of subclinical mastitis may be a proxy for some other breastmilk component, which might have an influence on intestinal permeability, although this would not appear to be dramatic, given the normal values for L:M.

To determine which other factors might influence L:M at each time point, univariate analysis was carried out. However, as L:M ratio was within the normal range at each time point it would be very difficult to develop a model which would reasonably predict any changes in L:M. Intestinal permeability may be influenced by feeding mode, but this was only found to be statistically significant by univariate analysis at 6 weeks (coefficient 0.539 for exclusive breastfeeding, $p=0.048$). A larger study in the McCord's Hospital population has indicated that HIV status of the infant is a major determinant of intestinal permeability (Filteau *et al.*, submitted 2000). Unfortunately HIV status is not known on all the infants included in the current study. Given the poor association between breastmilk factors and the normal intestinal permeability measurements in this study, it was decided not to analyse this further.

5.6 Summary of results

- Subclinical mastitis is common among HIV-1 infected women during the first 3 months of lactation, with few cases of bilateral subclinical mastitis after the first week. ***See table 5.4.3.***

- Different factors influence breastmilk Na/K ratio during lactation and unilateral subclinical mastitis may occur in different breasts at any time point. ***See section 5.5.1 and 5.5.3 respectively.***

- Breastmilk Na/K is associated with infant feeding mode and mothers who exclusively breastfed their infant had lower Na/K during early lactation compared with those who mixed fed their infant. ***See table 5.5.1.1.***

- Breastmilk Na/K was associated with breastmilk viral load and there was an interaction between feeding mode and Na/K. Mothers who exclusively breastfed their infant in early lactation had lower breastmilk viral load than those who mixed fed their infant. ***See section 5.5.2.***

- Breastmilk viral load is often unilateral and undetectable in one breast. Unilateral detectable viral load may occur in different breasts at each time point. ***See figure 5.5.3.2.***

- Multivariate models only predict a small proportion of breastmilk viral load and many other factors are involved. ***See section 5.5.2.***

- Infant L:M ratio was within the normal ranges and not associated with breastmilk IL-8. ***See section 5.5.4.***

6: The importance of subclinical mastitis, exclusive breastfeeding and breastmilk viral load for the breastfeeding dyad

6.1 Prevalence of subclinical mastitis among HIV-infected women and those of unknown HIV status

Disruption of mammary epithelia results in leakage of Na into milk via paracellular pathways, increasing Na/K ratio in breastmilk, making Na/K ratio an appropriate marker for subclinical mastitis. Raised Na/K may result from infectious or mechanical damage and a consequent inflammatory response, or milk stasis and mammary gland involution due to inadequate drainage of the breast. Na/K ratios >0.6 and >1.0 have been used as cut-offs to determine subclinical mastitis, but as the greatest impact on infant and breast health indicators would be expected at higher levels of Na/K, the >1.0 cut-off has been used for severely raised Na/K and >0.6 has been designated mildly raised Na/K in these studies. Breastmilk Na/K was first assessed in a cross-sectional study of breastfeeding women attending free Mother and Child Health clinics in Durban with an infant <1year of age and then longitudinally over the first 3 months of lactation in a cohort of HIV-infected women. Due to the stigma attached to HIV status, it was not possible to ascertain HIV status of women participating in the cross-sectional study; HIV seroprevalence among women attending antenatal clinics in KwaZulu-Natal at the time the study was conducted was 32.5% (6). The cohort study recruited only HIV-infected women.

Using breastmilk Na/K ratio, a high prevalence of subclinical mastitis was found in both women in the cross-sectional study (25.7%) and also in HIV-infected women in the cohort study (34.9% at 1 week, 22.4% at 6 weeks and 25.3% at 3 months). The similarity in prevalence of subclinical mastitis among women of unknown status and those known to be HIV-infected suggests that subclinical mastitis is not only associated with compromised immunological status, but is also common among healthy, uninfected women, who constitute the majority of the cross-sectional study population. The prevalence of subclinical mastitis was higher in early lactation in both studies.

Subclinical mastitis was found to often occur in only one breast in both the cross-sectional and cohort studies. Unilateral subclinical mastitis, with Na/K >1.0 in one breast and often ≤0.6 in the other occurred in 18.5% of women in the cross-sectional study and 23.8% at 1w, 19.0% at 6 w and 22.9% at 3m of HIV-infected women in the cohort study. Interestingly, bilaterally raised Na/K was more common in women of younger infants in both studies and rare in later lactation (12.8% at 0-2 weeks in cross-sectional study and 10.3% at 1 week in the cohort study; 4.3% at 14-20 weeks in cross-sectional study and 2.4% at 3 months in the cohort study). This would indicate that bilaterally raised Na/K may be a physiological phenomenon primarily associated with early lactation, whereas unilaterally raised Na/K may indicate a localised inflammatory response in one breast to either infectious damage, poor feeding practice and mechanical damage or milk stasis.

Overall, the prevalence of subclinical mastitis was slightly higher than that reported by other studies (97) (100), but this may be due to sampling from both breasts of each woman individually in this study, rather than taking only one or a pooled breastmilk sample. As has been shown in this study subclinical mastitis is often unilateral and sampling from only one breast or pooling samples may underestimate the true prevalence of this condition.

In the cohort study breastmilk samples were collected from both breasts of a group of women at 3 time points over the first 3 months of lactation, which provided a unique opportunity to describe the patterns of subclinical mastitis over time. A core group of 68 women provided breastmilk samples at each visit and in 50% of these breastmilk Na/K was bilaterally low at each time. However, in 35.7% of women who had more than 1 episode of unilateral subclinical mastitis Na/K was raised in different breasts during each episode. This indicates that unilateral subclinical mastitis is not simply a function of one "irritable" breast which has a tendency to produce milk with higher Na/K ratio, but rather subclinical mastitis and raised Na/K is a response to some insult, which may affect either breast at any given time point. The women of the core group (who attended every visit and were still breastfeeding at 3 months) were not different from those who dropped out or stopped breastfeeding with respect to the prevalence of subclinical mastitis. However, more women who subsequently dropped out had bilaterally high Na/K at 1 week. As most of these defaulted from the study it is not possible to determine if this was the factor for them deciding to stop breastfeeding.

The finding that unilateral subclinical mastitis is common and that different breasts may be affected at each episode indicates that breasts function independently of each other and the composition of breastmilk cannot be assumed to be the same in each breast of an individual women at any given time point. For these reasons, it is important in future research to collect breastmilk samples from each breast and analyse these separately.

6.1.1 Summary of prevalence of subclinical mastitis

- Subclinical mastitis common. ***(4.4 & 5.4)***
- Bilaterally severely raised Na/K may be phenomenon associated with early lactation. ***(4.5 & 5.4)***
- Unilaterally severely raised Na/K is common throughout lactation. ***(4.5 & 5.4)***
- Unilateral subclinical mastitis does not always occur in the same breast and there was no obvious pattern throughout lactation, indicating other processes are involved. ***(5.5.3)***
- Breastmilk should be collected from each breast and analysed separately in future studies, to take into consideration the considerable differences in breastmilk composition and that subclinical mastitis is often unilateral. ***(4.4 & 5.4)***

6.2 Determinants of subclinical mastitis

6.2.1 Influence of feeding mode on subclinical mastitis in the cross-sectional study

As poor breastfeeding practice was thought to be associated with subclinical mastitis, mothers were asked about infant feeding practice. Women in the cross-sectional study were asked what their infant had consumed in the previous 24 hours. Those who reported having given their infant nothing other than breastmilk (exclusive breastfeeding) had a lower Na/K than those who had also given the infant formula in addition to breastmilk (0.58 95%CI 0.53-0.62, n=160 vs. 0.95, 0.74-1.22, n=52). This would indicate that women who were supplementing breastmilk with formula might have suffered from milk stasis and mammary gland involution, due to reduced suckling and a subsequent decline in milk production. Alternatively, women may have chosen to supplement breastfeeding with formula because of difficulties with breastfeeding,

such as mechanical damage, which would also result in an inflammatory response and raised Na/K. Very few women reported breast or nipple pain when specifically asked about these, and a more in depth qualitative study would be required to ascertain the possible reasons for supplementation. It should also be noted that exclusive breastfeeding is not widely practised in this area, and early introduction of both formula and complementary foods is very common, often because breastmilk is felt to be "insufficient" (142). It is particularly interesting to note that women who reported having given their infant complementary foods in addition to breastmilk had the lowest Na/K (0.43, 0.38-0.47, n=83). These mothers may have been supplementing their infant with very small amounts ("tastes") of complementary foods very early in life, which would not necessarily interfere with full lactation, effective suckling and emptying of the breast. Alternatively, complementary feeds may have been given to very hungry infants, who were still suckling effectively at the breast and demanding enough breastmilk to ensure adequate emptying of the breast and the prevention of milk stasis.

The large number of women reporting exclusive breastfeeding in the previous 24 hours may be a result of a sampling bias, as many of the women with young infants in this study were recruited from McCord's Hospital, which had a very supportive approach towards breastfeeding. In addition, interviews at each of the sites were conducted in an area of the clinic, which may have resulted in women feeling obliged to report exclusive breastfeeding when they knew it was the preferred mode of feeding. Although the research assistant spoke Zulu and was not associated with the routine clinic in any way, it is important to recognise this potential source of bias with regards to infant feeding practice data. As it is also not currently known how rapidly breastmilk Na/K changes in response to feeding mode and milk stasis, recent infant feeding practices may not adequately explain breastmilk Na/K. In a recent prospective study of infant feeding practices in a rural district of KwaZulu-Natal, Bland *et al.* found that mothers often alternate periods of exclusive breastfeeding and using formula or complementary foods and that even once supplementation has been initiated many mothers return to EBF (Dr Ruth Bland, Africa Centre for Reproductive Health and Population Studies, personal communication 1999).

6.2.2 Summary of feeding mode and subclinical mastitis in the cross-sectional study

- Feeding mode influenced breastmilk Na/K ratio. ***(4.5.2)***
- Exclusive breastfeeding mothers had lower Na/K than those supplementing breastmilk with formula. ***(4.5.2)***
- Mothers who had given their infant complementary foods in the previous 24 hours had the lowest mean Na/K among the 3 feeding groups. ***(4.5.2)***
- This could be because formula displaces breastmilk to a greater extent and results in reduced breastmilk production, mammary gland involution and disruption of epithelia, whereas complementary feeding is given as either very small tastes, which do not displace breastmilk (often given at a very young age) or as true complementary feeding of a hungry infant who still suckles effectively and maintains breastmilk production.

6.2.3 Determinants of subclinical mastitis in HIV-infected women at different stages of lactation (cohort study)

The cohort study investigated the determinants of subclinical mastitis in more detail than could be achieved within the confines of the cross-sectional study. Unfortunately, many women either chose to stop breastfeeding during the study period, missed some follow-up visits or defaulted from the study. This drop-out had been anticipated, as many women stop breastfeeding in the first 3 months of lactation, and had been allowed for in the sample size calculations. Due to time and financial restrictions that necessitated terminating the study in December 1999, the desired sample size was unfortunately not achieved. Sample size calculations required 130 women to still be breastfeeding at 3 months of age, whereas only 90 returned for the final visit and were still breastfeeding in the study. However, a core group of 68 women who did breastfeed until at least 3 months and attended every study visit provide a unique opportunity to investigate longitudinal patterns of subclinical mastitis.

Lactation is a dynamic process and breastmilk composition is known to change with increasing infant age. The prevalence of bilateral and unilateral subclinical mastitis also varies throughout lactation and thus different factors would be expected to be important at each time. As women

were seen at regular intervals during the first 3 months postpartum detailed information on both maternal health and infant feeding could be collected. Infant feeding practice at the time of the follow-up visit was recorded, but this was not specifically limited to the previous 24 hours. In addition, as maternal health could be an important factor in the development of subclinical mastitis, particularly among HIV-infected women who might be immuno-compromised, maternal CD4 and CD8 cells counts were measured during pregnancy and maternal body temperature recorded at each visit. Detailed socio-economic data were collected, as this might also influence maternal health as well as feeding mode. Interestingly, there was no effect of socio-economic factors on breastmilk Na/K and these have not been included in any of the multivariate models developed to explain breastmilk Na/K. Maternal CD4 and CD8 cells counts during pregnancy and body temperature at each visit were also not important factors in determining breastmilk Na/K.

Consistent with the results from the cross-sectional study, women who reported exclusive breastfeeding (EBF, nothing other than breastmilk) had lower Na/K than those reporting mixed feeding (with formula) at 1 week of age, although this effect was no longer significant at 6 weeks and not included in the final multivariate model at 3 months. This could be related to better infant feeding practice and more effective emptying of breast by that time and therefore avoidance of milk stasis and mammary gland involution. The effect may be explained by the influence of feeding practice being strongest at 1 week of age, whereas in later lactation other external factors are more important in determining breastmilk Na/K.

Interestingly, infants who consumed breastmilk with a statistically significant lower Na/K at 6 weeks and 3 months demonstrated better growth between 6 weeks and 3 months. This pattern was similar with growth between 1 week and 6 weeks of age, but did not reach significance. This could be speculated that infants who received breastmilk with lower Na/K subsequently grew better, or that infants on a better growth trajectory were very efficient breastfeeders and their mothers less likely to suffer from subclinical mastitis. Percentage weight increase between 3 and 6 months was not included, as this is a period during which complementary feeding is normally initiated and it would have been difficult to determine the influence of breastmilk Na/K.

In addition, some unexpected factors were found to be associated with breastmilk Na/K. Mothers of female infants produced milk with higher Na/K than mothers of male infants at 1 week, but this was not statistically significant at 6 weeks. This sex bias may be related to more effective suckling among boys. This sex effect was not related to birthweight or gestational age at delivery.

Younger mothers aged <25 also had higher breastmilk Na/K than those over 25 at 1 week but this was not statistically significant by 6 weeks; this was not found to be related to parity (i.e. breastfeeding experience). One possible explanation might be that these women were more likely to have a systemic infection, such as a STD; although increased body temperature was associated with a slight increase in Na/K, this was not significant in the model.

Women who formed the core group that gave a breastmilk sample at each of the 3 visits had significantly lower breastmilk Na/K than those who had missed previous visits at 3 months. At 3 months 68/90 women who gave samples were part of the core group and most of those were still exclusively breastfeeding. However, it is important to note that there was no independent effect of feeding mode on breastmilk Na/K at this time point. At 1 week women who subsequently became part of the core group had a slight, but not statistically significantly lower Na/K. This may introduce a bias into the interpretation of these results, but it could be speculated that committed breastfeeding women had fewer problems and were part of an effective breastfeeding dyad and thus less likely to suffer from subclinical mastitis.

The type of subclinical mastitis might influence the explanatory factors for breastmilk Na/K; however, there were very few women with bilaterally high Na/K at each time point, making it difficult to determine the relationship between explanatory factors and breastmilk Na/K for unilateral versus bilateral subclinical mastitis.

It is important to emphasise that these multivariate models only explained between 5.7% and 7% of breastmilk Na/K , which is obviously influenced by other factors not considered in this study.

6.2.4 Summary of determinants of subclinical mastitis in HIV-infected women at different stages of lactation (cohort study)

- Exclusive breastfeeding was associated with lower breastmilk Na/K during early lactation. ***(5.5.1)***
- Lower Na/K concentration was associated with better growth. This could be an effect of effective breastfeeding by infants reducing the possibility of milk stasis, or infants exposed to breastmilk of lower Na/K achieving better growth. ***(5.5.1)***
- Mothers of female infants produced breastmilk with higher Na/K than those of male infants during early lactation. ***(5.5.1)***
- Younger mothers produced breastmilk with higher Na/K during early lactation, possibly as a result of a systemic inflammatory response disrupting the mammary epithelia tight junctions. Parity did not have an effect. ***(5.5.1)***
- The multivariate models developed only explain a small percentage of breastmilk Na/K and other factors may be involved which were not considered in this study. ***(5.5.1)***

6.3 Determinants of breastmilk viral load

Breastmilk cell-free HIV viral load in this study (median 408 at 3 months, range 200-208,929 copies/ml) was slightly lower than that reported in other studies (69). The percentage of breastmilk samples with viral load below the limit of detection of the analytical kit used was similar to that reported by other studies (60;61;64). Unfortunately breastmilk viral load was not analysed on every sample, as some women were unable to provide adequate breastmilk for all the laboratory analyses.

The factors influencing breastmilk viral load would be expected to change during the course of lactation and so were investigated at each time point separately. There was a correlation between Na/K and viral load and also between IL-8 and viral load. However, as Na/K and IL-8 are both indicators of subclinical mastitis and IL-8 is associated with leukocyte recruitment (and cell-free HIV load was measured), it was decided to concentrate the analysis on the relationship between Na/K and viral load only.

There was a strong linear association between the explanatory variable (Na/K) and breastmilk viral load in multivariate regression models, with an approximately 1 unit increase in log viral load for every 1 unit increase in ln Na/K at each time point. As socio-economic, maternal health and infant weight gain, feeding practices and birth characteristics could be important determinants of breastmilk viral load, in addition to the effect of subclinical mastitis, these potential confounders were tested in multivariate models, allowing for the effect of Na/K on breastmilk viral load.

Maternal CD4 during pregnancy was found to be a very important confounder in the relationship between Na/K and viral load at all time points. Women with CD4 ≤200 cell/ml during pregnancy had significantly higher breastmilk viral load than those with 200-500 or >500 cells/ml at both 1 week and 6 weeks. This was only borderline significant at 3 months postpartum, as CD4 cells counts may have changed since measured during pregnancy. CD4 is a proxy measure for maternal plasma viral load and disease progression, and as would be expected, women with more advanced immuno-supression and presumably higher plasma viral load had higher breastmilk viral load.

As was seen in the determinants of breastmilk Na/K, female infants consumed milk with slightly higher viral load at 1 week and 3 months, and slightly lower viral load at 6 weeks, but this was not statistically significant at any time point. There was a suggestion of an interaction term between Na/K and infant sex, but as the sample size was relatively small it would have been inappropriate to include more than one interaction term. For this reason sex was only included as confounder.

Mothers who were <25 years old also had higher breastmilk viral load at 1 week and 3 months. There was no influence of parity in the model, indicating that this is not simply a proxy for breastfeeding experience. As suggested for the influence of maternal age on Na/K, younger mothers could be more likely to have a systemic infections, such as an STD, or have acquired HIV more recently, and be actively shedding virus into breastmilk more than women with established infection.

The percentage weight increase since last visit was an important confounder at 3 months only, with those infants who had grown better since the 6 week visit consuming breastmilk with lower viral load. As the sample size of this study was insufficient to detect any differences in transmission, it is not possible to determine whether infants of mothers with a low CD4 count (and presumably a high plasma viral load) or those who had been exposed to breastmilk with a high viral load were more likely to have become infected either in utero, during delivery or during breastfeeding, and thus grew poorly between 6 weeks and 3 months of age.

Overall there was no confounding effect of subclinical mastitis type, but as discussed above, the number of women with bilateral subclinical mastitis was very small after the first week. Whether a woman belonged to the core group, who gave breastmilk samples at each of the 3 visits, or whether they subsequently stopped breastfeeding, missed a visit or defaulted was not associated with breastmilk viral load at any time.

One of the determinants of breastmilk viral load of greatest interest in this study is the effect of feeding practice, as this is potentially amenable to intervention and may be particularly important in situations where women have no access to antiretroviral drugs to reduce plasma viral load and choose to breastfeed because of social, hygiene and financial reasons. It should be noted that women who declared the intention to breastfeed during antenatal visits in this study were advised to exclusively breastfeed, and told the potential risks to the infant intestinal barrier and consequently possible increased risk of transmission due to mixed feeding. These messages were re-enforced at each postnatal visit. As a result exclusive breastfeeding was more common than in the general population and support and encouragement for exclusive breastfeeding was much greater than that which is currently available within the public health system in South Africa.

The association between feeding mode and breastmilk viral load changed during the course of lactation. At 1 week mothers who were mixed feeding had a statistically significantly higher breastmilk viral load than those who were exclusively breastfeeding after allowing for other factors associated with viral load. There was an interaction between feeding mode and Na/K, and among women who were mixed feeding for every 1 unit increase in ln Na/K there was an almost 1 unit increase in log viral load. Among women who were exclusively breastfeeding

(EBF) for 1 unit increase in ln Na/K there was a 0.07 unit decrease in log viral load, suggesting that exclusive breastfeeding was associated with a lower viral load during early lactation. However, by 6 weeks there was a non-significant increase in breastmilk viral load in women who exclusively breastfeed compared with mixed feeding. In the interaction term the coefficient for ln Na/K in mixed feeding and EBF groups were very similar. This indicates that the effect of feeding on the relationship between Na/K and viral load is not as important as at 1 week. As lactation is established and other external factors have a greater influence on breastmilk Na/K and viral load, feeding does not have such a strong predictive power as during early lactation. At 3 months women who were exclusively breastfeeding (the majority) had higher breastmilk viral load than those who were mixed feeding. In the interaction with Na/K, women who were mixed feeding had a much smaller (but not significant) increase in viral load than those who were EBF. It could be that viral shedding is not only determined by Na/K, but either influenced by other factors or random and occurs throughout lactation. Good breastfeeding practice early on in lactation may be protective, but it is not the only determinant, and as lactation progresses other factors become important too. The trend for higher breastmilk viral load in women who were exclusively breastfeeding at 6 weeks and 3 months should be investigated further, as the data in this study were inconclusive.

It is very important to note that these models only explain between 11.2 and 26% of breastmilk viral load and there are obviously many other factors involved in viral shedding during lactation. At 6 weeks it is difficult to explain breastmilk viral load as breastfeeding may be well established and not yet disrupted by weaning. Only CD4 count ≤200 remained a significant predictor of viral load at this age.

Analysis of samples from the women in the core group indicates that virus shedding is constant in some women, constantly undetectable in some, but in the majority is intermittent and can be unilateral, not always in the same breast. Of the 47 who had samples analysed for viral load, 16 had detectable virus at each time point and 8 had undetectable virus at each time point. The remaining 23 had a variety of unilaterally detectable, bilaterally undetectable and bilaterally detectable breastmilk viral loads during the 3 months. 70% of women who had more than 1 episode of unilaterally detectable viral load had detectable virus in different breast during each episode. Therefore, it is very important to collect breastmilk from both breasts in future studies.

It is not well established that the risk of MTCT is directly related to either cell-associated or cell-free breastmilk viral load concentration (143). The observation that women who have primary HIV infection (and therefore presumably high plasma and breastmilk viral load) are more likely to transmit the virus to their breastfed infant than women with established infection would suggest that the concentration of virus in breastmilk is an important determinant of transmission risk (17;52;53). Breastmilk viral load has also been shown to be associated with other known risk factors for transmission, such as low CD4 cell counts (64). Van de Perre *et al.* reported that women with HIV-infected cells in breastmilk samples taken at 15 days postpartum were 5.4 times more likely to transmit the virus to their breastfed infant than those with undetectable levels of virus in breastmilk (57). However, Guay *et al.* demonstrated no association between detectable viral load in breastmilk at 6 weeks and MTCT (60). In a study using archive breastmilk samples Semba *et al.* found that mothers of infants infected by 6 weeks of age had higher viral load in breastmilk samples given at 6 weeks of age (700 copies/ml vs. undetectable) compared to mothers of uninfected infants (69). Clearly, breastmilk viral load is not the only determinant of breastmilk transmission of HIV from mother to infant and any intervention aimed at reducing breastmilk viral load must recognise that many factors are involved in the complex aetiology of postnatal MTCT. However, the association between subclinical mastitis, feeding mode during early lactation and breastmilk viral load would indicate that there is potential to intervene, though with limited potential to decrease the overall risk of mother-to-child transmission. Lactation counselling to improve infant feeding practices would be a low-cost intervention that would not require knowledge or declaration of HIV status and would benefit the population as a whole, with particular advantages for HIV-infected women who choose to breastfeed.

6.3.1 Summary of determinants of breastmilk viral load

- Breastmilk Na/K is strongly associated with breastmilk viral load. ***(5.5.2)***
- Maternal CD4 count during pregnancy was a major determinant of breastmilk viral load, independently of Na/K. Maternal plasma CD4 is a proxy of plasma viral load, which is a known factor in determining breastmilk viral load. ***(5.5.2)***
- Female infants consumed breastmilk with higher viral load than male infants, possibly due to the interaction between Na/K and infant sex, although this was not included in the model. ***(5.5.2)***
- Younger mothers had higher breastmilk viral load than older mothers, possibly due to concurrent systemic infection, or more recent acquisition of HIV infection and higher breast viral shedding. ***(5.5.2)***
- Infants with better growth at 3 months consumed breastmilk with lower viral load, although it is not possible in this study to establish if those infants with poor growth were infected. ***(5.5.2)***
- Feeding mode interacted with Na/K and the effect of feeding on breastmilk viral load was associated with changes in breastmilk Na/K. Infants who were exclusively breastfed at 1 week consumed milk with lower viral load than those who were mixed fed. This may be through the establishment of optimal breast emptying through exclusive breastfeeding reducing the chances of inflammation and increased viral shedding. This effect was reduced and not significant by 6 weeks and 3 months of age, suggesting that only during early lactation is feeding mode a major determinant of breastmilk viral load. ***(5.5.2)***
- Breastmilk viral load is not the same in each breast of an individual woman at any time point and future studies of breastmilk viral load should collect and analyse samples from each breast separately. ***(5.5.3)***
- It is important to note that these models only explain a small proportion of breastmilk viral load and that a number of other factors are obviously involved in determining viral shedding in to breastmilk which were not investigated here. Also, breastmilk viral load is not the only determinant of postnatal MTCT. ***(5.5.2)***

6.4 Determinants of infant intestinal permeability

Infants in the cohort study underwent a test of intestinal permeability, using the lactulose:mannitol dual sugar absorption test. Intestinal permeability might be influenced by high levels of breastmilk cytokines and thus subclinical mastitis and high IL-8 could damage the intestine and allow HIV to enter the infant circulation. However, L:M values recorded in this study were within the normal range and it was difficult to determine what factors might influence intestinal permeability. There was no correlation between L:M ratio and IL-8, indicating that IL-8 does not pass through into the infant intestine intact and is probably denatured in the stomach. There was some correlation between intestinal permeability and breastmilk Na/K. Breastmilk Na/K may be a marker for some other breastmilk component which has some small effect on gut permeability, or a proxy for indicators of infant feeding, health and growth, which have been shown to influence breastmilk Na/K. It is also important to note that all infants were at least partially breastfed, and most were exclusively breastfed, which may explain the fact that most had a very healthy intestinal mucosa and so little likelihood of major disturbances in gut permeability. Data from the larger study of intestinal permeability in infants of HIV-infected mothers indicate that L:M ratios were only increased in infants given no breastmilk (Rollins *et al.,* in preparation). Goto *et al.* also conclude that in infants under 6 months of age non-breastfeeding is associated with increase L:M ratio (125). As the current study had as primary outcome breastmilk factors, infants who were no longer breastfed were excluded from the analysis. Results from this study combined with the original intestinal permeability cohort will enable the relationship between infant feeding and HIV status and intestinal permeability to be investigated more fully.

6.5 Recommendations for future research

HIV-infected women in resource poor settings will often continue to choose to breastfeed their infants, due to poor socio-economic conditions and hygiene and the high cost of infant formula. Therefore, it is important to make breastfeeding by HIV-infected women as safe as possible, to reduce the risk of breastmilk transmission of the virus to a previously uninfected infant where alternatives to breastfeeding are neither feasible nor affordable. The studies presented here indicate that subclinical mastitis is common among infected and uninfected women and in addition is associated with increased breastmilk viral load, which is known to be a risk factor for breastmilk transmission of HIV.

As current policy regarding infant feeding in the general population recommends exclusive breastfeeding to about 6 months of age, it would be important to investigate the association between subclinical mastitis and breastmilk viral load over the first 6 months of lactation. As we have demonstrated that breastmilk viral load varies between breasts of an individual woman, it may be that there is considerable variation in viral load in foremilk and hindmilk, or in breastmilk from a breast that has not been used for feeding for a long period of time, and this should be investigated further. As the primary concern is the risk of HIV transmission through breastmilk, it would be important to quantify the effect of subclinical mastitis and breastmilk viral load on the risk of breastfeeding transmission of HIV, which the studies presented here were unable to do, due to the large sample size that would be required.

As subclinical mastitis is a result of increased mammary gland epithelial permeability due either to an inflammatory response or mammary gland involution due to milk stasis and low milk production, it is important to consider interventions which could reduce the risk of subclinical mastitis in all women, independent of their HIV status. Also, in many situations women are unaware of their HIV status or do not wish to disclose this to health care staff or their community. An intervention to reduce breastmilk viral load which also improves breastfeeding practice and does not only target HIV-infected women would be of benefit to all women and their infants. Suboptimal breastfeeding practice has been shown to be associated with subclinical mastitis and this seems to be especially important in early lactation. Lactation counselling and support to exclusively breastfeed could reduce the incidence of subclinical

mastitis and reduce breastmilk viral load, thereby contributing to a reduction in risk of breastmilk transmission of HIV to previously uninfected infants. However, it must be emphasised that the models investigated in this study only explain a small percentage of breastmilk Na/K and viral load and many other factors obviously play an important part in determining breastmilk composition and infectivity.

6.6 Public health implications

Alternatives to breastfeeding are not available, affordable or acceptable to many women living with HIV, especially in developing countries where infant morbidity and mortality is high. In these cases it is important to find ways of making breastfeeding by HIV-infected women as safe as possible. The data presented here indicate that subclinical mastitis is common among HIV-infected and uninfected women and associated with breastmilk viral load and suboptimal infant feeding practices. However, the multivariate models were only able to predict a small proportion of breastmilk viral load and we do not have data to indicate whether this is directly associated with breastmilk transmission of the virus. Interventions to reduce the incidence of subclinical mastitis may benefit all women and their infants, regardless of HIV status by improving breast health and infant feeding practices. However, it must be recognised that prevention of subclinical mastitis will only have a limited effect on breastmilk viral load, and that this in turn may only result in a small reduction in the risk of postnatal MTCT.

The impact of an intervention to reduce subclinical mastitis could be estimated thus:

For example, assuming an overall MTCT rate of 25%, of which approximately 15% will occur in utero or intrapartum, then 10% will be due to breastmilk transmission. If subclinical mastitis predicts 20% of viral load and assuming viral load is the most important determinant of postnatal transmission, then 2% of infections could be avoided, thus reducing the MTCT rate to 23% (an 8% reduction). If antiretroviral therapy such as nevirapine (using HIVNET 012 regimen) becomes available and reduces intrapartum transmission by 50% and thereby overall transmission to 18%, the 2% reduction in breastmilk transmission would equate to a 12% reduction in MTCT. Of the 200,000 infants that become infected through breastfeeding every year, about 20% (40,000) of their mothers may have subclinical mastitis. Avoidance of

subclinical mastitis might reduce the risk of postnatal transmission in 20% of those cases, which would equate to 800 infants per year. The promotion of exclusive breastfeeding is currently thought to have no adverse effects in terms of MTCT and no extra cost, but could prove to be one means of making breastfeeding by HIV-infected mothers safer for their infants.

Annex 1: Questionnaires and subject information

A.1.1: Prevalence study

Breast Health Questionnaire	Informed verbal consent 1 A4 sheet, copied back-to-back (2 pages) Completed by Zulu-speaking research assistant

A.1.2: Cohort study

Examples of questionnaires from Cato Manor are included. Data collection sheets at McCord's were originally developed by Dr Anna Coutsoudis of the SAVITA study and Dr Nigel Rollins of the Infant Intestinal Permeability study. Additional questions regarding breast health and breastmilk collection were added. Forms adapted for use at Cato Manor were based on the format of the forms used at McCord's, to ensure continuity of data collection.

HIV testing consent form	1 page In Zulu Witnessed by HIV counsellor
Information to Patients	1 page In Zulu and English Study was referred to as the Vitamin A Study to avoid disclosure of HIV study by participation in the study
Informed consent	1 page In Zulu Witnessed by HIV counsellor and/or study clinician
Socio-economic data	1 page In English Completed by study clinician with the mother
Obstetric History	1 page In English Completed by study clinician with the mother
Delivery sheet	1 page In English Completed by study clinician with the mother
Morbidity and feeding history	1 A3 sheet, copied back-to-back (2 pages) In English Completed by study clinician with the mother
Permeability testing and breastmilk collection	1 A4 sheet, copied back-to-back (2 pages) In English Completed by the research nurse and/or research assistant with the mother

Breast Health Study

Annex 1

Today's date (dd/mm/yy) [ ]

Number [C]

Mother's name [ ]

Mother's age [ ]

Baby's date of birth (dd/mm/yy) [ ]

Baby's birth weight [g]

Baby's gestational age [weeks]

Do you have sore or tender breasts today? R Y N L Y N

Do you have cracked or sore nipples today? R Y N L Y N

Do you have any problems with breastfeeding? Y N

If yes, explain [ ]

Number C

What did your baby eat or drink since this time yesterday?

- water
- formula
- fruit juice
- mashed fruit or veg
- porridge
- rice
- commercial baby food
- bread
- other: what?

Breast skin temperature

R	L

Sample collected

R	L
Y N	Y N

Time hrs

Thank you for helping us

Mina.....

ngichazeliwe futhi ngiyaqonda nge HIV. Ngiyavuma ukuthatha igazi kuhlolwe iHIV.

Ukusayina.....

Date.....

Ufakazi.....

Date.....

Vitamin A Study: Information to patients

1. Lolu ucwaningo oluzosiza ukuthi kungabi yingozi ukuncelisa ingane yakho noma unegciwane lengculazi. Sicabanga ukuthi uVitamin A uzosiza ngakho ke bonke omama sizobanikeza uVitamin A ngenkathi besakhulelwe. Kumele ugwinye iphilisi elllodwa ngelanga uze uyobeletha.

2. Emuva kokubeletha kumele uzobona udokotela ngokushesha ukuze akunikeze elinye iphilisi elinamandla kakhulu nokuyilona elizosiza ubisi lwakho ukuthi lungakwazi ukudluiisela igciwane enganeni yakho. Udokotela uzobe esehlola ingane yakho, athathe igazi bese ekunikeza usuku ozoyibuyisa ngayo ekliniki.

3. Ingane usuzoyiletha eklinlki ekuseni ngehora lesishiyagalombili uma isinesonto elilodwa, inamasonto ayisithupha nalapho isinezinyanga ezintathu. Ingane izobe seyiphuziswa amanzi anohlobo oluthile lukashukela. Kuyobe sekuqokelelwa umchamo wengane amahora amahlanu. Udokotela uzoyihlola futhi ingane athathe negazi. Uzocelwa ukuba usinike ubisi luhlolwe. Ngenkathi ulindile uzonikezwa ukudla netiye bese unikezwa no R20.00 wokukulekelela kwimali yokugibela.

4. Lapho ingane isinezinyanga eziwu 6, nalapho seziwu 9 uzoyibuyisa ekliniki. Udokotela uzoyihlola bese ethatha igazi. Uyobesewunikezwa uR20.00 wokugibela.

5. Uma wena nengane yakho nigula noma inini, udokotela uzohlola ingane noma wena mahala.

Vitamin A Study: Information to patients

1. This is a study to find ways of making breastfeeding safer to protect your baby from getting HIV. We think Vitamin A will help and all mothers will get vitamin tablets during pregnancy. You take one tablet every day during pregnancy.

2. As soon as possible after delivery come to the clinic to see the doctor. She will give you a strong tablet of Vitamin A that we hope will boost your breastmilk. The Docotor will examine your baby, take blood and give you a date for your follow up appointment.

3. When the baby is approximately 1 week, 6 weeks and 3 months you will come for a morning visit at 8am to the clinic. The baby will be given a sugar drink. The urine is then collected from the baby for 5 hours. The baby's blood will be taken and the Doctor will examine your baby. You will be asked to give a breastmilk sample. Lunch and tea are provided and you will be given R20.00 for transport.

4. When your child is 6 months and 9 months you will come for a short visit to the clinic. The Doctor will examine your baby and blood will be taken. R20.00 will be given to you for transport.

5. If you or your baby are sick at any time the Doctor will see you free of charge at the clinic.

SIYABONGA **THANK YOU**

Cato Manor

Informed consent for inclusion in Vitmain A study

Mina, (Igama) _____

ngiyavuma ukuzibophezela kulenqubo kanye/noma uhlelo lokwelashwa olulandelayo, nolwenziwa kimina noma umntwana wami.

Mina ngiyavuma ukuthi ngiyayiqonda yonke imininingwane equkethwe yilefomu okuhlanganisa nolwazi olunikiwe encwajaneni esihloko "Information to Patients".

Ngiyazi ukuthi ngingakwazi ukukuhoxisa ukuzibophezela kwami nanoma yinini ngaphandle kokwesaba ukucwaseka ukuba nginakekelwe.

Signed: _____ Date: _____
(client)

Signed: _____ Date: _____
(witness)

Cato Manor

Vitamin A Study

Name: _____ Study no:_____

Address:_____

_____

_____

Tel: (h)_____(w)_____

Contact person:_____tel:_____

Socio-economic indicators

No. rooms in the house_____

No. people sleeping in house most of the week:_____

Where do you get water from	Inside	Outside tap	Other
Toilet	Flush	Pit	Other
Electricity in the house?	Yes	No	Don't know
Working TV in house?	Yes	No	Don't know
Education of mother	None	Primary	Secondary
Is mother currently employed?	Yes	No	Don't know

Occupation of mother:_____

Does the father of the child live here most of the week?

Yes No Don't know

Annex 1

Cato Manor

Obstetric History

Name: _____ Study no:_____

Age:_____ G:_____ P:_____ GA:_____

Rhesus: POS NEG Syphilis Serology: POS NEG

Past obstetric history:

Prem delivery
C/S
Miscarriage
Abortion
Perinatal death

Clinical Staging of AIDS (1-4): _____

Past medical history

TB
HPT
Cardiac disease
Diabetes
Epilepsy
Allergy (specify)
Other

New complications during pregnancy:

_____

_____

_____

Cato Manor

Vitamin A Clinic

Delivery Sheet

Name: _____ **Study No. :**_____

Date of delivery: _____

Place of delivery: _____

Mode of delivery:

Vaginal	Emergency C/S	Elective C/S
Singleton	Twin	

Duration rupture of membranes: _____**hrs**

Birth weight: _____**kg** **Sex:** _____

Apgar: **1min:** _____ **5 mins:** _____

Neonatal problems:

Annex 1

Cato Manor

Mother's name:_____ **Study no:**_____

	1 week	6 weeks	3 months	6 months	9 months	
Date						
Age						
Weight						
Length						
HC						
Illnesses **Thrush** **ARI** **Diarrhoea** **inc. severity, duration, hospital admission**						
Hepatosplenomegaly						
Lymphadenopathy						
Skin problems						
Developmental milestones						
Medication						
Blood tests						
Results						
Breastfeeding **Sore or tender breasts** **Cracked nipples**	Y N Y N Y N	Y N Y N Y N	Y N Y N Y N			
Mother's health						

Annex 1

Cato Manor

Mother's name:_____ **Study no:**_____

	1 week	6 weeks	3 months	6 months	9 months	
Date						
Age						
Breastfeeding?						
Date breastfeeding stopped						
Juice or water?						
Date introduced						
vol per day						
Before or after breast						
Formula/cow's milk (type)						
Date introduced						
No. bottles (b) or cups (c) per day						
vol per bottle or cup						
formula: no. scoops						
Cereal/porridge?	Y N	Y N	Y N			
Date introduced						
Mixed with						
No. times/day						
Estimate portion size						
Other foods 1 (name)						
Date introduced						
times/day						
Estimate portion size						
Other foods 2 (name)						
Date introduced						
times/day						
Estimate portion size						
Other foods 3 (name)						
Date introduced						
times/day						
Estimate portion size						
Other foods 4 (name)						
Date introduced						
times/day						
Estimate portion size						

Cato Manor

Annex 1

Permeability Testing

ENTRY DATA — **DATE**

1. Name	First	Surname
2. Study number		
3. Sex	F	M
4. Date of Birth dd/mm/yy	-	-
5. Birth weight	.	kg
6. Age	wks	
7. Mothers name		
8. Other contact		
9. Address		
10. Alternative contact		
11. Phone number		
12. Date of visit dd/mm/yy		
13. Visit number	1st	2nd / 3rd
14. Weight on visit No napkin	.	kg

18. **MORBIDITY HISTORY - GENERAL**

Well	No reported symptoms	0
Mild symptoms	Mild self-limiting diarrhoea, Feeding well, Mild URTI, Mild occasional fever	1
Moderate symptoms	Persisting diarrhoea, Feeding well / unwell intermittently, Significant recurring fever, Reported respiratory distress / cough	2
Severe symptoms	Persisting diarrhoea, Feeding poorly, Reported respiratory distress / cough and Earlier review than appointed or admission.	3

19. **MEDICAL HISTORY OVER PAST 3 DAYS)**

Diarrhoea	More than two loose stools per day	Yes	No
Fever		Yes	No
Fast Breathing		Yes	No
Other	Specify	Yes	No

20. Medicine (antibiotics) in past 1 wk	Antibiotic	Other	None
21. Which medicine - if known			
22. Enema? (nil or days pre visit)	days		

L:M Urine collection

23. Volume of lactulose:mannitol given (mls)		
24. Total volume of urine in 5 hours (mls)		**Date of 6 week review:**
25. Complications		**Date of 14 week review:**
26. Stools collected	Yes	No

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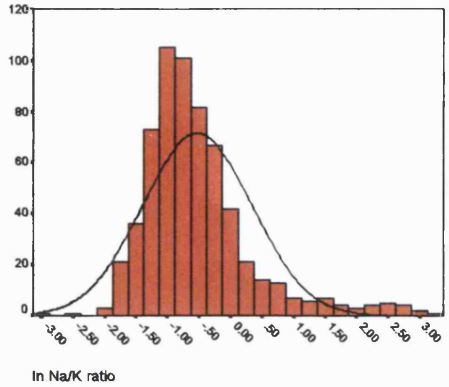
Annex 2: Tables and figures

Figure 4.4.1: Histograms of (a) ln Na/K and (b) ln IL-8, showing normal distribution curve

(a)



(b)

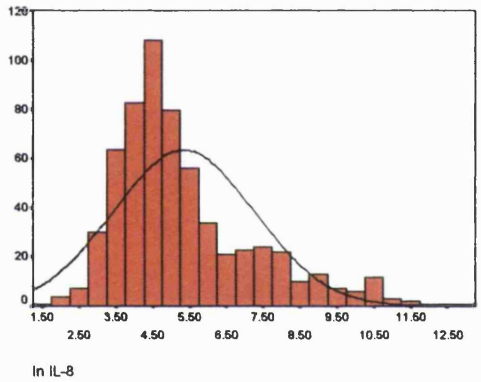
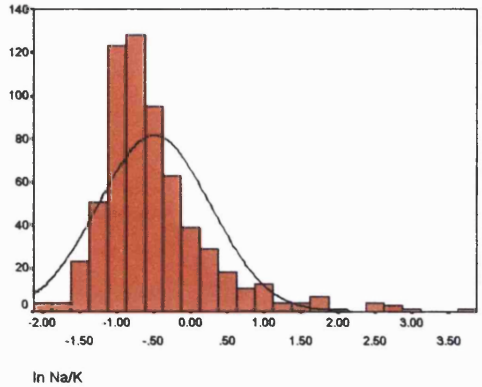
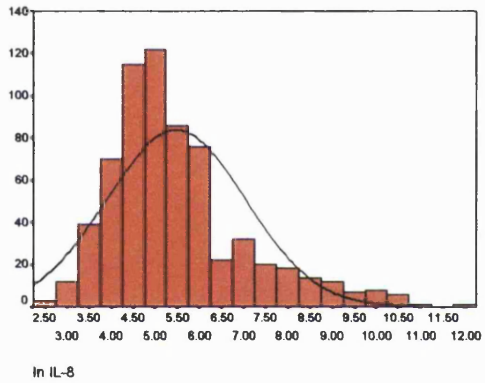


Figure 5.4.1: Histograms of (a) ln Na/K, (b) ln IL-8 and (c) log detectable (>200 copies/ml)viral load, showing normal distribution curves

(a)



(b)



(c)

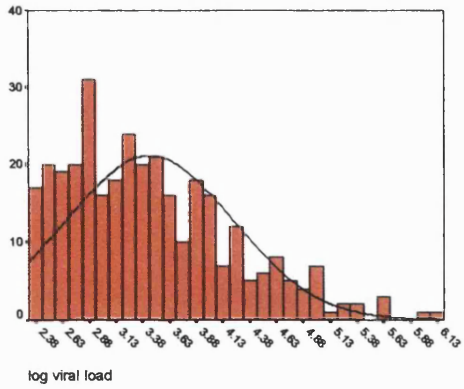


Table 5.3.2 Geometric mean breastmilk Na/K, IL-8 and viral load at each time point

	(n)	Right (95%CI)	Left (95%CI)	Both (95%CI, n)
Na/K ratio	1 wk (126)	0.71 (0.62-0.81)	0.64 (0.57-0.72)	0.67 (0.62-0.73)
	6 wks (116)	0.59 (0.52-0.67)	0.56 (0.49-0.64)	0.57 (0.52-0.63)
	3 mths (83)	0.52 (0.45-0.61)	0.60 (0.49-0.74)	0.56 (0.49-0.64)
IL-8 (pg/ml)	1 wk (128)	341 (247-471)	249 (187-331)	292 (235-362)
	6 wks (118)	208 (159-271)	178 (137-231)	192 (160-232)
	3 mths (86)	218 (169-283)	251 (181-347)	233 (190-287)
Viral load (copies/ml)	1 wk (92)	1142 (732-1785)	869 (597-1264)	995 (744-1132)
	6 wks (96)	968 (671-1396)	1116 (741-1678)	1039 (790-1366)
	3 mths (80)	950 (631-1436)	877 (597-1288)	914 (690-1210)

Note: not every woman was able to provide sufficient sample from both breasts for all laboratory analysis at each time point

Table 5.3.3 Prevalence of subclinical breast inflammation at each time point among the 68 women who provided breastmilk samples at each time point (core group) compared with those who did not.

	1 week (%) **n=68**	1 week (%) n=58	6 weeks (%) **n=68**	6 weeks (%) n=48	3 mths (%) **n=68**	3 mths (%) n= 15
Bilaterally normal	25 (37.8)	21 (36.2)	32 (47.1)	25 (52.1)	38 (55.9)	7 (46.6)
Unilaterally mildly raised	13 (19.1)	10 (17.2)	16 (23.5)	6 (12.5)	11 (16.2)	3 (20.0)
Bilaterally mildly raised	8 (11.8)	6 (10.3)	5 (7.3)	6 (12.5)	2 (2.9)	1 (6.6)
Bilaterally ≤1.0	**46 (67.6)**	**37 (63.8)**	**53 (77.9)**	**37 (77.1)**	**51 (75.0)**	**11 (73.3)**
Unilaterally severely raised	12 (17.6)	4 (6.9)	8 (11.8)	7 (14.6)	12 (17.6)	3 (20.0)
One mildly and one severely raised	7 (10.3)	7 (12.1)	6 (8.8)	1 (2.1)	4 (5.9)	0 (0)
Unilaterally >1.0	**19 (27.9)**	**11 (19.0)**	**14 (20.6)**	**8 (16.7)**	**16 (23.5)**	**3 (20.0)**
Bilaterally severely raised	3 (4.4)	10 (17.2)	1 (1.5)	3 (6.3)	1 (1.5)	1 (6.6)
Bilaterally >1.0	**3 (4.4)**	**10 (17.2)**	**1 (1.5)**	**3 (6.3)**	**1 (1.5)**	**1 (6.6)**

Note:Percentages may not add to 100 due to rounding. Not all women were able to provide sufficient sample from both breasts for all laboratory analysis. The women included here gave sufficient breastmilk from both breasts for Na/K analysis.

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