Immunological and virological determinants of HIV-1 transmission from mother-to-child via breastfeeding

Johannes Viljoen

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Spécialité : Biologie Santé

Présentée par Johannes VILJOEN

DETERMINANTS IMMUNOVIROLOGIQUES DE LA TRANSMISSION DU VIH-1 PAR L’ALLAITEMENT MATERNEL
IMMUNOLOGICAL AND VIROLOGICAL DETERMINANTS OF HIV-1 TRANSMISSION FROM MOTHER-TO-CHILD VIA BREASTFEEDING

Soutenue le 5 août 2015 devant le jury composé de

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Executive Summary

Breastfeeding is a most valuable source of nutrition for infants, especially in under-resourced areas, and has been a corner-stone for infant health through centuries. The anti-infective properties of breast milk are well documented and breastfeeding protects infants against gastrointestinal and respiratory illnesses. There is no disagreement that breastfeeding is the best form of nutrition for all infants everywhere, for a minimum of 6 months, and ideally to at least 2 years of life. Exclusive breast-feeding for 6 months is recommended for the general population primarily because human milk can satisfy all of an infants’ nutritional and hydration needs through the first 6 months of life.

New perinatal HIV infections in resource-rich countries have nearly been eliminated with the combination of universal, opt-out antenatal HIV testing, antiretroviral prophylaxis of the mother and infant, elective cesarean delivery, and avoidance of breastfeeding. Although effective interventions are available to reduce in utero and intrapartum transmission in resource-limited settings, postnatal transmission of HIV through breastfeeding has remained a significant problem. Acquisition of HIV through breast milk accounts for an estimated 40% of new infections in sub-Saharan Africa, where more than 90% of perinatal infection occurs.

The studies performed during this PhD were part of a larger intervention programme in KwaZulu-Natal that focused on the use of different forms of infant feeding within a rural setting. The Umkhanyakude district in northern KwaZulu-Natal, South Africa, is one of the areas worst affected by the HIV and AIDS pandemic, and has some of the highest prevalence figures in the world. Women were enrolled into this study prior to commencement of the South African national antiretroviral roll-out in 2005. This PhD research forms part of efforts to gain a better understanding of postnatal transmission of HIV-1 via breastfeeding, and to support the World Health Organization in their goal to reduce all forms of mother-to-child transmission (MTCT) to below five percent by the end of 2015.
In the first study performed, we provide for the first time evidence that cumulative exposure to HIV-1 RNA in breast milk is a key risk factor associated with postnatal mother-to-child transmission, independent of maternal CD4 and plasma HIV-1 viral load. Cumulative exposure is attributable to viral shedding in, rather than volume of, breast milk consumed. This data provides a better evaluation of the risk of HIV-1 MTCT and intra-breast viral load.

In the second study we confirm that cell-associated virus load in breast milk is a stronger predictor of the risk of early postnatal MTCT than cell-free virus, independent of HIV-1 replication in blood and breast milk. In contrast, cell-free virus load is a stronger predictor of later postnatal HIV-1 transmission. We provide evidence that the HIV-1 reservoir is a main risk factor for post-natal MTCT of HIV-1.

In the third study performed, we investigated the significance and impact of Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in breast milk from HIV-infected mothers, and MTCT of HIV-1. High levels of CMV is shed in breast milk, and frequently a significant level of EBV is shed in HIV-infected women. Hence, mothers whose breast milk contained high levels of CMV, were up to two and a half times more likely to transmit HIV-1 to her infant via breastfeeding compared to women with low levels. This is the first evidence of an association, independent of HIV-1 viral load, between CMV in breast milk and postnatal MTCT of HIV-1.

In contemporary breastfeeding populations with access to antiretroviral prophylaxis, the residual HIV-1 transmission risk, especially in the early postpartum period, is explained in part by the persistence of cell-associated virus in breast milk. More studies are needed to further knowledge on the mechanism of HIV-1 transmission during lactation, and factors associated with compartmentalized shedding of HIV-1 in breast milk, and to help develop more effective drugs for use in resource-limited populations where avoidance of breastfeeding is almost impossible.
Acknowledgements

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Bottlenecks to pMTCT of HIV-1 via breastfeeding

Incomplete understanding of the role of activated T cells
Incomplete understanding of the role of immune factors
Unclear efficacy of prophylaxis
Incomplete understanding of the role of co-viral infections in breast milk

Control of CMV infection/reactivation

CMV Vaccine
CMV Antiviral therapy

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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>ART</td>
<td>antiretroviral therapy</td>
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<td>BF</td>
<td>breastfeeding</td>
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<td>BM</td>
<td>breast milk</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>cCMV</td>
<td>congenital cytomegalovirus</td>
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<tr>
<td>DBS</td>
<td>dried blood spot</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ELISPOT</td>
<td>enzyme-linked immunospot</td>
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<tr>
<td>EpCAM⁺</td>
<td>epithelial cell adhesion molecule-positive</td>
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<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
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<tr>
<td>HEI</td>
<td>HIV-1 exposed infected</td>
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<tr>
<td>HEU</td>
<td>HIV-1 exposed uninfected</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
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<tr>
<td>MALT</td>
<td>Mucosal associated lymphoid tissue</td>
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<tr>
<td>MEC</td>
<td>mammary epithelial cells</td>
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<tr>
<td>MTCT</td>
<td>mother-to-child transmission</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>pMTCT</td>
<td>prevention of mother-to-child transmission</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RCT</td>
<td>randomized controlled trial</td>
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<tr>
<td>UNAIDS</td>
<td>Joint United Nations Programme on HIV/AIDS</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

In 2012, there were 35.3 million people living with Human Immunodeficiency Virus Type 1 (HIV-1) infection worldwide. New infections among children have declined by 52% since 2001. Worldwide, 260,000 children became newly infected with HIV-1 in 2012, down from 550,000 in 2001. Implementation of World Health Organization (WHO) guidelines has been associated with significant reductions in mother-to-child transmission (MTCT) of HIV-1, as well as improved child survival. Expanded access to services for prevention of mother-to-child transmission (pMTCT) prevented more than 670,000 children from acquiring HIV-1 from 2009 to 2012 [1, 2].

Risk of HIV-1 MTCT by breastfeeding (BF) and advantages of BF in resource constrained areas

In the absence of antiretroviral prophylaxis, breastfeeding (BF) accounts for almost one-third to one-half of mother-to-child transmissions of HIV-1 [3]. However, infant feeding in the context of HIV is complex because of the advantages of BF for infant survival in resource-limited settings [4]. The dilemma is to balance the risk of HIV transmission from mother-to-child through BF with the higher risk of death from malnutrition and serious illness among non-breastfed infants [5]. Hence, in Africa according to individual circumstances, environment and local situation, BF is frequently chosen as the most appropriate infant feeding option for HIV-infected mothers.

BF is known to have significant nutritional, immunological, and developmental advantages for the neonate and has been the foundation of child health and survival for centuries. BF in HIV-infected mothers may be efficiently circumvented in resource-rich countries by replacement feeding that is acceptable, safe and affordable. However, this is not currently possible in most of the developing world such as southern and eastern Africa where HIV/AIDS, diarrhea, pneumonia and malnutrition are leading causes of under 5-year mortality [4, 6]. There is thus a dilemma in predicting the risk of MTCT of HIV-1 to their infants via BF, versus the risk of infants dying from common infectious diseases by avoiding BF. Achieving an optimal balance would require reduced HIV-1 transmission through BF, improved child survival, and enabling HIV-
infected lactating mothers to benefit from interventions through ART. In recent years, research has identified ART interventions that significantly reduce HIV-1 transmission through BF. The WHO now recommends BF as well as the provision of ART to both mother and infant [7].

Exclusive breastfeeding (EBF), regardless of maternal HIV-1 status, is currently recommended by the WHO for the first 6 months of life [8]. The risk of postnatal MTCT of HIV-1 via breastfeeding (BF) is significantly higher in women with low CD4 T-cell counts and high plasma and breast milk (BM) viral load. In the absence of intervention using antiretroviral therapy (ART), postnatal MTCT of HIV-1 depends on maternal CD4 T-cell count with transmission rates of 1.57% versus 0.51% per month of breastfeeding when maternal CD4 T-cell count is less or more than 350 cells/µl, respectively [9]. This translates into a cumulative postnatal transmission risk of 14-20% when HIV-exposed infants breastfeed for 18-24 months. However, with the initiation of ART at diagnosis, intra-uterine (from time of initiation of ART) and intra-partum transmission are largely prevented, leaving postnatal MTCT via BF contributing to the majority of infections, especially in settings where rates of incident infections are high [10].

**Antiretroviral interventions that reduce postnatal MTCT of HIV-1**

A number of reports on the implementation of global recommendations are discussed and summarized in [4]. However, most of these recent reports focus on approaches for resolving implementation issues, rather than investigating new clinical interventions. Several investigators argue in favour of lifetime ART for all pregnant HIV-infected women in resource-limited settings [11-13]. In addition, from 2010 to 2013 WHO guidelines recommend that HIV-exposed infants be breastfed for 12 months [14], with mothers and infants receiving ART in the form of three pMTCT programmes; Option A, option B and option B+ [15]. Updated WHO guidelines in June 2013 endorse this approach [16], although there remains diverse opinion in southern Africa whether BF with ART is the appropriate method to reduce MTCT of HIV [17, 18].

WHO set a global target to reduce by at least 90% the number of new infections among children by 2015, to reduce all forms of MTCT of HIV-1 to a rate below 5% and to decrease the annual number of new paediatric infections to a value below 40,000 [19]. Achieving WHO goals
by 2015 will be challenging, and very few clinical trials that have evaluated the efficacy of either treatment option have shown a reduction in MTCT of HIV to levels below 5%. There is a need for a better understanding of what drives residual HIV MTCT in mothers receiving effective ART, more specifically to identify factors responsible for MTCT of infectious HIV-1 particles that remain cell-associated, and refractory to maternal ART intervention [20, 21]. In an observational cohort of ART-treated, HIV-infected pregnant women from the Kesho Bora trial with fewer than 200 CD4+ T-cells/µl, or with WHO stage 4 AIDS, the 18-month probability of HIV transmission was 7.5% [22-24]. In mothers on successful ART with undetectable HIV-1 RNA in blood and breastmilk, the risk of HIV-1 transmission through BM remain significant and is estimated at 0.2% per month of breastfeeding [9].

Impact of BM HIV reservoirs and coviral infections on HIV MTCT by BF

Potential HIV-1 reservoirs in BM include T-lymphocytes, macrophages, mammary epithelial cells (MEC) and stem cells. Although the importance of these reservoirs in HIV-1 transmission through BM has been demonstrated, the respective roles of each of these reservoirs still remain to be elucidated. The presence of these reservoirs poses a significant challenge to prevention of BM HIV-1 transmission. Indeed, these intra-cellular reservoirs of HIV-1 (both RNA and DNA) persist despite effective maternal ART, which could explain residual postnatal transmission [11, 12]. BM cell-free viral load is correlated with plasma viral load but may also vary with local intra-mammary factors. Coviral infections [13] and conditions such as clinical and subclinical mastitis [14, 15] have been shown to be associated with increased HIV-RNA shedding in BM, which in turn is associated with increased MTCT of HIV-1. Mastitis increases BM HIV RNA in the affected breast by up to 10-fold [16], and a similarly though less significant increase in BM HIV RNA is associated with subclinical mastitis [17]. In addition to HIV-1 viral load in BM, coviral infection with CMV (see below) and EBV, and BM composition (neutralizing antibodies, oligosaccharides and cytokines) play an important role. Innate immunity is an important concept in understanding why the majority of HIV-exposed breastfeeding infants never become infected with HIV.
Coviral infections in BM: The role of Human Cytomegalovirus

Human cytomegalovirus (CMV) is an ubiquitous herpesvirus that causes serious disease in congenitally infected neonates as well as in immunocompromised individuals suffering from AIDS and solid-organ and bone marrow transplant recipients [25]. It is the most common cause of congenital and perinatal viral infections throughout the world and is the leading non-genetic cause of congenital malformation in developed countries [26]. Congenital infection occurs in 1% of all live births in developed countries and in higher percentages in developing countries [27]. As a result of transmission during birth and by breast milk, perinatal infections are much more prevalent than congenital infections. The vast majority of these infections are chronic and subclinical, but symptomatic infections represent a major public health problem throughout the world [28]. CMV reactivates from latency sporadically throughout life but is enhanced by immunosuppression and allograft rejection in transplant recipients, in whom virus can be detected in the peripheral blood as well as in body fluids e.g. urine and breast milk. Studies have identified monocytes and their progenitors as major sites of lifelong latent infection [29] where the viral genome is maintained as an extrachromosomal plasmid at between 2 and 13 genome copies per infected cell [30, 31]. Maintenance of latency and reactivation in peripheral blood monocytes are linked to the cellular differentiation state and activated macrophages support reactivation and active replication [32, 33]. In addition to leukocytes of the hematopoetic cell lines, CMV can infect a broad range of other cell types including epithelial cells, endothelial cells, fibroblasts and smooth muscle cells [43]. In the United States primary CMV infection during pregnancy occurs in approximately 40 000 pregnant women every year and of these, approximately 8000 infants develop severe permanent neurologic damage, with neonatal death in utero in 10% of fetuses infected in utero. Neurologic damage include impaired development, mental retardation, and sensory hearing deficit [34] with a significant proportion (~15%) of initially asymptomatic CMV-infected babies, developing disease between birth and 5 years of age [35, 36].
HIV-1 and CMV coinfections have been shown to increase infant morbidity, mortality and AIDS-progression. Studies in the United States showed increased neurological disease and AIDS-progression in co-infected infants [37]. In HIV-endemic areas, maternal HIV-exposed but uninfected infants, which is the majority of children affected by HIV, also show poor growth and increased morbidity [38]. HIV-infected mothers can have reduced maternal passive immunity to protect their infants against CMV disease. Moreover, immunosuppression can lead to increased CMV levels due to reactivation or secondary infection. In a study performed in Zambia it was shown that in HIV-exposed uninfected infants, CMV infection was associated with increased prevalence to stunting, reduced head size and decreased psychomotor development [38]. It has also been shown that prenatal ART reduced perinatal and early postnatal CMV among HIV-infected as well as HIV-exposed uninfected infants [39]. Perinatal and postnatal CMV infection results in chronic viral excretion for years [28]. Although its sequelae are less severe than those of congenital infection, higher-risk infants, i.e. preterm or HIV-exposed, may develop hearing loss and clinical illnesses such as pneumonia, hepatitis, hepatosplenomegaly, anaemia, thrombocytopenia and abnormal hepatic function [40].

**Composition of BM and reservoirs of HIV-1**

To gain a better understanding of HIV-1 MTCT by breastfeeding it is critical to decipher the breast milk environment.

**Non-cellular components of the mammary gland environment – cell-free HIV-1 particles**

The stroma of the lactating mammary gland is an effector site for mucosal immunity that interacts with the mucosal associated lymphoid tissue (MALT) [41]. Its resident immune cells also have activation and cytokine profiles different from those of blood that can influence the dynamics of HIV-1 replication [42-44]. This environment could encourage initiation of the viral cycle or promote ongoing replication in CD4+ T lymphocytes harbouring HIV-1 DNA [45, 46]. In contrast, an antiviral Th1 environment in BM could limit HIV-1 replication through direct effects of cytokines such as interferon-γ (IFN-γ) and by promoting cytotoxic T cell responses [47, 48]. Cytokines of the CXC and CC chemokine families, are found in large amounts in BM, and act as mediators of inflammation that can activate leukocytes. These cytokines are secreted by both
mammary epithelial cells and BM leukocytes. Subclinical and symptomatic mastitis [49, 50] are both associated with an increase of pro-inflammatory cytokines in the mammary gland, as well as increased risk of MTCT of HIV-1 via breastfeeding [51, 52]. This could be related to an imbalance between antiviral and proinflammatory cytokines that facilitates HIV-1 replication in the mammary gland.

Seventy percent to 80% of HIV-1–infected, lactating women not treated by ART have detectable HIV-1 RNA in the whey, more if breast milk sampling is repeated because most women have intermittent viral shedding in breast milk [53-56]. In addition, up to one-third of the HIV-1 RNA in milk may be sequestered in the lipid fraction [57], and HIV-1 particles can be passively carried on the surface of breast milk cells. Thus, the frequency of HIV-1 RNA shedding in breast milk has probably been underestimated in studies testing only the liquid fraction of milk. The relationship between the level of HIV-1 RNA in breast milk and that in blood and the origin of cell-free HIV-1 particles in breast milk remain uncertain. Although correlated with plasma HIV-1 RNA levels [54], breast milk viral load is most frequently lower (by about 2 log10) than plasma viral load [58]. In addition, HIV-1 RNA levels may differ in milk collected from the right and left breasts [59], suggesting that local factors in the mammary gland contribute to viral production. The association of mammary gland inflammation (clinical or subclinical mastitis, breast abscess, engorgement, and systemic or multiorgan inflammation) with elevated breast milk HIV-1 RNA supports this conclusion [51, 54, 60-62]. Thus, cell-free HIV-1 particles in human milk, as measured by HIV-1 RNA, originate at least partly from local replication in the mammary gland [63, 64].

BM contains numerous soluble factors with antimicrobial, anti-inflammatory, and immunomodulatory activity, many of them yet to be characterized, that guards the integrity of the infant’s gut (Table 1).
Maternal mammary epithelial cells
Maternal immune cells
  Macrophages and dendritic cells
  Neutrophils
  Natural killer cells
  T cells
  B cells and their immunoglobulins
  Stem/progenitor cells
Cytokines
Nucleotides
Other immune components
  Chemokines
  Long-chain polyunsaturated fatty acids
  Anti-infective oligosaccharides
  Other anti-infective soluble factors
Compounds that promote microbial colonization of the infant’s colon
  Hormones, growth factors, and bioactive peptides

Table 1.
Constituents of human milk with potential to influence infants’ immune development and defenses (adapted from Blewett, 2008)

Non-cellular components include cytokines, nucleotides, and immune components such as immunoglobulins, chemokines, long-chain polyunsaturated fatty acids, anti-infective oligosaccharides, and numerous other anti-infective soluble factors. In addition there are compounds that promote microbial colonization of the infant’s colon including hormones, growth factors and bio-active peptides, all factors involved in the antimicrobial innate immune response. In broad, these substances protect the infant by:
I. bacterial lysis or inactivation. Well-characterized factors with a broad spectrum of antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, include lactoferrin that removes LPS from the outer microbial cell membrane [65, 66] and fatty acids that may damage bacteria by disrupting their cell membranes or by changing intracellular pH [67].

II. boosting the cellular immune response to bacteria. A soluble form of CD14 (sCD14) is present in BM in concentrations ~20 times higher than in serum [41], and along with maternal soluble Toll-like receptors [68], exerts its gram-negative antibacterial function. Abundant human β-defensin-1 expression in milk and mammary gland epithelium has also been described [69].

III. blockade of pathogen attachment and entry into host cells. Non-digestible oligosaccharides are important antibacterial constituents of human milk (6). Lactadherin has been shown to prevent rotavirus attachment [70], and similarly secretory leukocyte protease inhibitor (SLPI) prevents HIV-1 entry into cells [71]. In addition to Lactoferrin, several components and activation fragments of complement, likely to participate in the infant’s intestinal innate immune response against bacteria, have been described [72].

IV. mitigation of infant gut inflammation. BM downregulates inflammation in the infant’s gut. Immediately after birth, new antigens and LPS from pathogens and colonizing commensal bacteria, can induce an excessive mucosal inflammatory response, as shown in vitro [73]. In severe cases, this may contribute to necrotizing enterocolitis in preterm infants. Factors in BM may mitigate the T helper 1 (Th1) or inflammatory response and thereby preserve the gut mucosal barrier, likely accounting for the lower frequency of NE in breast-fed than formula-fed infants [74].
Cellular components

Mammary Epithelial cells
Epithelial cell adhesion molecule-positive (EpCAM⁺) epithelial cells from the mammary gland are the most abundant cells [75]. Stem and progenitor cells have also been identified in BM [76, 77]. The function of either these cell types, after ingestion via BF, remains unknown. Mammary gland epithelial cells, the major cellular component of breast milk [75], are also susceptible to HIV-1 infection [78]. These cells express CCR5, CXCR4 (co-receptors necessary for HIV-1 entry), galactosyl ceramide (GalCer), and, unexpectedly, CD4 surface markers [79]. When these cells are exposed to HIV-1 in vitro, HIV-1 is taken up into endosomal vacuoles. Co-culture of activated CD4⁺ T cells with HIV-1–exposed mammary epithelial cells can result in their productive infection, suggesting that epithelial cells can enhance infection in vivo, probably by transcytosis. Therefore, mammary epithelial cells may transport HIV-1 across the epithelial surface of the mammary gland acini and lactiferous ducts to contribute to HIV-1 shedding in breast milk, but because HIV-1 does not replicate in these cells, they are not likely to be an active reservoir for the virus.

Leukocytes (Lymphocytes and macrophages)
Mature breast milk, differing from colostrum and transition milk where leukocytes are abundant, contains a small and inconsistent concentration of leukocytes (~1-5 x 10⁵ cells/ml). Of these, neutrophils account for 80%, macrophages for 15%, and lymphocytes for less than 5% [75].

Lymphocytes
Various lymphocyte types coexist in breast milk: CD3⁺ T cells (representing about 83% of lymphocytes, almost equally distributed between CD4⁺ and CD8⁺ lymphocytes), γδ T cells (11%), CD16⁺ natural killer cells (3 to 4%), and B cells (2%). CD4⁺ T cells, one of the main target cells for HIV-1, represent almost 40% of the total lymphocyte population with 1 ml of breast milk containing about 2000 CD4⁺ T lymphocytes (by comparison, blood contains almost 1
During feeding, the mucosal area of the tonsil and the gut is exposed to, on average, 700 ml of maternal milk each day, exposing the infant to more than 1 million maternally derived CD4+ T cells. After 6 months of life, a baby will have ingested about $2 \times 10^8$ breast milk–derived CD4+ T cells. HIV-1 infection of the mother depletes CD4+ T cells more rapidly in blood than in breastmilk, so that CD4+ T cells, particularly CCR5+ CD4+ T cells, persist longer in breast milk than in other mucosal sites [80, 81].

Breastmilk T and B lymphocytes are distinct from circulating blood lymphocytes (Fig. 1 – adapted from [6]).

First, breast milk contains almost exclusively memory T and B lymphocytes, which have previously encountered antigens. Indeed, very few breast milk cells express the CD45RA receptor that characterizes naïve T cells [21]. Likewise, more than 70% of breast milk B cells are IgD− CD27+ memory B cells [82], most of which carry somatically mutated variable region genes and are class-switched B lymphocytes expressing surface IgG or IgA molecules. Therefore, most breast milk T and B cells are antigen-experienced and so can respond efficiently to bacterial and viral pathogens.

Second, many T and B lymphocytes from breast milk are activated, frequently expressing activation markers such as human leukocyte antigen (HLA)–DR, CD38, and CD69 [21, 80]. Many of these activated cells in breast milk are effector memory cells (which are therefore primed to respond to antigen exposure) [48], in contrast to blood T cells, which are primarily central memory cells. An average of 42% of CD4+ memory T lymphocytes are activated in breast milk [21], a proportion 5 to 10 times higher than in blood. HIV-1–specific CD8+ T cells are more frequent in breast milk than in blood, where they may help to limit HIV-1 production by infected CD4+ T cells [47]. The high frequency of activated immune cells in breast milk is paradoxical because human milk per se does not confer immune activation and is in fact anti-inflammatory. Breast milk lymphocytes most likely become activated through extravasation or during transepithelial migration [83]. In addition, breast milk B cells include mainly large-sized B
cells, plasmablasts, and plasmacells [82], which do not express complement receptor but are switched memory B cells primed to secrete antibodies.

Third, most breast milk T and B cells express the mucosal homing markers α₃ integrin (CD103), α₄ integrin (CD49d), β₇ integrin, and CCR9, confirming that they were primed in mucosal-associated lymphoid tissues (MALTs) and migrated to the mammary gland as an effector site. Milk B cells seem to have migrated preferentially from the gut-associated lymphoid tissues (GALTs).

Fourth, most breast milk CD4+ T lymphocytes express high levels of chemokine receptors CCR5 and CXCR4, the major co-receptors required for HIV-1 attachment and entry.

These characteristics of breast milk lymphocytes reinforce the idea that human milk provides neonates and infants with supplemental, highly immunologically active components designed to protect the mother-infant dyad from potential pathogens. Nevertheless, some of the same cells that provide these functions are ideal targets for HIV-1 infection and transmission: They are memory cells, of mucosal origin, with a high level of activation and abundant cell surface expression of HIV-1 co-receptors.
**Fig. 1. Comparison of breast milk and peripheral blood CD4+ T cells.**

[Fig 1 caption] Breast milk T lymphocytes have four characteristics that differentiate them from circulating blood lymphocytes: They express the CD45RO receptor almost exclusively (upper and lower panel), which is characteristic of memory T cells. They exhibit more markers of activation (upper and lower panels). The expression of HIV-1 co-receptors on the surface of breast milk cells is stronger than it is on T cells from blood (upper panel). Unlike blood cells, breast milk cells exhibit mucosal homing markers (upper panel) ((adapted from Van de Perre, 2012)).
Latently HIV-1–infected, resting CD4+ T lymphocytes harbor HIV-1 proviral DNA. These cells are very rare, estimated at $10^3$ to $10^7$ for an entire infected individual [84]. Although the decay characteristics of this latent reservoir remain uncertain, these cells have a very long half-life of about 44 months and are not affected by conventional ART ([85, 86]. These latently infected, resting CD4+ T cells in both breast milk and blood of HIV-1–infected women can transcribe HIV DNA and generate viral particles [87, 88]. Even when the HIV-1 DNA viral load is comparable in blood and BM, polyclonal activation results in 10 times more HIV-1 antigen–secreting cells (Ag SCs) in BM than in blood (500 versus 45 antigen secreting cells/$10^6$ cells). If one assumes that one to three copies of HIV-1 are integrated in latently infected cells, the efficiency of transcription and translation after activation is 1 to 2% in blood and 10 to 30% in breast milk [87]. Thus, the CD4+ T cells in breast milk are potentially 17 times more effective than their blood counterparts in producing HIV-1 antigens. The trafficking route and functional role of breast milk lymphocytes in the recipient infant remain unclear. Nevertheless, these cells likely produce HIV-1 if they become activated in the mammary gland or later in the infant’s digestive track. Indeed, latently infected, resting CD4+ T cells in breast milk are probably an HIV sanctuary from which the virus can be released after activation. The pronounced differences between CD4+ T cells in the blood and the breast milk may arise from several nonmutually exclusive causes.

a) First, as suggested by the absence of correlation between HIV-1 Ag SCs in blood and breast milk, T cells in milk may be a different functional cell population from those in peripheral blood. Most breast milk CD4+ T cells exhibit markers of the MALT system, showing that they originate from, differentiate within, or migrate through mammary gland tissue, where they may acquire properties different from those of blood T cells.

b) Second, the HIV-1 quasi species in milk may differ from their counterparts in peripheral blood [63]. At least some breast milk HIV-1 in CD4+ T lymphocytes originates from maternal epithelial cells; this HIV-1 can invade local CD4+ T lymphocytes with more accurate proviral integration than can blood HIV-1; and it is likely to be better adapted to mucosal transmission than is blood HIV-1 [89]. Thus, breast milk HIV-1 is particularly prone to transmission to the infant.
c) Third, the cytokines IL-1β, IL-6, TNF-α, and TNF in human milk [90] may stimulate latently infected lymphocytes to produce HIV-1 virions.

d) Fourth, protein S100, present in high concentrations in breast milk [91], may induce HIV-1 transcription from latently infected human CD4+ T lymphocytes by up-regulating NFκB through a viral enhancer sequence that positively modulates HIV replication [92].

In blood, almost all of the HIV-1 RNA originates from functional, activated CD4+ T cells that are in a productively infected state. These cells are short-lived, with a half-life of only 24 to 36 hours, and in viraemic subjects, they spontaneously secrete HIV-1 antigens, as measured by enzyme-linked immunospot (ELISPOT), and can produce HIV-1 RNA in culture [20]. Even in ARV-treated individuals, these functional, activated CD4+ T cells can support residual viral replication that can infect new susceptible cells and perpetuate infection [93]. In women with successful responses to ART, undetectable HIV-1 RNA in plasma and breast milk has been interpreted to mean that breast milk HIV-1 is no longer being replenished by lymphoid tissue viral replication [80] and that HIV-1 replication has been suppressed in the mammary gland [94]. But this may not be the case. Although ART causes a marked decrease of HIV-1 RNA and to a lesser extent HIV-1 DNA in breast milk [95], cell-associated HIV-1 RNA is not, or is only moderately, affected [96] Fig 2 – adapted from [6].
Fig. 2. HIV-1 reservoirs in breast milk and blood.

[Fig 2 caption] (A) In HIV-1–infected, lactating women without treatment with ART, activated CD4 T cells in blood and in the mammary gland are in a productively infected state, and new target cells become infected through ongoing cycles of viral replication (arrows).

(B) In HIV-1–infected, lactating women who have been successfully treated with ART, protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), and non-nucleoside reverse transcriptase inhibitors (NNRTI) suppress the release of mature infectious forms of the virus (virions) and inhibit the ongoing cycles of replication in blood. However, these cells become activated through extravasation or transepithelial migration in the mammary gland. After activation, virus from stable reservoirs such as the latent reservoir in resting CD4 T cells is released in the breast milk where PIs are present in low concentration, but NNRTI and NRTI inhibit ongoing cycles of replication. Small yellow spheres, HIV-1 virions. (adapted from Van de Perre, 2012).
Indeed, CD4+ T cells spontaneously secreting HIV-1 antigen can be detected by ELISPOT in both breast milk and blood of all HIV-1–infected women, whether untreated or virally suppressed by ART [21]. More than half of these patients also show cell-associated HIV-1 RNA in blood and breast milk. Further, when these cells are cultured, HIV-1 RNA could be detected and quantified in the supernatant, and this harvested HIV-1 is infectious. Thus, cells that can secrete HIV-1 antigens are present in breast milk of ART treated and untreated women, and these cells may be responsible for a residual mother-to-child viral transmission in the virally suppressed patients on ART [9].

The fact that HIV antigen-producing T cells can be identified in samples that have no detectable HIV-1 RNA suggests that these cells may only release tiny amounts of HIV-1 RNA or that their residence time in breast milk is very short. But these cells may be involved in cell-to-cell transfer of the virus. Thus, HIV-1–secreting CD4+ T cells in breast milk, which can be detected in vitro by their HIV-1 antigen or HIV-1 RNA production, are the most plausible source of HIV-1 transmission by breast-feeding from women successfully treated with ARV regimens [97].

**Macrophages**

BM macrophages differ from their blood counterparts in that they have a higher phagocytic capacity and a more effective defense against pathogens [98]. More frequently activated, their motility is also higher. BM macrophages and dendritic cells probably facilitate antigen transport, cell signaling, and cell-to-cell antigen trafficking (including HIV-1 and CMV, other). HIV-1 infection does not kill macrophages but severely impairs their function. It is not fully established whether HIV-infected macrophages in milk contribute to HIV-1 replication and release of viral particles. The DC-SIGN surface receptor, frequently expressed on breast milk macrophages [98], may bind HIV-1 and aid its transport in breast milk, and expression of DC-SIGN on the mucosal surfaces of the breast-fed infant could also facilitate transmission. In colostrum and transition milk from HIV-1–infected women, 0.1 to 1% of macrophages are infected, and some can actively produce viral particles. These macrophages have a longer half-life than T lymphocytes, are resistant to apoptosis, and could contribute to transmission [99].
is likely that breast milk macrophages contribute minimally to HIV-1 replication in the mammary gland. Nevertheless, breast milk macrophages expressing DC-SIGN may augment HIV-1 transport in the infant mucosa and cell-to-cell infection of infant T lymphocytes. Indeed, HIV-1 may behave similarly to other viruses transmitted through breast milk. The lentiviruses maedi-visna virus (MVV) and the related caprine arthritis-encephalitis virus (CAEV) are transmitted to newborn lambs through colostrum and milk. The virus is excreted from highly productive germinal centers in the vicinity of the lactiferous ducts and is propagated in macrophages [100]. The human T cell leukemia virus type I (HTLV-I) is also transmissible by breastfeeding; in this model, macrophages are thought to play a central role in viral propagation since an infected breast milk macrophage cell line can efficiently transmit the virus to activated T lymphocytes in vitro [101].

Cell-free and cell-associated HIV-1 in MTCT
High concentrations of cell-free HIV-1 RNA in breast milk, although an imperfect reflection of infectiousness, are associated with postnatal HIV-1 transmission by breast-feeding. We have shown that infants infected with HIV-1 by breast-feeding have been exposed to 17 times more cell-free HIV-1 RNA in milk than age-adjusted exposed but uninfected controls [58]. It has been shown that each log$_{10}$ increase in breast milk cell-free HIV-1 RNA doubles postnatal transmission risk [102]. Postnatal transmission risk also increases during the rebound of virus concentrations in milk after ARV treatment is interrupted in the mother [97]. Nevertheless, two studies show that 15% of HIV-1–infected mothers who transmitted the virus to their offspring by breast-feeding had undetectable HIV-1 RNA in the breast milk samples collected before transmission occurred [5, 97], indicating that cell-free HIV-1 in breast milk is not the sole viral reservoir that contributes to transmission. Indeed, we [103], and others [104-106], have shown that both cell-free and cell-associated HIV-1 mediate transmission events.

For HTLV-I as well as for bovine leukemia virus and other animal retroviruses transmissible by breast milk, cell-to-cell transfer is considered the predominant mechanism of transmission from mother to infant. One milliliter of human mature breast milk from an HTLV-I–infected mother contains 1000 infected cells but very few virions [107]. HTLV-I infection can be experimentally
transmitted to susceptible animals by ingestion of breast milk from infected mothers [108]. Converging arguments suggest that similar mechanisms apply to HIV-1. Detection of HIV-1 proviral DNA in human breast milk indicates that infants are exposed to HIV-1–infected cells and, indeed, proviral DNA is associated with breast milk transmission of HIV-1 [103-105]. The proportion of HIV-1–infected cells in breast milk is strongly and independently (from cell-free viral load) associated with postnatal transmission; each log_{10} increase in number of infected cells per milliliter triples the risk of transmission [106]. Therefore, cell-associated HIV-1 in milk is at least as important as cell-free virus in transmitting HIV-1 to infants. In fact, transmission probably arises from multiple pathogenic pathways of varying importance during the lactation process and according to breast-feeding practices.

For example, in a study conducted in Botswana, the comparison of C2 to C5 env fragment sequences among cell-free HIV-1, cell-associated HIV-1 in breast milk, and the virus transmitted to the infants suggested that before infants are 9 months old, HIV-1 is mainly transmitted by cells containing HIV-1 provirus, whereas cell-free virus is frequently the culprit later on [105]. We showed similarly that there were higher median levels of cell-free than cell-associated HIV-1 virus (per ml) in breast milk at 6 weeks and 6 months. By multivariable analysis, adjusting for antenatal CD4 count and maternal plasma viral load, at 6 weeks, each 10-fold increase in cell-free or cell-associated levels (per ml) was significantly associated with HIV-1 transmission but stronger for cell-associated than cell-free levels [2.47 (95% CI 1.33–4.59) vs. aHR 1.52 (95% CI, 1.17–1.96), respectively]. At 6 months, cell-free and cell-associated levels (per ml) in breast milk remained significantly associated with HIV-1 transmission, but was stronger for cell-free than cell-associated levels [aHR 2.53 (95% CI 1.64–3.92) vs. 1.73 (95% CI 0.94–3.19), respectively]. The findings suggest that cell-associated virus level (per ml) is more important for early postpartum HIV-1 transmission (at 6 weeks) than cell-free virus [103]. Some babies breast-fed by HIV-1–infected women taking ART or ARV prophylactic treatment become infected despite undetectable levels of HIV-1 RNA in their mother’s plasma and breast milk [22, 24, 109]. A stable HIV-1 reservoir in breast milk within CD4+ T lymphocytes, which have a much higher propensity to enter the viral cycle after activation than do blood CD4+ T cells [87], and within infected macrophages of HIV-1–infected mothers with immune activation,
likely fuels cell-to-cell transmission. *In vitro* infectivity of HIV-1 is 100 to 1000 times higher from cell-associated virus than from cell-free virus stocks [110]. HIV-1–secreting cells [21] in breast milk have direct access to infants’ intestinal and respiratory mucosae, and active immune cells from breast milk can infiltrate the intestinal mucosae of the breast-fed infant - Fig 3.

![Fig. 3. Mechanisms of HIV-1 transfer from breast milk to the infant’s intestinal mucosae.](image)

**Fig. 3. Mechanisms of HIV-1 transfer from breast milk to the infant’s intestinal mucosae.**

*[Fig 3 caption]* Cell-free HIV-1 and infected cells producing viruses encounter GalCer+ CCR5+ CXCR4+ epithelial cells of the gut mucosal surface. In the upper small intestine, cell-free virus enters epithelial cells through endocytosis at the luminal surface in a GalCer/CCR5 receptor–mediated mechanism (center of illustration). HIV-1–infected cells may also bind to the epithelial cell and induce the polarized budding of newly formed viruses that are rapidly endocytosed via GalCer (left side of illustration). HIV viruses able to penetrate into the lamina propria infect CCR5+CXCR4+CD4+ T lymphocytes. The capacity of human M cells to translocate HIV-1 remains unclear (right side of illustration). (adapted from Bomsel, 2002)
Cell-associated viral particles can also penetrate to the submucosa of the infant gut through mucosal breaches or via transcytosis. Viral transcytosis occurs through a virological synapse scaffold and integrin- and agrin dependent molecular machinery in epithelial cells [111-114]. Filopodia and nanotubes may also facilitate cell transfer of HIV-1 [115, 116]. HIV-1 transmission can occur across polysynapses between one infected cell and multiple recipient cells [117]. These structures may facilitate exponential viral growth and sustain sufficient viral propagation to establish infection from a very small inoculum. Virological synapses and polysynapses also allow the virus to avoid host immune cells and the innate protective substances present in breast milk. Indeed, although soluble factors in milk can prevent cell-free HIV-1 propagation \textit{in vitro}, they cannot prevent cell-associated virus propagation [118].

An infant breast-fed by an HIV-1–infected woman ingests an average of 178 HIV-1–secreting cells per day during the first 4 months of life [21]. Because one cell with replicating HIV-1 produces at least 1000 viral particles [110], the infant’s daily exposure could be as high as 178,000 cell-associated viruses, with a high capacity for cell-to-cell transfer. It is therefore likely that cell-associated HIV-1 in breast milk transferred by mother-infant cell-to-cell contact contributes substantially to transmission of HIV-1 from breast milk to infant.
Research Papers Personal Contribution

The general objectives of my work was twofold:

1. To describe the exposure of infants born to HIV-1 infected mothers to cell-free and cell-associated virus in breast milk and its relationship to breast milk transmission of HIV-1.
2. To evaluate the impact of shedding and/or reactivation of infectious agents other than HIV-1 in breast milk and their impact on HIV-1 viral load and transmission.

My contribution to these three papers were conception and design of laboratory protocols, contribution to reagents/materials/analysis tools, implementation of the study and performing the laboratory analyses, contribution to statistical analyses and interpretation of data, writing of the manuscripts.
The Vertical Transmission Study
The Vertical Transmission Study (VTS) which formed the basis for the laboratory analysis on BM presented in this thesis, has shown that EBF reduces transmission risk in the first 6 months by approximately half [5]. The mechanism by which exclusive BF protects against transmission is unclear as mixed BF practice is not associated with an increase in BM HIV RNA [18]. The three papers of BM laboratory analysis presented here were all nested in the VTS, a large infant feeding intervention cohort among both HIV-infected and HIV-uninfected mothers in KwaZulu-Natal, South Africa, with enrollment between August 2001 and September 2004. Rates of MTCT of HIV-1 via breastfeeding and detailed description of the study design and methods are published [5]. Study participants were ART naïve except for single dose nevirapine (sdNVP) provided to all HIV-infected women and their newborns during delivery as per national guidelines at the time [19]. Mothers and infants attended clinics for monthly follow-up and collection of an infant dried blood spot (DBS) sample by heel prick for HIV-1 molecular testing, and a BM sample (10 ml) from each breast of the mother. BM samples were maintained at 4°C and transported overnight to the Africa Centre Virology Laboratory in Durban, where it was stored as whole BM at -80°C until further analysis. Infants were considered to have been infected postnatally if they had a negative HIV-1 RNA viral load result at 6 weeks of age and a positive result at any time thereafter.
**Article 1:**
Background
We quantified the relationship between HIV-1 RNA shedding in BM, cumulative RNA exposure, and postnatal transmission, relating timing of infection in the infant to estimated total volume of milk exposure. This was a nested case-control study of 36 infants of HIV-infected mothers. Case patients were infants who acquired HIV infection through breastfeeding from age 6 through 28 weeks, and control subjects were uninfected infants matched on age at obtainment of a breast milk sample. Feeding data were collected daily and infant anthropometry was performed at 6 weeks and monthly thereafter. Volume of milk ingested was estimated using infant weight and feeding pattern.

Results
Before HIV acquisition in case patients, feeding pattern (exclusive breastfeeding; median duration, 65 vs 70 days; P = .6) and daily milk intake (mean volume, 638 vs 637 mL; P = .97) did not differ significantly between case patients and control subjects. Controls were exclusively breastfed for longer than cases (median duration 183 vs. 157 days; p = 0.003), although overall duration of any breastfeeding was not significantly different between the two groups (p = 0.17). The median duration of EBF before HIV acquisition was 65 days for cases and 70 days for controls, with nonsignificant difference in median duration of any breastfeeding for both groups. The estimated milk volume ingested at age 6-28 weeks or before HIV acquisition did not differ significantly between cases and controls: mean daily intake of 638 ml/day (cases) versus 637 ml/day (controls) (p = 0.97). Case mothers were more likely to shed virus (64% vs 9% always, 22% vs 20.5% intermittently, 14% vs 70.5% never shed; overall, P = .001). Case patients ingested ~15 times more HIV-1 RNA particles than did control subjects (196.5 vs 13 3 10^6 copies; P = .001). Allowing for maternal antenatal CD4 cell count and plasma HIV-1 load, child sex and duration of mixed breastfeeding, the association between HIV RNA exposure and infection remained statistically significant (P = .001).
Discussion

We showed that infants who become postnatally infected at 6-28 weeks ingested significantly more cell-free HIV RNA particles, and that it is independent of maternal CD4 cell count and plasma viral load. Postnatal acquisition of HIV-1 is more strongly associated with cumulative exposure to cell-free particles in breast milk than with feeding mode. Because estimated volume of breast milk ingested did not differ significantly between cases and controls, the difference in exposure to virus was driven by increased HIV shedding in breastmilk from mothers of case patients. Samples obtained from both breasts allowed comparison and we confirm a strong correlation in breastmilk HIV RNA load between breasts, and although there was differential shedding profiles between breasts, there was seldom a predominant breast throughout lactation, suggesting that breast milk samples may be collected from either breast for studies investigating HIV shedding patterns in breastmilk. There was no significant variation in HIV RNA load in breastmilk slope between last negative and first positive PCR in infants, suggesting transmission is not explained by an abrupt increase of HIV RNA load, and favours cumulative exposure as important predictor of transmission.
Cumulative Exposure to Cell-Free HIV in Breast Milk, Rather Than Feeding Pattern per se, Identifies Postnatally Infected Infants

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Background. We quantified the relationship between human immunodeficiency virus (HIV) RNA shedding in breast milk, cumulative RNA exposure, and postnatal transmission, relating timing of infection in the infant to estimated total volume of milk exposure.

Methods. Nested case-control study of 36 infants of HIV-infected mothers. Case patients were infants who acquired HIV infection through breastfeeding from age 6 through 28 weeks, and control subjects were uninfected infants matched on age at obtainment of a breast milk sample. Mothers and infants received peripartum single-dose nevirapine prophylaxis. Feeding data were collected daily; breast milk samples were collected and infant anthropometry was performed at 6 weeks and monthly thereafter. Volume of milk ingested was estimated using infant weight and feeding pattern.

Results. Before HIV acquisition in case patients, feeding pattern (exclusive breastfeeding; median duration, 65 vs 70 days; \( P = .6 \)) and daily milk intake (mean volume, 638 vs 637 mL; \( P = .97 \)) did not differ significantly between case patients and control subjects. Case mothers were more likely to shed virus (64% vs 9% always, 22% vs 20.5% intermittently, 14% vs 70.5% never shed; overall, \( P < .001 \)). Case patients ingested \( 15 \) times more HIV-1 RNA particles than did control subjects (196.5 vs \( 13 \times 10^6 \) copies; \( P < .001 \)). Allowing for maternal antenatal CD4 cell count and plasma HIV-1 load, child sex and duration of mixed breastfeeding, the association between HIV RNA exposure and infection remained statistically significant (\( P < .001 \)).

Conclusions. Postnatal acquisition of HIV-1 is more strongly associated with cumulative exposure to cell-free particles in breast milk than with feeding mode. Reducing breast milk viral load through antiretroviral therapy to mother or child can further decrease postnatal transmission in exclusively breastfed infants.
through breast milk is .00064 per liter of breast milk ingested [6]. However, this estimate does not account for breastmilk viral load, the intermittent nature of virus RNA shedding in milk, and the intensity of breastfeeding [7]. Three major HIV reservoirs coexist in breast milk: RNA, which represents cell-free viral particles; proviral DNA as cell-associated virus integrated in latent T cells; and intracellular RNA representing cell-associated virus in activated producing T cells [8–13]. Their respective role in breast milk transmission of HIV-1 is poorly understood.

Because of the low estimated probability of transmission through breast milk per liter of milk ingested and the intermittent pattern of cell-free virus shedding in milk, postnatal HIV transmission through breastfeeding likely depends on the cumulative HIV exposure (ie, the overall amount of cell-free and cell-associated viral particles ingested by the infant during breastfeeding) and pattern or intensity of feeding [exclusive vs mixed or partial] and possibly by factors other than HIV. We aimed to quantify the relationship between cell-free HIV shedding in breast milk, cumulative cell-free HIV exposure, and postnatal acquisition of infection at age 6–28 weeks.

**METHODS**

We nested a case-control study in a large infant feeding intervention cohort (Vertical Transmission Study) of women attending 9 clinics (8 rural and 1 urban) in KwaZulu-Natal, South Africa, which aimed to examine breastfeeding and HIV transmission in a community with a high prevalence of HIV infection [2, 14]. Single-dose nevirapine was provided to all HIV-1–infected women and their infants peripartum; women were counselled antenatally on infant feeding options in accordance with policy recommendations at the time: commercial formula feeds or exclusive breastfeeding for the first 6 months of life. Women were supported in their feeding choice by lay-workers who visited breastfeeding mothers at home. Maternal socioeconomic level was defined by education level and household water type [2]. Venous samples were taken from women at enrollment and at 6 months after delivery, for plasma RNA load assessment and CD4 cell count. Daily infant feeding data were collected at weekly home visits. Infant weight was collected at birth. Breast milk samples and dried blood spot samples from infants were collected and anthropometry was performed at 6 weeks and monthly thereafter; an additional dried blood spot sample was taken from infants after delivery [14, 15].

Postnatal transmission was defined as HIV infection acquired at age 6–28 weeks. The estimated age at HIV-1 infection was taken as the midpoint between the last negative RNA polymerase chain reaction (PCR) result and the first positive RNA PCR result [2]. Case patients were postnatally infected infants [2, 14]; 42 infants received a diagnosis of HIV infection at age 49.5–197 days. Control subjects were HIV-uninfected infants matched for infant age at the time of obtainment of breast milk samples that was closest to a case patient’s age at first positive PCR result and last negative PCR result (in a 1:1 ratio).

Breast milk lactoserum, including the lipid fraction, was collected from stored (−80°C) whole breast milk samples. RNA was isolated from 500 µL of lactoserum with use of the magnetic particle-based ASPS method (Abbott), and HIV load was quantified using the HIV Charge Virale assay (Biocentric) on the MJ MiniOpticon quantitative PCR detection platform (Biorad), with a sensitivity of 375 copies per mL of lactoserum [16]. This method enabled accurate assessment of cell-free viral load that is preferentially entrapped by lipids [17]. Feeding categories followed World Health Organization definitions [2, 14, 15].

The Vertical Transmission Study and breast milk analyses were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.

**Statistical Methods**

Case-control pairs with information on feeding pattern, infant weight, and breast milk viral load were included in the present analysis. Duration of breastfeeding was estimated using the Kaplan-Meier method and was compared between case patients and control subjects with use of the log-rank test. Half the value of the threshold (375/2 copies/mL of lactoserum; 50/2 copies/mL of plasma) was assigned to samples with undetectable HIV RNA load for the purpose of logarithmic transformation. Viral shedding in breast milk was categorized as never, intermittent, and permanent shredders [18].

The volume of milk ingested per day was estimated according to Arcus-Arth [19] as (−.312*age + 157.7*weight) in exclusively breastfed infants, with a multiplicative correction factor when, in addition to breast milk, the infant was given water (1), formula (.7), solids (.9), or at least 2 other foods (.7) [20]. Monthly weight measurements were linearly interpolated to obtain daily weight. In 3 control subjects and 1 case patient, missing birth weights were replaced by the median birth weight observed in the overall cohort (3100 g) [2]. The probability of transmission per liter of breast milk ingested was computed using estimated milk volume ingested and estimated risk of postnatal transmission through breastfeeding in the Vertical Transmission Study cohort [2]. Daily HIV RNA exposure (ie, the amount of cell-free viral particles in the volume of milk ingested) was equal to the product of linearly interpolated milk HIV RNA load and daily milk intake. By assuming that there was no predominant breast, mean daily HIV RNA exposure between breasts could be estimated. Cumulative HIV RNA exposure was estimated as the sum of daily RNA exposure between the first breast milk sample at ~6 weeks and HIV acquisition, with left and right truncating to elicitation summation over the same period in each case-control pair. For each woman, the slope of HIV RNA load between the

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last negative HIV PCR result and the first positive test result was computed as the ratio of HIV RNA load variation over the time between the 2 PCR tests.

Associations between parameters with non-Gaussian distributions were assessed using the Spearman correlation coefficient. The Wilcoxon signed-rank test for paired data was used to compare HIV RNA exposure and RNA load between case patients and their matched control subjects and between right and left breasts. To estimate the risk of postnatal transmission associated with cumulative HIV exposure in breast milk, we built a conditional logistic regression model with use of the PHREG procedure in SAS, version 9.1 (SAS Institute). Variables shown to be associated with postnatal transmission on the basis of a P value <.2 in univariate analysis were included in the multivariable model, after verifying the absence of multicollinearity.

All analyses were performed using SAS version 9.1 (SAS Institute).

RESULTS

Of 42 case-control pairs, 6 were excluded from further analyses because only 1 breast milk sample was available (5 pairs) or feeding data were collected after the estimated age of HIV acquisition (1 pair). In the remaining 36 pairs, the median estimated infant age at HIV acquisition was 89.5 days (interquartile range [IQR], 66–128 days; range, 49.5–186.5 days), with a last negative PCR test result at a minimum of 39 days; the median duration between the last negative and the first positive PCR test results was 28 days (IQR, 28–43 days). Case patients were mostly male, with a nonsignificantly higher birth weight, significantly higher maternal socioeconomic status, significantly lower maternal antepartum and postpartum CD4 cell count, and higher antepartum plasma RNA load (Table 1). Breast health problems, particularly serious breast pathologies, were rare (2 mothers of case patients) [21].

### Table 1. Maternal and Infant Characteristics of HIV-1–Infected Infants and HIV-1–Uninfected Infants

<table>
<thead>
<tr>
<th>Maternal characteristic</th>
<th>HIV-1–infected infants</th>
<th>HIV-1–uninfected infants</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at delivery, years</td>
<td>36</td>
<td>25.1 (22–28.2)</td>
<td>.88</td>
</tr>
<tr>
<td>Enrolment clinic, no. (%)</td>
<td></td>
<td></td>
<td>.77</td>
</tr>
<tr>
<td>Urban</td>
<td>9 (25)</td>
<td>8 (22.2)</td>
<td></td>
</tr>
<tr>
<td>Semi-urban</td>
<td>13 (36.1)</td>
<td>11 (30.6)</td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>14 (38.9)</td>
<td>17 (47.2)</td>
<td></td>
</tr>
<tr>
<td>Highest level of education, no. (%)</td>
<td></td>
<td></td>
<td>.59</td>
</tr>
<tr>
<td>No education</td>
<td>2 (5.6)</td>
<td>3 (8.3)</td>
<td></td>
</tr>
<tr>
<td>Some primary</td>
<td>9 (25)</td>
<td>13 (36.1)</td>
<td></td>
</tr>
<tr>
<td>Secondary and tertiary</td>
<td>24 (66.7)</td>
<td>20 (55.6)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (2.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water type, no. (%)</td>
<td></td>
<td></td>
<td>.06</td>
</tr>
<tr>
<td>Borehole, tank, well</td>
<td>1 (2.8)</td>
<td>3 (8.3)</td>
<td></td>
</tr>
<tr>
<td>River, stream</td>
<td>8 (22.2)</td>
<td>14 (38.9)</td>
<td></td>
</tr>
<tr>
<td>Piped water</td>
<td>27 (75)</td>
<td>17 (47.2)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>2 (5.6)</td>
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<tr>
<td>Previous liveborns, no. (range)</td>
<td>35</td>
<td>0 (0–2)</td>
<td>.07</td>
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<td>Mode of delivery, no. (%)</td>
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<td>.71</td>
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<tr>
<td>Vaginal</td>
<td>31 (86)</td>
<td>33 (92)</td>
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<tr>
<td>Caesarean</td>
<td>5 (14)</td>
<td>3 (8)</td>
<td></td>
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<tr>
<td>Duration of rupture of membranes, h</td>
<td>24</td>
<td>0.25 (1.1–9.5)</td>
<td>.83</td>
</tr>
<tr>
<td>Antenatal CD4 cell count, cells/µL</td>
<td>34</td>
<td>369 (223–556)</td>
<td>.037</td>
</tr>
<tr>
<td>Antenatal plasma HIV-1 RNA load, log10 copies/µL</td>
<td>32</td>
<td>4.38 (4.02–4.90)</td>
<td>.02</td>
</tr>
<tr>
<td>CD4 cell count at 6 months postpartum, cells/µL</td>
<td>28</td>
<td>376 (224–666)</td>
<td>.01</td>
</tr>
<tr>
<td>Plasma HIV-1 RNA load at 6 months post-partum, log10 copies/µL</td>
<td>27</td>
<td>4.45 (3.88–4.92)</td>
<td>.095</td>
</tr>
</tbody>
</table>

**Infant characteristics**

| Sex male, no. (%)                               | 22 (61)                | 15 (42)                  | .098    |
| Birth weight, g                                 | 35                     | 3200 (2800–3500)         | .37     |
| Age at last HIV negative test result (day)      | 36                     | 68 (44–116)              |         |
| Age at first HIV positive test result (day)     | 36                     | 115 (88–154)             | Not applicable |

* median (inter-quartile) are reported for quantitative variables
Breastfeeding Pattern

Overall, during the first 28 weeks of life, data on the breastfeeding pattern was collected up to 200 days for control subjects and for a slightly shorter period for case patients (median, 200 days; IQR, 198–200 days). Control subjects were exclusively breastfed for longer than case patients (median duration, 183 vs. 157 days; $P = .003$), although the overall duration of any breastfeeding was not significantly different between the 2 groups ($P = .17$). Before the age at HIV acquisition in the case patients (and matched age in the control subjects), cumulative feeding patterns did not differ significantly: 20 case patients (69%) and 25 control subjects (76%) were exclusively breastfed from birth ($P = .55$). The median duration of exclusive breastfeeding before HIV acquisition was 65 days (IQR, 51–95 days) for case patients and 70 days (IQR, 53–107) for control subjects ($P = .6$), with again nonsignificant difference in median duration of any breastfeeding for both case patients and control subjects (90 days; IQR, 67–128 days).

Milk Intake and Risk of Postnatal Transmission through Breast Milk

The estimated milk volume ingested at age 6–28 weeks (Figure 1A) or before HIV acquisition (Figure 1B) did not differ significantly between case patients and control subjects, with a mean daily milk intake of 638 mL in case patients and 637 mL in control subjects ($P = .97$).

The estimated risk of postnatal transmission through breastfeeding in this study was previously estimated at .032 per 100 child-days (95% confidence interval [CI], .0222–.0455 per 100 child-days) [2], which translates to an estimated probability of .0005 (95% CI, .00035–.00071) per liter of breast milk ingested.

HIV Shedding in Breast Milk in the First 6 Months of Life

From 34 days through 28 weeks postpartum, there were a total of 318 samples from both breasts taken at the same visit; median number of breastmilk samples per woman was 5 in case mothers (range, 2–6) and 4 in control mothers (range, 3–5). The mean breast milk HIVRNA load over the first 28 weeks per mother was inversely correlated with maternal antepartum CD4 cell count ($p = -.47$; 95% CI, $-0.63$ to $-0.26$; $n = 69$) and positively with maternal plasma HIV RNA level before ($p = .46$; 95% CI, $0.24$–$0.63$; $n = 66$) or 6 months after delivery (Appendix Tables A1 and A2, Figures A1 A and B).

Undetectable HIV RNA in milk was quasi-uniformly distributed over time (Appendix Figures A2 A and B), and there was no statistically significant variation in viral load slope (mean difference, $-0.0001$; $n = 33$ in right breast; $-0.0000$; $n = 34$ in left breast).

Figure 1. A, Daily milk volume before 28 weeks of age in HIV-infected infants (case patients) and in uninfected infants (control subjects). B, Daily milk volume before HIV acquisition in HIV-1–infected infants (case patients) and in uninfected infants (control subjects).
breast). Comparing left and right breast, breast milk HIV-1 RNA load was always at least .3 log_{10} copies/mL higher in the left breast in 2 case mothers (3%), intermittently higher in 1 breast in 44 (61%) mothers (33 case patient and 11 control subjects), and always similar in both breasts (difference, < .3 log_{10} copies/mL) in 26 mothers (36%); 1 case patient and 25 control subjects).

Breast milk viral load did not vary statistically significantly between breasts (mean difference, −.04 log_{10} copies/mL; P = .29; n = 318), and loads in breasts per woman were strongly correlated (ρ = .61; 95% CI, .54–.68). Further analysis assumed that there was no predominant breast.

By 28 weeks postpartum, mothers of case patients were more likely to shed virus from either breast than were mothers of control subjects (44% vs 3% always, 53% vs 35% intermittently, and 3% vs 62% never shed; overall P < .001).

Accounting for episodes with detectable breast milk viral load, either for all or only for those before HIV acquisition in case patients, the mean HIV RNA load was significantly higher in breast milk of case mothers (Table 2).

**Cumulative Cell-Free HIV Exposure Through Breast Milk Before HIV Acquisition**

Cumulative HIV RNA exposure was estimated from a median age of 44.5 days (range, 38–68 days) for a median duration of 41 days (IQR, 22–72 days). Infants ingested a median estimated amount of 231,325 free HIV particles daily (IQR, 138,439–4,166,627 particles; case patients: median, 1,349,530; IQR, 341,400–4,328,963; control subjects: median, 142,118; IQR, 125,116–179,289). We estimated that case patients ingested ~15 times more cell-free HIV RNA particles than did control subjects (196.5 × 10^6 vs 13.0 × 10^6; P < .001). To investigate whether the association remained after allowing for maternal disease progression, we analyzed the 12 case-control pairs in which both members had a maternal postpartum CD4 cell count >350 cells/μL. In this comparison, infected infants were still estimated to have been exposed to significantly more cell-free HIV particles than control subjects (22.4 × 10^6 vs. 8.05 × 10^6; P < .001) before HIV acquisition; maternal antepartum CD4 cell count (median, 518 vs 510 cells/μL; P = .66), and maternal antepartum plasma HIV-1 RNA load (median, 3.98 vs 4.04 log_{10} copies/mL; P = .56) did not differ significantly between case patients and control subjects in these pairs. After adjustment for infant sex, maternal antepartum CD4 cell count, maternal antepartum plasma viral load, and duration of mixed breastfeeding, a 1 × 10^7 increase in HIV-1 RNA ingested particles was associated with a 2-fold increased risk of postnatal infection in the infant (adjusted odds ratio, 2.06; 95% CI, 1.02–4.16) (Table 3).

**DISCUSSION**

We estimated the number of HIV cell-free particles in breast milk ingested by an infant before acquiring infection and showed that infants who became postnatally infected at 6–28 weeks of age ingested significantly more cell-free viral particles from breast milk than did uninfected infants, independently of maternal HIV CD4 cell count and plasma viral load. Because the estimated volume of breast milk consumed did not significantly differ between case patients and control subjects, the difference in exposure of the virus particles was driven by increased HIV shedding in breast milk from mothers of case patients. Our estimated probability of breast milk transmission (.0005 per liter ingested) was of the same order of magnitude as a previous estimate reported from a Kenyan study (.00064 per liter ingested) [6].

Our study was a case-control study nested in a well-designed prospective cohort, with intensive infant feeding support and follow-up and high-quality longitudinal data [2, 14, 22]. Mothers and infants were given single-dose nevirapine prophylaxis at or shortly after delivery only. Most importantly, the daily collected breastfeeding information, monthly collected maternal and child clinical data, infant HIV status, and breast milk samples from both breasts allowed the estimation of quantity of virus shedding in the breast milk, volume of milk intake, HIV RNA exposure, and assessment of the association of these factors with postnatal transmission. In addition,

<table>
<thead>
<tr>
<th>Table 2. Median HIV RNA Load in Breast Milk in Episodes with Detectable HIV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case patients</strong></td>
</tr>
<tr>
<td>No of samples</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td><strong>Before HIV-1 acquisition</strong></td>
</tr>
<tr>
<td>Right breast</td>
</tr>
<tr>
<td>Left breast</td>
</tr>
<tr>
<td><strong>Before 28 weeks</strong></td>
</tr>
<tr>
<td>Right breast</td>
</tr>
<tr>
<td>Left breast</td>
</tr>
</tbody>
</table>

Abbreviations: IQR, interquartile range.
comparison between the 2 breasts and adjustment on confounding factors was also possible.

However, our study presents some limitations. We used the midpoint between the last negative and the first positive HIV PCR test results to estimate timing of acquisition of postnatal infection. This assumption may underestimate variance [23]; however, because the length of the interval (median, 28 days) was relatively short, compared with the 22-weeks duration of follow-up, the potential bias is unlikely to be substantial. Furthermore, we estimated daily milk intake using a formula based on infant weight and feeding pattern that was validated with healthy, full-term European or northern American infants [19]. We used correction factors based on a survey conducted in Brazil [20] to account for introduction of food other than breast milk. Although our cohort differs from these populations, our daily milk intake estimates are close to age-specific standards ([25]). The plateau in estimated volume of breast milk intake that we observed from 8 weeks postpartum is similar to that reported in previous studies in which investigators directly estimated milk volumes by weighing infants before and after each feed [25].

Finally, although it has been suggested that subclinical mastitis may be associated with higher breast milk viral load [9, 26], with the intensive breastfeeding counseling, episodes of clinical and subclinical mastitis were rare [21].

We confirm a strong correlation in breast milk HIVRNA load between breast and breast [27], and although we show differential shedding profiles between breasts, there seldom was a persistently predominant breast throughout lactation, suggesting that breast milk samples may be collected from either breast for studies investigating HIV shedding patterns in breast milk. We also confirm the correlation between breast milk and maternal plasma HIV RNA loads, with lower values in breast milk, and an inverse correlation with maternal antepartum CD4 cell count [10].

Intermittent HIV RNA shedding was common, supporting the need for frequent breast milk sampling to identify underlying mechanisms of shedding. Mothers of case patients were more likely to shed virus and at higher levels than were mothers of control subjects, which confirms breast milk HIV RNA load as a strong predictor of postnatal HIV-1 transmission [9]. There was no significant variation in HIV RNA load in breastmilk slope between the last negative and the first positive HIV PCR test result in case infants of mothers, which suggests that transmission is not explained by an abrupt increase of HIV RNA load and favors cumulative HIV exposure as important predictor of transmission.

Mechanisms of HIV breast milk transmission remain poorly understood. Because of the dynamic nature of the relationship between the source of HIV reservoirs (breast milk) and the potential target host (the maturing gastrointestinal tract of the young infant), multiple mechanisms are likely to be at stake. A remaining question relates to the nature of HIV reservoirs in milk involved in transmission, and the association of HIV RNA exposure per se with postnatal transmission does not necessarily prove causation between the 2 events. Breast milk cellular reservoirs are likely to play a major role in transmission [28]. Both B and T lymphocytes in breast milk harbor homing markers strongly suggesting migration from mucosal sites, particularly from the gut [11, 29]. Recent studies identified latently infected CD4 T cells [13] and spontaneously activated CD4 T cells [12] in breast milk as likely reservoirs involved in transmission. These 2 reservoirs are unaffected by maternal antiretroviral therapy [12, 13, 30] and are likely responsible for residual transmission from antiretroviral-treated lactating women. In the present study, one transmitting mother never shed HIV-1 in breast milk, despite multiple measurements, which confirms that at least some breast milk transmission is attributable to cell-associated HIV reservoirs [8, 10].

In conclusion, higher cumulative exposure to cell-free HIV RNA in breast milk is associated with higher rates of postnatal infection in the infant, independent of maternal CD4 cell count and plasma viral load; cumulative exposure is attributable to viral shedding in, rather than volume of, breast milk consumed. The contribution of exposure to cell-associated HIV remains to be determined, as do factors associated with compartmentalized shedding of HIV in breast milk.

Table 3. Risk of Postnatal HIV Infection Associated with Cumulative HIV RNA Exposure in Breast Milk Between 6 Weeks of Age and Estimated Age of HIV Infection

<table>
<thead>
<tr>
<th>Variable</th>
<th>Adjusted OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative HIV-1 RNA exposure in milk (for each additional 10^7 copies)</td>
<td>2.06</td>
<td>1.02–4.16</td>
<td>.04</td>
</tr>
<tr>
<td>Maternal antepartum CD4 cell count (for each additional 100 cells/μL)</td>
<td>1.20</td>
<td>0.80–1.81</td>
<td>.37</td>
</tr>
<tr>
<td>Maternal antepartum plasma HIV load (for each additional log10 copies/mL)</td>
<td>1.05</td>
<td>0.45–2.46</td>
<td>.92</td>
</tr>
<tr>
<td>Duration of mixed breastfeeding (for each additional week)</td>
<td>1.04</td>
<td>0.94–1.15</td>
<td>.43</td>
</tr>
<tr>
<td>Male infants compared to female infants</td>
<td>3.40</td>
<td>0.44–26.40</td>
<td>.24</td>
</tr>
</tbody>
</table>

NOTE. The estimated age at mother-to-child HIV-1 transmission was taken as the midpoint between the last negative RNA PCR and the first positive RNA PCR tests. Estimated by conditional logistic regression with adjustment on the other factors reported in the table. Abbreviations: CI, confidence interval; OR, odds ratio.
Supplementary Material

Supplementary materials are available at Clinical Infectious Diseases online (http://www.oxfordjournals.org/our_journals/cid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Acknowledgments

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Potential conflicts of interest. All authors: no conflicts.

References

Article 2:
**Background**

We quantify the relationship between cell-free (RNA) and cell-associated (DNA) shedding of HIV-1 virus in breastmilk and the risk of postnatal HIV-1 transmission in the first 6 months postpartum. Thirty-six HIV-positive mothers who transmitted HIV-1 by breastfeeding were matched to 36 non-transmitting HIV-1 infected control mothers. RNA and DNA were quantified in the same breastmilk sample taken at 6 weeks and 6 months. Cox regression analysis assessed the association between cell-free and cell-associated virus levels and risk of postnatal HIV-1 transmission.

**Results**

There were no significant difference between left and right breast for DNA or RNA.

There were higher median levels of cell-free than cell-associated HIV-1 virus (per ml) in breastmilk at 6 weeks and 6 months. Multivariably, adjusting for antenatal CD4 count and maternal plasma viral load, at 6 weeks, each 10-fold increase in cell-free or cell-associated levels (per ml) was significantly associated with HIV-1 transmission but stronger for cell-associated than cell-free levels (2.47 vs. 1.52, respectively). At 6 months, cell-free and cell-associated levels (per ml) in breastmilk remained significantly associated with HIV-1 transmission but was stronger for cellfree than cell-associated levels (2.53 vs. 1.73, respectively).

**Discussion**

We show that at 6 weeks, DNA was more strongly associated with postnatal HIV-1 transmission than RNA while at 6 months, RNA was more strongly associated than DNA; few studies have compared RNA and DNA levels and the risk of postnatal HIV-1 transmission in the same population in the early postpartum period. Our results suggest that breastmilk cell-associated levels decrease earlier than noted in previous studies (9 months). The findings suggest that cell-associated virus level (per ml) is more important for early postpartum HIV-1 transmission (at 6 weeks) than cell-free virus. Our study also confirms a positive correlation between breastmilk
HIV-1 RNA and DNA and maternal antenatal plasma viral load and a negative correlation with maternal antenatal CD4 cell count. As cell-associated virus levels have been consistently detected in breastmilk despite antiretroviral therapy, this highlights a potential challenge to achieve the goal of eliminating vertical transmission.
Cell-Free (RNA) and Cell-Associated (DNA) HIV-1 and Postnatal Transmission through Breastfeeding

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Abstract

Introduction: Transmission through breastfeeding remains important for mother-to-child transmission (MTCT) in resource-limited settings. We quantify the relationship between cell-free (RNA) and cell-associated (DNA) shedding of HIV-1 virus in breastmilk and the risk of postnatal HIV-1 transmission in the first 6 months postpartum.

Materials and Methods: Thirty-six HIV-positive mothers who transmitted HIV-1 by breastfeeding were matched to 36 non-transmitting HIV-1 infected mothers in a case-control study nested in a cohort of HIV-infected women. RNA and DNA were quantified in the same breastmilk sample taken at 6 weeks and 6 months. Cox regression analysis assessed the association between cell-free and cell-associated virus levels and risk of postnatal HIV-1 transmission.

Results: There were higher median levels of cell-free than cell-associated HIV-1 virus (per ml) in breastmilk at 6 weeks and 6 months. Multivariably, adjusting for antenatal CD4 count and maternal plasma viral load, at 6 weeks, each 10-fold increase in cell-free or cell-associated levels (per ml) was significantly associated with HIV-1 transmission but stronger for cell-associated than cell-free levels [2.47 (95% CI 1.33–4.59) vs. aHR 1.52 (95% CI 1.17–1.96), respectively]. At 6 months, cell-free and cell-associated levels (per ml) in breastmilk remained significantly associated with HIV-1 transmission but was stronger for cell-free than cell-associated levels [aHR 2.53 (95% CI 1.64–3.92) vs. 1.73 (95% CI 0.94–3.19), respectively].

Conclusions: The findings suggest that cell-associated virus level (per ml) is more important for early postpartum HIV-1 transmission (at 6 weeks) than cell-free virus. As cell-associated virus levels have been consistently detected in breastmilk despite antiretroviral therapy, this highlights a potential challenge for resource-limited settings to achieve the UNAIDS goal for 2015 of eliminating vertical transmission. More studies would further knowledge on mechanisms of HIV-1 transmission and help develop more effective drugs during lactation.

Introduction

Globally, in 2010, an estimated 2.7 million people became infected with human immunodeficiency virus (HIV); 1.9 million (70%) of new infections occurred in sub-Saharan Africa (SSA) [1]. An estimated 390,000 (340,000–450,000) new infections occurred in children, 90% of these in SSA, mainly through mother-to-child transmission (MTCT) [1]. MTCT can occur before, during and after delivery, with postnatal transmission through breastfeeding which accounts for one-third to one-half of MTCT remaining an unresolved issue [2]. With maternal antiretroviral therapy (ART), the risk of MTCT can be substantially reduced [3–5]. However, ART is not always available in resource-limited settings with high HIV prevalence, where breastfeeding is the norm for infant survival, and where the provision of ART to the mother or the infant for up to one year of breastfeeding as per the current WHO guidelines [6] poses a challenge. Therefore, postnatal transmission of HIV-1 through breastfeeding is likely to remain an issue for the foreseeable future in resource-limited settings.

Although factors associated with MTCT have been quantified [7–10], the mechanisms underlying postnatal transmission remain poorly understood, in particular the relative roles of cell-free (RNA) and cell-associated (DNA) HIV-1 in breastmilk transmission. High levels of cell-free virus in maternal plasma and breastmilk are associated with a high risk of HIV-1 transmission during breastfeeding [11–16]. Similarly, an association has been observed with cell-associated virus in breastmilk, suggesting both cell-free and cell-associated are involved in breastmilk HIV-1 transmission.
transmission [11,17,18]. We previously showed that cumulative exposure to RNA particles in breastmilk significantly increased the risk of HIV-1 acquisition postnatally independently from maternal antenatal CD4 cell count, plasma HIV-1 load, child sex and duration of breastfeeding [19]. Recent studies observe that while ART leads to undetectable levels of cell-free HIV-1 virus, cell-associated virus levels are still detected in breastmilk [20,21]. Additionally, there are suggestions that cell-free and cell-associated virus vary in their prediction of HIV-1 transmission at early and late lactation stages [18]. If studies confirm such variations in HIV-1 transmission, and cell-associated virus levels are barely affected by maternal ART, this could account for the residual HIV-1 transmission during lactation.

This study examines the prevalence of, and quantifies the relationship between, cell-free and cell-associated shedding of HIV-1 virus in breastmilk and the risk of postnatal HIV-1 transmission, in both right and left breasts over the first 6 months postpartum.

**Materials and Methods**

**Study Population**

HIV-infected and HIV-uninfected women were enrolled in an intervention cohort study, between August 2001 and September 2004 [22,23], to investigate whether breastfeeding in a high HIV prevalence, poor rural setting in South Africa could be made safe in terms of both HIV-1 transmission and infant morbidity and mortality. Weekly home visits documented infant feeding and morbidity while clinic follow-up of the infants and mothers were scheduled monthly between 6 weeks and 9 months. Ten milliliters of breastmilk were collected from each breast for HIV-infected and uninfected breastfeeding mothers at each scheduled clinic visit. Samples were transported and maintained at 4 degrees Celsius overnight and stored long-term as whole breastmilk at minus 80 degrees Celsius until testing.

A dried blood spot for each infant was collected at each visit and stored at minus 20 degrees Celsius. HIV-1 RNA quantification was performed using the Nuclisens HIV-1 QT assay (Organon Teknika, Boxtel, Netherlands) and Nucliens EasyQ HIV-1 assay (Bioradlab, Boxtel, Netherlands) with a sensitivity of 80 copies HIV-1 RNA per mL of blood (equivalent to 1600 copies HIV-1 RNA per 50 μL dried blood spot) [24]. Rates of MTCT of HIV-1 during breastfeeding have been described previously [23]. Children were considered infected through breastfeeding if they had a negative HIV polymerase chain reaction (PCR) assay at 6 weeks of age and a positive PCR at any time thereafter. Single-dose nevirapine (sdNVP) for use during labour/delivery was provided for all HIV-infected women and to their newborns; ART for treatment or as MTCT prophylaxis from early in pregnancy or during the postnatal period was not available in the public health setting at the time of this study. Maternal viral load and CD4 count were collected antenatally. The project was approved by the Study Design

A case-control study was nested in this intervention cohort [22]. The primary study identified 42 babies who had acquired HIV infection postnatally (as diagnosed by PCR conversion) [23]. Our study includes 36 postnatally infected children who had both cell-free and cell-associated data on samples at 6 weeks and 6 months, and who were matched to controls. Cases and controls were matched (in a 1:1 ratio) on infant age at breastmilk sampling with a maximum allowance of 2 weeks of the sample date of the case to reduce potential bias of varying concentrations of breastmilk RNA and DNA over time [25]. Cases were mothers who transmitted HIV-1 to their infants through breastmilk between 6 and 28 weeks postpartum while controls were non-transmitting HIV-1 infected mothers. Transmission was estimated to have occurred at the midpoint between an infant’s last HIV negative PCR test and first positive result. Infants were included if they had at least one cell-free and one cell-associated breastmilk sample available close to the estimated time of transmission [F11]. Breastmilk samples from both breasts, for postnatal transmitters and controls had DNA quantified twice (at 6 weeks and 6 months) and RNA at multiple time points before 6 months. Thirty-six transmitting mothers had 85 samples tested for HIV-1 RNA and DNA in both left and right breast; 36 control mothers had 81 samples. This study differs from the previous study which investigated the association between postnatal HIV acquisition at 6–28 weeks and cumulative cell-free HIV exposure (i.e. the overall amount of cell-free viral particles ingested by the infant during breastfeeding, up to infection or equivalent age of control) [19]. The volume of milk ingested per day was estimated by pattern of feeding and the probability of transmission estimated per liter of breastmilk ingested. However, that study did not access the influence of cell-associated virus integrated in latent T cells on postnatal transmission. In contrast, the current study presents the association between cell-free and cell-associated shedding of HIV-1 virus in breastmilk and postnatal HIV-1 transmission.

**Quantification of HIV-1 Cell-free and Cell-associated Virus**

Cell-free HIV-1 quantification on breastmilk samples was performed as described previously [19]. Cell-associated HIV-1 quantification on whole breastmilk samples was performed using the Generic HIV DNA Cell assay (Biocentric, Bandol, France). Breastmilk samples were thawed at room temperature and vortex mixed. A maximum of 1.5 mL (range 0.5–1.5 mL) of breastmilk was aliquoted into a 2 mL microtube, centrifuged at 2000 g for 15 min and the lactoserum-lipid layer was removed to a 1.5 mL microtube. The lactoserum-lipid fraction was stored at −80°C. The remaining breastmilk pellet was used in the HIV DNA real time PCR (qPCR) assay. RNA was isolated from 500 μL of lactoserum cell, use of the magnetic particle-based ASPS method (Abbott), and HIV load was quantified using the Generic HIV Charge Virale assay (Biocentric, Bandol, France) on the MJ MiniOpticon quantitative PCR detection platform (Biorad), with a sensitivity of 375 copies per mL of lactoserum [26]. This method enabled accurate assessment of cell-free viral load entrapped by lipids [27]. The Qiagen DNA Mini Kit was used to isolate total DNA from the dry breastmilk pellet according to the manufacturer’s instructions. Total DNA concentration was measured with the Nanodrop instrument using 1 μL of sample. Samples with a DNA concentration of <50 ng/μL were tested neat. For samples with a DNA concentration of >50 ng/μL an appropriate dilution of up to 1:10 was performed. The total reaction volume was 50 μL with a 20 μL sample input volume, according to manufacturer’s instructions.

The human GAPDH housekeeping gene (Primer_F : 5’-AAGGTCTGAGTCACGGATT-3’; Primer_R R: 5’-CTCCTGGAAGATGTTGATGG-3’) was quantified by real-time PCR using SybrGreen to verify the integrity of the extracted DNA, to determine the presence or absence of inhibitors/contaminants, and to act as a reference gene for quantitative analysis [28–30]. Quantifying the host gene GAPDH provided an estimate of the number of cells per PCR, allowing expression of the number of copies of HIV per 10^6 cells in our sample despite not having a cell count.
Statistical Analysis

The analyses included transmitters and controls with both cell-free and cell-associated results available from the same breastmilk sample at 6 weeks and 6 months. When the 6 months results were more than 4 weeks after transmission, the RNA result closest to the transmission was used (RNA was quantified at multiple time points) while the average between the two DNA results was calculated, otherwise the result at 6 months was used. Breastmilk HIV-1 RNA viral load levels below the lower detectable limit (375 copies/ml) were assigned a value at the midpoint between this and zero [31,32]. Breastmilk HIV-1 DNA samples below the lower detectable limit were normalized for the amount of cells used to isolate the DNA (based on the GAPDH measurement which is different for each cell) [33]. No breastmilk samples were excluded because of low cell counts as all samples had DNA values above zero. Cell-free and cell-associated virus levels were analyzed on a decimal logarithmic scale to base-10 [11,18]. Counts of DNA quantified per million cells were converted to concentrations of DNA per milliliter by multiplying by 0.08 x 10^3 at 6 weeks and 0.05 x 10^3 at 6 months breastmilk cells per milliliter, as suggested in previous studies [11,34].

Chi-square test assessed differences in categorical variables while Wilcoxon rank-sum test was used for non-parametric analysis of continuous variables. Spearman rank correlation estimated correlation between continuous variables. Cox regression models, pooling multiple measurements from the left and right breastmilk samples, assessed the association between breastmilk cell-free and cell-associated virus levels and risk of postnatal HIV-1 transmission. Observation time was taken from 6 weeks of age (last negative HIV PCR assay) to the estimated time postpartum (Figure 1). Overall, cell-free and cell-associated virus levels were similar between breasts at both time points in the first 6 months postpartum (Figure 3). Overall, cell-free virus per milliliter and cell-associated virus levels per million cells were significantly positively correlated (r = 0.34, p < 0.001); these correlation was maintained at 6 weeks (r = 0.32, p < 0.001) and at 6 months (r = 0.32, p < 0.001).

Breastmilk cell-free virus levels were positively correlated with antenatal maternal plasma viral load (overall RNA: r = 0.49, p < 0.001; at 6 weeks r = 0.46, p < 0.001 and at 6 months r = 0.47, p < 0.001) and negatively with maternal CD4 count (RNA: r = -0.44, p < 0.001; at 6 weeks r = -0.43, p < 0.001 and at 6 months r = -0.46, p < 0.001). Similarly, breastmilk cell-associated virus levels per milliliter were positively correlated with antenatal maternal plasma viral load (overall RNA: r = 0.30, p < 0.001; at 6 weeks r = 0.35, p < 0.001 and at 6 months r = 0.26, p < 0.001) and negatively with maternal CD4 count (RNA: r = -0.33, p < 0.001; at 6 weeks r = -0.37, p < 0.001 and at 6 months r = -0.29, p < 0.001). Log10 cell-free and cell-associated virus levels in breastmilk were significantly higher in mothers with antenatal CD4 count below 500 compared to those with at least 500 cells per mm3 (median: 3.2 vs. 2.7, p = 0.001 for cell-free and 3.7 vs. 3.3, p = 0.001 for cell-associated virus levels).

Correlation with HIV-1 Transmission

In univariate Cox analysis, each 10-fold increase in the average (between 6 weeks and 6 months) cell-free and cell-associated levels was associated with a significant 2- and a 4-fold increase in breastmilk transmission (HR 2.18 (95% confidence interval [CI] 1.66–2.87) and 4.18 (95% CI 2.24–7.79) respectively). Multi-variably, adjusting for antenatal CD4 count and maternal plasma viral load, each 10-fold increase in cell-free or cell-associated levels was associated with a significant 2- and a 4-fold increase in breastmilk transmission ([adjusted hazard ratio (aHR) 1.96 (95% confidence interval [CI] 1.17–1.96) and 2.57 (95% CI 1.26–5.28) respectively). A Cox regression model was fitted for the HIV-1 RNA and DNA breastmilk samples collected at both 6 weeks and 6 months to control for potential variation in levels over time [11]. The trend suggested that at 6 weeks, HIV-1 DNA levels in breastmilk were more important for HIV-1 transmission than RNA (aHR 1.98 vs. 1.16, both P > 0.05). Conversely, at 6 months, RNA levels were more important than DNA (aHR 1.99 vs. 1.21, both p > 0.05). However, statistical power was lost, possibly due to collinearity between the 6 weeks and 6 months HIV-1 RNA and DNA levels.
DNA results. Therefore, we stratified the analysis by timing of the sample at 6 weeks and 6 months.

In univariate analyses, at 6 weeks, each 10-fold increase in breastmilk cell-free or cell-associated levels per ml was associated with significantly increased hazard of postnatal HIV-1 transmission [HR 1.63 and 3.38, respectively] (Table 2). Multivariably, DNA was more important for HIV-1 transmission than RNA [aHR 2.47 vs. 1.52].

At 6 months, each 10-fold increase in breastmilk cell-free or cell-associated levels per ml was univariately associated with an almost 3-fold significantly increased hazard of postnatal HIV-1 transmission (Table 2). However, multivariably, the association was stronger for RNA than DNA levels [aHR 2.53 vs. 1.73].

Table 1. Baseline characteristics of Infants and HIV-positive mothers transmitting HIV-1 through breastmilk and their controls.

<table>
<thead>
<tr>
<th>Infant or Maternal characteristic</th>
<th>Cases (n = 36)</th>
<th>Controls (n = 36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median (IQR)</td>
<td>25.5 (22.2–28.2)</td>
<td>27.3 (21.1–29.9)</td>
<td>0.502</td>
</tr>
<tr>
<td>Antenatal CD4 count cells/LL, median (IQR)</td>
<td>337 (198–540)</td>
<td>524 (369–697)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antenatal viral load log_{10} copies/mL, median (IQR)</td>
<td>4.5 (4.2–5.0)</td>
<td>4.0 (3.5–4.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Water source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-piped</td>
<td>13 (36.1)</td>
<td>11 (30.6)</td>
<td>0.714</td>
</tr>
<tr>
<td>Piped</td>
<td>23 (63.9)</td>
<td>25 (69.4)</td>
<td></td>
</tr>
<tr>
<td>Enrollment clinic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>16 (44.4)</td>
<td>14 (38.9)</td>
<td>0.764</td>
</tr>
<tr>
<td>Peri-urban</td>
<td>11 (30.6)</td>
<td>12 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>9 (25.0)</td>
<td>10 (27.8)</td>
<td></td>
</tr>
<tr>
<td>Maternal education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2 (5.6)</td>
<td>5 (13.9)</td>
<td></td>
</tr>
<tr>
<td>Some primary</td>
<td>12 (33.3)</td>
<td>14 (38.9)</td>
<td></td>
</tr>
<tr>
<td>Secondary and above</td>
<td>22 (61.1)</td>
<td>17 (47.2)</td>
<td>0.226</td>
</tr>
<tr>
<td>Birth weight (grams), median (IQR)</td>
<td>3200 (2800–3400)</td>
<td>3100 (2800–3500)</td>
<td>0.572</td>
</tr>
<tr>
<td>Birth head circumference, median (IQR)</td>
<td>34.8 (33.5–36.4)</td>
<td>35.0 (33.6–36.0)</td>
<td>0.828</td>
</tr>
</tbody>
</table>

Chi-square test assessed differences in categorical variables while Wilcoxon rank-sum test was used for non-parametric analysis of continuous variables.

Abbreviations: Cases, HIV-1 infected postnatal transmitters; Controls, non-transmitting HIV-1 infected mothers.

doi:10.1371/journal.pone.0051493.t001
**Discussion**

We examined the prevalence of detectable RNA and DNA and levels of cell-free and cell-associated HIV-1 and associated risk of postnatal transmission at 6 weeks and 6 months in a case-control study nested in a cohort of HIV-infected mothers in KwaZulu-Natal. We showed higher median levels of cell-free than cell-associated HIV-1 virus per milliliter in breastmilk at 6 weeks and 6 months, with similar levels between breasts. Both cell-free and cell-associated virus levels in breastmilk were significantly associated with HIV-1 transmission, with a suggestion that cell-associated virus levels per milliliter may be more strongly associated with transmission than cell-free virus levels per milliliter at 6 weeks and less so at 6 months.

The prevalence of detectable HIV-1 cell-free virus in all breastmilk samples was comparable to that in previous African...
studies [12,18,31] as was that of cell-associated virus [35,36]. Among the HIV-1 transmitting mothers, the prevalence of cell-associated virus in our study was similar to a Ugandan study at 6 weeks postpartum (77% vs. 80%, respectively) [37]. Our study showed no statistically significant difference between breasts in the levels of HIV-1 cell-free and cell-associated virus, and confirms a strong correlation of HIV-1 cell-free and cell-associated virus in breastmilk [19,38]. This would suggest that breastmilk samples can be collected from either breast in future studies investigating HIV-1 shedding in breastfeeding. Our study also confirms a positive correlation between breastmilk HIV-1 RNA and DNA and maternal antenatal CD4 cell count [11,12].

Previous studies have suggested that cell-associated virus levels in breastmilk decline over time [35,36] whereas cell-free virus levels increase [39]. Overall, we show a marginally declining trend in both cell-free and cell-associated virus levels in breastmilk starting at 6 weeks postpartum [12,36], although mothers transmitting HIV-1 had significantly higher levels of cell-free and cell-associated virus over time, as seen elsewhere [11].

Breastmilk cell-free and cell-associated levels [per ml] were significantly associated with postnatal HIV-1 transmission both univariately and multivariately. The overall adjusted model showed a 2-fold increased risk of HIV-1 transmission through breastmilk with each 10-fold increase in RNA or DNA levels as previously reported [12,18,19]. This is in line with results from a study in Nairobi which reported a significant association between the infected breastmilk cells and the risk of HIV-1 transmission during or after delivery [11]. Unlike our study, almost two-thirds of the first breastmilk samples in that study were collected less than 60 days after delivery [11].

Table 2. Risk factors for HIV-1 transmission through breastfeeding.

<table>
<thead>
<tr>
<th>Variable</th>
<th>First sample taken at 6 weeks</th>
<th>Second sample close to ETT (or at 6 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariable analysis</td>
<td>Multivariable analysis</td>
</tr>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>RNA load (for each additional log10 copies/ml)</td>
<td>1.63 (1.33–1.98)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DNA load (for each additional log10 copies/ml)</td>
<td>3.38 (1.92–5.93)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antenatal CD4 count (for each additional 100 cell/μl)</td>
<td>0.91 (0.79–1.04)</td>
<td>0.173</td>
</tr>
<tr>
<td>Antenatal viral load (for each additional log10 copies/ml)</td>
<td>2.11 (1.30–3.43)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Estimated by Cox regression with adjustment on the other factors reported in the table. Abbreviations: HR, hazard ratio; CI, confidence interval; aHR, adjusted hazard ratio; ETT estimated time of transmission.

doi:10.1371/journal.pone.0051493.t002

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Table 2 shows results from two separate clinical trials comparing HIV-1 cell-free and cell-associated virus in breastfeeding [12,18]. Among the HIV-1 transmitting mothers, the prevalence of cell-associated virus in our study was similar to a Ugandan study at 6 weeks postpartum (77% vs. 80%, respectively) [37]. Our study showed no statistically significant difference between breasts in the levels of HIV-1 cell-free and cell-associated virus, and confirms a strong correlation of HIV-1 cell-free and cell-associated virus in breastmilk [19,38]. This would suggest that breastmilk samples can be collected from either breast in future studies investigating HIV-1 shedding in breastfeeding. Our study also confirms a positive correlation between breastmilk HIV-1 RNA and DNA and maternal antenatal CD4 cell count [11,12].

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The current prevention of mother-to-child transmission (PMTCT) guidelines in South Africa recommend zidovudine (AZT) from 14 weeks of pregnancy, sdNVP and 3-hourly AZT intrapartum, and a single dose of tenofovir and emtricitabine postpartum, for women not eligible for lifelong ART. Their infants receive daily NVP for 6 weeks and then for up to one year during breastfeeding. Women with CD4 below 350 are eligible for lifelong ART and their infants get 6 weeks daily NVP only [41]. However, during the study period, only sdNVP was available for HIV-infected women during labour/delivery and for their newborns immediately postnatally [42]. Previous studies suggest that sdNVP may reduce early postnatal HIV-1 transmission [43], as the drug has a long half-life and can be found in maternal plasma and breastmilk up to 3 weeks postpartum [44], and may reduce cell-free virus levels in the early postpartum period [45]. NVP also has a good penetration in anatomic compartments leading to reduced levels of HIV-1 plasma viral loads [46]. In our study, the estimated risk of HIV-1 transmission associated with RNA relate to samples taken at 6 weeks after perinatal sdNVP exposure, while the 6 months samples are in the absence of ART, which may partly explain the higher risk of transmission associated with RNA at 6 months. In our primary study, without ART, the HIV-1 transmission rate was 14.1% at 6 weeks and 19.5% at 6 months in exclusively breastfed infants [23]. In the recent clinical trials, where HIV-infected pregnant women took triple-ARV regimen from about 28 weeks in pregnancy (or after delivery) to 6 months postpartum, HIV transmission ranged from 3.3%–4.2% at 6 weeks and from 1.1%–8.2% at 6 months [47–51]. These findings suggest that giving breastfeeding women a triple-ARV regimen is safe and feasible to reduce MTCT in resource-limited settings.

However, there are suggestions that the effect of ART is different on cell-free and cell-associated virus in breastmilk [20]. Results from two separate clinical trials comparing HIV-1 cell-free and cell-associated virus in breastmilk suggest that triple-ARV regimen during pregnancy or after delivery suppressed cell-free but not cell-associated HIV-1 loads in breastmilk [33,52]. The undetectable HIV-1 RNA in both plasma and breastmilk has been interpreted as reflecting the cessation of viral replication within maternal lymphoid tissues [53] and in the mammary gland [54]. As cell-associated HIV-1 virus in breastmilk is associated with HIV-1 transmission through breastfeeding [11,18], their detection in breastmilk of untreated as well as those receiving antiretroviral therapy might be responsible for a residual breastmilk transmission with maternal ART.

Opportunistic infections such as congenital cytomegalovirus during pregnancy or delivery, mastitis and breast abscess have
been found to be associated with the risk of HIV transmission intra partum or postpartum [16,55]. However, in our study, serious breast health problems were rare and there were no significant differences between HIV-infected and uninfected women [56].

The strengths of our study include the large number of breastmilk samples in the first six months postpartum and the concurrent measurement of cell-free and cell-associated virus in the right and left breasts. These findings, from a study conducted before ART was available in public health programmes in South Africa, increase understanding of the mechanisms of postnatal transmission, important for optimizing delivery of interventions in the current period.

In summary, cell-associated virus load in breastmilk is a stronger predictor of the risk of early postnatal HIV-1 infection than cell-free virus loads, independent of antenatal CD4 cell count and plasma viral loads. In contrast, cell-free virus load is a stronger predictor of later postnatal HIV-1 transmission. In contemporary breastfeeding populations with access to antiretroviral prophylaxis and ART, the residual HIV-1 transmission risk especially in the breastfeeding populations with access to antiretroviral prophylaxis.

The main limitation of this study was the small sample size of breastmilk samples, and therefore less precise measures for the estimates. Furthermore, this study was not designed to provide a risk assessment of breast milk viral load of mother to child transmission.

Another limitation was the lack of available data on the specific ART regimens that mothers in this study were undergoing. However, all women in this study received PI-based ART, which is the standard of care in South Africa.

A further limitation was that mothers in this study were from a single hospital in the province of KwaZulu Natal. The results of this study, therefore, might not be generalizable to other populations or settings.

The study was not blinded, but efforts were made to minimize bias. As the study was observational, there was potential for confounding by other factors such as maternal health, lifestyle, and breastfeeding practices. The study was not powered to detect differences between groups, and therefore the results should be interpreted with caution.

Acknowledgments

We would like to thank the women and children who participated in the VTS; the VTS staff for their dedication to this project; Africa Centre laboratory staff and Colin Newell for assisting in data management. We acknowledge the other VTS investigators: Hoozen M Coovadia, Nigel C Rollins and Anna Coutouaki.

Author Contributions

Conceived and designed the experiments: MLN RMB PVP. Performed the experiments: JN JD. Analyzed the data: JN CT MLN. Contributed reagents/materials/analysis tools: JN JD. Wrote the paper: JN. Supervised day-to-day running of the study: RMB. Provided oversight in lab procedures and quantification: PVP RMB MLN. Provided oversight in statistical data analysis and interpretation: CT MLN.

References


Article 3:

Background
Co-infections in BM with CMV and EBV are associated with increased HIV-1 shedding in this compartment [119]. Compartmentalized CMV replication and early MTCT in the mammary gland are associated with CMV levels in BM and CD4 cell count [120]. In our study we investigate the relationship between CMV levels and EBV detection in BM and the risk of MTCT of HIV-1 via breastfeeding. Cell-free HIV-1 RNA, cell-associated HIV-1 DNA, CMV and EBV DNA were quantified in BM from 62 HIV-infected mothers with proven postnatal MTCT of HIV-1 via breastfeeding. Controls were 62 HIV-positive mothers with HIV-uninfected infants.

Results
BM HIV-1 RNA level was significantly higher in cases than controls. HIV-1 MTCT was significantly associated with BM HIV-1 RNA detection. HIV-1 DNA was detected significantly more frequently in cases than in controls. CMV DNA was detectable in most BM samples of cases and controls. Median (IQR) CMV DNA viral load was significantly higher in cases than controls. CMV viral load in BM was significantly associated with a 2.5-fold increased risk of postnatal HIV-1 MTCT. Increased CMV levels in BM were associated with increased HIV-1 RNA shedding in BM. In further analysis, HIV-1 MTCT remained independently associated with CMV level after adjustment for BM HIV-1 RNA detection and plasma HIV-1 RNA levels. Univariately, EBV detection in BM was associated with a three-fold increase in risk of HIV-1 MTCT. However, in multivariable analysis adjusting for BM HIV-1 RNA detection, EBV DNA detection was no longer significantly associated with postnatal transmission of HIV-1.

Discussion
We show that CMV viral load in BM was significantly associated with MTCT of HIV-1 via breastfeeding, with this risk independent of HIV-1 RNA shedding in this compartment. This is the first study to demonstrate an independent association between CMV DNA in BM and postnatal MTCT of HIV-1. We hypothesize that this association could fuel persistent shedding of HIV-1 in BM in women receiving ART.
These data imply that an impairment in T-cell response leads to herpes virus reactivation with compartmentalized HIV-1 replication. Thus a specific anti-CMV default in T-cell response may facilitate intra-mammary CMV replication, which in turn may lead to an increase in HIV-1 replication and shedding in BM. EBV is less frequently detected in BM with EBV-memory B-cells that are the reservoir of EBV. The proportion of B-cells in BM is lower than in blood; B-cell count is also much lower than T-cell count in BM. This could explain in part why BM levels of EBV DNA were found to be lower than CMV DNA levels. Targeting CMV and perhaps EBV replication in the mammary gland may be of interest as an adjuvant to ART prophylaxis of HIV-1 MTCT, and may have the additional effect of reducing CMV burden overall.
Cytomegalovirus, and possibly Epstein–Barr virus, shedding in breast milk is associated with HIV-1 transmission by breastfeeding

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Objective: Postnatal HIV-1 mother-to-child transmission (MTCT) occurs in spite of antiretroviral therapy. Co-infections in breast milk with cytomegalovirus (CMV) and Epstein–Barr virus (EBV) are associated with increased HIV-1 shedding in this compartment. We investigated CMV levels and EBV detection in breast milk as potential risk factors for MTCT of HIV-1 via breastfeeding.

Methods: Cell-free HIV-1 RNA, cell-associated HIV-1 DNA, CMV and EBV DNA were quantified in breast milk from 62 HIV-infected mothers and proven postnatal MTCT of HIV-1 via breastfeeding. Controls were 62 HIV-positive mothers with HIV-uninfected infants.

Results: Median (interquartile range) CMV DNA viral load was significantly higher in cases [88 044 (18 586–233 904)] than in controls [11 167 (3221–31 152)] copies/10^6 breast milk cells (P < 0.001). Breast milk CMV DNA level correlated positively with breast milk HIV-1 RNA level in cases and controls. EBV DNA was detectable in a higher proportion of breast milk samples of cases (37.1%) than controls (16.1%; P = 0.009). HIV-1 MTCT was strongly associated with HIV-1 RNA shedding in breast milk and plasma. In multivariable analysis, every 1 log_{10} increase in breast milk CMV DNA was associated with a significant 2.5-fold greater odds of MTCT of HIV-1, independent of breast milk and plasma HIV-1 levels; the nearly three-fold increased risk of HIV-1 MTCT with breast milk EBV DNA detection did not reach significance.

Conclusion: We provide the first evidence of an independent association between CMV in breast milk, and postnatal MTCT of HIV-1. This association could fuel persistent shedding of HIV-1 in women receiving antiretroviral therapy. EBV DNA detection in breast milk may also be associated with MTCT of HIV-1, but only marginally so.

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Keywords: breast milk, cytomegalovirus, Epstein–Barr virus, postnatal HIV-1 mother-to-child transmission

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Marie-Louise Newell and Philippe van de Perre contributed equally to the writing of this article.

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Introduction

In the absence of antiretroviral prophylaxis, breastfeeding accounts for up to half of mother-to-child transmission (MTCT) of HIV-1 [1]. An estimated 260,000 children acquired HIV infection in 2012, of whom more than 90% were living in sub-Saharan Africa [2]. However, breast milk provides essential nutrition and immunological protection against mucosal pathogens, and breastfeeding remains crucial for infant survival in resource-limited settings [3,4].

Apart from HIV-1, breast milk is also a vehicle for the transmission to the infant of other viruses infecting leukocytes, such as cytomegalovirus (CMV) and human T-cell lymphoma/leukemia virus (HTLV-1) [5–7]. Epstein–Barr virus (EBV), although frequently shed in breast milk, is not significantly transmitted from mother to child via this route [8,9]. MTCT of HIV-1 via breastfeeding has been associated with levels of cell-free and cell-associated virus in breast milk [10], plasma HIV-1 RNA and maternal CD4+ levels [11,12], duration and pattern of breastfeeding [13,14], and subclinical [15] or symptomatic mastitis [16]. However, since the vast majority of breastfed infants of HIV-1-infected mothers escape infection even without maternal or infant prophylaxis, other factors may drive HIV-1 transmission by breastfeeding [17]. Co-infection with CMV and EBV may be associated with increased risk of HIV-1 transmission through mechanisms enhancing reciprocal viral replication, as has been shown for herpes simplex virus type 2 [18].

Cytomegalovirus is commonly excreted in breast milk with rates of detection estimated to be 88–99% in two studies on mothers with preterm infants [19,20]. Breast milk is a main source of MTCT of CMV and is estimated to occur in 40–66% of breast-fed infants in early life [21,22]. In HIV-1-infected mothers, breast milk CMV level and maternal CD4+ cell count have been shown to be independently associated with early infant CMV acquisition [23]. In a cross-sectional study of HIV-1–infected breastfeeding women in Zimbabwe, breast milk CMV and EBV levels were independently associated with detection of breast milk HIV-1 RNA after adjustment for indicators of mastitis and plasma HIV-1 RNA concentration [24].

Systemic CMV reactivation may be accompanied by CMV antigenemia/viruria; however, local CMV reactivation (intra-mammary, colon, lung, genital) is usually asymptomatic and without simultaneous detection of markers of systemic infection measured in peripheral blood or urine [25]. In-vitro studies have shown that interactions between HIV-1 and CMV may be bidirectional, with CMV and HIV-1 enhancing each other’s replication [26]. Indirect interactions may involve CMV-mediated T-cell activation, facilitating HIV-1 replication, and coexist with direct facilitating mechanisms involving CMV proteins such as chemokine receptor homologue (US28) acting as a co-factor for HIV-1 cell entry [27].

Epstein–Barr virus shedding in breast milk has similarly been described, but less so than for CMV, with detection rates of around 45% [8,24]. In addition, breast milk does not appear to be a significant source of MTCT of EBV [9]. In-vitro studies have demonstrated that EBV enhances replication of HIV-1 in CD4+ lymphocytes [28], and that EBV and HIV-1 co-infection of T cells increases HIV-1 production by transactivation of the HIV-1 long terminal repeats by Epstein–Barr nuclear antigen 2 [29]. An increase of cell-associated EBV DNA levels in blood is observed in HIV-viremic patients compared to HIV-nonviremic and healthy controls [30].

To optimize the management of HIV-1-infected mothers, it is important to understand the pathogenesis of MTCT of HIV-1, and identify factors associated with postnatal MTCT of HIV-1. Here, we report the findings of a case-control study that investigated breast milk CMV levels and breast milk EBV detection as factors associated with MTCT of HIV-1 via breastfeeding.

Materials and methods

Study design

The case-control study was nested in a large infant feeding intervention cohort among both HIV-infected and HIV-uninfected mothers in KwaZulu-Natal, South Africa – the Vertical Transmission Study (VTS), with enrollment between August 2001 and September 2004. Rates of MTCT of HIV-1 via breastfeeding and detailed description of the study design and methods are published [14,31]. Study participants were antiretroviral therapy (ART)-naive except for single-dose nevirapine (sdNVP) provided to all HIV-infected women and their newborns during delivery as per national guidelines at the time. Mothers and infants attended clinics for monthly follow-up and collection of an infant dried blood spot (DBS) sample by heel prick for HIV-1 molecular testing, and a breast milk sample (10 ml) from each breast of the mother. Breast milk samples were maintained at 4°C and transported overnight to the Africa Centre Virology Laboratory in Durban, where it was stored as whole breast milk at −80°C until further analysis. Infants were considered to have been infected postnatally if they had a negative HIV-1 RNA viral load result at 6 weeks of age and a positive result at any time thereafter. Maternal plasma viral load (Nuclisens EasyQ HIV-1 assay; Biomérieux, Boxtel, the Netherlands) and CD4+ cell count (Epics XL; Beckman Coulter, California, USA) were obtained from samples taken at enrollment, 6 weeks or 26 weeks postdelivery. All women provided informed consent, and the VTS and breast milk analyses were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.
Study population

Cases were all mothers included in the VTS with established HIV-1 infection and proven postnatal transmission of HIV-1 to their infants via breastfeeding [14]. Sixty-two mothers with breast milk sample available for HIV-1 and CMV/EBV testing met the criteria of inclusion. The estimated timing of infant HIV-1 acquisition was taken as the midpoint between the last negative and the first positive HIV-1 RNA test result. The breast milk sample selected for testing was that obtained immediately prior to the estimated timing of infant HIV-1 acquisition. Controls were 62 HIV-positive mothers with HIV-uninfected infants matched for infant age at the time of breast milk sample collection in a 1:1 ratio and randomly selected when several samples were available at the same date. Timing of sampling in days following delivery in cases and controls were similar, with a nonstatistically significant median time difference between groups of ±4.0 days (interquartile range (IQR) –18 to 12) (P = 0.4). The median time between last undetectable and first detectable HIV-1 RNA test in cases was 145 days (IQR 82–313).

Quantification of HIV-1 cell-free and cell-associated virus in breast milk

Cell-free HIV-1 RNA quantification in breast milk samples was performed as previously described [10,13]. In brief, RNA was extracted from 500 µl breast milk lactoserum, including the lipid fraction, to maximize detection of virus entrapped by lipids, using the magnetic particle-based Abbott Sample Preparation System (ASPS) assay (Abbott Laboratories, Wiesbaden, Germany). HIV-1 viral load was quantified using the Generic HIV Charge Virale assay (Biocentric, Bandol, France) on the MJ MiniOpticon quantitative PCR detection platform (BioRad, Hercules, California, USA); the lower limit of detection was 375 HIV-1 copies per ml.

DNA was extracted from cell pellet obtained from 1.5 ml of whole breast milk using the QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA). Cell-associated HIV-1 DNA quantification was performed using the Generic HIV DNA Cell assay (Biocentric) as previously described [10], with a sample input normalized through a human glyceraldehyde 3-phosphate dehydrogenase gene quantification assay to compensate for cell lysis [32]. HIV-1 DNA results were expressed as copies per 10^6 equivalent breast milk cells with a lower limit of detection of 560 HIV-1 DNA copies/10^6 breast milk cells.

Quantification of cytomegalovirus and Epstein–Barr virus DNA in breast milk

Cytomegalovirus and EBV DNA viral load quantification was performed on pooled left and right breast milk cell pellets. Although maternal CMV and EBV serostatus was not determined for individual breast milk samples, previous investigation into our study population using antenatal plasma samples yielded very high CMV (99.5%) and EBV (97%) prevalence rates (unpublished data). We used commercial quantitative PCR (qPCR) assays for CMV [PrimerDesign GeneSig qPCR Kit for Human Herpes Virus 5 (Cytomegalovirus), Southampton, UK] and EBV [PrimerDesign GeneSig qPCR Kit for Human Herpes Virus 4 (Epstein Barr), Southampton, UK] quantification. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen), and qPCR was performed using the LightCycler 480 system (Roche, Basel, Switzerland). The input volume into the qPCR assays for both viruses were 5 µl of a 5-ng/µl product (manufacturer’s instructions), with a total input of 25 ng DNA per reaction corresponding to an equivalent of 3.8 × 10^5 breast milk cells. Results were expressed as copies CMV or EBV DNA per 10^6 breast milk cells, with a lower limit of detection of 528 DNA copies/10^6 breast milk cells.

Statistical analysis

Continuous data were assessed with Student’s t test and Wilcoxon Mann–Whitney test when their distributions were normal or non-normal, respectively; categorical variables were assessed with chi-square or Fisher’s exact test if the number was small.

The Wilcoxon signed-rank test for paired data with non-Gaussian distributions and the Student’s t test for paired data with Gaussian distributions were used to compare breast milk viral loads of HIV-1 RNA and DNA, and for CMV and EBV DNA between cases and controls. The McNemar’s test was used for comparison of EBV DNA levels between cases and controls. Statistical significance was set at a P value less than 0.05.

To estimate the adjusted risk of postnatal transmission associated with CMV and EBV, we built a conditional logistic regression model. Variables associated with postnatal transmission on the basis of a P value less than 0.20 in univariate analysis were included in the multivariable model, and we used a backward selection. All analyses were performed using SAS Enterprise Guide version 5.1 (SAS Institute, Cary, North Carolina, USA). We used the mean value obtained between left and right breasts for HIV-1 RNA and DNA as the final value in breast milk. All specimens with undetectable levels of breast milk CMV or EBV DNA, breast milk HIV-1 RNA and DNA, and plasma HIV-1 RNA were assigned a value equal to half the lower limit of detection.

Results

Study population characteristics

Maternal HIV-1 plasma viral load was significantly higher in cases than in controls, and there was a trend towards lower CD4^+ cell counts (Table 1). The two groups were not significantly different regarding maternal age, socioeconomic and demographic characteristics, mode of delivery or duration of rupture of membranes, and infant
Table 1. Baseline socio-demographic, clinical, and laboratory characteristics of infants and mothers transmitting HIV-1 through breast milk and their controls.*

<table>
<thead>
<tr>
<th>Maternal characteristics</th>
<th>n Cases (n = 62)</th>
<th>n Controls (n = 62)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median (IQR)</td>
<td>62</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>CD4⁺ cell count cells/μl, median (IQR)b</td>
<td>62</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Plasma HIV-1 RNA (log 10 copies/ml), median (IQR)b</td>
<td>54</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal (%)</td>
<td>54</td>
<td>56</td>
<td>0.57</td>
</tr>
<tr>
<td>Caesarean (%)</td>
<td>8</td>
<td>6</td>
<td>9.7%</td>
</tr>
<tr>
<td>Duration of rupture of membranes (h)</td>
<td>44</td>
<td>44</td>
<td>0.389</td>
</tr>
<tr>
<td>Water source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piped water (%)</td>
<td>41</td>
<td>41</td>
<td>0.736</td>
</tr>
<tr>
<td>Borehole, tank, well (%)</td>
<td>5</td>
<td>6</td>
<td>8.1%</td>
</tr>
<tr>
<td>River, stream (%)</td>
<td>16</td>
<td>14</td>
<td>22.6%</td>
</tr>
<tr>
<td>Other (%)</td>
<td>–</td>
<td>2</td>
<td>3.2%</td>
</tr>
<tr>
<td>Enrollment clinic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural (%)</td>
<td>25</td>
<td>34</td>
<td>0.270</td>
</tr>
<tr>
<td>Peri-urban (%)</td>
<td>24</td>
<td>18</td>
<td>29.0%</td>
</tr>
<tr>
<td>Urban (%)</td>
<td>13</td>
<td>10</td>
<td>16.1%</td>
</tr>
<tr>
<td>Maternal education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (%)</td>
<td>2</td>
<td>3</td>
<td>4.8%</td>
</tr>
<tr>
<td>Some primary (%)</td>
<td>18</td>
<td>23</td>
<td>37.1%</td>
</tr>
<tr>
<td>Secondary and above (%)</td>
<td>41</td>
<td>36</td>
<td>58.1%</td>
</tr>
<tr>
<td>Unknown (%)</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Infant characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight (g), median (IQR)</td>
<td>61</td>
<td>59</td>
<td>0.981</td>
</tr>
<tr>
<td>Birth head circumference, median (IQR)</td>
<td>61</td>
<td>60</td>
<td>0.515</td>
</tr>
<tr>
<td>Child sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>35</td>
<td>29</td>
<td>0.281</td>
</tr>
<tr>
<td>Female (%)</td>
<td>27</td>
<td>33</td>
<td>51.2%</td>
</tr>
</tbody>
</table>

IQR, interquartile range.

*Median (interquartile range) are reported for quantitative variables.

**Majority (n = 105) of samples were collected at 26 weeks postdelivery; n = 8 samples were collected at 6 weeks postdelivery (cases = 5 and controls = 3); and n = 11 samples were collected at enrollment (cases = 10 and controls = 1).

characteristics at birth. There were no reports of clinical mastitis at sample collection in any mothers.

HIV-1 RNA and DNA in breast milk and association with HIV-1 transmission

Breast milk HIV-1 RNA level was significantly higher in cases than in controls [median (IQR) 1405 (188–6606) and 188 (188–188) copies/ml, respectively; P < 0.001] (Fig. 1a). HIV-1 RNA was more frequently detectable in the breast milk of cases [40/62 (64.5%)] than in that of controls [10/60 (16.7%); P < 0.001]. Since HIV-1 RNA was frequently undetectable from controls, the detection rate was used for further analysis. HIV-1 MTCT was significantly associated with breast milk HIV-1 RNA detection (Table 2).

Sufficient breast milk sample was available for HIV-1 DNA measurement in 29 of 62 of cases and 40 of 62 of controls, yielding 23 case-control pairs. HIV-1 DNA was detected significantly more frequently in cases (24/29, 82.8%) than in controls (10/40, 25%; P < 0.001). The median (IQR) breast milk HIV-1 DNA level was significantly higher in cases [5002 (1993–14890)] than that in controls [280 (280–462) copies/10⁶ breast milk cells; P < 0.001] (Fig. 1b). CD4⁺ cell count was marginally associated with MTCT of HIV-1 (P = 0.068).

Detection and quantification of cytomegalovirus DNA and Epstein–Barr virus DNA in breast milk

Cytomegalovirus DNA was detectable in most breast milk samples of cases and controls (both 96.8%, 60 out of 62). Median (IQR) CMV DNA viral load was significantly higher in cases [88 044 (18 586–233 904)] than in controls [11 167 (3221–31 152) copies/10⁶ breast milk cells; P < 0.001] (Fig. 1c).

Epstein–Barr virus DNA was detectable in a higher proportion of breast milk samples of cases than controls [23/62 (37.1%) and 10/62 (16.1%), respectively; P = 0.009]. Levels of EBV DNA were higher in cases than in controls (P = 0.023) (Fig. 1d). Since EBV DNA was frequently undetectable in controls, breast milk detection rate was used for further analysis. Feeding mode (exclusive and mixed breastfeeding) was not associated with significant differences in CMV viral load or EBV detection in breast milk.

Impact of cytomegalovirus DNA viral load and Epstein–Barr virus DNA detection in breast milk and mother-to-child transmission of HIV-1

Cytomegalovirus viral load in breast milk was significantly associated with a 2.5-folds greater odds of postnatal HIV-1 MTCT. Increased CMV levels were associated...
with increased HIV-1 RNA shedding in breast milk. In further analysis, HIV-1 MTCT remained independently associated with CMV level after adjustment for breast milk HIV-1 RNA detection and plasma HIV-1 RNA levels.

Univariately, EBV detection in breast milk was also associated with HIV-1 MTCT. However, in multivariable analysis, adjusting for breast milk HIV-1 RNA detection, EBV DNA detection was no longer significantly associated with postnatal transmission of HIV-1 (Table 2).

Association between cytomegalovirus and Epstein–Barr virus DNA in breast milk with HIV-1 replication and CD4⁺ depletion
Breast milk CMV levels correlated positively with breast milk HIV-1 RNA in cases and controls (Fig. 2a and b).

A strong correlation was observed between CMV and HIV-1 DNA levels in breast milk for cases, but not for controls (Fig. 2c and d). Breast milk CMV levels correlated positively with plasma HIV-1 RNA levels in cases and controls (Fig. 3a and b). A significant inverse correlation was observed between breast milk CMV level

Table 2. Univariate and multivariable analysis of mother-to-child transmission of HIV-1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Crude ORa (95% CI)</th>
<th>P</th>
<th>Adjusted ORb (95% CI)</th>
<th>P</th>
<th>Adjusted ORc (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA EBV (detection)³</td>
<td>3.17 (1.27–7.93)</td>
<td>0.014</td>
<td>–</td>
<td>–</td>
<td>3.17 (1.27–7.93)</td>
<td>0.014</td>
</tr>
<tr>
<td>DNA CMV (log)</td>
<td>2.49 (1.50–4.12)</td>
<td>&lt;0.001</td>
<td>1.94 (1.08–3.40)</td>
<td>0.0263</td>
<td>2.52 (1.28–4.99)</td>
<td>0.008</td>
</tr>
<tr>
<td>Breast milk HIV RNA (detection)</td>
<td>10.67 (3.27–34.83)</td>
<td>&lt;0.001</td>
<td>7.84 (2.28–27.00)</td>
<td>0.0011</td>
<td>1.58 (0.91–2.74)</td>
<td>0.104</td>
</tr>
<tr>
<td>Plasma HIV (log10 RNA)</td>
<td>1.897 (1.20–3.00)</td>
<td>0.0057</td>
<td>–</td>
<td>–</td>
<td>1.58 (0.91–2.74)</td>
<td>0.104</td>
</tr>
<tr>
<td>CD4⁺ T cells (cells/µl)</td>
<td>0.270 (0.07–1.10)</td>
<td>0.0678</td>
<td>–</td>
<td>–</td>
<td>0.270 (0.07–1.10)</td>
<td>0.0678</td>
</tr>
</tbody>
</table>

BM, breast milk; CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein–Barr virus; MTCT, mother-to-child transmission; OR, odds ratio.

aCrude odds ratio using univariate analysis.
bAdjusted odds ratio model 1: Analysis of HIV-1 MTCT according to BM CMV DNA levels taking into account BM HIV-1 RNA shedding.
cAdjusted odds ratio model 2: Analysis of HIV-1 MTCT according to BM CMV DNA levels taking into account plasma HIV-1 RNA level.
dThis variable was removed because it caused a loss of model quality.
Fig. 2. Correlation between BM CMV DNA and BM HIV RNA in (a) cases and (b) controls. Correlation between BM CMV DNA and BM HIV DNA in (c) cases and (d) controls. BM, breast milk; CMV, cytomegalovirus.

Fig. 3. Correlation between BM CMV DNA and plasma HIV RNA in (a) cases and (b) controls. Correlation between BM CMV DNA and blood CD4⁺ T-cell count in (c) cases and (d) controls. BM, breast milk; CMV, cytomegalovirus.
and maternal CD4+ cell count for cases, but not for controls (Fig. 3c and d).

No clear association was observed between EBV and HIV-1 RNA and DNA shedding in breast milk. Similarly, there was no association found between EBV DNA shedding in breast milk and CD4+ cell count (data not shown).

**Discussion**

We show that CMV viral load in breast milk was significantly associated with MTCT of HIV-1 via breastfeeding, independently of HIV-1 RNA shedding in this compartment. EBV detection in breast milk may also be associated with MTCT of HIV-1, but only marginally so. This is the first study to demonstrate an independent association between CMV DNA in breast milk and postnatal MTCT of HIV-1. Similar to previous studies, CMV DNA was detectable in the majority of breast milk samples studied from this population of untreated HIV-1-infected mothers, with measured CMV levels significantly higher in breast milk than levels previously reported in blood. Recently, Gianella et al. [33] reported median (IQR) values of 1.73 (0.12–4.54) log_{10} CMV DNA copies/10^6 peripheral blood mononuclear cells, compared to median (IQR) values of 4.4 (3.8–5.2) log_{10} CMV DNA copies/10^6 breast milk cells, and Slyker et al. [23] described median (IQR) values of 1.8 (1.8–2.0) log_{10} CMV copies/ml in plasma compared to 5.5 (5.0–6.4) log_{10} CMV copies/ml in breast milk. In contrast to HIV-1, which we previously found to be 1–2 log_{10} lower in breast milk compared to blood [13], CMV DNA level appears to be significantly higher in BM than blood, suggesting efficient compartmentalized CMV replication in the mammary gland. CMV DNA may be cell-associated in a variety of infected breast milk cells, including mammary epithelial cells, monocytes and T cells that traffic into breast milk [4,34].

Epstein–Barr virus is less frequently detected in breast milk [24]. EBV-memory B cells are the reservoir of EBV. The proportion of B cells in breast milk is lower than that in the blood; B-cell count is also much lower than T-cell count in breast milk. This could explain in part why breast milk levels of EBV DNA were found to be lower than that of CMV DNA levels.

HIV-1 DNA and RNA levels in breast milk appeared strongly predictive of postnatal HIV-1 MTCT. We further observed a greater than two-folds greater odds of HIV-1 MTCT with every 1 log_{10} increase in breast milk CMV DNA, independently of breast milk HIV-1 RNA level. It has been shown that a short-course prophylaxis of HIV-1 MTCT using NVP strongly impact on plasma and breast milk HIV-1 viral load 1 week after delivery with rebound levels peaking at 4 weeks, significant for plasma but not in breast milk, to prior levels [35]. Hence, at the time of breast milk sample collection, NVP traces were most likely to have disappeared with replication levels in breast milk rebounding to levels comparable to pre-entry in this study.

HIV-1 DNA, reflecting cell-associated HIV-1, was not included in the MTCT model since this parameter was only available for some breast milk samples. However, a strong association was observed for cases when CMV DNA was compared to HIV-1 DNA. High CMV and HIV-1 DNA levels in breast milk may be associated with an influx of leukocytes due to local or systemic inflammatory processes [36], and consequently of both CMV and HIV-1-infected cells in breast milk. A second link between the two viruses may involve immune activation since high levels of CMV shedding in breast milk may target T-cell activation and facilitate reactivation of CD4+ cells latently infected by HIV-1 and located in the mammary gland. Thus, CMV shedding in breast milk may be one factor driving the high level of immune activation observed in breast milk cells [37], coupled with the high capacity of breast milk HIV-1-infected CD4+ cells to produce HIV particles [38]. Impairment of the immune response against CMV may be a third mechanism involved in the poor control of both CMV and HIV-1 shedding in the mammary compartment. Hence, our data have shown that CMV DNA level in breast milk was associated with CD4+ depletion in cases. We did not observe the same association in the control group, which may suggest that T-cell responses were somewhat more preserved in nontransmitters. Consistent with this observation, MTCT of CMV was recently shown to be strongly associated with levels of CMV DNA in breast milk and CD4+ depletion in HIV-infected women [23]. Similarly, a decrease in T-cell population due to HIV-1 infection has been associated with the loss of control of herpes simplex type 2 replication in the female genital compartment [39]. These data imply that an impairment in T-cell response leads to herpes virus reactivation with compartmentalized HIV-1 replication. Thus, a specific anti-CMV default in T-cell response may facilitate intra-mammary CMV replication, which, in turn, may lead to an increase in HIV-1 replication and shedding in breast milk.

The study had several limitations. Firstly, due to the observational character, it is difficult to infer causality of the reported associations as CMV and EBV viral load might be a consequence of increased immunosuppression in transmitters. Secondly, since CMV and EBV shedding is mostly intermittent, interventional clinical trials are needed to describe longitudinal patterns of intermittent versus continuous shedding, and its effect on MTCT of HIV-1. Thirdly, we did not investigate the potential confounding influence in cases with high breast milk CMV shedding associated with the different immune milieu as found in subclinical mastitis.
The age of the infant was chosen as the only matching criterion for controls because age is associated with dramatic changes in breast milk composition over time. Since breast milk HIV-1 viral load and CD4⁺ T-cell count were important risk factors for HIV-1 transmission, we preferred not to use them as matching criteria. Further studies on breast milk in women on ART, using alternative ratios for case-control numbers, and matching criteria are needed to confirm the findings of this study.

The demonstrated association between CMV and EBV DNA and HIV-1 levels in this study may explain persistent shedding of HIV-1 in breast milk. Cell-associated levels of HIV-1 RNA and proviral DNA in breast milk have been shown to be refractory to treatment with ART [38]. We hypothesize that compartmentalized CMV and EBV replication may contribute to residual HIV-1 MTCT via breastfeeding for women receiving ART, as previously described [40].

Targeting CMV and perhaps EBV replication in the mammary glands may be of interest as an adjuvant to ART prophylaxis of HIV-1 MTCT, and may have the additional effect of reducing CMV burden overall. Recent data, as to the role of CMV in breast milk cells and risk of mother-to-child transmission of HIV-1 transmission: association with maternal plasma HIV-1 RNA load, genital HIV-1 DNA shedding, and breast infections. J Infect Dis 2001; 183:206–212.

Acknowledgements

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Author contributions: J.V., E.T., R.B., N.R., M.L.N. and P.V. designed and supervised the study. J.V., E.T., S.D., PP., and V.F. implemented the study and performed the laboratory analyses. Data management was organized by N.N., M.P., M.L.N. and P.V., who also provided input into statistical analyses. The manuscript was written by J.V., E.T., M.L.N. and P.V. All authors reviewed and approved the final version of the manuscript.

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Conflicts of interest

We declare that we have no conflicts of interest.

References


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Cytomegalovirus, Epstein–Barr virus and postnatal HIV-1 transmission Viljoen et al.


38. Valea D, Tuailion E, Al Tabaa Y, Rouet F, Rubbo PA, Meda N, et al. CD4+ T cells spontaneously producing human immunodeficiency virus type 1 in breast milk from women with or without antiretroviral drugs. Retrovirology 2011; 8:34.


GENERAL DISCUSSION

Although HIV-1 can enter breast milk by transudation from the vascular compartment, HIV-1 can also replicate in mammary gland tissues and breast milk. Transmission of HIV-1 by breast-feeding is the result of multiple factors: the nature and size of the viral reservoir, host susceptibility, and the complex interplay of numerous breast milk factors that may be anti-infectious, immunomodulatory, and anti- or proinflammatory. Although cell-free HIV-1 particles can mediate HIV-1 transmission from breast milk to infant, especially late in lactation [103, 105], cell-associated HIV-1, either latently infected or activated virus-producing T cells, is predominantly responsible for breast milk–mediated HIV transmission. Compared with those in blood, breast milk B and T cells are activated more frequently and express higher levels of memory and mucosal homing markers. Activation of latently infected immune cells favors HIV-1 replication and release of viruses from these persistent, stable reservoirs in the mammary gland. It is likely that cell-to-cell transfer of viruses from this cell-associated HIV-1 reservoir to cells in the infant is a key element during mother-to-child transmission. This mechanism can explain the residual risk of HIV transmission to infants by mothers taking combined ARV therapies with no or minimal HIV-1 RNA in their body fluids. Indeed, the equation “no detectable HIV-1 RNA equals no transmission,” which correctly applies to sexual transmission [121, 122] and perinatal transmission of HIV-1 [123], does not apply to breast-feeding transmission. The residual HIV-1 cell-associated reservoir in breast milk, which is not eliminated by maternal ART—in conjunction with the vulnerability of the infant’s gut mucosal barrier—are consistent with this mechanism of maternal-to-infant HIV transmission.

It is therefore unlikely that mother-to-child transmission of HIV-1 can be eliminated by maternal ART alone [124]. In contrast, infant pre-exposure prophylaxis, administered during the entire duration of breast-feeding, is more likely to protect exposed babies against all possible routes of breast milk transmission, including cell-to-cell viral transfer. To achieve optimal adherence during infant pre-exposure prophylaxis, long-acting drugs that can be more practically given to infants and that have a good safety profile are urgently needed.
Bottlenecks to pMTCT of HIV-1 via breastfeeding

(i) Incomplete understanding of the role of activated T cells

Although it is clear that both T cells latently infected with HIV-1 and activated HIV-producing T cells persist in breast milk and contribute to transmission of HIV to breast-feeding infants, we do not understand the respective roles of these two reservoirs. This is important to clarify because maternal ART only minimally reduces HIV in these cells. Consequently, because these reservoirs contribute to HIV transmission, approaches other than maternal ART should be considered to eliminate this source of paediatric infection. For example, if immune activation facilitates HIV-1 transmission, strategies such as prevention of inflammation and subclinical mastitis in the breast, both causes of immune cell activation, could prove useful. To test whether activated CD4+ T cells from breast milk contribute to HIV-1 transmission, these cells should be enumerated in breast milk samples from transmitting and nontransmitting mothers. Such studies are ongoing on limited numbers of frozen samples, but conclusive findings may require fresh cells and rigorous freezing procedures. Identifying a proxy of cell activation by measuring the activation-prone environment in breast milk and soluble factors could well prove more informative.

(ii) Incomplete understanding of the role of immune factors

Innate, anti-infectious factors such as lactoferrin, lactadherin, mucins, and anti-secretory lectins, may prevent bacterial adherence to the gut epithelial surface and therefore protect against alteration of the vulnerable newborn’s gut mucosal barrier. Because bacterial translocation and consequent immune activation may boost HIV-1 replication in CD4+ T cells and maybe macrophages, it is important to know whether breast-fed HIV-1–infected infants
have a slower disease progression or a better ART response than infants deprived of their mother’s milk. Other factors, such as SLPI, lysozyme, or lactoferrin, are under scrutiny in studies comparing breast milk composition in transmitting and non-transmitting mothers. If proven protective, these factors could be included in an intervention package aimed at defending the infant’s mucosae against HIV-1.

Finally, we need to determine whether the humoral immune response, mainly sIgA and sIgM, or the local T cell response protects against HIV-1 transmission; positive findings would indicate that maternal immunization eliciting such responses may prove beneficial in preventing breast milk–mediated transmission.

(iii) Unclear efficacy of prophylaxis
In HIV-1–infected mothers not eligible for ART, triple combination ART administered during lactation reduces transmission by only 50 to 60%. This poor response will hamper considerably efforts to achieve the WHO objective of reducing mother-to-child transmission of HIV-1 worldwide to ~10% of present levels. Estimates of residual risk of transmission has been provided by meta-analyses and mathematical modelling and are provided in the table 2 below—adapted from [9]. For example, the residual transmission by breastfeeding from a mother on ART is estimated to be 0.2% per month by breastfeeding.

The prophylactic efficacy of the WHO-recommended option B (maternal triple prophylaxis) has been assessed by the Kesho Bora trial [125]. In this randomized trial, prophylactic ARV therapy with three drugs during pregnancy and breast-feeding for a maximum of 6 months was compared to a short perinatal AZT/single-dose NVP prophylaxis to prevent mother-to-child transmission of HIV-1. In infants whose mothers declared they intended to breast-feed, the cumulative rate of HIV-1 transmission at 12 months was 5.6% in the triple ARV group and 10.7% in the AZT/single-dose NVP group, corresponding to an intervention efficacy of 52%. This lower than expected efficacy of the triple combination prophylaxis could be a result of suboptimal maternal adherence, breast milk exposure after maternal prophylaxis had been stopped, or transmission via cell-associated viruses not suppressed by maternal prophylaxis.
### Postnatal transmission per month of any breastfeeding* (except incident infection)

<table>
<thead>
<tr>
<th>Regimen</th>
<th>CD4&lt;350</th>
<th>CD4&gt;350</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incident infections (range of reported transmission probabilities)</td>
<td>28% (14.3%-56%)</td>
<td></td>
</tr>
<tr>
<td>No prophylaxis (range of reported transmission probabilities)</td>
<td>1.57%/mBF</td>
<td>0.51%/mBF</td>
</tr>
<tr>
<td>sdNVP (range of reported transmission probabilities)</td>
<td>1.57%/mBF</td>
<td>0.51%/mBF</td>
</tr>
<tr>
<td>WHO 2006 dual prophylaxis (range of reported transmission probabilities)</td>
<td>1.57%/mBF</td>
<td>0.51%/mBF</td>
</tr>
<tr>
<td>Option A+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Option B§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ART (range of reported transmission probabilities)</td>
<td>0.2%/mBF</td>
<td></td>
</tr>
<tr>
<td>ART (before pregnancy)</td>
<td>0.16%/mBF</td>
<td></td>
</tr>
</tbody>
</table>

Shading indicates transmission probabilities that are not estimated for a particular regimen either because the regimen is not recommended for women with a particular CD4 count, for example, Option A or B for women with CD4 counts less than 350 cells/ml, or because transmission data were not available for a regimen by CD4 count, for example, sdNVP in women with CD4 350–500 cells/ml.

*For the transmission probabilities associated with breastfeeding the values are given to two decimal places since rounding these values up or down would result in significantly greater or lesser transmission rates when multiplied according to the duration of breastfeeding.

+A in Option A, HIV-positive pregnant women who are eligible for lifelong ART should be started on treatment in the first trimester of pregnancy. HIV-positive pregnant women who are not eligible for ART should receive daily AZT from 14 weeks gestation until delivery, single dose nevirapine during labour and AZT+3TC during labour and for 7 days post partum. HIV-exposed infants would receive AZT or NVP until 6 weeks of age and if breastfeeding then NVP would continue until 1 week after all breast feeding has stopped.

§In Option B, HIV-positive pregnant women who are eligible for lifelong ART should be started on treatment in the first trimester of pregnancy. HIV-positive pregnant women who are not eligible for lifelong ART should receive one of
four combinations of ARVs during pregnancy throughout the breastfeeding period and 1 week after. Exposed infants would receive either AZT or 3TC, lamivudine; ART, antiretroviral therapy; ARV, antiretroviral drug; AZT, zidovudine; BF, breastfeeding; sdNVP, single dose nevirapine.

Table 2.

Summary of breast milk transmission probabilities by antiretroviral regimen and maternal CD4 count (adapted from Rollins 2012)

An unexpected adverse effect of the “option B” maternal triple prophylaxis is development of a high rate of resistance to multiclass ARV drugs in babies that become infected despite maternal prophylaxis [126]. In a study from Uganda in which mothers initiated ART immediately after delivery while breast-feeding, six of seven HIV-1–infected babies harbored multiclass-resistant viruses at 12 months of age, jeopardizing the success of further ARV therapies [127]. This high rate of resistant mutants in these untreated babies is likely a result of exposure to suboptimal concentration of ARV drugs in ingested milk caused by variable diffusion of maternal drugs into breast milk [128, 129].

The prophylactic efficacy of WHO option A (infant peri-exposure prophylaxis) has been demonstrated in two proof-of-concept trials [130, 131]. Prophylaxis by treatment of the infant with daily NVP from 6 weeks to 6 months has been evaluated in South Africa in a randomized placebo-controlled trial [132]. Of the infants receiving this treatment, 1.1% acquired HIV-1 between 6 weeks and 6 months, whereas 2.4% of the placebo controls became infected, a 54% reduction in transmission. However, mortality at 6 months did not differ between the two groups.

The optimal drug of choice for infant prophylaxis remains unclear. The ideal drug should have excellent efficacy and a very good safety profile because the vast majority of infants will not be infected with HIV and so cannot ethically be given drugs with problematic side effects. The drug should not compromise or complicate the future HIV-1 treatment of infants who may acquire HIV despite the treatment. NVP satisfies the first two points (although its efficacy could be improved), but most infants who acquire HIV will become resistant to the whole class of non-
nucleoside reverse transcriptase inhibitor (NNRTI) drugs. Lamivudine (3TC), which proved as efficacious and safe as NVP [133], with a similar rate of resistance, may be a better choice. In this case, resistance would be limited to 3TC, and so use of this drug would not compromise the successful use of other nucleosidic reverse transcriptase inhibitors. Finally, other drugs could prove useful, such as lopinavir/ritonavir (LPV/r), which is more potent and has a high genetic barrier to resistance, with a good safety profile in preliminary studies of young infants [134].

Lopinavir/Ritonavir versus Lamivudine peri-exposure prophylaxis to prevent HIV-1 transmission by breastfeeding is being investigated in a multicenter trial: the PROMISE-PEP trial Protocol ANRS 12174 [135]. The ANRS 12174 study aims to compare the risk of HIV-1 transmission during and safety of prolonged infant PEP with LPV/r (40/10 mg twice daily if 2-4 kg and 80/20 mg twice daily if >4 kg) versus Lamivudine (7.5 mg twice daily if 2-4 kg, 25 mg twice daily if 4-8 kg and 50 mg twice daily if >8 kg) from day 7 until one week after cessation of BF (maximum 50 weeks of prophylaxis) to prevent postnatal HIV-1 acquisition between 7 days and 50 weeks of age. Among the 1273 infants randomised in this trial (636 in LPV/r arm and 637 in 3TC arm), 17 HIV-1 infections were diagnosed, giving HIV-1 infection rates of 1·4% and 1·5%, respectively. At week 50, HIV-1-free survival was not different between the two arms. Clinical or biological severe adverse events were not different between arms. Infant prophylactic LPV/r or 3TC for up to 50 weeks of breastfeeding achieved very low rates of HIV-1 postnatal transmission, without demonstrated superiority of one drug over the other (Nagot N et al, InPress)

(iv) Incomplete understanding of the role of co-viral infections in breast milk

Although maternal antiretroviral therapy reduces milk HIV-1 RNA load and postnatal transmission, its impact on milk CMV load is unclear. We provide the first evidence of an independent association between CMV DNA in BM and postnatal MTCT of HIV-1. Every 1 log10 increase in breast milk CMV DNA, was associated with a significant 2.5-fold greater odds of MTCT of HIV-1, independent of BM and plasma HIV-1 levels. This association could fuel persistent shedding of HIV-1 in breast milk in women receiving ART. HIV-1 and CMV are important pathogens transmitted via breastfeeding. Perinatal CMV transmission may impact growth and disease progression in HIV-exposed infants.
It is important to know if impairment of CMV host virus control in the mother impacts on MTCT of CMV, in both HIV-exposed-infected (HEI) and HIV-exposed-uninfected (HEU) children. In a study done in Kenya, it was shown that CMV DNA levels in plasma correlated strongly with HIV status and HIV viral load but not with CD4 count [120]. In this study there was a trend for women who were CMV DNAemic to transmit CMV to their (HIV-1 infected) infants earlier than women who were not CMV DNAemic, thus there was early transmission and higher CMV VL in both HEU/HEI from CMV DNAemic mothers. In another cohort in Malawi, it was found that CMV DNA levels in BM correlated with HIV viral load, but CMV DNA in BM was similar in ART-treated and untreated women [136]. It has also been shown that CMV MTCT is higher in mothers not receiving ART treatment and in those with lower CD4 count 2012 [39].

It is not known whether early CMV MTCT is associated with the formation of a larger CMV reservoir in children. CMV viral load kinetics studied in the Kenyan cohort, HEU (n=20) and HEI (n=44), showed that 90% of children had been infected at 3 months, with no difference between HEU and HEI; that CMV viral loads were highest in the 1-3 months following the first detection of virus and declined rapidly thereafter; that CMV peak viral loads were significantly higher in the HIV-infected infants compared with the HIV-exposed uninfected infants; and that the detection of CMV DNA persisted to 7-9 months post-CMV infection in both the HIV-exposed uninfected (8/17, 47%) and HIV-infected (13/18, 72%, P = 0.2) children [120].

There is also the question of whether early MTCT of CMV or a large reservoir of CMV in the infant contributes to infant morbidity and mortality. Slyker et al comparing HEU vs HEI and children from CMV DNAemic mothers vs not, showed that maternal CMV DNAemia remained a significant risk factor for mortality in HEI infants after adjusting for maternal CD4 T-cell count, HIV viral load or maternal death, with HIV-1 infected infants born to CMV DNAemic women having a 4-fold increased risk of mortality during 24 months of follow-up. [120]. In a cohort in Zambia it has been shown that human CMV infant infection adversely affects growth and development in both HEI and HEU children. In this study human CMV seropositive infants had decreased length-for-age development by 18 months of age compared to CMV seronegative
infants. In addition HIV-exposed infants positive for CMV serology status and levels of CMV DNA, had reduced head size and lower psychomotor development, and was more commonly referred for hospital treatment than CMV negative infants. [38]. Impact on infant growth characteristics was confirmed in the Malawian cohort, where higher milk CMV load was associated with lower length-for-age and weight-for-age Z score at six months in exposed, uninfected infants. As the impact of maternal antiretroviral therapy on the magnitude of postnatal CMV exposure may be limited, the findings of an inverse relationship between infant growth and milk CMV load highlight the importance of defining the role of perinatal CMV exposure on growth faltering of HIV-exposed infants [136].

Slyker et al showed that CMV viral loads peak in the 1-3 months following the first detection of virus and declined rapidly thereafter. CMV peak viral loads were significantly higher in the HIV-infected infants compared with HIV-exposed uninfected infants. In another study conducted in Cape Town on infants with severe pneumonia (HEU=297 and HEI=120), the level of CMV viraemia was significantly higher in a subset of infants diagnosed with CMV pneumonia with prevalence and level of CMV viraemia peaking at 3-4 months of age [137].

In a study looking at the effect of prenatal ART on CMV MTCT in the perinatal/early postnatal, it was found that MTCT of CMV was associated with the presence or absence of ART, and that For HIV-uninfected infants with CMV, symptoms including splenomegaly, lymphadenopathy, and hepatomegaly were associated with no maternal ART. These findings confirm the importance of prenatal ART for all HIV-infected pregnant women [39].

Control of CMV infection/reactivation

CMV Vaccine

Pass et al [138] reported on a phase II placebo-controlled, RCT where (n=225) CMV-seronegative women received 1-3 intramuscular injections/vaccinations of glycoprotein B adjuvanted with MF59. Infection occurred in 8% of the vaccine group and in 14% of the placebo group, which equates to a vaccine efficacy of 50%. The effect of vaccination in CMV-
seropositive women, and whether it could modify shedding, especially in BM, has not been studied thus far.

Hamilton et al [26] identified a significant lack of robust clinical data examining either prophylaxis or treatment interventions for congenital CMV. Furthermore, high-quality evidence from RCTs will be required before any interventions can be recommended and will agree to cover the associated costs.
**CMV Antiviral therapy**

Ganciclovir (six studies - [140-145]) and valganciclovir (2 studies - [146, 147]) therapy have been extensively studied in infants who presented with symptomatic congenital CMV disease. All were done on symptomatic cCMV except Lackner et al, who initiated 23 asymptomatic but with proven congenital cytomegalovirus infection within the first 10 days of life. All but one showed some association with neutropaenia. This class of agent currently remains the treatment of choice for symptomatic cCMV infections. However, its use as an antiviral to reduce CMV-associated HIV-1 transmission, through both its association to DNA and/or RNA, as we have shown, is unlikely. No study has investigated the effect of ganciclovir or valganciclovir on BM CMV and HIV transmission. This would also be difficult to study as large-scale treatment using this class of agent could never be justified as an HIV intervention, due to its extensive side-effect profile and prohibitive cost. Valacyclovir may have a direct effect on HIV-1 and Valacyclovir therapy has been shown to decrease the risk of MTCT of HIV-1 even in HSV-uninfected women [148].

Thus, the goal to decrease CMV levels in BM remains an elusive one in the absence of an effective vaccine or drug, that can provide protection against primary infection (prevent seroconversion, especially during pregnancy), but more importantly can modify CMV replication and compartmentalized shedding (breast milk, renal, vaginal, semen, oral), in HIV-infected mothers.

Three double-blind randomized placebo-controlled phase 2 proof-of-concept studies have each identified a novel antiviral drug with activity against CMV infection in bone marrow transplant patients. One of these (brincidofovir) inhibits the DNA polymerase that is the target of the currently licensed drug ganciclovir) [149]. Another new drug (maribavir) inhibits a protein kinase which, coincidentally, is the enzyme responsible for activating ganciclovir through phosphorylation [150]. The third drug (letermovir) inhibits the terminase enzyme complex responsible for packaging unit length DNA into assembling virions [151]. In addition, in a double-blind randomized placebo-controlled trial in neonates with symptomatic congenital CMV infection, a 6-month course of valganciclovir was superior to the standard 6-week course
of the same drug [152]. In pregnant women with primary CMV infection, administration of hyperimmune immunoglobulin did not significantly reduce transmission of CMV across the placenta.

**Perspectives**

Effective ART is imperative to minimize perinatal and postnatal HIV transmission from mother-to-child. However, residual transmission is still occurring mostly due to cell-associates HIV reservoirs which remain refractory to ART. We have demonstrated that the cumulative dose of HIV RNA particles remains one of the most important risk factors. In addition we have shown that HIV DNA is also a very important factor especially in the first 3 months postnatally.

However there is still residual HIV shedding despite ART. It appears that it could be a very important adjunctive to administer prophylaxis to the breastfeeding infant (Promise PEP). Thus treating only the mother is only partially effective and treatment of the infant is important. Further information is needed on immune profile and morbidity of HEU children.

The relationship between blood and BM HIV remains complex. Inflammation and coviral infections in the mammary gland contributes significantly to intra-mammary compartmentalization.

What is the best therapy to avoid HIV MTCT? Option B+? A long-acting drug for infant prophylaxis? A combination of these strategies?

Targeting CMV to reduce MTCT is an attractive complementary option, but there is no vaccine available yet and antiviral therapy for prophylaxis is not available. The next decade should see major advancement on these questions.
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Appendix

There is an urgent need to assess the accuracy/feasibility of using dried blood spots (DBS) for monitoring of HIV-1 viral load in resource-limited settings. A total of 892 DBS from HIV-1–positive pregnant women and their neonates enrolled in the Kesho Bora prevention of mother-to-child transmission trial conducted in Durban (South Africa) and Bobo-Dioulasso (Burkina Faso) between May 2005 and July 2008 were tested for HIV-1 RNA. The combination Nuclisens extraction method (BioMe´rieux)/Generic HIV Viral Load assay (Biocentric) was performed using one DBS (in Durban) versus 2 DBS (in Bobo-Dioulasso) on 2 distinct open real-time polymerase chain reaction instruments. DBS HIV-1 RNA results were compared with plasma HIV-1 RNA and HIV serology results used as the gold standards. The limits of detection of assays on DBS were 3100 and 1550 copies per milliliter in Durban and Bobo-Dioulasso, respectively. DBS HIV-1 RNA values correlated significantly with plasma levels (n = 327; R = 0.7351) and were uniformly distributed according to duration of DBS storage at 220_C (median duration, 280 days). For early infant diagnosis, the sensitivity and specificity were 100% (95% confidence interval: 97.2 to 100.0 and 96.5 to 100.0, respectively). HIV-1 viral load kinetics in DNase-pretreated DBS were similar to those obtained in plasma specimens among 13 patients receiving antiretroviral treatment. HIV-1 RNA findings from serial infant DBS collected prospectively (n = 164) showed 100% concordance with HIV serology at 18 months of life. Our findings strongly advocate the implementation of DBS HIV-1 RNA testing in remote areas from low-income and middle-income countries.
Dried Blood Spot HIV-1 RNA Quantification Using Open Real-Time Systems in South Africa and Burkina Faso

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Abstract: There is an urgent need to assess the accuracy/feasibility of using dried blood spots (DBS) for monitoring of HIV-1 viral load in resource-limited settings. A total of 892 DBS from HIV-1–positive pregnant women and their neonates enrolled in the Kesho Bora prevention of mother-to-child transmission trial conducted in Durban (South Africa) and Bobo-Dioulasso (Burkina Faso) between May 2005 and July 2008 were tested for HIV-1 RNA. The combination Nuclisens extraction method (BioMérieux)/Generic HIV Viral Load assay (Biocentric) was performed using one DBS (in Durban) versus 2 DBS (in Bobo-Dioulasso) on 2 distinct open real-time polymerase chain reaction instruments. DBS HIV-1 RNA results were compared with plasma HIV-1 RNA and HIV serology results used as the gold standards. The limits of detection of assays on DBS were 3100 and 1550 copies per milliliter in Durban and Bobo-Dioulasso, respectively. DBS HIV-1 RNA values correlated significantly with plasma levels (n = 327; R = 0.7351) and were uniformly distributed according to duration of DBS storage at −20°C (median duration, 280 days). For early infant diagnosis, the sensitivity and specificity were 100% (95% confidence interval: 97.2 to 100.0 and 96.5 to 100.0, respectively). HIV-1 viral load kinetics in DNase-pretreated DBS were similar to those obtained in plasma specimens among 13 patients receiving antiretroviral treatment. HIV-1 RNA findings from serial infant DBS collected prospectively (n = 164) showed 100% concordance with HIV serology at 18 months of life. Our findings strongly advocate the implementation of DBS HIV-1 RNA testing in remote areas from low-income and middle-income countries.

Key Words: DBS, HIV-1 RNA quantification, real-time PCR, sub-Saharan Africa

(†) Acquir Immune Defic Syndr 2010;55:290–298)

INTRODUCTION

At present, laboratory capacity for biological monitoring of HIV-1 infection in sub-Saharan Africa remains insufficient. Without individually scheduled HIV-1 RNA viral load (VL) measurements (as done in developed countries), the recent gains in HIV treatment in developing countries might fade in the coming years, given the important risks of virological failure and subsequent spread of HIV-1 drug-resistant strains. To make HIV-1 RNA VL measurements more accessible in Africa, dried blood spots (DBS) may be a “field-friendly” tool for sample collection and transport from remote resource-limited settings to central testing laboratories. Compared with standard plasma specimens, DBS offers a simplified sampling method eliminating many logistical and technical limitations, as they are much easier to collect, transport, and store. However, studies evaluating the usefulness and reliability of filter papers focused mainly on the role of DBS in public health HIV-1 drug resistance surveillance. Studies using DBS for clinical HIV-1 RNA VL monitoring on an individual patient basis are fewer, and testing was mainly performed in laboratories in developed countries where DBS were shipped, prohibiting technology transfer to low-income countries. Studies performed in African laboratories remain scarce, with limited sample sizes. Apart from an assay developed by Mehta et al, expensive Food and Drug Administration–approved HIV-1 RNA tests on closed platforms have been used, further reducing the potential for implementation in low-income countries.

We report the accuracy and feasibility of using long-term stored DBS for HIV-1 RNA VL measurements in 2 African laboratories. We used the combination of the Nuclisens extraction method (BioMérieux) and the Generic

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HIV Viral Load assay (Biocentric) performed on 2 distinct open real-time polymerase chain reaction (PCR) instruments. The rationale for the choice of this combination was the excellent performance of the BioMérieux technique for DBS extraction and the affordability of the Biocentric assay which accurately amplified non–B HIV-1 subtypes.

SUBJECTS AND METHODS

Study Design and Selection Criteria for Specimens

Our study was carried out at two African sites, Bobo-Dioulasso (Burkina Faso) and Durban (South Africa), where the Kesho Bora trial aimed at preventing mother-to-child transmission of HIV-1 was conducted in 2005–2010. Written informed consent was obtained from all participants. The protocol was approved by the World Health Organization (WHO) ethics committee and by the Institutional and National Review Boards in both countries.

The study consisted of 2 parts in which a total of 892 DBS and 69 dried plasma spots (DPS), collected within the Kesho Bora trial, were tested for HIV-1 RNA (Table 1). Part 1 was a large-scale retrospective survey evaluating VLs obtained with DBS and DPS, collected at both sites between May 2005 and July 2008. Results were compared with those obtained on paired plasma specimens used as the gold standard. All maternal DBS collected at enrollment in Durban were assessed, whereas in Bobo-Dioulasso, cases were selected in a blinded manner. DBS from all HIV-1–infected children in Bobo-Dioulasso, taken between 6 weeks and 18 months, were tested for HIV-1 RNA. For each infected child, 3 uninfected infants were randomly selected. Their DBS samples, taken between 6 weeks and 12 months, were also tested for HIV-1 RNA. Forty-six pregnant women with CD4 count, 200 cells per cubic millimeter at inclusion received highly active antiretroviral therapy (HAART) in Bobo-Dioulasso. From this group, we selected sequential DBS from all women (n = 4) who showed moderate (>300 copies/mL) or major (>5000 copies/mL) plasma viral rebounds at month 6 and/or 12 of follow-up. We also randomly selected 9 women who had a successful treatment response according to their plasma values. Part 2 of the study, from August 2008 to April 2010, was a prospective cohort study in Durban, where all field-based DBS collected consecutively in infants were used prospectively for the early diagnosis of pediatric HIV-1 infection and finally compared with HIV serology results (SD BIOLINE HIV 1/2 3.0, Standard Diagnostics, Inc. Kyonggi-do, Korea) obtained at 18 months of life.

DBS/DPS Collection

In part 1, DBS were prepared in reference laboratories using venous blood collected by venipuncture in 5.0 mL EDTA-anticoagulated tubes. Briefly, 5 spots of whole blood (50 μL each) were spotted onto filter specimen collection paper (Whatman no. 903; formerly SS903, Schleicher & Schull, Kenne, NH), dried overnight at room temperature, placed in individual zip-lock bags containing a silica desiccant, and stored at −20°C until further testing. The remaining blood sample was centrifuged, and plasma was used for preparing DPS (50 μL each). Remaining plasma was stored at −80°C until further testing.

In part 2, DBS were prepared by health professionals in 2 rural antenatal clinics in South Africa (KwaDabeka and KwaMsane), from finger or heel prick. Samples were processed in a similar way as above and transported by road at ambient temperature within 24 hours to the reference laboratory in Durban.

HIV-1 Molecular Techniques

All assays were performed at the Africa Centre Virology Laboratory for specimens from South Africa and at the

<table>
<thead>
<tr>
<th>TABLE 1. Selected Populations and Samples Tested</th>
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<td>Tested Subjects (n)</td>
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<tr>
<td>Part 1</td>
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<tr>
<td>HIV-1–positive mothers</td>
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<td>HIV-1–exposed infants†</td>
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<td>HIV-1 infected</td>
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*Evaluation conducted in Durban only.
†Evaluation conducted in Bobo-Dioulasso only.
‡41 DBS measurements without DNase pretreatment, and 41 DBS measurements with DNase pretreatment.
PL, plasma; Incl, inclusion; D, day; W, week; M, month.
Virology Laboratory of Centre Muraz for samples from Burkina Faso. The NucliSENS miniMag extraction method (BioMérieux, Boxtel, The Netherlands) was used for DBS/DPS RNA extraction.15 To assess the sensitivity of DBS HIV-1 RNA VL testing according to the number of spots used, the pre-extraction procedure differed between laboratories: in Durban, 1 spot of DBS or DPS was eluted in 9.0 mL of NucliSens lysis buffer19 compared with two spots in 2.0 mL of the same buffer in Bobo-Dioulasso.12 Samples were rotated for 60 minutes and subsequently processed according to the manufacturer’s instructions. For paired plasma specimens, RNA was isolated from 200 mL of plasma using the QIAGEN procedure (QIAamp Viral RNA Mini Kit, Qiagen, Courtaboeuf, France).

HIV-1 RNA was quantified in all extracts using the long-terminal repeat–based Generic HIV Viral Load assay (Bio-centric, Bandol, France), with a limit of detection (LOD) of 300 copies per milliliter for plasma using an input volume of 200 μL.16,18 For standardization in DBS results, customized DBS standards were prepared as follows: the liquid standard with a known HIV-1 RNA concentration (6,200,000 copies/mL) (Optiquant quantification panel HIV RNA N°6 (Acrometrix Inc, CA) included in the kit) was diluted 1:1 with HIV-seronegative blood, and spotted onto filter papers (each spot 50 μL). A DBS for the low-positive control (LPC) included in the kit (quantified at 6200 copies/mL) was similarly prepared. DBS standard and LPC were then extracted together with DBS clinical specimens. Extracted DBS standard was serially diluted (10-fold) to concentrations from 3,100,000 to 310 copies per milliliter. DPS standard and LPC were spotted onto filter papers and extracted with DPS clinical specimens. Extracted standard DPS was 10-fold diluted. All DBS values from clinical specimens were corrected for hematocrit as follows: result in copies per milliliter of blood × 100/100—hematocrit.9 For patients receiving HAART, we compared DBS results obtained with or without prior DNase treatment (DNase I, Applied Biosystems/Ambion Inc, Austin, TX). Treatment with DNase may prevent co-extraction and co-amplification of proviral HIV-1 DNA, due to the presence of white blood cells in whole blood, which can interfere on HIV-1 amplification of proviral HIV-1 DNA, due to the presence of T-cell counts and DBS HIV-1 RNA levels. The Bland–Altman method22 was used to assess the agreement between HIV-1 RNA values obtained with DBS versus plasma and with DPS versus plasma, and to study the impact of duration of DBS storage on DBS assay accuracy. For monitoring HAART efficiency, individual DBS results were compared with plasma results and analyzed according to a threshold of 5000 copies per milliliter. The WHO currently recommends this value for conservation of first-line HAART (or a switch to second-line regimen) in resource-limited settings.23 These data were also analyzed by using a Bland–Altman representation. If plasma and DBS samples were undetectable (<300 and <1550 copies/mL, respectively), the difference (d) was assigned to zero.

RESULTS

Retrospective Laboratory Study on DBS Performance

Analytical Sensitivity, Reproducibility, and Repeatability of the DBS Assays

In both configurations (1 DBS with the MiniOpticon versus 2 DBS with the ABI PRISM), the assay was shown to be linear over the entire range of 3,100,000–3100 copies per milliliter, with detection rates of 100% at 3100 copies per milliliter (Fig. 1). The fitted slope was marginally greater in Durban. At 310 copies per milliliter (dotted sections of the lines), the assays’ sensitivities decreased to 38% with 1 spot and 45% with 2 spots. To estimate the LOD more precisely, 10 additional measurements at 1 550 and 775 copies per milliliter were performed (2-fold dilutions of 3100 copies/mL). Using 1 spot yielded detection rates of 70% and 40% versus 100% and 70% using 2 spots. Therefore, LODs were set at 3100 and 1550 copies per milliliter in Durban and Bobo-Dioulasso, respectively.

In Durban, the DBS-LPC yielded repeatability and reproducibility mean values of 3.42 (SD: ±0.27) and 3.47 (SD: ±0.43) log10 copies per milliliter, with coefficients of variation of 7.9% and 12.3%, respectively. In Bobo-Dioulasso, they were 3.65 (SD: ±0.23) and 3.32 (SD: ±0.30) log10 copies per milliliter, with coefficients of variation of 6.3% and 8.9%.

Clinical Sensitivity of the DBS Assay Compared With Plasma Specimens

Overall, of 353 positive (≥300 copies/mL) maternal plasma samples obtained at inclusion from 353 pregnant antiretroviral (ARV)-naïve women, 327 paired DBS [327 of 353, 92.6%, 95% confidence interval (95% CI): 89.5 to 95.0] tested positive (Table 2). The sensitivity decreased significantly (χ² test, P < 0.001) for HIV-1 RNA levels approximately equal to or below the assays’ LODs. Contrary to Bobo-Dioulasso, where the sensitivity was 100% from 3.51 log10 copies per milliliter, there were 7 discordant pairs in Durban (plasma positive range, from 3.53 to 4.47 log10 copies/mL but

Statistical Analyses

In each laboratory, the DBS standard serial dilutions were tested in >10 independent runs to determine the analytical sensitivity and linearity of the DBS assay.21 Repeatability (intra-assay variance) was assessed by testing DBS-LPC in 10 replicates in the same run. Reproducibility (intersay variance) was calculated by testing DBS-LPC in >10 separate runs. Clinical sensitivity was calculated as the number of positive results divided by the total number of plasma HIV-1 RNA VL results ≥300 copies per milliliter from infected subjects. Clinical specificity was calculated as the number of negative results divided by the total number of negative results from uninfected individuals with plasma VL measurement <300 copies per milliliter. Spearman correlation coefficients were calculated to determine the relationship between HIV-1 RNA concentrations in DBS versus plasma specimens or in DPS versus plasma samples, and between CD4 T-cell counts and DBS HIV-1 RNA levels. The Bland–Altman method22 was used to assess the agreement between HIV-1 RNA values obtained with DBS versus plasma and with DPS versus plasma, and to study the impact of duration of DBS storage on DBS assay accuracy. For monitoring HAART efficiency, individual DBS results were compared with plasma results and analyzed according to a threshold of 5000 copies per milliliter. The WHO currently recommends this value for conservation of first-line HAART (or a switch to second-line regimen) in resource-limited settings.23 These data were also analyzed by using a Bland–Altman representation. If plasma and DBS samples were undetectable (<300 and <1550 copies/mL, respectively), the difference (d) was assigned to zero.
For 327 concordantly positive results, plasma and hematocrit-corrected DBS levels were well correlated (Fig. 2A) ($R = 0.7351$; $P < 0.001$). The overall mean difference ($d$) in the HIV-1 RNA values obtained with DBS and plasma samples was $+0.28 \log_{10}$ copies/mL (Fig. 2B). When considering low-level viremia ($<4.0 \log_{10}$ copies/mL), $d$ reached $+0.39 \log_{10}$ copies per milliliter. As shown in Figure 3, DBS levels were negatively correlated with absolute CD4+ T-cell counts ($R = -0.3861$; $P < 0.001$).

Data comparing DPS with plasma from 69 consecutively enrolled women in Durban revealed a lower sensitivity (62 DPS positive, sensitivity = 89.9%) (Table 2) compared with DBS but with good correlation (Fig. 2C) ($R = 0.7683$; $P < 0.001$). The overall $d$ between DPS and plasma results was $+0.07 \log_{10}$ copies per milliliter (Fig. 2D). Thus, we decided to focus our work on field-friendly DBS only.

**Impact of DBS Storage Duration on the Reliability of the DBS Assay Results**

The 327 maternal DBS with concordant positive plasma results were stored for a median duration of 280 days (range: 1–1599 days) at $220^\circ C$. No statistically significant difference was observed in the mean VL difference ($d$) (DBS minus plasma results) obtained between short/medium-term ($\leq 280$ days; $n = 163$; $d = +0.30 \log_{10}$ copies/mL) and long-term (>280 days; $n = 164$; $d = +0.26 \log_{10}$ copies/mL) stored DBS and plasma specimens (Wilcoxon signed rank test, $P = 0.65$) (Fig. 4).

**Clinical Evaluations**

**Early Diagnosis of HIV-1 Infection in Children**

In Bobo–Dioulasso, 106 samples from 33 HIV-1–infected children had detectable RNA in plasma and paired DBS, leading to a DBS sensitivity of 100% (106 of 106, 95% CI: 97.2 to 100) at 6 weeks ($n = 20$), 3–6 months ($n = 34$), and 9–18 months ($n = 52$) of life. In 105 HIV-1–uninfected children, all DBS collected at 6 weeks ($n = 94$), 3–6 months ($n = 4$), and 12 months ($n = 7$) of life were concordantly negative with plasma, yielding a DBS specificity of 100% (95% CI: 96.5 to 100).

**Kinetics of DBS Measurements Among Patients Who Received HAART**

Nine women (numbered 1–9, Fig. 5) were treated successfully with HAART and maintained plasma HIV-1 RNA levels <5000 copies/mL during their entire follow-up (except

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**TABLE 2.** DBS and DPS Clinical Sensitivity Among Untreated Pregnant Women Enrolled in the Kesho Bora Trial in Durban and Bobo–Dioulasso (2005–2008)

<table>
<thead>
<tr>
<th>Plasma HIV-1 RNA Class (in $\log_{10}$ copies/mL)</th>
<th>DPS*</th>
<th>DBS†</th>
<th>DPS‡</th>
<th>DBS‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5–3.0</td>
<td>0/2</td>
<td>5/11</td>
<td>2/4</td>
<td>7/15</td>
</tr>
<tr>
<td>3.01–3.5</td>
<td>3/5</td>
<td>22/28</td>
<td>12/12</td>
<td>30/41</td>
</tr>
<tr>
<td>3.51–4.0</td>
<td>12/14</td>
<td>48/51</td>
<td>25/26</td>
<td>60/63</td>
</tr>
<tr>
<td>4.01–4.5</td>
<td>25/26</td>
<td>69/73</td>
<td>30/30</td>
<td>94/98</td>
</tr>
<tr>
<td>4.51–5.0</td>
<td>41/41</td>
<td>21/21</td>
<td>44/44</td>
<td>71/71</td>
</tr>
<tr>
<td>5.0</td>
<td>8/8</td>
<td>206/225</td>
<td>121/128</td>
<td>327/353</td>
</tr>
</tbody>
</table>

*The sensitivity for DPS was calculated using a theoretical LOD equal to 1200 copies/mL.
†The sensitivity for DBS was calculated using the LOD experimentally determined in Durban (ie, 3100 copies/mL).
‡The sensitivity for DBS was calculated using the LOD experimentally obtained in Bobo–Dioulasso (ie, 1550 copies/mL).
for woman 9 at delivery). In 17 DBS taken during follow-up, and not pre-treated with DNase, 10 (10 of 17, 59%) HIV-1 VL values were discordantly.

5000 copies per milliliter. After DNase treatment that prevented HIV-1 proviral DNA co-amplification, all (17 of 17, 100%) DBS values became concordant ([5000 copies/mL]). The mean difference ($d$) in log$_{10}$ copies per milliliter between DBS HIV-1 VL results and plasma values obtained from the 28 samples taken during follow-up is summarized in Figure 6. The overall $d$ between crude DBS results and plasma values was almost $+1.0$ log$_{10}$ copies per milliliter (Fig. 6A). This difference was particularly marked for samples showing undetectable (<300 copies/mL) plasma HIV-1 RNA values. When considering DBS pre-treated by DNase, the overall $d$ with plasma concentrations was nil, strongly suggesting that the DBS enzymatic treatment allowed HIV-1 RNA levels not to be affected by coquantification of HIV-1 proviral DNA (Fig. 6B).

Prospective Field Study on DBS Feasibility

In Durban, from August 2008 to April 2010, 220 DBS samples were negative for HIV-1 RNA in 153 infants at 6 weeks of life or thereafter. All of them were HIV antibody negative at months 18 of life. By testing DBS, 11 children were diagnosed as HIV-1 infected, including 6 cases of postnatal transmission by breastfeeding. All of these were confirmed positive by HIV serology at 18 months of life.
DISCUSSION

Our open real-time systems using DBS showed excellent performance characteristics for HIV-1 RNA VL measurements in South Africa and Burkina Faso. DBS HIV-1 RNA levels displayed good concordance with plasma values and were inversely correlated with CD4+ T-cell counts. Long-term stored DBS (as long as 4 years) at 220°C was accurate as a repository. Under basic field conditions, DBS were useful for clinical applications such as early infant diagnosis. Monitoring the efficiency of HAART was achieved after a prior DNase treatment step. The cost per test was ~US $12 which made this strategy cost-effective.

This large-scale study was carried out in 2 African laboratories, reflecting real-life conditions of HIV-1 RNA monitoring and the relevance of its implementation to similar settings, despite difficulties in human resources, reagents supply, and laboratory infrastructure/maintenance. As previously demonstrated,\(^8,24,25\) the use of robust and highly flexible real-time PCR instruments was a significant advantage, compared with the restrictions of expensive closed platforms. For instance, the long-life light-emitting diode-based MiniOpticon, used in Durban, was affordable (~US $20,000) and required no maintenance.

Compared with liquid plasma-based methods, DBS nevertheless have some disadvantages. First, the extraction of nucleic acids from DBS was manual and required extra hands on time, including their excision with scissors and a potential DNase pretreatment step. Future operational research studies should be directed toward automation of both DBS excision (with automated punchers, instead of scissors) and nucleic acid extraction by using automated extractors (such as the EasyMag from BioMérieux), to increase throughput.

Second, as found by others,\(^10–13,15\) our study revealed a reduced DBS sensitivity in comparison with plasma. However, whatever the number of DBS used (1 or 2), the sensitivities of our assays fit with the national guidelines in South Africa and Burkina Faso which recommend, in accordance with WHO guidelines,\(^23\) an ARV therapy switch above a level 5000 copies per milliliter. Thus, in our clinical context where VL measurements are required in conjunction with targeted adherence monitoring for conserving first-line ARV drugs regimen,\(^26\) both methods are acceptable and show distinct advantages: given its better sensitivity (LOD 1000 copies/mL), the use of 2 DBS represents the standardized extraction protocol, currently recommended by BioMérieux. Tubes containing 2.0 mL of lysis buffer are ready made and seem more convenient than handling 9.0 mL. Using only 1 DBS, as historically performed at the Africa Center Virology Laboratory in Durban,\(^19,27\) allows usage of additional spot for other purposes (such as HIV serology, ultrasensitive p24 antigen, HIV DNA PCR or resistance genotyping).

Seven of 186 (3.7%) DBS false negative results were obtained in clinical specimens from Durban, despite corresponding plasma results higher than the LOD. These discrepancies could be explained by impaired efficiency...
of nucleic acid extraction (in our experience, some silica-based reagents were substandard) and nucleic acid degradation during DBS preparation and/or storage. Given that the Generic HIV Viral Load assay amplifies a small HIV-1 long-terminal repeat fragment (123 base pairs), it is less likely to be affected by degradation than techniques amplifying longer ones.

Third, because the Nuclisens extraction method is not RNA specific but isolates cell-associated DNA as well, archived proviral HIV-1 DNA may interfere with results generated by DBS, yielding false-positive results as previously documented. In our study, an overall \( +0.3 \log_{10} \) copies per milliliter difference was obtained between DBS and plasma levels from untreated subjects. The impact of DNA was more significant in low-viremia specimens where positive DBS values could be due to the presence of detectable HIV-DNA. The presence of HIV-1 DNA in crude DBS interfered with

![FIGURE 5](image)

**FIGURE 5.** Individual kinetics of HIV-1 VL measurements obtained by using plasma specimens, DBS without DNase treatment, and DBS with DNase treatment among 13 patients from Bobo–Dioulasso receiving HAART. For each patient, the threshold of 5000 copies per milliliter is indicated by a dotted line. The median time between inclusion and delivery was 2 months. Patients 1–9: plasma virological success; Patients 10–13: plasma virological failure (moderate for patients 10 and 11 and major for patients 12 and 13).

![FIGURE 6](image)

**FIGURE 6.** Difference between the viral loads measured on DBS and plasma specimens among 28 samples taken during the follow-up of 13 ARV-treated patients from Bobo–Dioulasso. A, DBS not pretreated by DNase. B, DBS pretreated by DNase. Undetectable (<300 copies/mL) plasma specimens (n = 17) are represented by gray circles. Detectable (>300 copies/mL) plasma samples (n = 11) are depicted by distinct colored symbols.
monitoring of successfully ARV-treated patients (with undetectable plasma results), whereas this interference was not significant in individuals with plasma virological failure. The specificity of our assay was very low (~40% at a threshold of 5000 copies/mL) when using crude DBS in ARV-treated patients showing plasma virological success. In addition, the difference between crude DBS and plasma results was high (oscillating from +0.87 to +1.76 log_{10} copies/mL). Thus, in our survey, performing a prior DNase treatment step on DBS was a prerequisite for accurate monitoring of HAART efficiency. After enzymatic treatment, DBS specificity reached 100%, and the difference between DBS and plasma values was virtually nil. Other solutions may be to use DPS, to perform extraction with the Abbott method which is more RNA specific than the BioMérieux technique, or to resort to the NASBA technology which is designed specifically for RNA, as reported recently by Johannessen et al.

We have shown in 2 African countries that DBS HIV-1 RNA measurements, using open real systems and long-term stored spots, are reliable and feasible. These data should prompt other reference laboratories from similar settings to revisit and expand DBS HIV-1 RNA monitoring strategies. They should also help to strengthen the commitment of health care providers, physicians, and all public health stakeholders who are not sufficiently aware of this affordable, simple, and robust sampling method, ideal for HIV-1 infection monitoring in remote areas from middle-income (such as South Africa) and low-income (such as Burkina Faso) countries. Considering the difference between DBS and DPS/plasma values, clinicians should be informed that it is recommended not to switch between these 2 formats of testing during monitoring of efficiency of HAART. It is our view that plasma specimens should remain the gold standard for adults living in African cities where tertiary reference laboratories and transportation facilities are available. However, in remote rural areas, besides rapid VL testing strategies (such as the BioHelix Express Strip (BESS) and lab-in-a-tube (LiAT) technologies), which need to be further evaluated, DBS can pave the way for expanded access to HIV-1 VL testing for millions of ARV-treated adults and babies born to seropositive mothers. Such efforts are imperative to meet the high demand encountered presently in sub-Saharan African countries endemic for HIV-1.

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REFERENCES


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Résumé

L'allaitement maternel est la modalité idéale d'alimentation du nourrisson. Les propriétés anti-infectieuses du lait maternel sont bien documentées. L'allaitement maternel protège les nourrissons contre les infections intestinales et respiratoires. L'allaitement maternel exclusif est recommandé pendant les 6 premiers mois, principalement parce que le lait maternel satisfait de façon optimale à tous les besoins nutritionnels et hydriques du nourrisson. Les nouvelles infections périmatérales par le VIH dans les pays riches ont presque été éliminées grâce à la combinaison du dépistage prénatal du VIH, à la prophylaxie antirétrovirale de la mère et de l'enfant, à la césarienne élective et l'évitement de l'allaitement maternel. Bien que les interventions efficaces soient disponibles pour réduire la transmission in utero et intrapartum dans les pays à ressources limitées, la transmission postnatale du VIH par l'allaitement demeure un enjeu de santé publique. L'acquisition du VIH par l'allaitement maternel est responsable d'environ 40% des nouvelles infections en Afrique subsaharienne. Les études effectuées au cours de cette thèse faisaient partie d'un programme d'intervention qui a porté sur l'utilisation des différentes formes d'alimentation du nourrisson dans un environnement rural, à Umkhanyakude, dans le nord du KwaZulu-Natal, en Afrique du Sud. Les femmes ont été incluses dans cette étude avant le début de l'accès universel aux antirétroviraux en Afrique du Sud (2005). Le travail de doctorat visait à acquérir une meilleure compréhension de la transmission postnatale du VIH-1 par l'allaitement maternel, indispensable pour atteindre l'objectif de l'Organisation mondiale de la Santé de réduire toutes les formes de transmission du VIH de la mère à l'enfant (TME) à moins de 5% d'ici la fin de 2015. Dans la première étude, nous apportons la preuve que l'exposition cumulée à l'ARN VIH-1 par le lait maternel est un facteur de risque associé à la transmission postnatale de la mère à l'enfant (TME) à moins de 5% d'ici la fin de 2015. Dans la première étude, nous apportons la preuve que l'exposition cumulée à l'ARN VIH-1 par le lait maternel est un facteur de risque associé à la transmission postnatale de la mère à l'enfant, indépendamment du taux de CD4 maternels et de la charge virale plasmatique du VIH-1. Ces données fournissent une meilleure évaluation du risque de transmission mère-enfant du VIH-1 et de la charge virale dans le compartiment mammaire. Dans la seconde étude, nous confirmions que la charge virale associée aux cellules dans le lait maternel est un meilleur facteur prédictif du risque de TME postnatale précoce que la charge virale libre. En revanche, la charge virale libre est un facteur prédictif de transmission postnatale tardive (au-delà de 6 mois). Dans la troisième étude, nous avons étudié l'impact sur la TME du VIH-1 du cytomégalovirus (CMV) et du virus d'Epstein-Barr (EBV) dans le lait maternel des mères infectées par le VIH. Des niveaux élevés de CMV sont excrétés dans le lait maternel, et un niveau significatif de l'EBV est fréquemment observé. Les mères dont le lait maternel contient des niveaux élevés de CMV étaient jusqu'à deux fois et demi plus susceptibles de transmettre le VIH-1 à leur enfant par l'allaitement maternel comparativement aux femmes ayant un faible niveau de réplication de CMV. Nous apportons donc la preuve d'une association, indépendante de la charge virale du VIH-1, entre l'excrétement du CMV dans le lait maternel et la transmission postnatale du VIH-1. Chez les femmes allaitant infectées par le VIH-1 et sous traitement antirétroviral, le risque de transmission résiduelle par l'allaitement est expliqué en partie par la persistance du virus associé aux cellules dans le lait maternel. D'autres études sont nécessaires pour approfondir les connaissances sur le mécanisme du VIH-1 transmission pendant l'allaitement, et les facteurs associés à l'excrétement compartimentée du VIH-1 dans le lait maternel, et pour aider à développer des médicaments plus efficaces pour une utilisation dans les populations à ressources limitées où l'évitement de l'allaitement maternel est souvent impossible.