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REVIEW ARTICLE

Characterization of Early Viral Populations in Infants Acquiring HIV Through Perinatal and Breastmilk Transmission: A Review of what is Currently Known and the Gaps that Need to be Addressed to Guide Passive HIV Immunization of Breastfeeding Infants

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Abstract: Newborns represent only 1% of the population. Yet, HIV vertical transmissions represent 10% of all new infections globally, even though antiretroviral therapy (ART) has been shown to reduce the risk of vertical transmission to less than 2%. While vaccines still represent the most efficient and cost-effective intervention to eradicate new infections, HIV immunogens that can effectively elicit broad-spectrum protection are still at least a decade away. In contrast, passive immunization with broadly neutralizing antibody (bnAb) combinations has the potential to provide a more immediate pathway to HIV prophylaxis. Early-phase infant trials are underway to establish the safety and pharmacokinetics of bnAb combinations selected for their potency against viruses acquired *via* adult transmissions. However, the specific characteristics and phenotypic differences of vertically transmitted viruses in infants compared to those in adults remain uncertain, including their susceptibility to known broadly neutralizing antibodies (bnAbs). We review the current knowledge of vertically transmitted HIV viruses, including their genetics and phenotypic features. Differences in immunity between adults and infants lead us to hypothesize that distinct selection and evolutionary pressures act on the virus at the time of transmission and during the early phases of infection, and these may in turn affect the choice of bnAb combinations needed for protection against vertical transmission of HIV.

Keywords: Antiretroviral therapy, HIV viruses, phenotypic features, transmissions of HIV, bnAbs, HIV prophylaxis.

1. INTRODUCTION

While antiretroviral therapy (ART) in pregnant and breastfeeding persons living with HIV (PLWH) has substantially decreased the incidence of vertical transmission, infants continue to be disproportionally affected [1]. In 2023, an estimated 120,000 new pediatric HIV infections occurred, representing 10% of all new HIV infections globally, even though newborns represent only 1% of the global population [2]. Persistence of pediatric HIV transmissions can be attributed to numerous factors, including poor access to care, late diagnosis of maternal infection, incomplete adherence to treatment, resistance to ART, and maternal infection during pregnancy or breastfeeding [1, 3]. We note here that not all birthing or lactating parents identify as female; however, for simplicity, we will refer to birthing and/or lactating parents as "women" and "mothers" throughout this document.

In the absence of ART, the rate of vertical HIV trans-mission is estimated to be 30-45% among breastfed infants [3, 4]. Postnatal transmission via breastfeeding accounts for almost half of pediatric infections [5] and is correlated with the duration of breastfeeding [6], with an estimated transmission rate of 1% per month of breastfeeding [7]. A randomized,

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controlled trial conducted in Nairobi, Kenya, estimated that in the absence of ART, the rate of HIV transmission via breast milk over the first 24 months of life was 16.2%, with the majority of infections occurring early during breastfeeding [8]. While replacing breastfeeding with formula feeding is associated with a substantial reduction in HIV acquisition risk in the absence of antiretroviral treatment [8, 9], breastfeeding has critical short- and long-term benefits for the infant, including reduced morbidity, reduced mortality, and improved growth and development. Additionally, there are benefits of breastfeeding for the mother, such as reduced postpartum bleeding, improved birth spacing, and reduced risk of breast and ovarian cancer [10].

High maternal viral load (VL) is strongly and directly associated with the risk of vertical transmission [11, 12] and infant disease progression [13]. Viral suppression attained through antiretroviral (ARV) therapeutic use during pregnancy and postnatally in conjunction with infant ARV prophylaxis has been shown to reduce the risk of vertical HIV transmission to less than 2% [14, 15]. However, a recent cross-sectional study of 10 sub-Saharan countries showed that ART coverage and adherence among pregnant and breastfeeding women remain sub-optimal [16]. In high-incidence countries, newly diagnosed maternal HIV infections account for an increasing proportion of infant HIV infections [17, 18]. Therefore, interventions that either replace or complement daily maternal ART and/or infant ARV prophylaxis are needed to eliminate vertical transmission of HIV, including addressing the need for infant prophylaxis during pregnancy and breastfeeding. In addition, while several HIV candidate vaccines are under study, an effective vaccine is still likely to be a decade or more away.

On the other hand, passively administered HIV broadly neutralizing antibodies (bnAbs) have shown promise for the prevention of sexually acquired HIV in adults. Notably, the landmark Antibody Mediated Prevention (AMP) trials [19] established that passive infusion of the CD4 binding site bnAb VRC01 can prevent the acquisition of HIV viruses sensitive to this antibody, with an estimated vaccine efficacy of 75% against viruses with a VRC01 $IC_{50} < 1$ μg/mL [20]. This result pointed to the need for bnAb combinations, especially with bnAbs that target distinct epitopes on the HIV envelope (*env*), to protect against the diversity of circulating HIV. Accordingly, dual and triple combination bnAb regimens are currently under investigation in early-phase studies in adults [21-26], including several studies within the HIV Vaccine and Prevention Trials Networks (HVTN/HPTN).

BnAbs as a prophylactic and therapeutic intervention against HIV are being evaluated in early phase trials in infants to establish their safety and pharmacokinetics. A recent prospective clinical trial conducted in Botswana tested the use of the dual bnAb combination VRC01LS and 10–1074 in children living with HIV [27] and found that infusion of two bnAbs may have prolonged virologic control following interruption of ART in infants with low initial viral DNA levels [27]. On the prophylaxis side, a phase I trial of single bnAb administration, either VRC01, VRC01LS, or VRC07LS in HIV-exposed infants is underway (NCT02256631) [28, 29]. Initial results indicate that VRC01 and VRC01LS have a good safety and pharmacokinetic (PK) profile [28, 29]. Trials testing bnAb combinations are also underway: IMPAACT 2037 will evaluate one and two doses of PGT121.414.LS and VRC07-523LS in HIV-exposed infants (impaactnetwork.org /studies/impaact2037). PedMAB1/2 is evaluating VRC07-523LS and CAP256V2LS alone and in combination at birth and at age 3 months in HIV exposed infants. SAMBULELO is a phase 2 study evaluating VRC07-523LS in HIV exposed and infected newborns. An additional IMPAACT study under development will evaluate VRC07-523LS, ePGT121v1-LS, and PDGM1400LS, alone and in combination, in HIV-exposed and unexposed infants. Most studies have evaluated subcutaneous administration; however, IMPAACT 2048 will also evaluate intramuscular administration.

Many stakeholders have called for additional programmatic investment in bnAbs to prevent vertical transmission of HIV, highlighting attributes that make bnAbs especially suitable in the pediatric setting: the short window of HIV exposure, the existing infrastructure for getting infants into care during the first 1-2 years of life, and the small doses needed for infant *vs*. adult HIV prophylaxis, which may help overcome antibody production and cost barriers [30, 31]. Additionally, the potential for less frequent administration is advantageous compared to the daily dosing of ARVs [32] and may improve adherence. Investment in local capacity to manufacture novel biologics and immunotherapies will lend further support for the program. The advent of Nirsevimab, a long-acting monoclonal antibody that prevents severe disease in infants caused by respiratory syncytial virus (RSV) infection, serves as a guiding example [33].

A barrier to advancing bnAb-based prevention of vertical transmission is the paucity of data on the phenotypic and genotypic characteristics of HIV transmitted to infants. As discussed below, a few panels of vertically transmitted viruses currently exist, and all were collected over a decade ago. Over the past decades, the genetic diversity of HIV has greatly expanded, with circulating recombinant forms (CRFs) alone globally increasing in prevalence by 8% between 2010 and 2021 [34]. Furthermore, HIV has also become more resistant to autologous sera [35] and bnAbs [36, 37]. The AMP trials [20] demonstrated that bnAb susceptibility varies by clade and geography [36]. In the pediatric setting, where infants are exposed to both viruses and antibodies from the mother through pregnancy or breastfeeding, vertically transmitted viruses may undergo additional selection pressures in addition to the genetic drift observed globally. Therefore, it is imperative to evaluate the sensitivity of bnAb neutralization to vertically transmitted viruses in areas with the highest infection burden. A multidisciplinary and global task force convened by the International AIDS Vaccine Initiative (IAVI) identified this as a key step necessary for advancing the infant bnAb program [30].

In this manuscript, we review the available data on vertically transmitted viruses, including risk factors for pediatric HIV infection, and identify knowledge gaps that remain to be addressed. We also characterize known virus features as well as the diversity of published sequence data from transmitted lineages. We use the term “transmitted lineage” instead of the more common “transmitted founder” to acknowledge that these viruses are samples from larger virus populations, where each sequence set represents a genetic lineage or family that shares a unique common ancestor.

2. SEQUENCE AND LITERATURE SELECTION

Because the focus of this review was characterizing HIV env sequences sampled from infants, one of the criteria we used to base our literature search was the availability of such sequences. Therefore, both literature and sequence searches were conducted together, using the sequence search interface available on the LANL sequence database (www.hiv.lanl.gov/components/sequence/HIV/search/search.html) as follows:

1. Under "Genomic region," select "Env CDS".
2. Select "Patient Information -> Risk Factor -> Mother->Child".
3. Submit query.
4. At the time of drafting the paper (June 2024), this search yielded 4,167 records.
5. We further selected "One record per patient" and obtained 271 records, sampled between 1985 and 2019.
6. Next, we manually reviewed all records and down-selected based on the following criteria:

a. Infant sequences had been sampled within one year of birth;

b. Most of gp120 was included in the sequence (*i.e.*, at least all five variable regions);

c. sequence(s) were linked to a PMID, retrievable using the accession number(s).

1. Using the above criteria, we collected the PMIDs of 74 papers from the sequence records, of which only 30 were mother-infant studies.
2. Of the 30, 10 had full env sequences from infants, of which 6 were confirmed postnatal transmissions. These were from the following mother/infant cohorts: WITS, MPH, ZEBS, VTS, CH009, and BAN. From the references of some of these papers, we later found two additional mother/infant cohorts: PACTG076 and NBT. See Table **1** for details and references.
3. Five out of the 30 papers had maternal sequences only.

3. SEQUENCE ANALYSIS

Analyses in this study used 139 full env nucleotide sequences from 13 infants and 226 from 13 mothers from Zambia [38], GenBank accession numbers: GU939124-GU939142, HM036739-HM037037, KY229265-KY229682; 195 V1-V5 env region sequences from 9 infants from Malawi [39], GenBank accession numbers: JN108036-JN108067, JN108203-JN108257, JN108284-JN108383, JN108481-JN108520, JN108549-JN108649, and JN108670-JN108760; and 119 V1-V4 env region sequences from 7 infants from Kenya [40], GenBank accession numbers: AY174897-AY175103. All infants tested negative at birth, and all were breastfed. The infants from Zambia and Malawi tested positive at 6 weeks of age, with no other testing done between birth and week 6, and were considered intra-partum infections. However, transmission via breastmilk could not be excluded. In addition to testing negative at birth and positive at week 6, the 13 infants from Zambia also tested negative at 1 month of age and were therefore considered breastmilk infections. While sequencing from later time points was also available for some infants, these analyses only considered sequences sampled at 6 weeks of age. All studies were conducted before the availability of ART; however, women and their newborn infants in the Malawi cohort received a single dose of Nevirapine. In the Zambia cohort, all women received Nevirapine, and all infants received cotrimoxazole prophylaxis from 6 weeks to 12 months of age.

3.1. Env Diversity and Mutation Rate Analysis

Sequences were aligned using the LANL tool Gene Cutter (www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html) and then manually revised to correct for software artefacts. Phylogenetic trees were obtained using IQ Tree software with ultrafast bootstrap branch support [41], and Highlighter plots were obtained using the Highlighter tool from the LANL database (www.hiv.lanl.gov/content/ sequence/HIGHLIGHT/highlighter_top.html). Viral diversity was measured via mean pairwise Hamming distances, defined as the number of mutations between every sequence pair and averaged over the total number of sequence pairs within each sample, per nucleotide site. Highlighter plots for each infant sample were used to identify the presence of recombinants and multiple transmitted lineages. When present, recombinants were excluded from the diversity analysis. When multiple lineages were detected, Hamming distances were calculated within lineages.

3.2. Fitting a Poisson Model

To estimate the early viral mutation rate in infants, we used the LANL tool Poisson Fitter (www.hiv.lanl.gov/ content/sequence/POISSON_FITTER/pfitter.html) with the inferred consensus as reference sequence and an estimated time of infection of 42 days for the infants from the Kenya and Malawi cohorts, and 14 days for the infants from the Zambia cohort. The model and methods for the Poisson Fitter tool are described elsewhere [42, 43].

4. RESULTS

4.1. Differences between Adult and Pediatric HIV Infections

There are immune features unique to the context of vertical transmission that need to be considered when evaluating the phenotypic and genotypic characteristics of the transmitted virus lineages. HIV acquisition by infants occurs in the presence of antibodies derived from the mother, either during pregnancy or lactation, and infants are exposed to viruses that have evolved and adapted to the maternal immune system and HLA repertoire. The latter overlaps with the infant's HLA repertoire and hence, at least in part, shapes the infant's immune responses [13, 44]—an important difference from adult transmissions, where the likelihood of sharing HLA alleles is much lower. Indeed, mother-infant HLA concordance has been found to increase the risk of vertical transmission [45], and, additionally, certain maternal HLA types and/or mutations have also been associated with an increased risk of vertical transmission [46, 47]. In adults, transmitted viruses tend to be more infectious compared to viruses sampled from chronic infections [48] but are generally more sensitive to autologous plasma from the transmitting partner [49]. On the other hand, in vertical transmissions, several studies have found that viruses transmitted either perinatally or via breastfeeding are generally resistant to parental autologous plasma [50-53], compared to non-transmitted parental variants. However, other studies have also found no difference [39].

The immune landscape in infancy differs from that in adults, likely affecting the viral genetic bottleneck as well as post-infection virus evolution and disease prognosis. Compared to adults living with HIV, young children living with HIV tend to have higher CD4 T cell counts, higher viral load peaks and set points, and higher rates of viral replication [54-57]. There are also notable differences in the nature and dynamics of autologous antibodies in pediatric HIV: a higher proportion of infants and children living with HIV generate bnAbs compared to adults [58, 59], and infants with HIV generate bnAbs faster than adults [58]. Infant bnAbs may therefore be induced more rapidly compared to adults [60, 61], and indeed they tend to be less mutated than those in adults [60].

The three possible routes of vertical transmission — in utero, intrapartum, and postnatal — involve biologically distinct mechanisms. In utero acquisition of HIV is believed to occur primarily through the placenta [62], whereas intra-partum infection likely occurs either through transplacental microtransfusion or viral exposure during passage through the birth canal and subsequent absorption of the virus through the infant's digestive tract [63]. On the other hand, postnatal transmissions *via* breastmilk are unique in that infants are exposed to both cell-free and cell-associated virus in the oral and gastroenteric tract [62, 64]. Multiple studies have demonstrated that, although viral exchange occurs between plasma and breast tissue [65-67], limited compartment-talization in the breast results in local replication and clonal amplification of nearly identical viral quasispecies within the mammary gland [66-70]. Therefore, multiple yet very similar transmitted lineages may initiate infection in the infant.

In summary, compared to sexual HIV transmission, vertical HIV transmission is unique: viruses replicate in the breast milk compartment, which comprises cell-free and cell-associated HIV particles; the infant's immune landscape is shaped by exposure to maternal antibodies and virus and potentially shared HLA alleles, and infants produce antibodies faster and with fewer mutations. These differences may mean that a bnAb combination selected to prevent sexual HIV transmission may have different efficacy at preventing vertical transmission.

4.2. Passive Immunization *via* Pregnancy and/or Lactation

Maternal immunization has been used as an effective means to protect the mother, the fetus, and the infant during the first few months of life against several pathogens [71]. Immunization of pregnant women living with HIV could potentially provide benefits to both the mother and the baby, as maternal antibodies are transferred across the placenta and to the gastrointestinal tract during breastfeeding, which could contribute to the prevention of vertical HIV transmission. This strategy for blocking transmission to the infant is potentially easier than immunizing the infant against the entire diversity of circulating HIV strains, as the enhanced maternal immunity would only need to target the autologous maternal viruses to block transmission effectively. In fact, autologous virus-neutralizing antibodies have been achieved by current HIV vaccine candidates [72]. They could leverage the phenomenon of immunologic imprinting by raising humoral immune responses against the original infecting HIV strain using a targeted immunogen [73].

Two phase I clinical trials conducted by the *AIDS Vaccine Evaluation Group (AVEG), AVEG104 and 102,* evaluated the safety and immunogenicity of recombinant HIV Env subunit vaccines administered to pregnant women living with HIV [74]. This gp120-based vaccine strategy did not enhance the ability of the plasma of pregnant women to neutralize autologous viruses compared to those that received placebo immunization [75]. However, in a non-human primate model of maternal immunization with Simian-Human Immunod-eficiency Virus (SHIV)- infected dams, heterologous gp120 immunization led to the enhancement of binding antibodies that predominantly recognized the Env of the originally infecting SHIV, indicating that antigenic seniority was leveraged by this vaccination in previously infected animals [73]. Thus, future perinatal HIV vaccine studies could further explore a strategy to boost maternal immune responses specifically against the virus that the infant is exposed to and prevent vertical transmission. While this is promising as a form of infant passive immunization, maternal vaccines have major challenges. There is emerging evidence that antibodies are not transferred equally across the placenta [76-78], and the extent and type of antibody responses transferred can differ by antigen [79].

Additionally, and partly related to this antibody “placental sieve”, viral escape from autologous virus neutralization needs to be considered to ensure complete transmission prevention and avoid selection of pre-existing resistant strains [51]. Recent studies indicate that antibody glycosylation may impact IgG transplacental transfer [77-79]. Therefore, a maternal vaccine would need to induce antibody specificity and glycoforms that not only fully block the maternal viral population but can also be efficiently transferred across the placenta.

4.3. HIV *Env* Gene Sequences from Mother-Infant Cohorts

Vertical transmissions happen over a known window of exposure. In theory, a prospective study of infants at risk of vertical transmission that applied frequent HIV testing during this exposure window would afford the unique opportunity to: (i) identify characteristics of vertically transmitted lineage(s), and (ii) design and evaluate targeted interventions, such as passive immunization with bnAbs to which most transmitted variants are susceptible. However, historically, such studies have been challenging to conduct, and to date, few studies have sequenced and characterized vertically transmitted viral lineages. Current GenBank entries of full *env* sequences in particular, where bnAb escape mutations are found, have been obtained from mother-infant cohorts that date from 2010 or earlier, and most include only clade C viruses (Table **1**). While subtype C is still the most prevalent subtype globally [80], other clades such as A, D, G, and many CRF forms are found

Table 1. Mother-infant study cohorts with published *env* sequences for infants acquiring HIV vertically. Note that only a small subset of the enrolled participants had *env* sequences generated.

-	Participants (Transmission Mode)	Cohort Name	Subtype	Country	Years	Enrolled	Primary Reference (PMID)
Mother/Infant Pair Studies	Mother/Infant (in utero and intra-partum)	Pediatric AIDS Clinical Trials (PACTG 076)	CRF01_AE	Thailand	1991-1993	477 women, 415 infants	Connor 1994 (7935654)
	Mother/Infant (postnatal)	Nairobi Breastfeeding Trial (NBT)	A, C, A/D, C/D	Kenya	1992-1998	425 women, 410 pairs	Nduati 2000 (10703779)
	Mother/Infant (in utero and intra-partum)	WITS: Women and Infants Transmission Study	B	US	1988-2004	788 women, 657 infants	Landesman 1996 (8628356)
	Mother/Infant (in utero and intra-partum)	MPH: Malaria and HIV-1 in Pregnancy	C	Malawi	2001-2003	480 women	Mwapasa 2004 (15096809)
	Mother/Infant (postnatal)	Zambia Exclusive Breastfeeding Study (ZEBS)	C, G	Zambia	2001-2004	1,200 women	Thea 2004 (15296810)
	Mother/Infant (postnatal)	Vertical Transmission Study (VTS)	C	KwaZulu-Natal (South Africa)	2001-2006	2,722 women, 1,132 infants	Coovadia 2007 (17398310)
	Mother/Infant (postnatal)	CHAVI CH009	C	Malawi	2008-2009	41 women	Salazar-Gonzalez 2011 (21191008)
	Mother/Infant (postnatal)	Breastfeeding, Antiretrovirals, and Nutrition (BAN)	C	Malawi	2004-2010	2,369 women	Chasela 2010 (20554982)
	Mother/Infant (postnatal)	SAMBULELO (Phase II VRC07-523LS clinical trial)	C	South Africa	2024-	Ongoing	McFarland 2021 (34009371)
Infant/Child Only Studies	Perinatal infections only	Perinatal/LILAC (NISDI)	B, F, F1, F2	AR, BR, PE, MX, JM	2002-2011	922 infants	Hazra 2009 (19036797)
	Infants, children, and adolescents	Pediatric/PLACES (NISDI)	B, F, F1, F2	AR, BR, PE, MX, JM	2007-2011	1,629 children	Hazra 2009 (19036797)
	Infants only, perinatally infected, early ART	Children with HIV Early Antiretroviral (CHER)	C	South Africa	2005-2013	377 infants	Cotton 2013 (24209829)
	Infants only, perinatally infected/exposed, early ART	TARA (Toward AIDS Remission Approaches)	C	Maputo (Mozambique)	2017-2020	89 infants	Lain 2022 (36360495) Dinh 2023 (38045254)

at a frequency of 9% or higher in countries like Kenya, Nigeria, and the DRC, where vertical transmission rates are still unacceptably high [81-85].

Two historical mother-infant cohorts contributed insights into the biology and diversification of vertically transmitted HIV in the pre-ART era: the Nairobi Breastfeeding Trial [8], conducted in Kenya from 1992 to 1998, and the Women and Infants Transmission Study (WITS) [86], conducted in the US from 1988 to 2004. The former cohort led to the publication of subtype A, C, and recombinant A/D and A/C *env* sequences from *in utero,* intra-partum and breastmilk transmission pairs [52, 53] (Table **2**), whereas *env* sequences collected from mother-infant intra-partum transmission pairs enrolled in the WITS cohort were all subtype B [51, 87]. Additional clade B full env sequences sampled in the early 1990s (1990-1995) were published by Kishko *et al*. from five mother-infant pairs, all of whom were infected at birth (Table **2**) [88]. Two of these mothers had been treated with ZDV at the time of delivery.

Through a GenBank search, we identified two additional sets of non-subtype C env sequences obtained from vertical transmission pairs, both of which were collected during clinical trials conducted in the 1990s. Samleerat *et al*. published gp120 CRF01_AE sequences from 17 mother-infant pairs infected perinatally (either *in utero* or at birth) [89]. All mothers had been enrolled in PACGT 076, a phase III randomized clinical trial part of the Pediatric AIDS Clinical Trials Group (PACTG) clinical trial network conducted in Thailand from 1991 to 1993 [90]. Verhofstede *et al*. [40] published partial env sequences (regions V1-V5) from subtypes A1, C, and D in 14 mother-infant pairs enrolled in the nonintervention arm of a prospective study conducted in Mombasa, Kenya, from 1996 to 1999 [91].

Table 2. Subtype, cohort, publication year, and reference, transmission route, number of transmission pairs, median number of env sequences, and subtype for the studies listed in section 2. Transmission route abbreviations: PP = peripartum, IU = *in utero*, and BM = breastmilk.

Subtype	Reference (PMID)	Year	Transmission Route	No. Mother/ Infant Pairs	Median No. Sequences per Infant (Range)	Cohort (Country)
A, C, D	Verhofstede (12584330)	2003	PP or BM	14	20 (4-36)	Kenya
A, C, A/D, C/D	Wu (16378985)	2006	PP or BM	8	3 (1-5)	Nairobi BF (Kenya)
A, C, A/D, C/D	Rainwater (17346133)	2007	PP, IP, BM	4	3 (1-5)	Nairobi BF (Kenya)
C	Kwiek (18427205)	2008	IU, PP	2	1, 9	MHP (Malawi)
C	Samleerat (21593171)	2011	IU, PP	19	27 (11-43)	MHP (Malawi)
CRF01_AE	Russell (18700833)	2008	IU, PP	17	11 (6-15)	PACGT 076 (Thailand)
A, C	Baan (21916748)	2012	IU, PP	7	12 (10-17)	Rwanda
C	Russell (23075434)	2013	BM	3	4 (2-6)	MHP (Malawi)
A, C, A/D, C/D	Mabuka (23856624)	2013	BM	12	2 (1-5)	Nairobi BF (Kenya)
C	Danaviah (25793402)	2015	BM	11	12 (3-16)	VTS (South Africa)
C, G	Nakamura (28122636)	2017	IU, BM	22	12 (4-23)	ZEBS (Zambia)
B	Kumar (29672607)	2018	PP	16	27 (15-44)	WITS (US)
B, C	Martinez (32156815)	2020	IU, PP	4	25 (20-47)	WITS/CH009 (US/Malawi)
B, C	Marichannegowda (34337555)	2021	IU	12	33 (7-53)	WITS/CH009 (US/Malawi)

Abbreviations: PP = peripartum, *in utero*, intra-partum, and breastmilk.

The Zambia Exclusive Breast-Feeding Study (ZEBS) was conducted in Zambia between May 2001 and September 2004 and enrolled close to 1,500 pregnant women living with HIV [92]. Mother-infant pairs received single-dose nevirapine (NVP). Nakamura *et al*. sequenced full-length *envs* (Table **2**) from 22 mother-infant transmission pairs enrolled in the ZEBS cohort, of which 13 were confirmed breast milk transmissions, six were in utero transmissions, and three were undetermined [38].

Three mother-infant cohort studies were conducted in Malawi throughout the first decade of the 2000s, all of which provided *env* sequences from *in utero* and peripartum transmissions, and two of which sequenced infant viruses acquired *via* breastmilk. The first cohort was the Malaria and HIV in Pregnancy study (MPH), a cross-sectional study of pregnant women living with HIV with and without placental malaria, conducted from 2001 to 2003 [93]. All sequences collected from other infant pairs enrolled in the MPH cohort were subtype C [39, 94].

Also from Malawi, the CHAVI 009 cohort study was conducted between 2008 and 2009 and enrolled 41 lactating women chronically infected with subtype C HIV [67]. Full-length *env* sequence analysis from 12 women allowed the identification of the transmitted lineages from seven infants who acquired HIV postnatally [95]. This study also highlighted the importance of sampling mother-infant viral populations as closely as possible to the time of transmission to make the most accurate inference of the vertically transmitted viruses. For one infant, the identification of the transmitted lineage was unambiguous, as both the mother and infant were sampled at the same time point, which was also the first time the infant tested positive for the infection. Additionally, one of the maternal sequences was identical to the transmitted lineage found in the infant. However, this was not the case for a second infant for whom sequencing was not done until 12 months of age, despite testing positive at 6 months of age. Within six months, the infant and maternal viral populations had significantly diverged, and the best inference of the transmitted lineage was made by selecting the closest maternal sequence via phylogenetic analysis [95].

The third and most recent mother-infant cohort from Malawi was the Breastfeeding, Antiretrovirals, and Nutrition (BAN) cohort, conducted between 2004 and 2010 [96, 97]. A final relevant study is the Vertical Transmission Study (VTS), which enrolled pregnant women with and without HIV in KwaZulu-Natal, South Africa, between 2001 and 2006 [98]. C2–V5 *env* region sequences were obtained from the infants' dried blood spot samples (DBS), all of whom had acquired HIV via breastfeeding [68].

In summary, HIV *env* sequences for infants who acquired HIV vertically are few in number and inadequately representative: they date from more than 10 years prior, do not represent all circulating subtypes, have uncertain attribution in terms of acquisition route, and, across all studies that published *env* sequences, fewer than forty sequences from transmitted lineages were from confirmed breast milk transmissions (Table **2**).

4.4. Env Mutation Rates and Viral Diversity in Early Infant Infection

The immune selection pressure exerted by the infant and the subsequent adaptation of the mother to the infant's immune environment play a role in the evolution of vertically transmitted viruses. In particular, vertically transmitted viruses replicate in an immune climate for which they have already been, at least partially, pre-selected: this can happen by escaping maternal autologous antibodies that are also passed on to the infant, and by having adapted to immune responses shaped by maternal HLA genes that are also, at least in part, shared by the infant. Antibodies passively acquired from the mother may further shape viral evolution in the infant. Additionally, compared to adults, infants and children who acquire HIV tend to have higher viral load peak and set point, even after ARV initiation [54-57]. In non-human primate (NHP) models of neonatal infection, higher viral loads correlated with higher viral diversity, suggesting a higher mutation rate compared to adults [99, 100]. On the other hand, mathematical modeling indicates that the replication rate in children is comparable to that observed in adults [101].

Over the past decades, several studies have measured the evolutionary rate of HIV in infants. Among studies that focused on the rapidly evolving *env* gene, early reports that sequenced the *env* C2-V5 region in infants from the US indicated the presence of highly homogeneous viral populations within the first two months of infection [102]. Subsequent studies that studied longitudinally sampled *env* sequences from vertically infected infants did not always report consistent findings. However, between-study comparisons are challenging due to differences in measures of viral evolutionary rates and/or divergence across studies. To fairly compare the estimated evolutionary rates in *env* found in the literature, when available, we downloaded the infant sequences and performed the same analysis across all studies. All together, we considered 139 full-length *env* sequences from 13 infants from Zambia; 195 V1-V5 *env* region sequences from nine infants from Malawi; and 119 V1-V4 *env* region sequences from seven infants from Kenya [38-40]. All sequences were from the infants who tested negative at birth. Subtype, country, and number of sequences per infant are outlined in Table **3**.

The infant cohort from Zambia was the group that had been infected for the least amount of time overall, as all infants tested negative at one month of age. Yet this cohort exhibited the highest within-lineage median diversity, 0.26% (range 0.09%-1%), compared to the other two cohorts, whose median diversity was 0.1% for both (range 0.01%-0.37% for the Malawian infants, and 0.05% and 0.7% for the Kenyan ones)—less than half that of the Zambia cohort (Table **3**). Additionally, only four of 13 infants in this cohort had at least one sequence that was identical to the inferred consensus sequence. In all other infants, all sequence pairs differed at one or more sites.

We measured the HIV evolutionary rate in these infants by estimating the *env* mutation rate under a Poisson model of random accumulation of mutations [42, 43] and assuming an infection time of 6 weeks for the infants who last tested negative at birth, and 2 weeks for those who last tested negative at 1 month of age (the time from the last negative test). The assumptions of such models were usually met in early infection, within the time window when these infants had acquired HIV. However, there were two infants in each cohort whose *env* diversity either did not conform to a Poisson distribution or there were too many recombinants to reliably subset the sequences into distinct lineages. For the remaining infants, the estimated evolutionary rate ranged from 1.2×10^{-6} and 1.2×10^{-4} mutations per site per generation cycle, with the highest values observed in the Zambian cohort. The median rates for the Malawian and Kenyan cohorts were 8.5×10^{-6} and $8.7 \times 10^{-6,}$ respectively, and 7.6×10^{-5} for the Zambian cohort (range 2.5×10^{-5} – 1.2×10^{-4} mutations per site per generation cycle; Table **3**).

In adult acute infections, the estimated mutation rate is of the order of 10^{-5}, while a mutation rate of the order of 10^{-4} is on the high end of the plausible spectrum [103-107]. Under a model of random accumulation of mutations in the very early phase of infection, Keele *et al*. estimate it at 2.16×10^{-5}, which was derived from Mansky and Temin after excluding APOBEC mutations and recombinants [42, 104]. The estimated mutation rates from the Kenyan and Malawian infants are at most half of this value (Table **3**). Notably, these rates would be underestimated if the infants had been truly infected post-birth, rather than at birth, which is possible given that all infants had been breastfed. However, examining the infants from Zambia, all of whom tested negative at birth and at 1 month of age and were therefore most likely to have acquired the virus *via* breast milk, only one had an estimated mutation rate similar to the adult estimate. In contrast, all other estimates yielded values ranging from one to two orders of magnitude higher (Table **3**). Among six infants, five of whom had a higher mutation rate than adults, no single sequence was identical to the overall consensus, suggesting that the common ancestor of the viral lineage occurred in the maternal population.

Table 3. Env characteristics and estimated mutation rates from studies of infants acquiring HIV vertically. Sequences were obtained from three published studies (38-40) according to the following criteria: all infants tested negative at birth and positive at one month of age or later; a region of approximately 1,000 nucleotides or more of *env* was sequenced from these infants.

Study	Infant ID	Subtype	Country	Env Region	No. Seq.	Last HIV Negative (All Positive at 6 Weeks)	Mean %Diversity**	Estimated Mutation Rate***
Verhofstede *et al.*	IP1	A	Kenya	V1-V4	11	Birth	0.05	5.3×10^{-6}
	IP2	A/D	Kenya	V1-V4	17	Birth	NA	NA
	IP3	A	Kenya	V1-V4	21	Birth	0.09	8.5×10^{-6}
	IP4	A	Kenya	V1-V4	16	Birth	0.05	4.5×10^{-6}
	IP5	A	Kenya	V1-V4	21	Birth	0.61	NA
	IP6*	A	Kenya	V1-V4	16	Birth	0.14	1.3×10^{-5}
	IP7	A	Kenya	V1-V4	20	Birth	0.12	1.1×10^{-5}

(Table 3) Contd…

Study	Infant ID	Subtype	Country	Env Region	No. Seq.	Last HIV Negative (All Positive at 6 Weeks)	Mean %Diversity**	Estimated Mutation Rate***
Russell *et al.*	312*	C	Malawi	V1-V5	32	Birth	0.37	NA
	819*	C	Malawi	V1-V5	40	Birth	0.06	NA
	874	C	Malawi	V1-V5	31	Birth	0.2	2.0x10^{-5}
	1100	C	Malawi	V1-V5	16	Birth	0.01	1.2x10^{-6}
	1846	C	Malawi	V1-V5	36	Birth	0.19	1.8x10^{-5}
	1945	C	Malawi	V1-V5	36	Birth	0.06	5.9x10^{-6}
	2038*	C	Malawi	V1-V5	43	Birth	0.09	8.7 x10^{-6}
	2684*	C	Malawi	V1-V5	26	Birth	0.06	7.8x10^{-6}
	2909	C	Malawi	V1-V5	32	Birth	0.09	1.3 x10^{-5}
Nakamura *et al.*	1B	C	Zambia	gp160	15	1 month	0.38	3.7 x10^{-5}
	2B*	C	Zambia	gp160	10	1 month	0.73	NA
	3B	C	Zambia	gp160	16	1 month	0.15	1.4x10^{-5}
	4B	C	Zambia	gp160	6	1 month	0.36	3.4x10^{-5}
	5B	C	Zambia	gp160	16	1 month	0.42	4.1x10^{-5}
	6B	C	Zambia	gp160	12	1 month	0.25	2.4x10^{-5}
	7B	C	Zambia	gp160	15	1 month	0.33	3.1x10^{-5}
	8B	G	Zambia	gp160	14	1 month	0.25	2.4x10^{-5}
	9B	C	Zambia	gp160	12	1 month	0.24	2.3x10^{-5}
	10B	C	Zambia	gp160	6	1 month	0.26	2.5x10^{-5}
	11B*	C	Zambia	gp160	6	1 month	1.00	NA
	12B	C	Zambia	gp160	7	1 month	0.09	8.5x10^{-6}
	22B	C	Zambia	gp160	4	1 month	0.13	1.3x10^{-5}

Note: * Multiple lineages detected (analyzed separately when over 3 sequences, otherwise minor lineages excluded from analysis). ** Defined as the mean percent number of mutations across all sequence pairs within an individual. Calculated on the most abundant lineage when multiple lineages were detected. NA when it was not possible to isolate recombinants. *** Defined as the number of new mutations from the founder lineage, per nucleotide, per day of infection. Calculated when diversity followed a Poisson distribution, assuming an infected time of 42 days (since birth) for the infants who last tested negative at birth and 14 days for those who last tested negative at 1 month. NA when the sample did not fit a Poisson distribution.

While the mutation rate estimates from the Zambian cohort trend higher compared to similar estimates from acute adult infections sampled within 2-4 weeks from infection, they are compatible with a scenario where multiple low-diversity viral variants, originating from a common ancestor in the mammary gland, are then transmitted to the infant *via* breastfeeding. In such a scenario, the most recent common ancestor originated in the maternal population, not the infant, and the increased diversity from mutations accumulated during replication in the mammary gland, rather than post-transmission, artificially inflates the estimates of the evolutionary rate.

Taken together, these results suggest that intra-partum infections have lower evolutionary rates compared to adult acute infections. On the other hand, the viral population associated with early breast milk transmission can be more complex, comprising multiple low-diversity variants that seed the infection. This causes the Poisson model to yield overestimated mutation rates, as the high similarity between variants makes it difficult to separate the distinct transmitted lineages. Finally, we should note that while variation across individuals is expected, additional factors, such as disease progression and the severity of immunosuppression, have been found to affect evolutionary rates in infants [13, 102, 108, 109].

4.5. Genetic Characterization of Vertically Transmitted Viruses

We have mentioned the importance of characterizing the phenotypic features of the transmitted lineages that initiate HIV infection across the different transmission routes. In infancy, the window of exposure is well understood. Under ideal conditions, frequent sampling from birth throughout lactation would enable early detection of the virus and higher precision in identifying the actual transmitted lineages. With less frequent sampling, the chance of sampling the virus early in the infection is lower, and the longer the time from infection, the greater the viral divergence from the initial founders. Vertically transmitted viruses undergo a genetic bottleneck, resulting in a more homogeneous viral population compared to the virus circulating in the mother. In subtype C infections, vertically transmitted viruses have fewer glycosylation sites in the env gene and shorter variable loops compared to maternal variants [53, 95, 110, 111]. While this genetic bottleneck has also been observed in sexual transmissions, the different biological pathways involved in vertical transmissions suggest that different strategies should be employed when identifying transmitted lineages [42]. For example, in the 13 established breast milk transmissions published by Nakamura *et al*. the *env* mean percent diversity ranged from 0.1% to 1% [38]. This is significantly lower than the diversity measured in the paired maternal viruses (1%-4.5% range, *p*=0.0002 by paired Wilcoxon test; Fig. **1**). In sexual transmissions sampled within a similar exposure window, and with similarly low viral diversity and comparable number of sampled sequences, the majority of infections were found to have been initiated by a single transmitted lineage [42, 112]. This was demonstrated by the fact that early viral diversity, within the first two months from viral exposure, increased over time following a Poisson distribution [42, 43].

In contrast, when looking at the breastmilk transmission pairs from Nakamura *et al*., while all had low viral diversity, eight out of 13 infants had one or more subgroups of sequences that either shared one or more mutations at a few distinct sites from the rest of the sequences, or a higher number of mutations than expected by random variation alone [38]. We chose mother-infant pairs 3 and 5 to illustrate these two different viral patterns in Fig. (**2**): both infants clearly show much lower diversity compared to the maternal sequences; however, in infant 3, we see a bulk of identical sequences and randomly scattered mutations across the remainder of the sequences (Fig. **2A**), whereas no two sequences are identical in Infant 5. In fact, if we were to calculate a consensus sequence out of this sample, it would not match any of the actual *env* sequences found in this infant (Fig. **2B**).

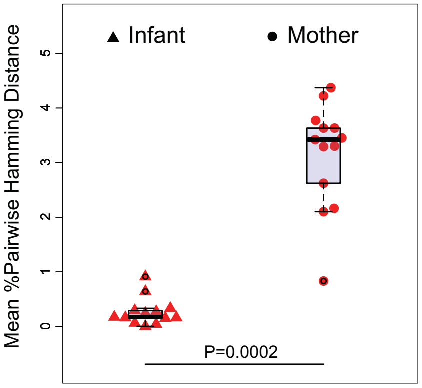

Fig. (1). Mean Percent Viral Diversity in Breastmilk Vertical Transmissions from 13 Mother-Infant Pairs in the ZEBS Cohort. Mean pairwise Hamming distances (defined as the number of mutations between every pair of env sequences within an individual and averaged over the total number of pairs of sequence for an individual) per nucleotide are shown for 13 mother-infant transmission pairs described in Nakamura *et al*. [38]. All infants tested negative at birth and at 1 month of age and positive at 6 weeks of age. Infant viral diversity is shown in red triangles on the left and maternal viral diversity on the right in red-filled circles. The *p*-value was obtained *via* a paired Wilcoxon test. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Fitting a Poisson model to the sequence diversity distribution allows us to infer an estimated time since the first viral replication in the new host, as previously described [42, 43]. Of the 11 infant sequence sets from these breastmilk transmissions that did follow a Poisson distribution, only one (infant 12B, with the lowest mean diversity; Table **3**) yielded an estimated time of infection within the time window between the sequence sampling time and the last negative time point. The other infant *env* sequences yielded estimated times of infection that were 2-10 weeks longer, suggesting that the true common ancestor of these lineages is to be found in the maternal viruses, not the infant's. Taken together, these findings indicate that the majority of these breastmilk infections were initiated by multiple transmitted lineages with genetically similar common ancestors, consistent with the infant being exposed and infected multiple times.

Nakamura *et al*. also compared the sequences from the 13 postnatal transmissions with those from six additional in utero transmission pairs [38]. They found that *in utero*-transmitted clade C gp160 sequences were shorter and less glycosylated than their corresponding maternal variants (*p* = 0.008 and 0.001, respectively), yet this was no longer true when comparing gp160 sequences transmitted via breast milk. A different study, which examined only the V1-V5 region of env, found that intra-partum transmitted clade C viruses had shorter env V1-V5 regions with fewer putative N-linked glycosylation sites compared to matched maternal sequences; however, the same finding was not observed for in utero transmitted viruses [39]. While these findings are not necessarily contradictory, given that Nakamura *et al*. compared in utero transmission with breast milk transmission, whereas Russell et al. compared in utero and peripartum transmission of viruses, the different results emphasize the need to obtain more *env* viral sequence data from vertical transmission pairs.

The issue of genetic diversity is particularly relevant in light of the latest deep sequencing technology, the Pacific Biosciences single-molecule real-time platform, also known as PacBio [113]. Until now, vertically transmitted virus sequences have only been studied using bulk PCR and cloning, or single-genome amplification (SGA), which, on average, yield 10-50 sequences per individual [42]. Utilizing unique molecular identifiers (SMRT-UMI), the PacBio technology was employed to sequence study participants enrolled in the AMP trials [20], generating an average of 270 sequences per sample. This sequencing depth led to the discovery of a higher rate of rare "minority" variants and an increased power to detect multiple transmitted lineages [Mullins J., *et al*., manuscript in preparation]. Studies employing PacBio sequencing technology to generate data on vertical transmission are underway. If consistent with the findings from the AMP trials, they will significantly enhance our ability to detect minority variants that would otherwise remain undetected [30]. It remains to be seen whether there are considerably more minority variants among vertically transmitted viruses and thus a wider range of viral phenotypes and features than previously described.

4.6. Role of Antibodies in Vertical Transmission

In the absence of ART, less than half of infants born to mothers living with HIV acquire infection, suggesting that

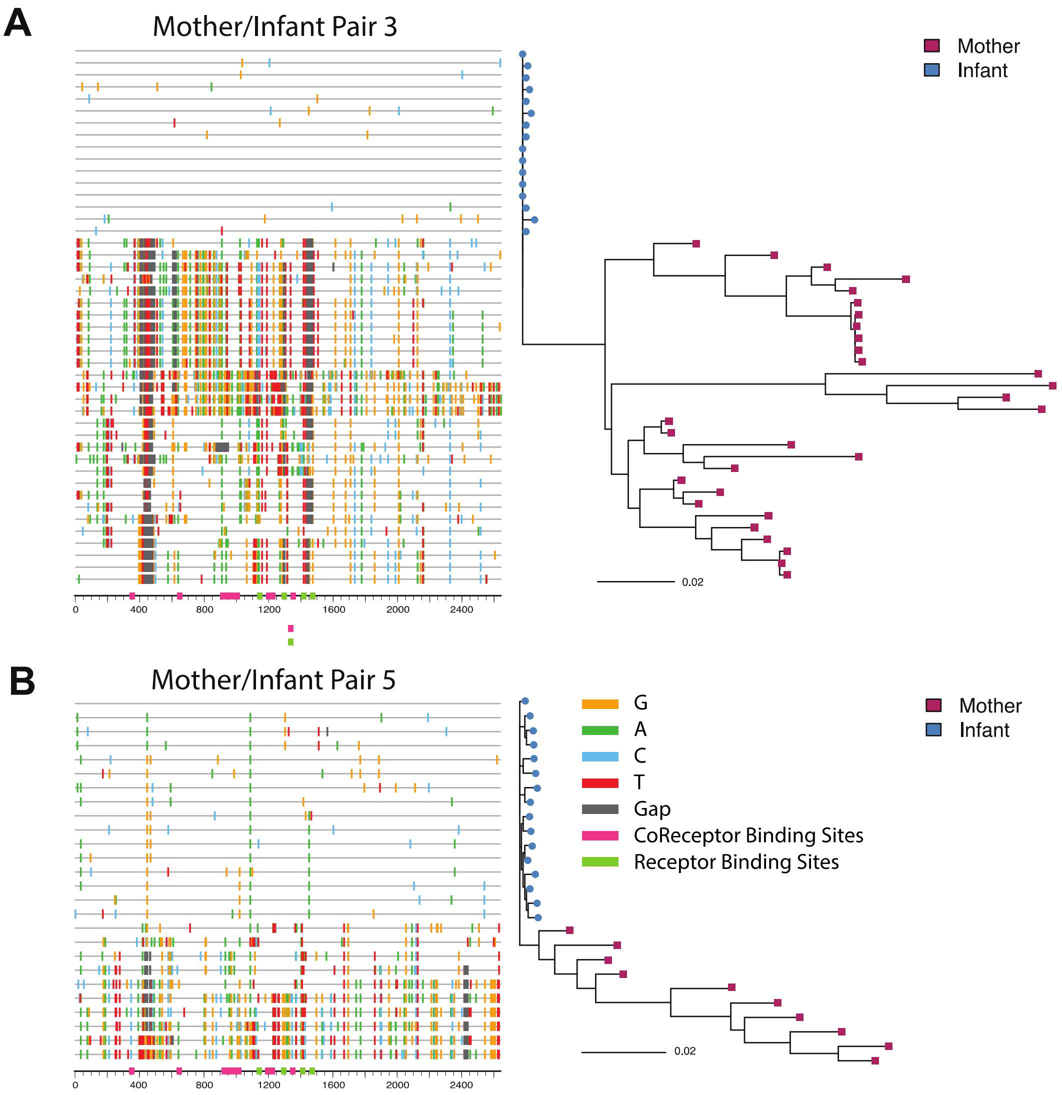

Fig. (2). Highlighter and Phylogenetic Tree of env Sequences from Two Breastmilk Transmission Mother-infant Pairs in the ZEBS cohort. Phylogenetic trees (right) were obtained using IQ-TREE software with ultrafast bootstrap branch support (41) and depict infant sequences as blue-filled circles and maternal sequences as filled red squares. Highlighter plots (left) were obtained using the Highlighter tool from the LANL database (www.hiv.lanl.gov) and depict each sequence as a line in the same order from top to bottom as shown in the phylogenetic tree on the right. Color-coded vertical ticks indicate mutations from the consensus sequence (shown at the top): green for A, aquamarine for C, orange for G, red for T, and gray for gaps. Green and magenta-filled squares at the bottom indicate the positions of the receptor and co-receptor binding domains, respectively. Both pairs exhibit far fewer mutations and shorter branch lengths in the infant compared to the mother. However, infant 3 (**A**) exhibits a more homogeneous viral population, with most sequences identical to the consensus. In contrast, infant 5 (**B**) shows that no two sequences are identical, and all infant sequence pairs share high similarity, with 3-10 mutations separating them from one another. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

maternal IgG responses may play a protective role [8-10]. In fact, vertical transmissions are the only HIV transmissions that occur in the presence of antibodies that co-evolved with the transmitted virus in the recipient. HIV Env-specific IgG antibodies are present not only in the maternal serum, but also in breastmilk, though at lower concentrations than plasma [114]. Characterizing maternal antibody responses and their role in vertical transmission is crucial in designing immunization strategies for use during pregnancy or lactation, as well as in assessing the impact of population-level immune-zation on vertical HIV transmission. Passive immunization strategies to prevent vertical transmission must not only block viruses from initiating infection, but also quell any viral escape route to avoid selecting for more resistant, hard-to-neutralize viruses that can potentially evade immunity.

Several studies have reported that viruses transmitted either perinatally or via breastfeeding are generally resistant to maternal autologous serum neutralization, suggesting that neutralization escape variants are preferentially transmitted [50, 52, 53, 75]. Shorter variable loops and fewer glycos-ylation sites have been generally observed in transmitted viruses when compared to later stage viruses [115-118]. In studies of subtype C adult transmission pairs, these charac-teristics also appear to make viruses more sensitive to antibodies from the transmitting partner [49]. This paradigm is reversed in some, but not all, studies of vertical transmission, where several studies have found that viruses transmitted either perinatally or *via* breastfeeding are generally resistant to maternal autologous plasma compared to non-transmitted maternal variants (although other studies have also found no difference [39, 50-53]).

Importantly, an inverse association between vertical transmission risk and the overall breadth or magnitude of maternal IgG responses has not always been confirmed [119, 120]. Some studies have reported an inverse association between maternal antibody responses and the risk of transmission, and have also shown evidence that the specificity of maternal reactions may play a role in protection. Using plasma samples from mothers whose infants acquired HIV and matched mothers whose infants did not acquire HIV from the WITS study, Permar *et al.* found that the magnitude of maternal IgG responses specific for the V3 variable loop was predictive of a reduced risk of vertical transmission [86, 121]. Subsequent studies from the same group found that the protective V3-specific IgG responses were directed at the C-terminal flank of the V3 crown, in particular, which could be a response generated *via* vaccination [122, 123]. However, the same association was not observed in Malawian mothers living with HIV who received ARV at delivery [124]. A subset of mothers from the same study (BAN), whose infants acquired HIV, was found to have significantly higher heterologous neutralization activity compared to mothers whose infants did not acquire HIV [125]. Interestingly, when looking specifically at pregnant women living with HIV with broad and potent neutralization activity, multi-specific bnAb responses or bnAb responses that target uncommon epitopes are more common among mothers whose infants did not acquire HIV [97].

The role of maternal HIV specific IgA in vertical transmission is also unclear. Kuhn *et al.* analyzed IgA concentrations in breast milk from 26 transmitting and 64 non-transmitting mothers living with HIV [126]. They detected HIV-specific IgA more often in transmitting mothers compared to non-transmitting ones. However, Pollara *et al.* found no significant difference in the magnitude of total plasma or breast milk IgA binding against a multiclade panel of HIV-1 Env antigens [127]. Analysis of 19 transmitting and 57 non-transmitting mothers from the International Maternal-Pediatric-Adolescent AIDS Clinical Trials Network Promoting Maternal-Infant Survival Everywhere (PROMISE) trial, Hompe et al. found no association between breast milk transmission risk and antibody responses, including breast milk Env-specific secretory IgA and plasma Env-specific IgA [128]. Overall, the impact of maternal HIV-specific IgA responses on vertical transmission deserves further investigation.

Several studies have looked at the neutralization sensitivity of vertically transmitted viruses against first and second-generation HIV bnAbs. Mabuka *et al.* tested 107 *envs* from mothers participating in the Nairobi Breastfeeding Trial and 10 infants (all of whom tested negative at birth, with nine testing positive at 6 weeks and one at 6 months of age) against a panel of 7 bnAbs: NIH45-46W, VRC01, b12, PGT128, PGT121, PG9, and PGT145 [129]. While they found no differences in bnAb sensitivity when comparing to *envs* from mothers or matched non-transmitting mothers, they did find that the vertically transmitted *envs* were significantly more sensitive to neutralization by V3 bnAbs PGT128 and PGT121 compared to heterosexually transmitted viruses sampled from the same region (Table **4**). No differences were found for the other bNAbs. They also found significant differences in neutralization sensitivity among vertically transmitted viruses when comparing clade A *envs* to clade C *envs*, consistent with previous observations that bnAb sensitivity varies across different HIV subtypes [130].

When comparing bnAb sensitivity of transmitted vs. non-transmitted viral variants isolated from the same mother, findings vary across studies (Table **4**). Rainwater *et al.* examined four breastmilk transmission pairs, all infected with subtype A, and found no difference in sensitivity against autologous plasma or monoclonal bnAbs 2G12 and b12 between the transmitted viruses and maternal non-transmitted viruses [52]. This negative result was likely due to the small sample size as later studies with a larger number of transmission pairs found significant differences: Wu *et al.* studied 12 mother-infant pairs infected with subtype A or C viruses, and a few with either A/D or C/D recombinant viruses, and found breastmilk transmitted *envs* to be among the most resistant variants against b12, 2G12, 2F5 and 4E10 compared to non-transmitted maternal variants [53]. Kumar *et al.* replicated these results for b12 and 2F5 (Table **4**), examining intra-partum transmission pairs infected with subtype B, and additionally found that intra-partum transmitted *envs* were more sensitive to V3 bnAb PGT121 and CD4bs bnAbs VRC01 and NIH45-46W [51].

Nakamura *et al.* [38] found no significant difference in sensitivity to V2 bnAbs PG9 and PG16 between transmitted and non-transmitted variants for *in utero* transmission pairs. However, postnatally transmitted *envs* (*via* breastmilk) were more sensitive to both bnAbs when compared to non-transmitted maternal variants. This latter result was consistent with Russell *et al.* who also found that breastmilk-transmitted variants were generally sensitive to VRC01 and resistant to 2F5 and 2G12, in line with previous reports [51, 53, 94]. All viruses tested in both studies were subtype C, except one infant in Nakamura *et al.* who was infected with subtype G.

The sensitivity of vertically transmitted variants to non-neutralizing functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC), may be another important determinant of the success of passive immunization strategies in preventing vertical transmission. Mabuka *et al.* [131] examined ADCC in the breastmilk of a small subset of Kenyan women living with HIV whose babies acquired (n=9) or did not acquire (n=10) the virus. ADCC-mediating antibodies were frequently detected in breast milk, and high breast milk ADCC capacity was associated with a lower risk of infant virus acquisition [131]. However, in a larger cohort of 72 mother-infant pairs, no association was observed between ADCC activity in maternal plasma and the infant's acquisition risk. Nevertheless, ADCC activity in the plasma of infants who became infected was associated with a lower risk of infant morbidity [132]. In the BAN study, no association was observed between breastmilk ADCC activity against a heterologous virus strain and infant transmission [127]. Still, Thomas *et al.* reported that infants with high ADCC activity against their maternal virus strains had a lower risk of acquiring the virus [133]. Subsequently, the latter group reported that infants with

Table 4. Summary of studies that compared the sensitivity of vertically transmitted viruses *vs*. heterosexually transmitted viruses or non-transmitted maternal viruses to individual HIV bnAbs. Studies are: Wu *et al*., Mabuka *et al*., Rainwater *et al*., Nakamura *et al*., Russell *et al*., Kumar *et al*.

bnAb	Epitope	Vertically Transmitted Viruses *vs*. Heterosexually Transmitted Viruses	Vertically Transmitted Viruses *vs*. Non-transmitted Maternal Viruses
NIH45-46W	CD4bs	no difference (Mabuka, BM)	more sensitive (Kumar, IP), no difference (Mabuka, BM)
VRC01	CD4bs	no difference (Mabuka, BM)	sensitive (Russell, BM; Kumar, IP) no difference (Mabuka, BM)
b12	CD4bs	-	more resistant (Wu*; Kumar, IP), no difference (Rainwater, BM)
PG9	V2	no difference (Mabuka, BM)	more sensitive (Nakamura, BM), no difference (Nakamura, IU; Russell, BM)
PGT145	V2	no difference (Mabuka, BM)	no difference (Mabuka, BM)
PG16	V2	-	more sensitive (Nakamura, BM), no difference (Nakamura, IU; Russell, BM)
PGT128	V3	more sensitive (Mabuka, BM)	no difference (Mabuka, BM)
PGT121	V3	more sensitive (Mabuka, BM)	sensitive (Kumar, IP) no difference (Mabuka, BM)
2G12	Glycan	-	more resistant (Wu*, Russell, BM), no difference (Rainwater, BM)
2F5	MPER	-	more resistant (Wu*, Russell, BM; Kumar, IP)
4E10	MPER	-	more resistant (Wu*; Kumar, IP)
10E8	MPER	-	sensitive (Kumar, IP)

Abbreviations: BM = breastmilk transmitted viruses, IP = intrapartum transmitted viruses. IU = *in utero* transmitted viruses [38, 39, 51-53, 129].

a combination of high ADCC activity and exposure to more ADCC-sensitive strains were less likely to acquire HIV [134]. Thus, it will be important to assess the sensitivity of vertically transmitted viruses to bnAb-mediated ADCC when developing bnAb combinations to prevent HIV vertical transmission.

Taken together, these results suggest that vertically transmitted viruses tend to be more sensitive to V2 and V3 bnAbs and potent second-generation CD4bs bnAbs, such as VRC01 and NIH45-46W, while being more resistant to membrane-proximal external region (MPER) bnAbs, inclu-ding 2F5 and 4E10, compared to maternal non-transmitted variants. However, as pointed out earlier, all viruses from these studies lack adequate representation and were obtained from cohorts sampled at least a decade ago; more recent studies will be needed to confirm the findings.

CONCLUSION

With infants still disproportionally affected by new HIV infections despite the advent of ARV therapy, new interventions are needed to eradicate vertical transmission of HIV. Following the AMP trials, which established that passive infusion of the CD4 binding site bnAb VRC01 can prevent sexual acquisition of VRC01-sensitive viruses [19, 20], dual and triple combination bnAb regimens are under early phase study in adults and infants (*i.e*., IMPAACT 2037) to establish their safety and pharmacokinetics [21-26]. Combination bnAbs targeting distinct Env epitopes have been advanced for testing based on their potent and broad coverage of multi-clade viral panels. However, these panels are comprised of viruses exclusively sampled from adult transmissions.

HIV susceptibility to bnAbs varies by clade, geography, and over time [130]. In fact, over the past decades, HIV has greatly diversified, with CRF forms alone increasing in global prevalence, becoming more resistant to autologous sera and bnAbs. [34-37]. Because vertically transmitted viruses are derived from adults, it is reasonable to hypothesize that they have undergone the same genetic drift. However, many factors differentiate the context of vertical *vs*. sexual transmission: (i) each vertical transmission route, whether the placenta, the birth canal, or the mammary gland, is biologically different than that of the genital mucosa; (ii) transmission *via* all three routes occurs in the presence of autologous antibodies that the infant passively acquires from the mother; and (iii) fetal and infant immune systems are distinct from the mature adult immune system. Therefore, vertically transmitted viruses undergo distinct transmission bottlenecks and early evolutionary pressure compared to viruses acquired sexually. Little is known about how these unique features affect virus phenotypes, particularly their neutralization sensitivity to candidate bnAbs and bnAb combinations. The vertically transmitted viruses that have been characterized were sampled and isolated more than a decade ago. Some of the viruses were sampled before the advent of maternal ART; they are not geographically representative, and they only represent a subset of the clades responsible for vertical transmission. Many phenotypic features, such as variable loop lengths and the number of glycosylation sites, have only been evaluated in clade C transmission pairs and may not necessarily apply to other subtypes.

The AMP trials have highlighted the need to update viral panels with more recent *env* sequences to represent better the global diversity of globally circulating HIV [36]. Additionally, sequences obtained using PacBio SMRT-UMI technology far surpassed the sampling depth of previous sequencing strategies, such as SGA [113]. It revealed a more diverse viral population in adults than previously observed during early infection. This sequencing depth, applied to infant samples, is likely to reveal minority variants that would have otherwise been undetected. Identifying and testing for *in vitro* neutralization of such variants is crucial in preparation for bnAb combination studies, as missing low-frequency viruses that carry pre-existing bnAb resistance could lead to the selection of more resistant strains. Deep sequencing will also better inform the differences between maternal transmitted and non-transmitted variants and across the three routes of vertical transmission-in utero, intra-partum, and *via* breastfeeding. Here, based on the env diversity of sequences sampled between 1996 and 2008, we uncovered striking differences in breastmilk-transmitted viruses compared to intra-partum-transmitted viruses: breastmilk transmissions were characterized by multiple low-diversity founder lineages. However, low-diversity minor variants may be present in all infants at frequencies so low that sampling sizes of 10-20 sequences per infant are insufficient to detect them. It is also possible that PacBio SMRT-UMI [113] technology will reveal even higher diversity in breastmilk infection and deepen the complexity of such infections.

In conclusion, we highlight the urgent need to collect more recent panels of vertically transmitted HIV viruses, across different subtypes, sampling these as early as possible in the course of infant infection and using accurate deep sequencing technology to characterize their genetic diversity, phenotypic traits, and, in particular, their neutralization sensitivities to HIV bnAbs that are candidates for future clinical testing.

AUTHORS' CONTRIBUTIONS

The authors confirm their contribution to the paper as follows: Manuscript conceptualization: EEG, MRA, GF, GJS, AG, SRP, HJ, TMM. Data collection and analysis: EEG. Literature collection and review: EEG, MRA, GF, GJS, AG, JIM, SRP, HJ, TMM. Figure and table preparation: EEG. Manuscript writing: EEG, MRA, GF, AG, JIM, SRP, HJ. Manuscript editing: EEG, MRA, GF, GJS, AG, JIM, SRP, HJ, TMM.

LIST OF ABBREVIATIONS

ADCC = Antibody Dependent Cell-mediated Cytotoxicity
AMP = Antibody Mediated Trials
ART = Antiretroviral Therapy
ARV = Antiretroviral
bnAb = Broadly Neutralizing Antibody
CRF = Circulating Recombinant Form
DBS = Dried Blood Spot
HIV = Human Immunodeficiency Virus
PLWH = People Living with HIV
SHIV = Simian- Human Immunodeficiency Virus
VL = Viral Load

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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