

## ABSTRACT

**Background:** Transmission through breastfeeding is an important cause of infant HIV-1 infections in developing countries; however, its mechanism remains largely unknown. We have explored the association between cell-free virus (CFV) and cell-associated virus (CAV) levels in breast milk, as reflected by viral RNA and proviral DNA, respectively, and the risk of infant HIV-1 infection after 6 weeks postpartum.

**Methods:** We matched 61 HIV<sup>+</sup> mothers who transmitted HIV-1 by breast milk to 61 HIV<sup>-</sup> non-transmitting mothers, based on infant's age at sample collection. CFV and CAV were quantified in a single breast milk specimen per mother, preceding the infant's first HIV<sup>+</sup> result and close to the estimated time of transmission. The C2-C5 *env* locus was amplified from the first positive blood sample and from breast milk HIV-1 CFV and CAV. Phylogenetic analysis was used to identify any preferential clustering of the transmitted infant viral sequences with either CAV or CFV in maternal milk. The role of N-linked glycosylation of the C2-C5 *env* region for transmission of either CFV or CAV through breastfeeding was also examined.

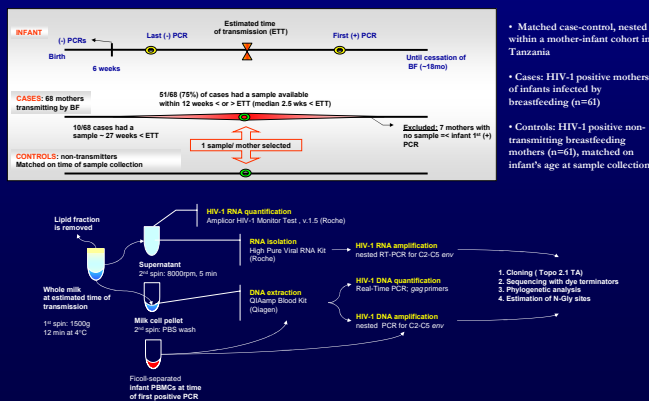
**Results:** CAV and CFV loads in breast milk were positively correlated (Spearman's rho = 0.46, p < 0.00001). In a conditional logistic regression model adjusting for maternal CD4<sup>+</sup> cell counts and HIV disease stage, each 10-fold increase in CFV or CAV load was significantly associated with about a 3-fold increase in breast milk transmission (OR = 3.24, CI 95% 1.5 to 6.76 and OR = 2.83, CI 95% 1.43 to 5.58, respectively). While CAV load was predictive of breast milk transmission both before and after 9 months postpartum (OR = 5.0, p = 0.03 and OR = 5.0, p = 0.01, respectively), CFV was a significant predictor of transmission occurring only after 9 months (OR = 0.08 and OR = 5.33, p = 0.008, respectively). Phylogenetic analyses of the C2-C5 *env* region showed that 85% (11 of 13) of infants harboring viruses that clustered with CFV in their mother's milk were infected after 9 months postpartum. A significantly larger number of milk HIV-1 CFV sequences had low N-linked glycosylation density in cases with phylogenetic evidence of CFV transmission (11 of 13) compared with cases transmitting CAV (6 of 16), Fisher's exact test p = 0.02.

**Conclusions:** A reduction in milk CAV and CFV loads might significantly decrease HIV-1 transmission by breastfeeding. Transmission of CFV seems to be facilitated at later lactation stages and might be associated with lower envelope glycosylation.

## BACKGROUND

Transmission of HIV-1 by breastfeeding accounts for a third of infant infections and cannot be prevented by current perinatal drug regimens. HIV-1 is present in breast milk of infected mothers both as cell-free viruses (CFV) and as infected cells (CAV); however, their relative role in transmission has been controversial and not well studied. Since the main carriers of HIV-1 in breast milk are long-lived cells that are also the major reservoirs of ongoing viral replication during HAART, determining the role of CAV in HIV-1 transmission might help design effective strategies for prevention of postnatal infections. Although CAV and CFV have been previously quantified in breast milk, no studies have specifically compared their effects on the risk of transmission through breastfeeding in the same population.

## DESIGN AND METHODS



• Matched case-control, nested within a mother-infant cohort in Tanzania

• Cases: HIV-1 positive mothers of infants infected by breastfeeding (n=61)

• Controls: HIV-1 positive non-transmitting breastfeeding mothers (n=61), matched on infant's age at sample collection

## RESULTS

**Table 1.** Both breast milk HIV-1 CAV and CFV loads are significantly associated with MTCT by breastfeeding

	Cases	Controls	Regression analysis of continuous CAV/CFV <sup>a</sup>		Regression analysis of binary CAV/CFV <sup>b</sup>	
			OR	95% CI	OR	95% CI
CAV load <sup>c</sup>	5.91 (19.94)	1.25 (4.23)	OR=2.83, p=0.003	CI <sub>low</sub> , 1.43-5.03	OR=5.00, p=0.001	CI <sub>low</sub> , 1.59-11.85
mean (SD) <sup>d</sup>						
CFV load <sup>e</sup>	2561 (5138)	618 (1780)	OR=3.24, p=0.002	CI <sub>low</sub> , 1.55-6.76	OR=5.50, p=0.002	CI <sub>low</sub> , 1.90-15.96
mean (SD) <sup>d</sup>						

<sup>a</sup> HIV-1 proviral copies per 10,000 cells;

<sup>b</sup> HIV-1 copies per ml;

<sup>c</sup> SD= standard deviation

<sup>d</sup> Univariate conditional logistic regression;

<sup>e</sup> CAV and CFV loads analyzed after a log<sub>10</sub> transformation

<sup>f</sup> CAV and CFV loads analyzed as high versus low using the respective median values among cases as cut off

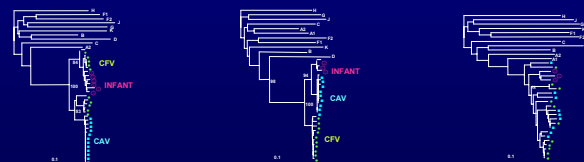
**Table 2.** Differences in the association of breast milk HIV-1 CAV and CFV levels with postnatal HIV-1 transmission occurring before and after 9 months postpartum.

	Transmission ≤ 9 months		Transmission > 9 months	
	Crude OR	Adjusted OR <sup>b</sup>	Crude OR	Adjusted OR <sup>b</sup>
High CAV <sup>a</sup>	5.0, p=0.038	6.09, p=0.04	5.0, p=0.011	5.29, p=0.05
95% CI	1.10-22.82	1.06-34.94	1.45-17.27	0.98-28.61
High CFV <sup>a</sup>	2.75, p=0.083	2.57, p=0.11	5.33, p=0.008	4.73, p=0.02
95% CI	0.88-8.64	0.81-8.21	1.55-18.30	1.25-17.86

<sup>a</sup> HIV-1 milk CAV and CFV levels analyzed as high (≥ median among cases) versus low (< median among cases)

<sup>b</sup> OR adjusted for CD4<sup>+</sup> cell counts at delivery and HIV disease stage at baseline

**Table 3.** Differences in phylogenetic clustering between HIV-1 clones from infected infants and HIV-1 clones from breast milk CFV versus CAV of their mothers according to time of postnatal transmission.



	HIV-1 CFV transmission	HIV-1 CAV transmission	Indeterminate
≤ 9 months postpartum	2	8	6
> 9 months postpartum	11	8	5
Total mother/infant pairs	13	16	11

one sided test of proportions p=0.03

• Mothers with phylogenetic evidence for transmission of CAV had higher CD4<sup>+</sup> cell counts at delivery (Fisher's exact p= 0.031)

• No preferential CFV vs CAV clustering was observed among HIV-1 subtypes A, C, D and their recombinants

**Table 4.** Low levels of virion C2-C5 *env* N-linked glycosylation are associated with higher risk of CFV versus CAV transmission by breastfeeding

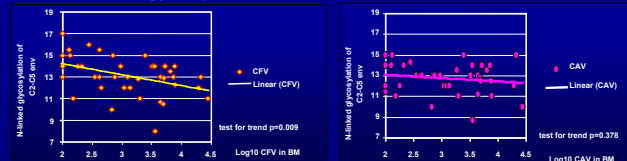
	CFV		CAV	
	N-Gly levels <sup>a</sup>	N-Gly levels <sup>a</sup>	N-Gly levels <sup>a</sup>	N-Gly levels <sup>a</sup>
	Low	High	Low	High
HIV-1 CFV transmission	11	2	9	4
HIV-1 CAV transmission	6	10	9	7
Fisher's exact test	p=0.02		p=0.07	
Mann Whitney test <sup>b</sup>	p=0.05		p=0.9	

<sup>a</sup> The number of N-Gly sites was estimated for each C2-C5 *env* clone within a transmitting mother and their average was used in subsequent analyses.

<sup>b</sup> N-Gly levels of CFV and CAV C2-C5 *env* clones from breast milk of potentially transmitting mothers were analyzed as high versus low using the mean level of glycosylation in each group as the cut-off

<sup>c</sup> N-gly levels of CFV and CAV C2-C5 *env* clones were analyzed as continuous variables

**Figure 1.** N-gly density of CFV C2-C5 *env* was inversely correlated with CFV load in breast milk. No trend was observed between the N-gly density of CAV C2-C5 *env* and CAV load in breast milk.



## INTERPRETATION

Some of the factors that could be responsible for enhanced CFV transmission via breast milk over time

- Decrease in anti-HIV IgM in milk over time.
- Drop in passive anti-HIV IgG in the infant after 9 months postpartum.
- Decrease in the concentration of antiviral compounds that inhibit CFV adsorption and cell entry (e.g., SLPI, MBL)
- Variation in cell types in milk over time (macrophages vs Ls) may lead to differences in the source of CFV and its characteristics such as *env* glycosylation.
- Variation in susceptibility of infant mucosa related to feeding practices, expression of coreceptors, etc.
- Variability of CFV shedding in breast milk

Some of the factors that could be related to enhanced transmission of CFV with low N-Gly *env* density

- Low N-gly density may reflect poor immunity
- Facilitated transmission of neutralization-sensitive virions in the absence of Ab titers in milk and/or infant blood.
- Improved access to receptor-binding domains and facilitated interaction with a particular cell type.
- Escape from innate immunity conferred by MBL.
- Facilitated productive infection of CD4<sup>+</sup>ve gingival keratinocytes.
- Low N-gly density may be a marker for local CFV production by a specific cell type (e.g., Ls by macrophages) that results in CFV with increased infectiousness.

## CONCLUSIONS

• Both HIV-1 CFV and CAV can be transmitted by breastfeeding. Each ten-fold increase in CAV and CFV levels in breast milk was associated with about a three-fold higher risk of HIV-1 transmission through breastfeeding.

• Breast milk transmission of CAV was associated with higher blood CD4<sup>+</sup> cell counts and could occur at any time during lactation; however, transmission of CFV seemed to be facilitated after the 9 month postpartum.

• Concentration of CAV and/or CFV was not the only determinant of transmission since no threshold could be detected and since infection with CAV could occur in the presence of low CAV/high CFV levels and vice versa.

• The mechanism of CFV transmission by breastfeeding is not clear; however, it seems to be associated with low levels of N-Gly of the virion envelope.

• A decrease in both CAV and CFV in breast milk is likely to be needed in order to prevent postnatal MTCT.