Original Article

Increased Cytomegalovirus Secretion and Risks of Infant Infection by Breastfeeding Duration From Maternal Human Immunodeficiency Virus Positive Compared to Negative Mothers in Sub-Saharan Africa

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Background. Breastfeeding imparts beneficial immune protection and nutrition to infants for healthy growth, but it is also a route for human immunodeficiency virus (HIV) and human cytomegalovirus (HCMV) infection. In previous studies, we showed that HCMV adversely affects infant development in Africa, particularly with maternal HIV exposure. In this study, we analyzed infants risks for acquisition of HCMV infection from breastfeeding and compared HIV-positive and HIV-negative mothers.

Methods. Two cohorts were studied in Zambia. (1) Two hundred sixty-one HIV-infected and HIV-uninfected mothers were compared for HCMV deoxyribonucleic acid (DNA) loads and genotypes (glycoprotein gO) in milk from birth to 4 months postpartum. (2) Maternally HIV-exposed and HIV-unexposed infants were compared for HCMV infection risk factors. The second cohort of 460 infants, from a trial of micronutrient-fortified complementary-food to breastfeeding, were studied between 6 and 18 months of age. Human cytomegalovirus seroprevalence was assayed, and logistic regression was used to calculate risk factors for HCMV infection, including maternal HIV exposure and breastfeeding duration.

Results. Human cytomegalovirus was detected in breast milk from 3 days to 4 months *postpartum*, with significantly raised levels in HIV-positive women and independent of genotype. In infants, HCMV antibody seroprevalence was 83% by 18 months age. Longer breastfeeding duration increased infection risk in maternally HIV-unexposed (odds ratio [OR] = 2.69 for 18 months vs <12 months; 95% confidence interval [CI], 0.84–8.59; $P = .03$) and HIV-exposed infants (OR = 20.37 for >6 months vs never; 95% CI, 3.71–111.70; $P < .001$).

Conclusions. Prolonged breastfeeding, which is common in Africa, increased risk of HCMV infection in infants. Both HIV-positive and HIV-negative women had extended milk HCMV secretion. Women who were HIV-positive secreted higher HCMV levels, and for longer duration, with their children at increased infection risk. Human cytomegalovirus control is required to maintain health benefits of breastfeeding.

Key words. breast milk; HCMV; HIV-exposed infants; infant HIV; maternal HIV.

Human cytomegalovirus (HCMV) is a cause of serious disease in infancy particularly with immunosuppression from human immunodeficiency virus (HIV) [1]. Congenital HCMV, acquired in utero, is the main infectious cause of mental retardation and neurodevelopmental impairment in neonates [1]. Postnatal infection, mainly via breast milk, can cause severe morbidity in some preterm or low birthweight babies [2–10]. Human immunodeficiency virus and HCMV coinfected children have increased neurological and respiratory disease, acquired immune deficiency syndrome (AIDS) progression, and death [11–13]. In sub-Saharan Africa where HIV is endemic, maternal

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HCMV plasma deoxyribonucleic acid (DNA) was linked to increased mortality in both mother and child in Kenya [14]. Human cytomegalovirus secretion in milk is associated with infant HIV transmission in South Africa and Malawi [15, 16]. In maternally HIV-exposed Zambian children, who themselves remained HIV-negative, we showed that HCMV was associated with lower infant growth and psychomotor development [17]. In Zimbabwe, HCMV milk secretion was related to growth faltering in maternally HIV-exposed children [18]. West African children infected with HCMV already express the HCMV "aged" immune phenotype, present in older Europeans, which may alter immune responses to infections and vaccines [19, 20].

Although maternal HIV exposure has been shown to increase HCMV congenital infection prevalence (from 1% to 4% [21–25]), the effects of maternal HIV on postnatal HCMV transmission via breastfeeding, the predominant route of infection, is unknown [26]. In women who are HIV-positive, a correlation has been made among milk HCMV loads, lower CD4 counts, reduced growth, and infant transmission [18, 27]. However, because comparisons have not been made to mothers who are HIV-negative, it is not known whether maternal HIV causes greater HCMV reactivation, load, or extended secretion in milk. In addition, the effects of breastfeeding duration, which varies greatly in women who are HIV-positive, are unknown. In Europe, breastfeeding of infants up to 3 months of age and raised HCMV viral load in breast milk increased risks of HCMV infection [3, 28]. In Africa, extended breastfeeding into the second year of life is common practice. In order to apply any intervention against HCMV, it is important to determine infection risk factors and their timing in HIV-positive versus HIV-negative women, especially in endemic regions of Africa where comorbidity is increased.

In this study, we examined breast milk directly for HCMV DNA loads and genotypes. In addition, we examined the association of breastfeeding duration with HCMV infant infection risks in Zambia, where maternal HIV exposure is frequent. To our knowledge, there is no previous research comparing the effects of maternal HIV status on secretion and transmission of HCMV in breast milk. In this study, we have compared both HIV-positive and HIV-negative mothers in order to understand the effects of breastfeeding practice and HIV on secretion and transmission of HCMV.

METHODS

Ethical Approval

This study was approved by the ethics committees of both the University of Zambia and the London School of Hygiene and Tropical Medicine, University of London.

Study Population and Protocol

Studies were conducted at Chilenje clinic in Lusaka, Zambia, in 2 cohorts: in the first cohort, studies examined extended HCMV secretion in breast milk; the second cohort focused on the physiological relevance in the longer term. Both cohorts were recruited from a similar region served by the clinic, many mothers participated in both cohorts, and there were similar antiretroviral therapy (ART) treatment protocols. At the time of the studies, local care standards included single-dose of nevirapine for HIV-positive mothers and their newborns. In the second, later cohort, ART was available to mothers with CD4 count <200 cells/μL, and towards the end of the study this was changed to <350 cells/μL, in accordance with revised National HIV treatment guidelines (Ministry of Health, Zambia). Only a few mothers in the study were on ART.

Breastfeeding Cohort. The Breastfeeding and Postpartum Health study investigated *postpartum* health among 387 (198 HIV negative, 189 HIV positive) women, from 2001 to 2003 [29]. Breastfeeding was a recruitment criterion, and all women were breastfeeding exclusively or predominantly. Milk samples were collected on 11 scheduled visits during the first 16 *postpartum* weeks and stored at −80°C. Two hundred sixty-one milk samples (from 118 HIV-positive and 143 HIV-negative mothers), collected at *postpartum* week 16, were available for our study. For a subset of 40 women (20 HIV-negative and 20 HIV-positive), we also analyzed samples collected at 5 earlier time points: day 3, and weeks 2, 4, 9, and 12 *postpartum*. Maternal HIV serostatus was determined antenatally using a serial testing algorithm, per local care standards [28].

Infant Cohort. The Chilenje Infant Growth, Nutrition and Infection Study (CIGNIS) was a randomized double-blind controlled trial of micronutrient-fortified infant foods (ISRCTN37460449; www.controlled-trials.com/mrct) and was conducted from 2005 to 2009. Infants (n = 811) were enrolled at 6 months of age and observed for 12 months [30]. Socio-demographic information was obtained using a questionnaire at recruitment. At recruitment and monthly, mothers were asked whether they were still breastfeeding or when they stopped [31]. Infant venous blood was collected in plain vacutainers, serum was separated, and antibody was assayed for HIV and HCMV at study completion (18 months age). Human immunodeficiency virus serostatus was determined using the local Ministry of Health-approved serial testing algorithm as described previously [17]. The ETI-CYTOK-G PLUS ELISA Kit (DiaSorin) was used to test for HCMV immunoglobulin (Ig)G, with standard curves plotted using control sera provided and then used to interpolate each sample IgG

titer; we considered HCMV IgG positive above a cutoff of 0.4 IU/mL.

Deoxyribonucleic Acid Extraction, Qualitative and Quantitative Polymerase Chain Reaction

Deoxyribonucleic acid was extracted from 200 µL homogenized milk using the QIAamp DNA Mini kit (QIAGEN) and eluted in 50 µL H_2O. Human cytomegalovirus glycoprotein *gB* gene was used for qualitative polymerase chain reaction (PCR) screening and quantification, human *GAPDH* gene was used as internal control, and hypervariable marker HCMV glycoprotein gO (gO) was used for genotyping [17, 32, 33]. Human cytomegalovirus DNA copy numbers were computed by TaqMan real-time assay, performed in triplicate on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems Inc.) [32]. Standard curves were generated from 10-fold serial dilutions of plasmid-cloned amplification products (pGEM-T Easy Vector Systems, Promega), normalized with an internationally certified clinical reference standard (National Institute of Biological Standards and Control, UK Medicines and Healthcare products Regulatory Agency, Potters Bar, United Kingdom). Levels detected below the sensitivity of the virus standard were recorded at a value of half the limit of detection. Each quantitative PCR (qPCR) reaction had 10 µL KAPA PROBE FAST Universal qPCR Master Mix (Kapa Biosystems), 1 µL probe (5 mM), 1 µL each of both forward and reverse primer (10 mM), 0.4 µL ROX Low, 7 µL dH_2O, and 5 µL template DNA. Cycling conditions were as follows: 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. All PCR assays included both positive and negative controls (reagents and water-only).

Genotyping

Human cytomegalovirus gO (*UL74* gene) genotyping was performed on a subset of 34 milk samples with sufficient remaining volume. Based on translated amino acid sequences, HCMV gO (*UL74* gene) has 8 distinct genotypes: gO1a, gO1b, gO1c, gO2a, gO2b, gO3, gO4, and gO5 [32, 33]. We used PCR-based genotyping with the gOup/gOlw primers, which detected all 8 gO genotypes. Nucleotide sequences were determined using Sanger methods [32, 33] and compiled using ChromasPro software. Multiple alignments used CLUSTAL and phylogenetic analysis via maximum-likelihood methods in MEGA6 [34].

Statistical Analysis

Programs Prism (version 6; GraphPad Software, Inc.) and SPSS (version 20.0; IBM Corp., Armonk, New York) were utilized to analyze milk HCMV DNA data by Student's *t* test and Mann–Whitney *U* test with 2-tailed *P* values and alpha = 0.05. Stata (version 11.1; StataCorp, College Station, Texas) was used in analyses of the infant cohort data, and odds ratios (ORs) and 95% confidence intervals (CIs) were obtained by logistic regression. We assessed associations of breastfeeding duration with HCMV antibody at 18 months, in a multivariable model. Analyses were adjusted for maternal education and socioeconomic status, as main effect modifiers as described [17], and stratified by maternal HIV status. To account for missing data, mainly from limited infant serum sample volumes, we also imputed missing HCMV results using multiple imputations with chained equations. The results imputed to account for missing data were similar to the main analyses (data not shown).

RESULTS

Increased Loads and Duration of Human Cytomegalovirus Milk Excretion in Human Immunodeficiency Virus-Positive Women

Human cytomegalovirus DNA measurements were made in 405 breast milk samples from 261 mothers at week 16 *postpartum* and a subset of 40 mothers at multiple time points. In the longitudinal subset (Figure 1), all mothers screened were HCMV positive by qualitative assay from day 3, and the median HCMV DNA in day 3 milk (colostrum or transitional milk) was not significantly different in HIV-infected compared with HIV-uninfected mothers (3.9 and 4.1 $\log_{10}$ copies/mL, respectively; $P = .90$). Deoxyribonucleic acid lactia then increased in both groups, reaching peak levels at week 4. In the HIV-negative

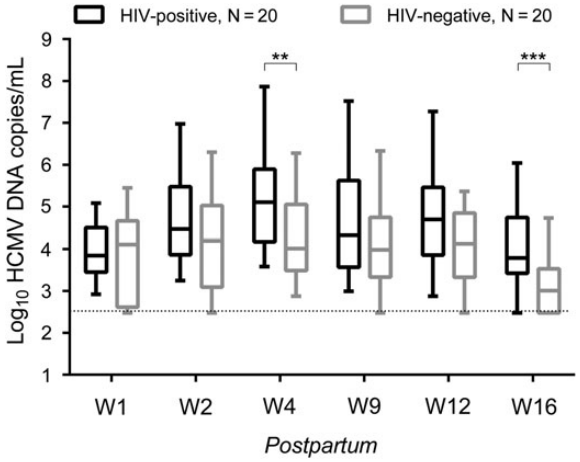

Figure 1. Human cytomegalovirus (HCMV) shedding kinetics in milk of human immunodeficiency virus (HIV)-positive and HIV-negative women. Comparison of milk HCMV deoxyribonucleic acid (DNA) kinetics between HIV-positive (black lines) and negative women (gray lines) over the first 16 *postpartum* weeks (n = 40, 20 in each group). Human cytomegalovirus DNA load, log copies/mL milk, increased from comparable levels in the 2 groups from day 3 (week 1 [W1]) to peak levels by week 4 (W4) postpartum. Sensitivity cut-off is indicated by the lower dotted line. Milk DNA loads from HIV-positive women were raised compared with HIV-negative women from W4. Box plots show the median and interquartile range using a Mann–Whitney *U* test, $^{**}P = .026$ and $^{***}P < .001$ indicate significant differences from W4 and week 16 (W16), respectively.

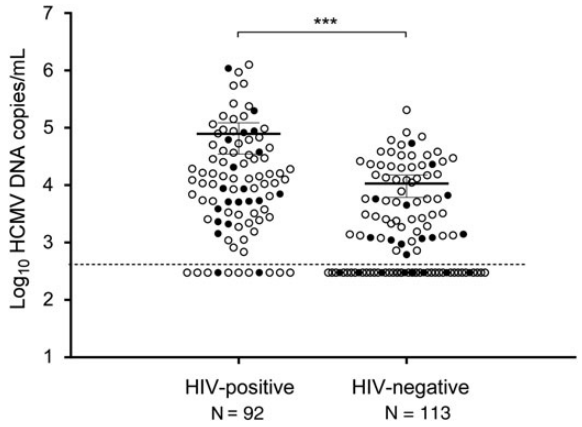

Figure 2. Human cytomegalovirus (HCMV) deoxyribonucleic acid (DNA) loads in week 16 milk samples, stratified by human immunodeficiency virus (HIV) serostatus. Scatter plot showing HCMV DNA levels in all available milk samples, at week 16 (W16) (92 HIV-positive and 113 HIV-negative). Human immunodeficiency virus-positive women had a significantly higher mean milk HCMV DNA load, approaching 1 log higher, compared with their HIV-negative counterparts. Furthermore, a higher proportion of milk samples from the HIV-positive group remained with detectable HCMV at this late time point (88.0% [81 of 92] vs 59.3% [67 of 113]; $P < .001$), with correspondingly decreased proportions below the limit of detection, indicated by the dashed line, in the HIV-positive compared with HIV-negative group (12% [11 of 92] vs 40.7% [46 of 113]). Means were 7.9×10^4 and 1.1×10^4 copies/mL in HIV-positive and HIV-negative women, respectively ($P < .001$, 2 sample, 2 tailed Student's t test with 95% confidence interval indicated by error bars), with similar differences in the W16 values for the subset with the complete kinetics from Figure 1 as indicated by black circles, mean 8.2×10^4 and 0.5×10^4 copies/mL for HIV-positive compared with HIV-negative women.

mothers, these loads declined gradually by week 16 to below day 3 levels, whereas in HIV-infected mothers, the DNA loads remained elevated. From week 4 to week 16, the median DNA loads had sustained increases in HIV-infected compared with the HIV-uninfected women ($P < .001$ by week 16), with over a 10-fold difference at peak levels recorded at week 4 ($P = .026$).

At week 16, HCMV was detected by the *gB* screening assay in 83.9% (99 of 118) of milk from HIV-infected women, versus 63.6% (91 of 143) in HIV-negative women ($P < .001$). Human cytomegalovirus DNA loads were quantified by real-time qPCR in 205 samples, all of which contained sufficient sample (with no demographic differences). Of these, there was a significantly higher proportion of HIV-infected women with DNA levels above the assay detection limit (88.0% [81 of 92] of HIV-infected versus 59.3% [67 of 113] among HIV-uninfected; $P < .001$). The mean DNA load was significantly higher in the HIV-infected group (7.9×104 copies/mL; 95% CI, 3.5×10^4 to 1.2×10^5) compared with the HIV-uninfected group (1.1×10^4 copies/mL; 95% CI, 0.6×10^4 to 1.5×10^4; $P < .001$) (Figure 2).

Human Cytomegalovirus Glycoprotein O Genotypes in Breast Milk

Human cytomegalovirus gO (*UL74* gene) genotyping was performed in a subset of W4 (peak HCMV DNA loads)

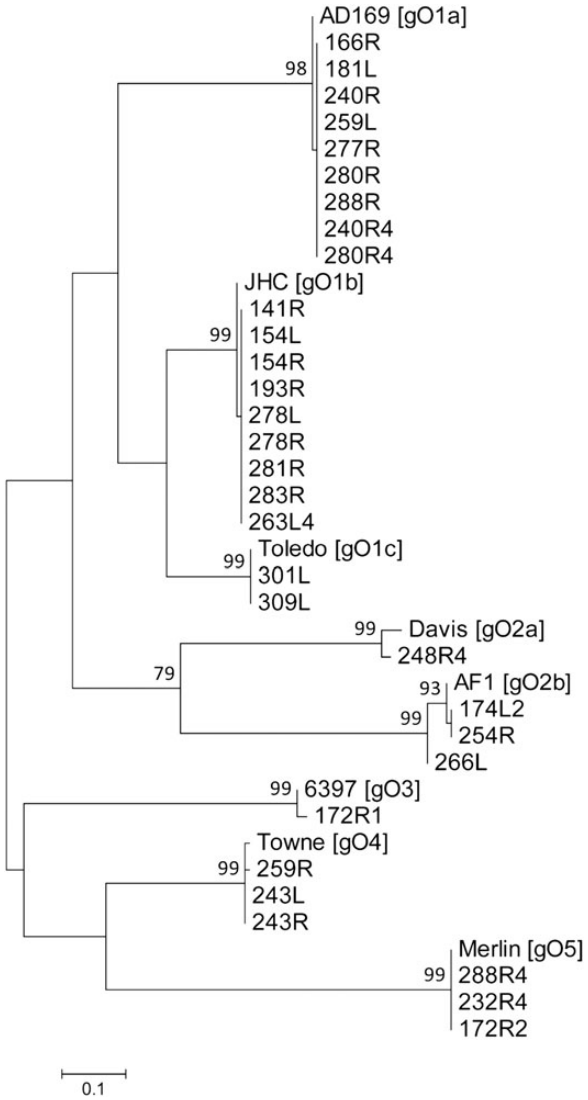

Figure 3. Phylogenetic analyses glycoprotein O (gO) genotypes. Representatives of genotype groups defined here were analyzed in comparison to reference strains, as described previously [32, 33]. Multiple alignments were performed using CLUSTAL in MEGA6 [34], followed by phylogenetic constructions inferred using the Maximum Likelihood method based on the JTT matrix-based model. The analysis involved 39 amino acid sequences and 154 positions in the final dataset. Reference strains for gO genotypes are indicated. Bootstrapping analyses indicate that major nodes are well supported.

and W16 (latest time point) milk samples. This included all samples with sufficient DNA and 7 samples paired at both time points. On the basis of encoded amino acid sequences, all 8 HCMV gO distinct genotypes—gO1a, gO1b, gO1c, gO2a, gO2b, gO3, gO4, and gO5 [32, 33]—were detectable in the milk samples. These were predominantly genotypes 1a, 1b, and 5, as represented by reference strains AD169, TR, and Merlin, respectively (Figure 3); genotype prevalence differences in HIV-positive compared with HIV-negative women did not reach significance (Supplementary Table S1). There was no evidence for higher viral load with any one genotype, although all appeared

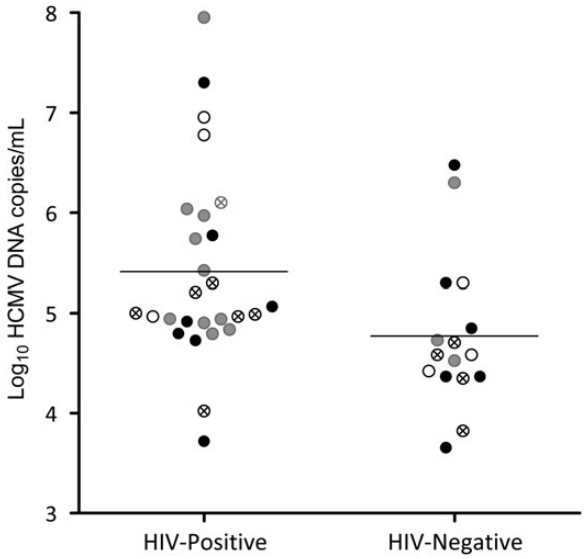

Figure 4. Genotype-independent increases in human cytomegalovirus (HCMV) load in milk from human immunodeficiency virus (HIV)-positive women. Viral loads per genotype were examined for HIV-positive and HIV-negative women. The main 3 glycoprotein O (gO) genotypes (gO1a, gO1b, and gO5) were plotted, and all the remaining genotypes were grouped together. All genotypes appear increased in HIV-positive women compared with HIV-negative women. Genotype gO1a (black circle), gO1b (gray circle), gO5 (white circle), other gO genotypes (crossed circle) are shown. Mean values are as follows: 2.6×10^5 and 5.9×10^4 for HIV-positive and HIV-negative women at both maximal and minimal secreted levels, at 4 and 16 weeks postpartum. Abbreviation: DNA, deoxyribonucleic acid.

raised in milk samples from HIV-positive women compared with HIV-negative women (Figure 4). Of the 7 paired samples, 3 had different genotypes detected at W4 and W16 (HIV-positive and HIV-negative).

Prevalence of Infant Human Cytomegalovirus Antibody at 18 Months of Age and Breastfeeding Duration

The effects of breastfeeding duration on infant HCMV infection were compared between HIV-positive and HIV-negative women in the CIGNIS infant cohort. Overall, 460 of 811 (57%) infant samples were available for HCMV antibody testing at 18 months age. Most infants were seropositive (384 of 460; 83%). As shown previously, we found no effect of micronutrient fortification (trial intervention) on HCMV antibody at 18 months, either overall or by maternal HIV status, but the prevalence of HCMV antibody significantly increased with decreasing maternal socioeconomic conditions or education and increased with longer breastfeeding duration, which were all measures adjusted in analyses of HCMV effects on growth [17]. Breastfeeding duration differed markedly between HIV-infected and HIV-uninfected women in this cohort, as shown previously [31]. Of the HCMV study subgroup analyzed here, only 3 HIV-negative women, compared with 29 HIV-positive women, never breastfed; and only an additional 6 HIV-negative women breastfed for less than 6 months. Therefore, to further analyze this result, we examined the effect of breastfeeding duration on HCMV antibody stratified by maternal HIV status. Among children of HIV-negative mothers, those who were still breastfeeding at 18 months had nearly 3 times the odds of HCMV antibody as those who had breastfed for <12 months (OR = 2.69; 95% CI, 0.84–8.59; $P = .03$) (Table 1). Even though it was a relatively small group who were uninfected by 18 months age, the children of HIV-positive mothers were now at significantly greater risk of early HCMV infection, as detected by antibody at 18 months, with prolonged breastfeeding (OR for breastfeeding >6 months compared with no breastfeeding = 20.37; 95% CI, 3.71–111.70; $P < .001$) (Table 1).

Table 1. Effects Maternal HIV and Breastfeeding Duration on HCMV Infection

	HCMV Infant Infection (Antibody)		
Months Breastfeeding	Antibody N (%)	Adjusted OR[a] (95% CI)	*P* Value
HIV-Negative Mothers			
<12[b]	25/32 (78.1%)	1	
12–17	128/161 (79.5%)	0.94 (0.35–2.53)	
18+	110/119 (92.4%)	2.69 (0.84–8.59)	.03
HIV-Positive Mothers			
Never	13/26 (50.0%)	1	
<6	31/35 (88.6%)	6.83 (1.69–27.6)	
6+	42/44 (95.5%)	20.37 (3.71–111.7)	<.001

Abbreviations: CI, confidence interval; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; OR, odds ratio.
[a] Adjusted for socioeconomic status and maternal education.
[b] Only 3 HIV-negative mothers never breastfed and only 6 for <6 months.

DISCUSSION

The study shows widespread HCMV infections in Zambian infants, from both HIV-positive and HIV-negative women. Human cytomegalovirus seropositivity was 83% by 18 months age, which is both higher and earlier than in many regions. Although similar to some other regions in Africa, this population has the added effect of endemic HIV. In Africa, the common practice of extended breastfeeding was identified as a risk for HCMV infection, which was increased for maternally HIV-exposed children. Furthermore, HIV-positive women had strikingly higher loads of HCMV secreted in their breast milk, with extended periods of raised levels, compared with HIV-negative women. There was some overlap so other factors may also influence secretion. This is comparable to studies from East Africa that reported high HCMV loads in HIV-infected women, although comparisons to HIV-negative women were not made [27]. Our studies have now compared both HIV-positive and HIV-negative mothers as well as considered breastfeeding duration in this sub-Saharan African setting. Similar to European and South East Asian surveys, our studies show

peak HCMV DNA levels at 4 weeks *postpartum* but differences in shedding duration. More HIV-positive than HIV-negative women had detectable HCMV secretion in breast milk, with initial reactivated levels equal at day 3, then raised from 2 to 16 weeks *postpartum*. Recent studies show HCMV-susceptible $CD14^+$ leukocytes increasing in breast milk at this time [35]. Thus, in milk secreted from HIV-positive women, there appears to be decreased immune regulation or increased susceptible cells, possibly from immune activation or inflammation. Studies in this cohort showed increased mastitis in HIV-positive women [29], and we have demonstrated increased secretion of HCMV. We further show that, in addition to increased risk of congenital infection from intrauterine HCMV infection [21-23], infants of HIV-infected women have increased odds of HCMV infant infection from breastfeeding. This can be a confounder for determining congenital infection, because diagnostic tests by saliva in those under 2 weeks of age may detect HCMV in saliva from breast milk. Our studies of birth prevalence of HCMV in Zambia using newborn saliva show 1% (1 of 100) prevalence in normal labor ward (K.G.M. and U.A.G, unpublished), lower than studies in the neonatal intensive care unit where breast milk could also be a source of early infection [25].

Low socioeconomic status and level of education were associated with HCMV seroprevalence in analyses of risk factors, similar to those reported elsewhere [17, 36]. With adjustment for these risk factors, breastfeeding up to 18 months remained significantly associated with HCMV infection. Studies in other continents show that almost all HCMV-positive women excrete HCMV in breast milk from local tissue reactivation, which is distinct from detection in plasma. Even with breast milk secretion in HCMV-positive mothers, the transmission rate varies, and 58%–80% infants were found to be seropositive by 1 year of age [26]. In Europe and Asia, studies have shown breast milk HCMV secretion up to 2–3 months *postpartum*, with higher milk viral loads and prolonged secretion linked to infant transmission [3, 28, 37]. In Zambia, we showed sustained breast milk HCMV secretion for over 4 months. Furthermore, increased breastfeeding over 6 months among HIV-positive women, or over 18 months among HIV-negative women, increased risk of infant HCMV infection. This shows that HCMV is secreted (or possibly more transmissible in breast milk) for longer duration than that reported in Europe or Asia. In HIV-negative women, longer duration of lactation may increase HCMV local reactivation and secretion. In HIV-positive women, secreted levels may be further raised through both HIV and HCMV immune dysregulation and amplified with breastfeeding duration.

Increased transmission during breastfeeding also allows for reinfections with multiple strains and widens the total population exposure to HCMV infection. We previously showed complex mixtures of HCMV strains, a potential factor for severe HCMV disease (currently under assessment) and a marker for burden of infection in this population, from blood or lung samples of HIV-positive infants [32]. We used gO for genotyping, one of the most variable genes in HCMV. Our genotype analyses showed that all 8 gO genotypes were detected in breast milk, demonstrating that genotypes were not constrained in this tissue compartment. There was also evidence for mixed-infections indicative of reinfection, which was confirmed using gN genotyping (data not shown). In HIV-positive women, viral load seemed to be raised independent of the genotypes secreted. The main milk gO genotypes 1a, 1b, and 5 were similar to prevalences we previously described in blood and respiratory compartments of primarily HIV-infected children in the same region and in other tissues from different global sources; differences in minor genotypes did not reach significance, although gO3 was greater in other tissues (Supplementary Table 1 and Supplementary Figure S1) [32, 33]. The analyses were limited by the small proportion of samples available for genotype analyses. These genotype ratios require further study, because the gO trimeric or alternate pentameric complexes with gH/gL glycoproteins affect host transmission and candidate vaccines [38, 39].

In the infant cohort, almost all HIV-negative women breastfed their infants, whereas a quarter of HIV-positive women never breastfed their infants, and overall HIV-positive women had significantly shorter breastfeeding durations than HIV-negative women (median 6 months vs 15 months, $P < .01$). Various reasons, including trying to limit infant HIV, were presented by the mothers [31]. This may have provided some protection from HCMV infection to children of HIV-positive mothers because those who never breastfed were only 50% HCMV positive, whereas breastfeeding at least to 6 months increased the prevalence of HCMV-positive children to 88.6% and over 6 months up to 95.5%. These differences in breastfeeding behavior between HIV-positive and HIV-negative women also meant that comparisons could only be made using different infant breastfeeding duration categories and are a limitation of the study. The 6-fold and 20-fold increase in odds of infection in adjusted analyses with breastfeeding to 6 months and over 6 months age, respectively, is in agreement with the viral loads measured in breast milk. Although limited from 2 different cohort studies, these were from the same residential area and study clinic. A study in Kenya showed that HCMV transmitters had a median of

5.4 compared with 4.5 logs copies/mL in milk for nontransmitters at 2 weeks *postpartum* [27]. Although it is difficult to apply exact thresholds, at similar times *postpartum*, in our Zambian cohort, HIV-positive compared with negative women had a median of 5.1 versus 4.0 logs copies/mL in breast milk. Furthermore, between day 3 and week 16, HIV-positive women had 23 measurements above 5.5 logs copies/mL compared with only 7 for HIV-negative women. This would indicate clinical relevance for the log differences in HCMV milk secretion of increased risk for transmission and at earlier times in the HIV-positive group.

In a separate analysis, we showed that HCMV adversely affected this infant cohort's growth and psychomotor development, particularly in maternally HIV-exposed children [17]. In Kenya, HCMV DNA in maternal plasma is a predictor of mortality in HIV-infected women and their infants [14], and lower CD4 levels were related to lower levels of milk HCMV required for transmission [27]. Although it was not measured, HCMV could be a factor in studies in Zambia where women with advanced HIV/AIDS who breastfed less than 4 months had improved infant survival [42]. In Zimbabwe, HIV-positive women with HCMV coinfection excreted both pathogens in breast milk, and HCMV infection correlated with higher levels of HIV ribonucleic acid [43]. Further analyses show HCMV milk secretion in HIV-positive mothers correlates with infant growth-faltering [18]. In United States, studies assessing earlier ART during pregnancy in HIV-positive women showed reductions in peri- or postnatal HCMV infection [44]. Although this was a non-breastfeeding population, the results support use of interventions to improve maternal health, such as earlier use of ART in HIV-positive women, which is now being applied in Zambia. However, recent studies in African breastfed populations showed that the use of antiretrovirals in HIV-positive mothers did not restrict HCMV transmission to their infants and therefore remained a risk for increasing numbers of HIV-exposed uninfected children [16, 18].

There are several limitations to our studies. The HCMV serostatus of the mothers was not known, although breast milk DNA PCR showed that all mothers were HCMV positive. We did not have CD4 levels, and these can affect milk secretion of CMV, as shown in studies of only HIV-positive mothers in Kenya [27], and may have contributed to varying HCMV levels in the HIV-positive mothers. We did not include day care status as a possible source for infant transmission via saliva [40]; however, in Zambia, infant day care is minimal to nonexistent. Other HCMV secretory routes, particularly saliva and also urinary [41] from siblings, can affect transmission, and 50% of infants became HCMV seropositive from HIV-positive women who did not breastfeed. However, we could not compare this result to HIV-negative mothers because almost all of them breastfed. Furthermore, most of our cohort mothers had children under age 5 who could be secreting HCMV; therefore, in general, background exposure was equal. We did not screen for prenatal HCMV, but this result would be only approximately 1% based on previous studies on newborns. The strengths of this study are the size of the cohorts and the comparison between both HIV-negative and HIV-positive groups, which clearly show raised levels of HCMV milk secretion and duration, with increased risks for infant infection in HIV-positive compared with HIV-negative women.

The World Health Organization recommends 6 months exclusive breastfeeding for ideal infant nutrition and immune protection. In Zambia, Demographic Health Survey data show that 73% of infants are exclusively breastfed for 6 months, and, overall, 98% of children breastfed with a median duration of 20.1 months [45]. Possible interventions against HCMV need to retain breastfeeding benefits. Direct HCMV inactivation in milk using ultrashort heat treatment has been evaluated in Europe, particularly for at-risk, premature, and underweight infants, and was found to be effective while preserving the nutritional and immunological qualities of breast milk [46]. This intervention warrants assessment in the sub-Saharan Africa setting, where it could be useful for concurrent inactivation of HCMV and HIV in breast milk, for at risk groups. Administration of anti-HCMV drugs to mothers to lower milk HCMV load is another potential intervention, but this requires efficacy and safety trials; no anti-HCMV drug to date is licenced for use during lactation. Improved hygiene could lower complementary routes of transmission, including saliva or urine [47]. Furthermore, there are several promising vaccines currently in development [48, 49].

CONCLUSIONS

We conclude that longer breastfeeding duration over 6 to 18 months increases HCMV infant infection. We also showed that HIV-positive compared with HIV-negative women had both raised breast milk secretion and likelihood for infant infection. Interventions to reduce HCMV infection could be considered, particularly in countries with high HCMV and HIV prevalence, because breastfeeding remains critical for infant health.

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Supplementary Data

Supplementary materials are available at the *Journal of The Pediatric Infectious Diseases Society* online (http://jpids.oxfordjournals.org).

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