Morbidity, mortality, and gut virome ecology of Kenyan children exposed to versus not exposed to maternal HIV

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Abstract

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Mother-to-child transmission of HIV has decreased rapidly with the expansion of optimized antiretroviral therapy (ART) regimens for all individuals living with HIV, resulting in a growing population of children who are HIV-exposed but uninfected (CHEU). Historically, CHEU have experienced greater morbidity and mortality than children who are HIV-unexposed uninfected (CHUU). The etiology of these differences remains unclear, but likely includes a combination of maternal and child biological, socio-behavioral, and environmental factors. With universal ART, many mothers living with HIV initiate optimized ART before pregnancy, which has been shown to improve maternal immunology and general health, and to lower overall morbidity and mortality among CHEU in the first years of life. However, few studies have directly compared the incidence and mechanisms of morbidity and mortality among CHEU and CHUU in the current era of optimized, universal ART to assess whether historic differences in these outcomes persist. Such data are needed to inform new directions of investigation and intervention that support the health of CHEU worldwide.

To address this gap, this dissertation leveraged data from the Linda Kizazi Study—a Nairobi, Kenya-based cohort of healthy mother-infant pairs followed from the third trimester of pregnancy through two years postpartum—to describe and compare outcomes among CHUU and CHEU born to women living with HIV on ≥6 months of optimized ART. In Chapter 2, we used survival analysis methods to compare the risk of acute diarrhea, respiratory tract infections, malaria, hospitalization, and all-cause mortality between CHEU and CHUU. In Chapter 3, we used survival analysis methods to determine the probability, timing, and correlates of primary CMV infection among all infants and by HIV exposure status. In Chapter 4, we described the ecology of the infant gut "virome" (collection of all viruses) and assessed whether the richness (number of unique viruses), relative abundance, and alpha and beta diversity of viruses differed between CHEU and CHUU.

In the Linda Kizazi cohort, most mothers living with HIV initiated ART before pregnancy and most CHEU received antiretroviral and cotrimoxazole prophylaxis. The incidence of respiratory tract infections, including pneumonia, in the first two years of life was lower among CHEU than CHUU; this association may have been mediated by exclusive breastfeeding since a significantly greater proportion of CHEU than CHUU were exclusively breastfed for 6 months. However, there were no significant differences between CHEU and CHUU in mortality or other measures of morbidity (acute diarrhea, malaria, hospitalization). There also was no association between HIV exposure and the probability or timing of primary CMV infection, though both CHEU and CHUU had a two times greater risk of CMV acquisition for every $\log_{10}$ increase of CMV DNA in their mother's breast milk. Compared to CHUU, CHEU had lower gut virome richness and a lower relative abundance of several Anelloviruses (a family of commensal viruses), but the alpha and beta diversity and relative abundance of other viral families were similar between HIV exposure groups.

Findings from this dissertation suggest that universal optimized ART can successfully reduce morbidity and mortality among otherwise healthy CHEU, resulting in outcomes similar to their CHUU peers. Our results differ from historic cohorts in the pre-ART era, which showed greater morbidity and mortality and earlier CMV acquisition among CHEU than CHUU. To our knowledge, this is the first study to describe the gut virome of CHEU; our data suggest that the overall gut virome composition in infancy is not substantially altered by HIV exposure despite some differences in the presence versus absence of specific taxa, but further study is needed to understand why there were some differences in overall richness and the relative abundance of Anelloviruses. Overall, these results emphasize the need to continue expanding access to optimized ART for all women living with HIV and provide additional evidence that supports current recommendations to exclusively breastfeed all infants for 6 months.

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CHAPTER 1. INTRODUCTION

Epidemiology & Health Consequences of HIV Exposure

Mother-to-child transmission of HIV has decreased since the 2015 WHO recommendation for all individuals living with HIV to initiate lifelong optimized antiretroviral therapy (ART) (1), resulting in a growing population of children that are HIV-exposed, uninfected (CHEU). From 2000 to 2018, the number of CHEU ages 0-14 years increased from 6.8 million to 14.8 million, about 90% of whom reside in countries across sub-Saharan Africa where rates of new HIV infection are highest; in comparison, an estimated 1.7 million children live with HIV worldwide (2). Throughout the HIV pandemic, CHEU have experienced higher incidence of developmental delays, adverse health outcomes, and death in the first years of life compared to children that are HIV-exposed, uninfected (CHUU) (3–5). Mechanisms for these differences between CHEU and CHUU remain unclear, and few studies have assessed whether these differences persist in the current era of optimized, universal ART.

The etiology behind the differences in morbidity and mortality between CHEU and CHUU is likely multifactorial, resulting from various maternal and infant social-behavioral, environmental, and biological factors. To further decrease global incidence of morbidity and mortality in children under age 5, there is a need for novel research that explores how HIV exposure contributes to increased morbidity and mortality among CHEU. Morbidity and mortality among mothers living with HIV may affect their ability to care for an infant. HIV infection can be associated with poverty, food insecurity, and housing instability, which increase malnutrition and exposure to pathogens for both mothers and infants (4,5). Uptake of effective ART and infant antiretroviral (ARV) prophylaxis allows mothers living with HIV to safely breastfeed, which encourages initiation of breastfeeding and longer duration of exclusive breastfeeding (EBF). EBF is associated with improved infant growth and development compared to formula feeding, which without clean water

sources, increases risk of diarrheal disease in many global regions (6). However, disparities remain in breastfeeding initiation and duration between CHEU and CHUU (7,8).

Additionally, direct exposure to HIV through breastfeeding or while *in utero* may instigate adverse immune responses among CHEU. Individuals with HIV have high levels of immune cell activation and inflammation; similar immunological characteristics have been observed among CHEU (9,10). Exposure to maternal immune dysregulation and/or HIV during pregnancy may "prime" the immune system to be pro-inflammatory and have altered adaptive immune biology. In turn, these changes may exacerbate CHEU's risk of both infectious and non-infectious disease.

HIV Exposure & Cytomegalovirus

HIV infection and exposure are associated with increased risk of infection with cytomegalovirus (CMV), a ubiquitous virus that is often acquired in early childhood and that infects most individuals by adulthood (11). Though disease is generally mild or asymptomatic, congenital CMV infection is possible and is a leading cause of childhood hearing loss and neurodevelopmental delays (12). CMV also is pro-inflammatory and may exacerbate other causes of childhood morbidity (11). Reinfection with new strains or reactivation of latent CMV is possible throughout life, thwarting efforts to develop an effective vaccine for prevention (13) and current antiviral treatments for children with congenital or severe disease are limited (14).

Improved understanding of the correlates of postnatal CMV infection will facilitate development of non-medical interventions to delay or reduce CMV infection among all infants until effective treatments and/or vaccines are available. Additionally, CHEU have had higher incidence of congenital CMV and earlier acquisition of postnatal CMV infection than CHUU throughout the HIV pandemic (15) because women living with HIV shed higher levels of CMV in saliva, the genital tract, and breast milk than women without HIV (16). This difference in risk may contribute to the historically higher morbidity and mortality among CHEU than CHUU. Immune reconstitution

mediated by long-term, optimized ART may reduce maternal CMV viral load compared to historical cohorts, thus reducing the risk of vertical transmission to infants in the current era of universal, optimized ART (15). However, new studies are needed to test this hypothesis, and to identify and compare the most relevant correlates of CMV among today's CHEU and CHUU.

HIV Exposure & the Gut Virome

The consequences of HIV on infant immune development may also interrupt the carefully balanced interactions between immune cells and the body's microbial communities. Such disruptions in the gut mucosa can lead to microbial dysbiosis, elevated enteric inflammation, and greater susceptibility to disease (17). Data on how maternal HIV infection affects the infant gut microbiota, either directly through HIV exposure or indirectly from immune consequences of infection, are limited (18), but this pathway could be a mechanism by which CHEU's health, growth, and development are altered.

The human microbiome is composed of bacterial, viral, fungal, protist, and archaeal communities that coexist with our own cells in nearly every biological compartment of the body (19). Recent improvements to nucleic acid extraction and sequencing technologies have led to an explosion of microbiome research, with most focus directed to the bacterial microbiome—the collection of all bacteria. However, attention is turning toward these other microbes and their consequences on health. The virome—the collection of all prokaryotic and eukaryotic viruses—is of particular interest due to the known effects of viruses on human health, the potential for bacteriophages to disrupt the bacterial microbiome, and the ubiquity of viruses in our environments (20).

Most virome studies to date have explored gut viruses. Current data suggest that infants are born without a gut virome (21), but that the gut is quickly seeded by environmental exposures in the first weeks of life and becomes more complex over the first few years (22–24). Specifically,

the number of different viruses and their abundance increase into adulthood when the gut virome becomes relatively stable (25,26). However, there is a need for larger, longitudinal studies from diverse populations to support the consistency of these findings.

Factors such as diet, environmental exposures, health conditions, immunology, and genetics may influence gut virome composition over the life course (27). Research quantifying the associations between the gut virome and health is nascent, but early evidence suggests shifts in viral ecology are associated with diseases such as malnutrition, ulcerative colitis, diabetes and colorectal cancer, likely due to interaction between viruses and intestinal immune cells (28). Among adults living with HIV, low CD4 count was associated with reduced gut microbial diversity regardless of ART (29), suggesting the immune effects of HIV may contribute to changes in gut virome composition. To date, no research has characterized the virome of CHEU or compared virome ecology between CHEU and CHUU, but such data could help uncover biological mechanisms by which morbidity occurs among CHEU.

Dissertation Rationale & Aims

The rapid evolution of the HIV pandemic and our tools to combat it has limited the relevance and comparability of existing data on health differences between CHEU and CHUU. While undoubtedly one of the greatest public health successes, ARVs, drug doses and regimens, and ART implementation policies at global and national levels have changed extensively over time. The resulting heterogeneity in HIV treatment and prevention obscures our ability to identify potential causal mechanisms explaining higher morbidity in CHEU by violating the consistency assumption of children's HIV exposure.

Observational research is further complicated by selection bias and unmeasured confounding that is inherent to many study designs. Before universal ART, women living with HIV were more likely to differ from women not living with HIV in risk factors and health outcomes,

which reduced exchangeability between groups of CHEU and CHUU. Additionally, existing studies of CHEU have limited concurrently-collected longitudinal data on maternal HIV treatment, EBF, and health outcomes (e.g., 30–32) that are needed to assess time-varying confounding by the significant developmental and behavioral changes in the first years of life. Together, these limitations in the existing literature impede our ability to advance research on CHEU. To inform new directions of investigation and intervention, there is a need for additional prospective longitudinal studies with rigorous epidemiologic methods conducted in the current era of universal, optimized ART.

This dissertation is nested in the Linda Kizazi Study, a Nairobi, Kenya-based cohort of mother-infant pairs that originally was designed to characterize how the virome is transmitted vertically when mothers are taking long-term, optimized ART regimens. Women living with HIV who had ≥6 months of ART and women without HIV from the same neighborhood were recruited prospectively in the third trimester of pregnancy and followed through two years postpartum. This cohort provides a unique opportunity to compare morbidity, mortality, and the gut virome of CHEU whose mothers have received long-term ART and their CHUU peers through the following aims:

Aim 1: To assess whether incidence of morbidity and mortality in the first two years of life differ between children born to women living with HIV on optimized, lifelong ART and children born to women not living with HIV among a cohort of 211 Kenyan infants. We used survival analysis methods to compare the risk of acute diarrhea, respiratory tract infections (including pneumonia), malaria, hospitalization, and all-cause mortality between CHEU and CHUU from birth to two years. *Hypothesis: CHEU will have a higher incidence of morbidity and mortality compared to their CHUU peers.*

Aim 2: To evaluate correlates and timing of CMV infection in the first two years of life among Kenyan CHEU and CHUU. We tested infant saliva/urine and maternal saliva/breast milk for CMV DNA using quantitative PCR. We used survival analysis methods to compare the overall probability and timing of primary, postnatal CMV infection between CHEU and CHUU, and to identify correlates of among all infants and among CHEU and CHUU separately. *Hypothesis: CHEU will acquire CMV earlier than CHUU and have higher probability of primary CMV infection. Higher maternal breast milk CMV viral load will predict infant CMV infection.*

Aim 3: To compare longitudinally the taxonomic richness and diversity of the gut virome in Kenyan CHEU versus CHUU over the first two years of life. We sequenced the DNA virome in infant stool samples to characterize the gut virome of CHEU and CHUU. We used generalized estimating equations to compare the richness and Shannon (alpha) diversity of viral taxa, and we used principal coordinates analysis and PERMANOVA to compare Bray Curtis distances (beta diversity) between CHEU and CHUU. *Hypothesis: CHEU and CHUU will have similar gut virome richness and diversity at birth. By 18 months, CHEU's gut virome will have lower richness and diversity than CHUU.*

Impact & Innovation

Findings from this dissertation will direct new research on the biological mechanisms by which HIV exposure affects infant outcomes. To our knowledge, this project was the first to describe the gut virome of CHEU and to compare the gut virome between CHEU and CHUU. Few prospective, longitudinal studies have evaluated whether there is a relationship between HIV exposure and CMV infection in healthy infants in the era of universal ART. Results from our analysis on the correlates of primary CMV infection will guide development of new interventions to reduce CMV transmission in early life when infants are most at risk of adverse outcomes from

infection. In all aims, we conducted well-designed prospective, longitudinal analyses to compare CHEU and CHUU outcomes *in the era of universal, optimized ART*, an assessment for which data are needed urgently to inform development of new interventions that improve children's health.

The parent study (Linda Kizazi Study) design overcomes several limitations of prior investigations. Our participants were enrolled from a small catchment area with similar socioeconomic and environmental exposures, so we expect to have comparable groups of CHEU and CHUU to minimize confounding by these factors. Information on maternal ART, infant ARV prophylaxis, maternal viral load and CD4 count, and infant feeding were captured concurrently with clinical data and specimens, which allowed us to account for the effect of and explore interactions between these factors. Additionally, all enrolled mothers received ≥6 months of ART prior to enrollment, most initiated ART prior to pregnancy, most received the same ART regimen during pregnancy and the neonatal period (TDF+3TC+EFV for 89% of mothers), and nearly all infants received ARV and cotrimoxazole prophylaxis, allowing for similar experiences with HIV exposure among the CHEU subgroup.

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CHAPTER 2. The Linda Kizazi Study: A comparison of morbidity and mortality from birth to two years between children who are HIV-exposed, uninfected and HIV-unexposed in the era of universal ART

The Linda Kizazi Study: A comparison of morbidity and mortality from birth to two years between children who are HIV-exposed, uninfected and HIV-unexposed in the era of universal ART

ABSTRACT

Background: Historically, children who are HIV-exposed, uninfected (CHEU) have been found to have greater morbidity and mortality than children who are HIV-unexposed, uninfected (CHUU). To assess whether this difference persists in the era of universal antiretroviral therapy (ART), we conducted a cohort study to compare the risk of acute diarrhea, respiratory tract infections (RTI), malaria, hospitalization, and all-cause mortality between Kenyan CHEU and CHUU from birth to two years.

Methods: From December 2018-March 2020 at Mathare North Health Centre in Nairobi, we recruited pregnant women living with HIV on ART for ≥6 months and pregnant women without HIV from the same community. We followed the mother-infant pairs for 2 years postpartum and collected data on symptoms of illness, clinical visits and diagnoses, and infant feeding every 3 months; a self-selected subset of participants also received weekly data collection for up to 1 year. We compared the risk of each outcome between CHEU versus CHUU using hazard ratios (HR) from Andersen-Gill (recurrent morbidity outcomes) and Cox proportional hazards (mortality) regression models adjusted for maternal age, marital status, and education level.

Results: Among 187 mother-infant pairs with postpartum data, 86 (46%) infants were CHEU and 101 (54%) were CHUU. All initiated breastfeeding, and 88% of CHEU and 57% of CHUU were exclusively breastfed (EBF) for ≥6 months. There was no significant difference in risk of diarrhea (HR=0.79, 95% CI 0.52-1.22), malaria (HR=0.44, 95% CI: 0.16-1.21), hospitalization (HR=1.11, 95% CI 0.30-4.14), or mortality (HR=1.87, 95% CI 0.17-20.5). However, CHEU had lower risk of any RTI (HR=0.60, 95% CI 0.44-0.82) and pneumonia (HR=0.29, 95% CI 0.091-0.89).

Conclusions: In this healthy cohort, CHEU born to women on effective long-term ART experienced similar overall morbidity and mortality as CHUU in the first two years of life. However, CHEU had substantially lower risk of pneumonia and other RTI, possibly due to longer EBF in this group.

INTRODUCTION

Over the last decade, expanded access to antiretroviral therapy (ART) for all people living with HIV has increased the population of children who are HIV-exposed but uninfected (CHEU) (1). About 90% of CHEU reside in sub-Saharan Africa where overall HIV burden and pediatric morbidity and mortality are high (2). Though CHEU are at lower risk of adverse health outcomes than children living with HIV, historically studies consistently have found them to have greater morbidity and mortality than their HIV-unexposed peers (1,3).

The etiology of worse outcomes among CHEU relative to children who are HIV-unexposed, uninfected (CHUU) is often unclear and likely arises from various combinations of exposures including antigenic exposure to HIV virus and maternal immune dysregulation from HIV (4,5), *in utero* and early life infections (6,7), exposure to antiretroviral drugs (8–10), and environmental and social factors associated with HIV (11). Multiple studies have shown that CHEU born to mothers with lower CD4 count, higher HIV viral load, and/or co-infections such as CMV during pregnancy had greater risk of diarrhea, pneumonia, hospitalization, and death than CHUU, especially in the first year of life (12–14). Risk of these outcomes appears to be mitigated when mothers initiate combination ART before or during pregnancy, mothers and CHEU receive cotrimoxazole preventive therapy (CPT), and infants are breastfed (1,11), especially if exclusively for at least six months as recommended by the WHO (15).

Understanding the relative contribution of individual risk factors to CHEU's morbidity and mortality has been complicated by rapid changes to HIV testing, treatment, and prevention strategies (1). Consequently, data from existing studies are heterogenous across time and global region, and typically do not reflect recommendations for the current era of universal ART. Today, over two-thirds of children in sub-Saharan Africa are born to women receiving ART during pregnancy, an increasing number of whom initiated ART before conception (16). Mothers receiving long-term optimized ART regimens are more likely to achieve viral suppression, have less inflammation and immune dysregulation, and experience fewer co-infections (17), which may

reduce risk of atypical immune development and susceptibility to infection among CHEU (4,5,18). In limited resource settings, mothers with HIV are also encouraged to breastfeed since optimized ART regimens suppress HIV viral replication and prevent transmission through breast milk (19,20). Breastfeeding decreases infants' risk of mortality, diarrhea, and respiratory disease and is associated with healthy growth and development (19,21–23), benefits that can now be extended to more CHEU. Together, these interventions are changing our understanding of CHEU's health; however, current data on morbidity and mortality among CHEU are limited.

To address this gap, this study leverages data from the Linda Kizazi Study—a prospective cohort of mother-infant pairs in Nairobi, Kenya—to compare morbidity and mortality in the first two years of life between CHEU and CHUU born to women living with HIV that received ≥6 months of stable ART initiated before or during pregnancy.

METHODS

Cohort Design and Participants

Human subjects approvals for Linda Kizazi Study procedures and analyses of study data were obtained from the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (P472/07/2018) and the University of Washington Institutional Review Board (STUDY00004006). Participants provided written informed consent for all procedures.

Enrollment took place from December 2018-March 2020 at Mathare North Health Centre, a maternal and child health (MCH) clinic in a densely populated, low-income neighborhood of Nairobi, Kenya. Women in their third trimester of pregnancy (28-42 weeks gestation) attending antenatal or HIV care visits were recruited and eligible for participation if they were 18-40 years old, planned to breastfeed, and if living with HIV, had received ART for ≥6 months. Women were excluded if they had planned a Caesarean section, had a serious medical condition, or had taken antimicrobial (within 2 months) or immunosuppressive (ever) medications other than those recommended for HIV prevention or treatment.

Women and their infants were followed from delivery through two years postpartum. All mother-infant pairs received a home visit approximately four days postpartum, followed by clinic visits in our established research clinic at Mathare North Health Centre. Clinic visits were timed to coincide with Kenya's pediatric immunization schedule (24) at week 6, week 10, month 6, and every three months thereafter. At enrollment, a subset of participants self-selected to receive additional weekly home visits from delivery through 12 months, and a second, overlapping subset of participants later self-selected to receive additional weekly visits between September-December 2021 for data collection related to COVID-19.

Data Collection

Information on participant sociodemographic characteristics, current health, and obstetric history was collected at enrollment. For women living with HIV, the study performed CD4 testing at enrollment and every 6 months postpartum. Delivery information was abstracted from delivery facility medical records and/or the participant's MCH booklet, which serves as a portable pregnancy health and infant immunization record in Kenya.

At all visits, women were asked to self-report recent (since the last study visit) and current symptoms of illness, diagnoses, and medications for themselves and their infant. Women were also asked to describe infant immunizations and feeding, including duration of exclusive breastfeeding (EBF) and when new foods were introduced. To reduce self-report bias, study clinicians asked women to respond yes or no to a specific list of possible symptoms, checked medication containers, and compared infant information with the MCH booklet when possible. At clinic visits, mothers and infants also received a physical exam. If needed, new diagnoses were made, medications were prescribed, and/or participants were referred for further care. Information about referrals and other outside medical care was captured at the next study visit. Hospitalizations and deaths were self-reported and recorded in adverse events reports to the ethics committees; no hospitalizations or deaths were related to study procedures.

Patient and Public Involvement

A community advisory board (CAB) was convened during study development to solicit input on all aspects of the cohort's design and implementation including: best practices for participant recruitment, consent, retention, and reimbursement; the anticipated acceptability and feasibility of study procedures to the study population, including the frequency and length of follow-up visits; approaches to encourage partner and family support for women's participation; expectations for maintaining participant privacy and confidentiality, especially during home visits; and ways to facilitate and enhance collaboration between the research team and the local community. CAB members included local chiefs, community health workers, representatives from health organizations working in the Mathare North neighborhood, healthcare administrators, and Mathare North Health Centre leadership, clinicians, and laboratory staff. Study participants and other patients at Mathare North Health Centre were not involved in study design.

Quarterly meetings with the CAB and Kenya-based study team members were held throughout follow-up for additional feedback and to share study progress and findings. Feedback from the initial meeting established procedures for recruitment, informed the design and content of data collection forms, and directed the standard operating procedures for study visits. Feedback from subsequent meetings was incorporated into study modifications to address concerns, facilitate participation, and collect new information in response to scientific and logistic questions from CAB members. As study results are finalized, the study team will meet with CAB members to develop appropriate plans for results dissemination to study participants and other key local and national stakeholders.

Mathare North Health Centre staff introduced the study to women attending routine antenatal or HIV care appointments and referred those interested to study staff for eligibility screening and enrollment. Facility staff notified the study team of deliveries and supported abstraction of delivery information from medical records. The study team met with facility leadership monthly to ensure these processes were appropriate and did not interfere with facility

work, adjusting procedures as needed. These meetings were also leveraged to facilitate referrals and other support for participants' healthcare.

Statistical Analysis

All singleton infants and firstborn twins with ≥1 postpartum follow-up visit were included in this analysis. Infant HIV exposure was defined by their mother's HIV status at enrollment; no mothers or infants acquired HIV during follow-up. Infant morbidity was assessed via several outcomes: maternal report and/or clinician diagnosis of acute diarrhea, defined as ≥3 loose stools in a 24-hour period for <14 days; clinician-diagnosed pneumonia, defined as cough and/or difficulty breathing with rapid respiration or chest indrawing; maternal report and/or clinician diagnosis of any respiratory tract infection (RTI), defined as cough with phlegm, wheezing, or shortness of breath (lower RTI) or with runny nose or nasal congestion (upper RTI); clinician-diagnosed malaria; and hospitalization for any cause. Cases of pneumonia were also included in the case definition of "any RTI". Infant mortality included deaths from any cause. Infants were considered EBF for 6 months if mothers reported breastfeeding and did not report giving the infant anything other than breast milk or prescribed medications for the first six months of life.

Kaplan-Meier survival analysis was used to assess the probability and time-to-event of outcomes, overall and stratified by HIV exposure and EBF for 6 months. Incidence was calculated as the number of events per 1000 person-months at risk. Infants' time at risk began at birth and ended when they exited the study due to completion of or loss to follow-up, voluntary withdrawal, or death; analyses of EBF were restricted to infants followed for ≥6 months. Morbidity onset was estimated as either the midpoint between the infant's last visit and the visit at which the outcome was reported, if the outcome occurred between visits, or as three days prior to a visit at which current symptoms were reported or a new diagnosis was made by study clinicians. To avoid double-counting ongoing events, we excluded the two weeks after acute diarrhea events and four weeks after pneumonia/any RTI events from an infant's time at risk. Dates of hospitalization and

death were abstracted from adverse events reports and used as the time of event for these outcomes.

To account for recurrent morbidities, Andersen-Gill regression was used to obtain hazard ratios for the associations between HIV exposure or EBF for ≥6 months and each morbidity outcome; the associations for mortality were assessed with Cox proportional hazards regression. Models were based on the person-time at risk described above and adjusted for *a priori*-determined confounders identified via a directed acyclic graph (DAG) approach (Figure S1). The DAG was constructed with factors known to be associated with HIV exposure and/or infant morbidity/mortality for which we had study data; the minimal set sufficient for adjustment included maternal age and socioeconomic status, which we defined by partnership status (married or with a steady partner vs not) and education level (secondary or higher vs primary or lower). All analyses were conducted in Stata (version 17; StataCorp, College Station, TX, USA) using two-sided tests with a significance level of 0.05.

RESULTS

Of 211 mother-infant pairs enrolled in the Linda Kizazi Study, 187 (89%) infants remained in follow-up after delivery, of which 86 (46%) were children who were HIV-exposed, uninfected (CHEU) and 101 (54%) were children who were HIV-unexposed, uninfected (CHUU). CHEU and CHUU did not differ in duration of follow-up (median 24 months for both, Wilcoxon rank-sum p=0.601) or number of follow-up clinic visits attended (median 8 for both, p=0.880), and a similar proportion of mothers with and without HIV elected to receive any weekly visits (63% vs 59%, Chi-square p=0.636).

As shown in Table 1, mothers living with HIV versus without HIV were slightly older at enrollment (median 30 vs 26 years), fewer were married/with a steady partner (88% vs 96%), and fewer had received secondary or higher education (45% vs 57%). Though more mothers living with HIV were employed (47% vs 41%), they had a lower median weekly income (12.50 vs 17.50

US dollars). Two-thirds of participants experienced household crowding. Most lived in homes with metal roofing (69%) that had electricity (97%), though only 24% of HIV-affected and 19% of HIV-unaffected mother-infant pairs had running water. Few participants (13%) had a toilet inside their home and 85% reported sharing their toilet with other households; more HIV-exposed than HIV-unexposed mother-infant pairs had a toilet at home (17% vs 9%) and did not share a toilet with other households (19% vs 12%). No mothers reported smoking cigarettes during pregnancy, but more HIV-affected mother-infant pairs lived with someone that smoked inside the home (6% vs 1%). Nearly all (97%) mothers living with HIV were diagnosed before pregnancy (Table 1); all but one of these mothers also reported initiating ART before pregnancy. The median duration of ART use at enrollment was 5 years and median baseline CD4 count was 523 cells/μl (interquartile range 413-641); 94% had a baseline CD4 count ≥200 cells/μl. Ninety-one percent reported receiving TDF+3TC+EFV for ART during pregnancy, of which 45/52 (87%) followed through 24 months switched to TDF+3TC+DTG after delivery per updates to Kenya National Guidelines (25). About three-quarters (74%) of all mothers reported receiving cotrimoxazole preventive therapy (CPT) during pregnancy.

Data from the delivery were available for 158 (84%) infants; there were 7 (4%) preterm births (<37 weeks gestation) and 3 (2%) infants with low birth weight (LBW; <2.5 kg). The proportion of preterm and LBW infants was similar among CHEU and CHUU (3 [4%] vs 4 [4%] preterm and 1 [1%] vs 2 [2%] LBW). A similar proportion of CHEU and CHUU were assigned male sex at birth (56% vs 53%; Table 1). While all mothers initiated breastfeeding, substantially more CHEU than CHUU were EBF for 6 months (88% vs 57%). However, mothers without HIV reported any breastfeeding for a longer duration than mothers living with HIV (median 19 vs 12 months). All but one CHEU received ARV prophylaxis and 95% received CPT.

Table 1. Selected participant characteristics

		All		HIV-unexposed uninfected		HIV-exposed uninfected
	N	Median (IQR) or n (%)	N	Median (IQR) or n (%)	N	Median (IQR) or n (%)
Maternal Characteristics						
Age (years) at enrollment	187	28 (24-32)	101	26 (23-31)	86	30 (26-33)
Married or steady partner	187	173 (92.5)	101	97 (96.0)	86	76 (88.4)
Highest education level	187		101		86	
Secondary or higher		97 (51.9)		58 (57.4)		39 (45.4)
Primary or lower		90 (48.1)		43 (42.6)		47 (54.7)
Employed	187	81 (43.3)	101	41 (40.6)	86	40 (46.5)
Weekly income (USD)[1]	63	15.00 (7.50-30.00)	34	17.50 (10.00-30.00)	29	12.50 (7.50-21.00)
Lifetime number of pregnancies	187	3 (2-4)	101	2 (2-3)	86	3 (2-4)
Number of living children[2]	157	2 (1-3)	80	2 (1-2)	77	2 (1-3)
HIV diagnosis before pregnancy		--		--	86	83 (96.5)
Started ART before pregnancy					86	82 (95.4)
Years on ART at enrollment		--		--	86	5 (2-7)
ART regimen at enrollment					86	
TDF + 3TC + EFV		--		--		78 (90.7)
TDF + 3TC + DTG						1 (1.2)
Other[3]						7 (8.1)
CD4 count (cells/μl) at enrollment		--		--	84	523 (413-641)
Reported receiving CPT during pregnancy		--		--	86	64 (74.4)
Sociodemographics						
Experienced crowding (>3 people/room) in home	186	122 (65.6)	101	68 (67.3)	85	54 (63.5)

Home roofing material	187		101		86	
Metal		128 (68.5)		69 (68.3)		59 (68.6)
Concrete		54 (28.9)		29 (28.7)		25 (29.1)
Other		5 (2.7)		3 (3.0)		2 (2.3)
Toilet is outside the home	187	163 (87.2)	101	92 (91.1)	86	71 (82.6)
Toilet is shared with other households	187	159 (85.0)	101	89 (88.1)	86	70 (81.4)
Home has running water	187	40 (21.4)	101	19 (18.8)	86	21 (24.4)
Home has electricity	187	181 (96.8)	101	98 (97.0)	86	83 (96.5)
Someone smokes cigarettes inside home	187	6 (3.2)	101	1 (1.0)	86	5 (5.8)
Infant Characteristics						
Male sex assigned at birth	186	101 (54.3)	100	53 (53.0)	86	48 (55.8)
Vaginal delivery	158	143 (90.5)	90	83 (92.2)	68	60 (88.2)
Gestational age at birth (weeks)	157	38 (38-40)	90	38 (38-40)	67	38 (38-39)
Birthweight (kg)	156	3.3 (3.0-3.5)	89	3.4 (3.0-3.6)	67	3.2 (3.0-3.5)
Ever breastfed	187	187 (100.0)	101	101 (100.0)	86	86 (100.0)
Exclusively breastfed for 6 months[4]	163	117 (71.8)	83	47 (56.6)	80	70 (87.5)
Months of exclusive breastfeeding[4]	162	6 (5-6)	83	6 (4-6)	79	6 (6-6)
Months of any breastfeeding[5]	121	14 (12-20)	63	19 (16-24)	58	12 (12-13)
Ever received antiretrovirals for HIV prophylaxis		--		--	86	85 (98.8)
Ever received CPT		--		--	86	82 (95.4)

IQR = Interquartile range. USD = US dollars. TDF = Tenofovir. 3TC = Lamivudine. EFV = Efavirenz. DTG = Dolutegravir. CPT = Cotrimoxazole preventive therapy. [1] If employed. [2] If ≥1 pregnancy prior to pregnancy at enrollment. [3] Three women reported receiving AZT+3TC+ATV/r and one woman each reported receiving AZT+3TC+NVP, AZT+3TC+EFV, TDF+FTC+EFV, and TDF+3TC+ATV/r. [4] Among mother-infant pairs followed for ≥6 months. [5] Among mother-infant pairs followed for 24 months.

As recommended by the Kenya Pediatric Immunization Schedule, all infants received a BCG vaccine at birth; all of 170 infants followed for ≥14 weeks received ≥1 dose each of the oral polio, pneumococcal conjugate-10 (PCV-10), DPT-HepB-HiB, and rotavirus vaccines; and all of 157 infants followed for ≥9 months received their first dose of the measles/rubella vaccine. Coverage was also high for the inactivated polio vaccine at 14 weeks (167/170, 98%; all but 3 CHUU).

Acute diarrhea (143 cases) and any RTI (238 cases) were the most common morbidities among all infants with an overall incidence of 39/1000 person-months and 66/1000 person-months, respectively (Table 2). Among infants with events, there was a median of 1 (interquartile range [IQR] 1-2; range 1-5) diarrhea event and a median of 2 (IQR 1-3; range 1-6) RTI events. Infants were a median 8.3 months old (IQR 4.0-15.7) at the first diarrhea event and a median 7.5 months old (IQR 3.5-16.7) at the first RTI. There were substantially fewer cases of pneumonia (19 cases, 5.2/1000 person-months), malaria (18 cases, 5.0/1000 person-months), and hospitalization (11 cases, 3.0/1000 person-months), all of which were single events except for two children with a second case of pneumonia, one child with a second case of malaria, and one child that was hospitalized twice. Median age at the first event was 11.9 months (IQR 4.4-15.6) for pneumonia, 18.8 months (IQR 15.2-23.1) for malaria, and 7.4 months (IQR 1.6-15.2) for hospitalization. There were five deaths (3 CHEU, 2 CHUU; 26.7/1000 live births), all of which were among infants born at term and a normal birth weight. Median age at death was 7 months (IQR 7-8 months). Of two infants that died after 6 months and had feeding data, both were EBF for 6 months. The overall incidence of mortality was 1.4/1000 person-months.

Table 2. Incidence of morbidity and mortality in CHEU and CHUU.

	Overall		HIV-unexposed uninfected		HIV-exposed uninfected	
	Events	Cases/1000 person-months (95% CI)	Events	Cases/1000 person-months (95% CI)	Events	Cases/1000 person-months (95% CI)
Acute diarrhea	143	39.4 (33.4-46.4)	79	42.0 (33.7-52.4)	64	36.5 (28.6-46.6)
Pneumonia	19	5.2 (3.3-8.2)	15	7.9 (4.8-13.2)	4	2.3 (0.9-6.1)
Any respiratory tract infection	238	65.9 (58.0-74.8)	152	81.1 (69.2-95.1)	86	49.4 (40.0-61.0)
Malaria	18	5.0 (3.1-7.9)	12	6.4 (3.7-11.3)	6	3.4 (1.5-7.6)
Hospitalization	11	3.0 (1.7-5.5)	6	3.2 (1.4-7.1)	5	2.8 (1.2-6.8)
Mortality	5	1.4 (0.6-3.3)	2	1.1 (0.3-4.3)	3	1.7 (0.6-5.3)

CI = Confidence interval. Includes recurrent events for morbidity outcomes.

There was no significant difference in risk of acute diarrhea, malaria, hospitalization, or mortality between CHEU and CHUU before or after adjusting for maternal age, partnership status, and education level (Table 3). However, CHEU had 71% lower risk of pneumonia (aHR=0.29, 95% CI: 0.091-0.89; p=0.031) and 40% lower risk of any RTI than CHUU (aHR=0.60, 95% CI: 0.44-0.82; p=0.001). Among CHEU, risk of diarrhea or any RTI did not differ by maternal report of CPT during pregnancy or by visit-level maternal CD4 count, ART regimen, or infant ARV and CPT exposure (Table S1); however, there was insufficient statistical power to assess the risk of other outcomes in this group.

Table 3. Hazard ratios comparing morbidity and mortality outcomes in the first two years of life between CHEU and CHUU.

	Unadjusted		Adjusted[1]	
	HR (95% CI)	p-value	aHR (95% CI)	p-value
Acute diarrhea	0.86 (0.59-1.27)	0.452	0.79 (0.52-1.22)	0.289
Pneumonia	0.29 (0.095-0.86)	0.026	0.29 (0.091-0.89)	0.031
Any respiratory tract infection	0.61 (0.46-0.81)	0.001	0.60 (0.44-0.82)	0.001
Malaria	0.53 (0.20-1.39)	0.194	0.44 (0.16-1.21)	0.114
Hospitalization	0.89 (0.25-3.23)	0.859	1.11 (0.30-4.14)	0.872
Mortality	1.57 (0.26-9.43)	0.621	1.87 (0.17-20.5)	0.607

HR = Hazard ratio. CI = Confidence interval. [1] Adjusted for maternal age, marital/partnership status, and education level.

CHEU and CHUU experienced similar timing of events for mortality and most morbidity outcomes, despite the differences in RTI incidence (Figure 1). The probability of diarrhea was slightly higher among CHEU before 4 months and higher among CHUU after 4 months (Figure 1A). However, there remained no significant association between HIV exposure and diarrhea when stratifying the analysis by time <4 months (aHR=1.13, 95% CI: 0.47-2.72; p=0.782) versus ≥4 months (aHR=0.74, 95% CI: 0.45-1.20; p=0.220).

Because significantly more CHEU than CHUU were EBF for 6 months (Table 1, Chi-square p<0.001), we assessed whether EBF for 6 months was associated with post 6-month morbidity, regardless of HIV exposure. Infants who were EBF for 6 months had 40% lower risk of any RTI (aHR=0.60, 95% CI: 0.44-0.82; p=0.001) and there was a trend for lower risk of pneumonia (aHR=0.31, 95% CI: 0.093-1.02; p=0.054). EBF for 6 months was not associated with the risk of acute diarrhea (aHR=1.17, 95% CI: 0.74-1.85; p=0.504), malaria (aHR=1.72, 95% CI: 0.49-5.97; p=0.395), or hospitalization (aHR=0.59, 95% CI: 0.10-3.48; p=0.558).

We also conducted a sensitivity analysis excluding weekly visit data to evaluate whether the more frequent intervals and/or self-selection into the weekly visit subgroups biased our analysis. Without the weekly data, there were fewer observed events, reducing incidence of each morbidity overall and among both CHEU and CHUU (Table S2). Acute diarrhea and any RTI remained the most common outcomes (72 events, 20/1000 person-months and 151 events, 42/1000 person-months, respectively), but 7/19 pneumonia cases and 2/11 hospitalizations were missed, reducing incidence to 3.3/1000 person-months and 2.5/1000 person-months, respectively. The timing of events remained similar between CHEU and CHUU (Figure S2) and there was still no significant association between HIV exposure and acute diarrhea, malaria, or hospitalization in either unadjusted or adjusted analyses (Table S3). CHEU had a similar lower risk of any RTI when excluding weekly visits (aHR=0.62, 95% CI: 0.42-0.91; p=0.014); however, the association between HIV exposure and pneumonia was reduced to a trend in the adjusted analysis (aHR=0.25, 95% CI: 0.049-1.26; p=0.092).

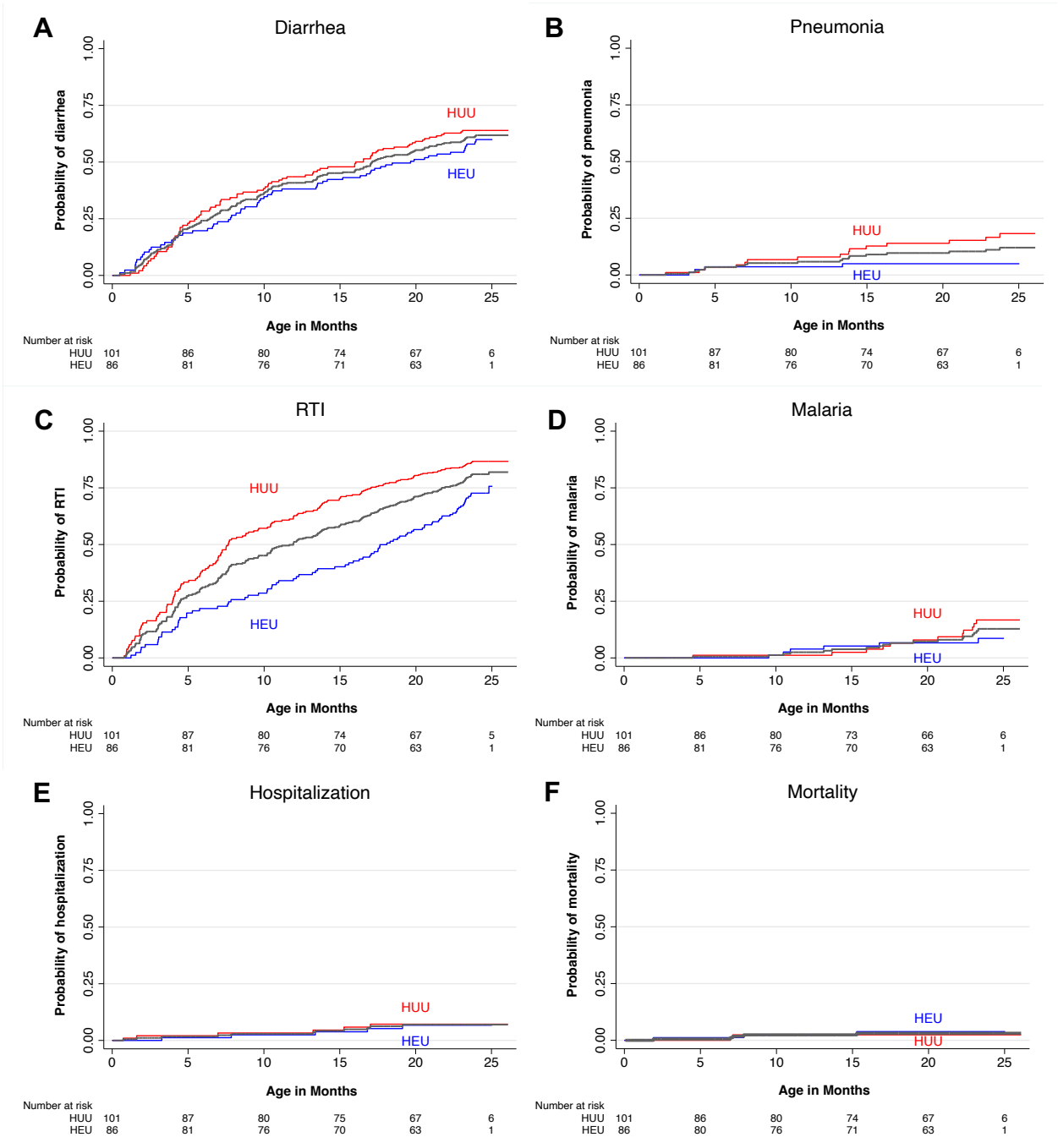

Figure 1. Kaplan-Meier curves comparing probability of (a) acute diarrhea, (b) pneumonia, (c) any respiratory tract infection, (d) malaria, (e) hospitalization, and (f) mortality over the first two years of life between CHEU versus CHUU.

DISCUSSION

In this cohort of healthy mother-infant pairs in Nairobi, Kenya, acute diarrhea and RTI were common, but there were few cases of pneumonia, malaria, and hospitalization. Though there were few deaths, mortality in the study was similar to the national average infant mortality rate in Kenya (27/1000 vs 28/1000 live births, respectively) (26). CHEU and infants that were EBF for 6 months had lower incidence of RTIs, including pneumonia, than CHUU or infants not EBF for 6 months. Compared to the Kenya national average of 61%, more women living with HIV (88%) but fewer women without HIV (57%) EBF their infant for 6 months (26).

The frequency of RTI in this cohort was unsurprising given that acute respiratory illnesses are among the leading causes of mortality under age five (27), but our finding that CHEU were less likely to experience RTI, including pneumonia, contrasts with earlier reports. Both before and after availability of lifelong ART for all pregnant women (Option B+), most studies demonstrated higher incidence of pneumonia (28,29) and other lower RTIs (30–32), any RTIs (33,34), and hospitalization for respiratory infections (7,35–38). However, our findings are similar to those of a study in western Kenya from 2011-2016, which observed 23% lower risk of upper RTI and 20% lower risk of any respiratory illness among CHEU who received ARVs and CPT and were born to women on ART during pregnancy (39).

Published studies suggest respiratory morbidity consistently is highest among CHEU whose mothers had low CD4 count or high HIV viral load (40–42) and are not breastfed, while infants EBF for 6 months tend to have fewer RTIs (43–45). A study conducted in South Africa between 2017-2019 observed CHEU to be at higher risk of hospitalization due to RTI, despite 53% of mothers starting ART before conception, though fewer CHEU than CHUU were EBF for ≥6 months in that cohort (28% vs 52%, respectively) (37). However, a study from Malawi between 2019-2021 also noted higher risk of lower RTI among CHEU despite similarities in breastfeeding to CHUU (32). While data from the current era of universal optimized ART remain limited, our findings suggest that initiation of maternal ART before conception reduces respiratory morbidity

among CHEU in the first two years of life. Possible mechanisms for this outcome include reduced infectious exposures for the HIV-exposed fetus and infant due to stronger maternal immunity from ART-induced immune reconstitution, and longer duration of breastfeeding due to ART's protection against HIV transmission in breast milk.

In our Kenyan cohort followed between 2018-2022, 97% of mothers started ART before pregnancy and 88% of CHEU were EBF for 6 months; hence, we speculate the protective effects of early ART, higher rates of EBF, and CPT may together explain why we saw lower risk of RTI among CHEU than CHUU, in contrast to other recent studies. Compared to EBF for <6 months or replacement feeding, EBF for ≥6 months has been consistently associated with reduced risk of pneumonia and other RTI in both low-and-middle and high-income settings, regardless of maternal HIV status (46). Breast milk contains maternal immunoglobulin A and other immune factors that protect the infant against bacterial and viral infections, particularly in mucosal cells of the respiratory and gastrointestinal tracts (47). The immunological and nutritional composition of breast milk also changes in response to the infant's needs, which can help reduce the risk and severity of infection both directly through antibody transfer and indirectly by preventing malnutrition (22). Breastfeeding and human milk oligosaccharides also help establish the infant's gastrointestinal microbiome and immune system, which in turn affects overall susceptibility to infections (48). EBF in the first six months of life, when the infant immune system is most vulnerable, maximizes these benefits while simultaneously reducing exposure to dietary sources of infection (49). With the expansion of access to effective ART, women living with HIV are increasingly encouraged to breastfeed their infants and may receive more frequent messaging about or support for breastfeeding than women without HIV (20), which may explain why more CHEU were EBF in our cohort.

CPT is recommended for all HIV-exposed infants to reduce mortality from bacterial infections in early life (15). In addition to direct antibiotic effects, cotrimoxazole also has been found to reduce infectious morbidity caused by malaria and viral pathogens, possibly due to its

immunomodulatory and anti-inflammatory properties that could reduce host susceptibility to infection (50). Together, these benefits of CPT could have lowered CHEU's risk of RTI. However, many studies comparing the risk of RTI between CHEU who did versus did not receive CPT did not demonstrate a significant protective benefit, possibly because many infections were viral in origin (51–54). Since nearly all CHEU in this cohort received CPT, we did not have sufficient power to compare RTI risk by this exposure, and further study is needed.

Conversely, numerous studies have shown that CPT can decrease the risk of malaria among CHEU (55). Because malaria is not endemic to Nairobi (56), we had few malaria diagnoses in this cohort, which may explain why there was no significant difference in incidence between CHEU and CHUU. We expect the observed cases were acquired during travel to endemic regions. Our small study population and restrictive enrollment criteria (healthy women with no recent antimicrobial use, ≥6 months ART for women with HIV) also may have contributed to the low number of events of malaria, hospitalization, and death. Though our data are encouraging because CHEU historically have had a greater risk of hospitalization and death than CHUU (57,58), we did not have sufficient statistical power to rule out a difference in these outcomes between CHEU and CHUU.

CHEU in this study did not have higher risk of acute diarrhea than CHUU, which is consistent with other studies of breastfed CHEU. Incidence of and hospitalization for diarrhea has historically been higher among CHEU than CHUU, and were highest before the availability of any maternal ART (29,31,59). These risks declined as ART became more effective and widely available, in part because women with HIV previously had been discouraged from breastfeeding or counseled to wean their infants at younger ages as an HIV prevention strategy (60); replacement feeding and introduction of other foods and liquids before six months are associated with higher incidence of diarrheal disease in all infants (46). Today, women on lifelong ART who have undetectable HIV viral load can—and are encouraged to—safely breastfeed for the same

duration as women without HIV (20), which reduces differences in feeding and nutrition between CHEU and CHUU that may translate into lower risk of diarrhea for CHEU.

The primary limitation of this study is its small sample size. While we hypothesize that EBF and CPT may mediate the improved outcomes observed among CHEU in this cohort, further study of the relationships between HIV exposure, breastfeeding, CPT, and morbidity is needed in larger, contemporary cohorts with greater heterogeneity of CPT. While it is possible that CHEU and CHUU differed by unmeasured behavioral or biological characteristics, bias from these factors may have been minimized by enrolling participants from a small catchment area with consistent socioeconomic and environmental exposures—for example, we observed similar household characteristics and vaccination access between HIV exposure groups. We also decided *a priori* to adjust for mothers' partnership status and education level to reduce confounding by differences in socioeconomic status. Additionally, we showed previously that CHEU and CHUU in this cohort experienced SARS-CoV-2 infection at a similar rate and most infections were asymptomatic (61), so it is unlikely that the COVID-19 pandemic affected our results.

We acknowledge that the results of this study are not generalizable to all populations of CHEU due to high rates of maternal ART and infant HIV prophylaxis in our cohort. All mothers living with HIV received ≥6 months of optimized ART regimens, which more effectively suppress HIV viral load and reconstitute the immune system compared to earlier regimens (15). Though we did not have systematically collected HIV viral load data, most mothers living with HIV had had normal CD4 counts at enrollment and throughout follow-up, which suggests their HIV was well-treated. Nearly all CHEU received nevirapine and zidovudine prophylaxis and CPT while breastfeeding, which is not possible in many settings with high HIV burden. Additionally, there was little variability in maternal ART regimen, though mothers living with HIV switched from tenofovir/lamivudine/efavirenz (TDF+3TC+EFV) to tenofovir/lamivudine/dolutegravir (TDF+3TC+DTG) at variable times during follow-up. Trial data show that DTG-containing

regimens during pregnancy are associated with lower risk of preterm birth and neonatal mortality than the EFV-containing regimens (62), but we could not assess differences in birth outcomes in this cohort because all switches to TDF+3TC+DTG occurred postpartum. It remains unclear whether DTG-containing regimens affect infant outcomes after the neonatal period independently of preterm birth; we did not have sufficient sample size to assess this relationship and more research is needed.

CONCLUSION

Overall, this analysis suggests universal optimized ART can successfully reduce morbidity and mortality among otherwise healthy CHEU, resulting in outcomes similar to their CHUU peers. However, it is important to acknowledge these interventions are not implemented uniformly or equitably in all areas affected by HIV and our findings underscore the importance of ensuring all women living with HIV and their infants have access to effective HIV treatment and prophylaxis with support for EBF

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SUPPLEMENTARY INFORMATION

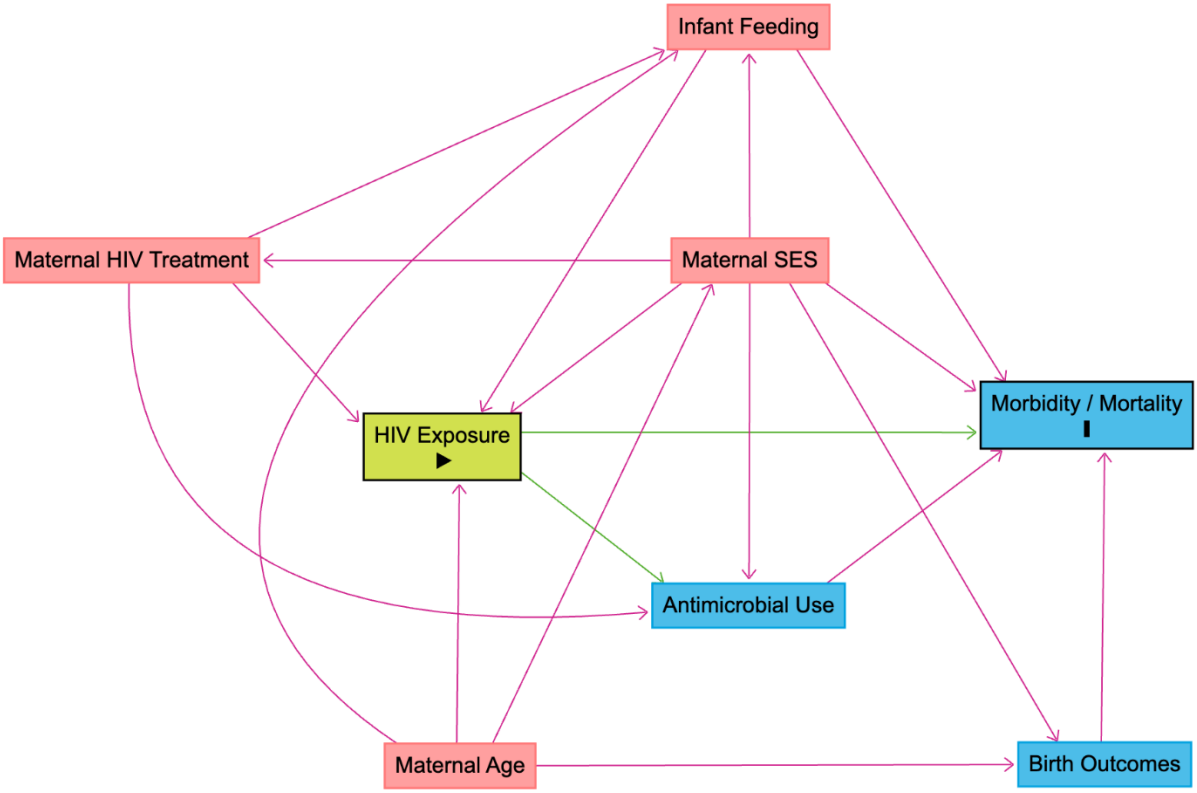

Figure S1. Directed acyclic graph of hypothesized relationships between infant HIV exposure, morbidity/mortality outcomes, and other factors known to be associated both the exposure and outcomes of interest. Arrows indicate the hypothesized direction of association. Confounders are defined as variables that are ancestors of both exposure and outcome. DAGitty (1) was used to prepare the figure and identify a minimal set of variables needed for adjustment.

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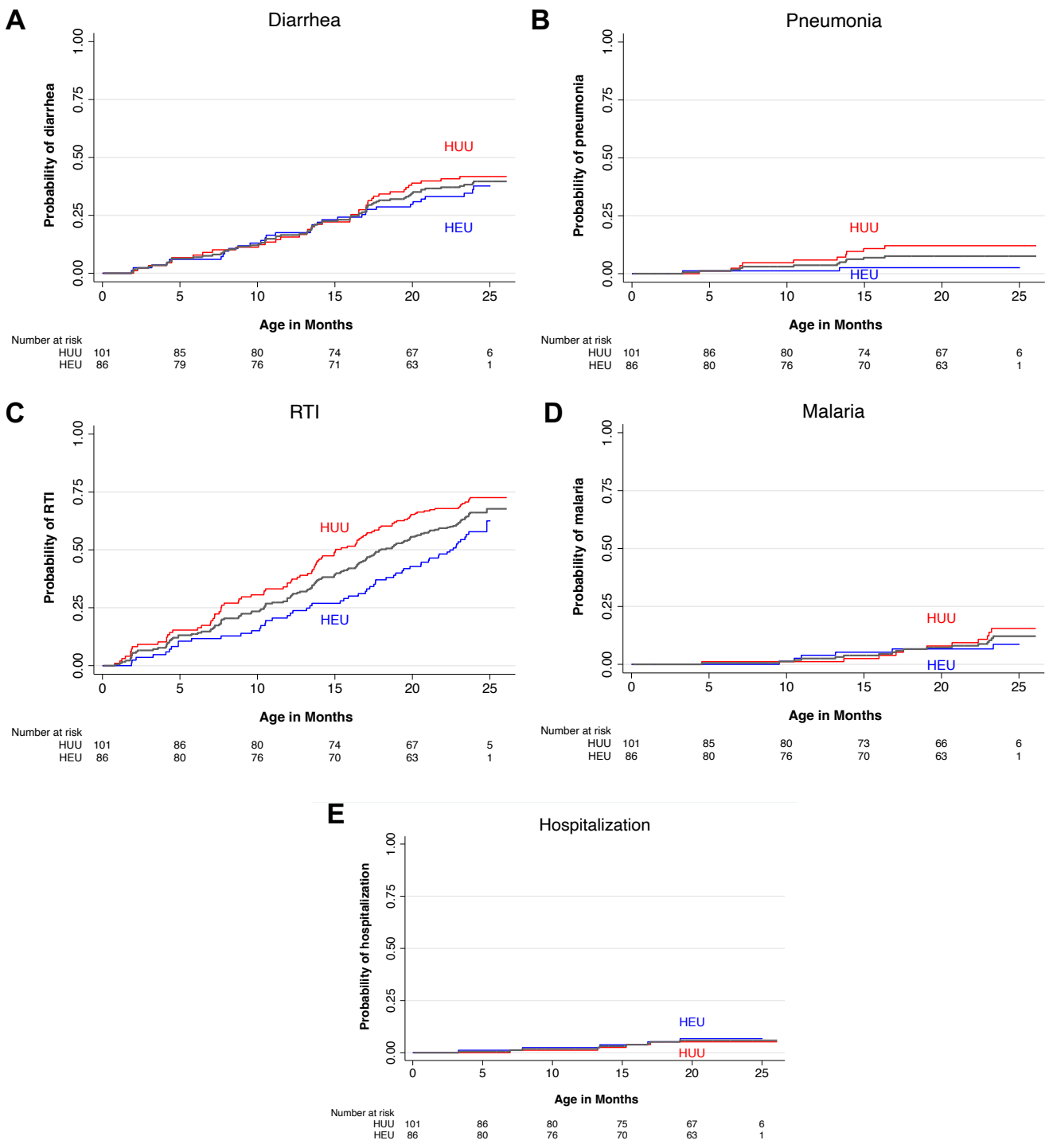

Figure S2. Kaplan-Meier curves comparing probability of (a) acute diarrhea, (b) pneumonia, (c) any respiratory tract infection, (d) malaria, and (e) hospitalization over the first two years of life between CHEU versus CHUU, as measured only at clinic follow-up visits.

Table S1. Adjusted hazard ratios for the associations between maternal HIV treatment and infant HIV prophylaxis characteristics and acute diarrhea and any respiratory tract infection in the first two years of life among CHEU (N=86).

	Acute Diarrhea		Any Respiratory Tract Infection	
	aHR[1] (95% CI)	p-value	aHR[1] (95% CI)	p-value
Maternal report of CPT in pregnancy	0.89 (95% CI: 0.48-1.67)	0.724	1.29 (95% CI: 0.66-2.52)	0.465
Visit-level maternal CD4 count (per 100 cells/μl)	1.10 (95% CI: 0.95-1.26)	0.199	1.02 (95% CI: 0.89-1.17)	0.823
Visit-level maternal ART regimen				
TDF+3TC+EFV	*ref*	--	*ref*	--
TDF+3TC+DTG	1.91 (95% CI: 0.90-4.06)	0.094	1.18 (95% CI: 0.67-2.06)	0.570
Other regimens	0.78 (95% CI: 0.23-2.71)	0.699	1.27 (95% CI: 0.59-2.75)	0.537
Visit-level infant ARV exposure	1.29 (95% CI: 0.66-2.52)	0.460	1.87 (95% CI: 0.86-4.07)	0.113
Visit-level infant CPT exposure	1.11 (95% CI: 0.58-2.13)	0.754	1.44 (95% CI: 0.87-2.38)	0.153

HR = Hazard ratio. CI = Confidence interval. CPT = Cotrimoxazole preventive therapy. ARV = Antiretroviral.
[1] Adjusted for maternal age, marital/partnership status, and education level.

Table S2. Incidence of morbidity and mortality in CHEU and CHUU, measured only at clinic follow-up visits.

	Overall		HIV-unexposed uninfected		HIV-exposed uninfected	
	Events	Cases/1000 person-months (95% CI)	Events	Cases/1000 person-months (95% CI)	Events	Cases/1000 person-months (95% CI)
Acute diarrhea	72	19.9 (15.8-25.1)	40	21.4 (15.7-29.2)	32	18.3 (13.0-25.9)
Pneumonia	12	3.3 (1.9-5.8)	10	5.3 (2.9-9.9)	2	1.1 (0.3-4.6)
Any respiratory tract infection	151	42.0 (35.8-49.3)	94	50.5 (41.2-61.8)	57	32.9 (25.4-42.7)
Malaria	17	4.7 (2.9-7.6)	11	5.9 (3.3-10.7)	6	3.4 (1.5-7.6)
Hospitalization	9	2.5 (1.3-4.8)	4	2.1 (0.8-5.7)	5	2.9 (1.2-6.9)
Mortality	5	1.4 (0.6-3.3)	2	1.1 (0.3-4.3)	3	1.7 (0.6-5.3)

CI = Confidence interval. Includes recurrent events for morbidity outcomes.

Table S3. Hazard ratios comparing morbidity and mortality outcomes between CHEU and CHUU in the first two years of life, as measured only at clinic follow-up visits.

	Unadjusted		Adjusted[1]	
	HR (95% CI)	p-value	aHR (95% CI)	p-value
Acute diarrhea	0.85 (0.49-1.49)	0.568	0.70 (0.36-1.34)	0.276
Pneumonia	0.21 (0.045-0.99)	0.048	0.25 (0.049-1.26)	0.092
Any respiratory tract infection	0.65 (0.46-0.94)	0.021	0.62 (0.42-0.91)	0.014
Malaria	0.57 (0.22-1.49)	0.253	0.49 (0.18-1.31)	0.157
Hospitalization	1.31 (0.32-5.43)	0.705	1.60 (0.37-6.87)	0.528
Mortality	1.57 (0.26-9.43)	0.621	1.87 (0.17-20.5)	0.607

HR = Hazard ratio. CI = Confidence interval. [1] Adjusted for maternal age, marital/partnership status, and education level.

CHAPTER 3. Breast milk cytomegalovirus DNA levels increase risk of cytomegalovirus acquisition among HIV-exposed, uninfected and HIV-unexposed infants in the setting of optimized maternal antiretroviral therapy

Breast milk cytomegalovirus DNA levels increase risk of cytomegalovirus acquisition among HIV-exposed, uninfected and HIV-unexposed infants in the setting of optimized maternal antiretroviral therapy

ABSTRACT

Background: Children who are HIV-exposed, uninfected (CHEU) have had higher incidence and earlier cytomegalovirus (CMV) acquisition than children who are HIV-unexposed, uninfected (CHUU). To determine whether this difference persists in the era of optimized, universal ART, we assessed the probability, timing, and correlates of CMV acquisition within a birth cohort of CHEU and CHUU.

Methods: Women without HIV and women living with HIV on ART for ≥6 months were recruited in pregnancy (28-42 weeks) from a maternal-child health clinic in Nairobi, Kenya between December 2018-March 2020; 89% of mothers with HIV initiated ART before pregnancy. Mother-infant pairs were followed for 12 months postpartum with weekly data and sample collection. Quantitative PCR was used to measure $\log_{10}$-CMV DNA levels in infant urine/saliva and maternal saliva/breast milk. The probability and timing of infant CMV acquisition were determined by Kaplan-Meier survival analysis. Correlates of CMV acquisition were assessed using Cox proportional hazards regression models stratified by exclusive breastfeeding (EBF) status and adjusted for infant sex, HIV exposure, and household crowding.

Results: Fifty-three (74%) of 72 infants (29% CHEU, 61% CHUU) acquired CMV. The cumulative probability of postnatal CMV acquisition was 79%. Median time to infection was 2.4 months (IQR 2.0-4.7). HIV exposure did not affect the hazard of CMV acquisition when infants were EBF (adjusted hazard ratio [aHR]= 1.24, 95% CI: 0.69-2.23, p=0.471) or not EBF (aHR=0.52, 95% CI: 0.10-2.68, p=0.438). However, the hazard was two times greater per $\log_{10}$ increase in breast milk

CMV DNA both when infants were EBF (aHR=2.09, 95% CI: 1.48-2.95, p<0.001) and received mixed feeds (aHR=1.98, 95% CI: 1.26-3.10, p=0.003); this finding did not differ by HIV exposure.

Conclusions: CMV acquisition was similar between CHUU and CHEU whose mothers initiated optimized ART before pregnancy. Breast milk remains a primary source of CMV acquisition for all infants.

INTRODUCTION

Cytomegalovirus (CMV) is a human herpesvirus that infects between 40-100% of people worldwide, with the highest seroprevalence in low- and middle-income countries (1,2). Primary CMV infection often occurs in childhood and lifelong latency is established with the first infection (3). Inflammation and immune deficiency can lead to reactivation of latent virus, and reinfection with new strains is possible despite a robust adaptive immune response to initial infection (3,4). Following infection and reactivation, infectious CMV is shed in mucosal tissues for long periods and is transmitted through body fluids including saliva, nasal and cough secretions, urine, cervicovaginal secretions, and breast milk (5,6).

These characteristics facilitate mother-to-infant CMV transmission, which is a common source of primary infection. Globally, 40-60% of infants with a CMV-seropositive mother will acquire CMV by 12 months, though this proportion can be greater in populations with high seroprevalence (7). CMV can cross the placenta and intrauterine transmission occurs in 0.2-1% of live births when women experience CMV infection or reactivation during pregnancy (4). Intrapartum transmission also occurs in about half of vaginal deliveries when the mother is shedding infectious virus (7). Postpartum transmission is more frequent, especially during breastfeeding. After infection or reactivation of latent virus, which occurs with the onset of lactation in 90-95% CMV-seropositive individuals (8,9), CMV is transmitted throughout breastfeeding via infected breast milk cells and as cell-free virus (6,10). Close interpersonal contact between mother and infant also facilitates transmission in saliva and nasopharyngeal secretions (5), though the frequency of infection by these routes is difficult to measure since it is so ubiquitous.

Most CMV infections among healthy individuals are asymptomatic or cause mild flu-like symptoms (3,4). However, congenital CMV is a leading cause of hearing loss and neurodevelopmental delay in children (11,12), and both congenital and early life infection can exacerbate other causes of infant morbidity and mortality, such as adverse birth outcomes, malnutrition, and pneumonia (13–17). CMV has wide tropism and is highly immunomodulatory,

and a growing body of evidence suggests CMV infection may alter immune responses to other pathogens (3,4) and vaccines (17,18). While less common in the era of universal, optimized ART, CMV remains an important opportunistic infection among individuals who are ART naïve and severely immunocompromised (3,4).

While preventing CMV transmission is an important public health priority, there are currently no scalable biomedical interventions to prevent CMV infection. Behavioral interventions focused on minimizing infant exposure to infectious fluids, including breast milk and saliva, often are neither ethical nor practical to implement (19,20). Given the poor ability of natural immunity to prevent reinfection and our limited knowledge of viral or immunologic correlates of transmission, CMV vaccine development has largely focused on the prevention of congenital transmission rather than the prevention of postnatal infection and trial results to date have been disappointing (20).

Historically, children who are HIV-exposed, uninfected (CHEU) have had higher incidence of congenital CMV and earlier acquisition of postnatal CMV infection than children who are HIV-unexposed, uninfected (CHUU), and these differences persisted through the early ART era (21). This higher incidence is likely explained by both immunologic differences in CHEU versus CHUU (22,23) and increased exposure to CMV shedding in breast milk and saliva from mothers living with HIV (24). Antiretroviral therapy (ART)-mediated immune recovery among women living with HIV may reduce or delay CMV transmission to CHEU. However, no studies have compared vertical CMV transmission between CHEU and CHUU in the setting of optimized maternal ART initiated before pregnancy, which could more substantially normalize maternal immune status and reduce transmission risk to approximate that of CHUU.

To address this research gap, we sought to determine whether the timing and incidence of primary CMV infection differed between HIV-unexposed infants and infants born to women with HIV who initiated ART pre-conception, which is more likely to optimize maternal immunity and CMV control compared to historic cohorts. Additionally, we defined correlates of CMV acquisition

among the CHEU and CHUU and compared maternal breast milk levels between women living with and without HIV.

METHODS

Study Population

Data and samples for this analysis were collected as part of the Linda Kizazi Study, a cohort of mother-infant pairs from the Mathare North neighborhood of Nairobi, Kenya followed from delivery to 24 months postpartum as previously described (25). Participants were recruited at the Mathare North Health Centre, a high-volume maternal and child health clinic providing antenatal and postnatal care for most women and children in the densely populated neighborhood. Women living with HIV and without HIV were eligible for the parent cohort if they were 28-42 weeks pregnant, 18-40 years old, planned to breastfeed their infant, did not have a planned Caesarean section, were not diagnosed with a serious medical condition, and had received ≥6 months of ART if living with HIV. All participants received a home visit approximately 4 days postpartum followed by visits at the study clinic approximately every 3 months between week 6 and month 24. At enrollment, participants were offered the option to participate in weekly home visits between delivery and 12 months postpartum. Mother-infant pairs were eligible for this nested analysis if they self-selected to receive the weekly home visits; if a mother had twins, only the firstborn child was included in the analysis.

Mothers provided written informed consent for themselves and their infant. All Linda Kizazi Study procedures and data analyses were approved by the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (P472/07/2018) and the University of Washington Institutional Review Board (STUDY00004006).

Data & Specimen Collection

At enrollment, study clinicians recorded participants' sociodemographic characteristics, obstetric history, current health, and, if living with HIV, HIV diagnosis and treatment history. Beginning the first week after delivery, study staff visited the participant's home weekly to collect data on whether the mother or infant had any symptoms of illness, diagnoses, or medications, including antiretrovirals and cotrimoxazole for HIV prevention, and what the infant was fed in the last week; the same data were also captured at the 3-monthly clinic visits.

At weekly home visits, a study clinician collected samples of the infant's urine and saliva and the mother's saliva and breast milk; only infant and maternal saliva samples were collected at clinic visits. Urine was collected into an adhesive collection bag worn by the infant during the visit, then transferred to a sterile specimen cup and stored at 4°C during transfer to the laboratory where samples were aliquoted into 2ml vials and stored at -80°C without preservatives. Saliva was collected from the infant's or mother's cheeks, palate, gum line, and posterior mouth wall using sterile Dacron swabs ≥1 hour after breastfeeding or ≥15 minutes after eating other foods. Swabs were immediately placed in 1ml of preservative (see Supplementary Information), the plastic swab handle was broken off, and samples were stored at room temperature. To collect breast milk, mothers were asked to do the following for each breast: clean the breast with an alcohol wipe, express and discard the first drops of milk for a "clean-catch" sample, and then express about 10ml of breast milk into a sterile 50ml collection tube. Breast milk samples were stored at 4°C during transfer to the laboratory, then centrifuged at 2000rpm at 4°C for 25 minutes to separate and remove the lipid layer. The resulting sample was aliquoted into 2ml vials and stored at -80°C without preservatives.

CMV DNA PCR

CMV DNA levels in urine, saliva, and breast milk were measured using quantitative polymerase chain reaction (qPCR) testing. CMV DNA was extracted from 500µl of each sample using the AltoStar® Purification Kit 1.5 and qPCR was performed using the AltoStar® CMV PCR Kit 1.5 via the AltoStar® Automation System AM16 according to manufacturer instructions (altona Diagnostics). The limit of detection was 285 log IU/ml (95% CI: 2.62-3.1) for urine, 2.604 log IU/ml (95% CI: 2.434-2.925) for saliva, and 2.275 log IU/ml (95% CI:2.073-2.632) for breast milk; samples with CMV DNA above the limit of detection were considered CMV-positive. The first detectable CMV DNA level in saliva and/or urine followed by ≥3 subsequent, consecutive visits with an average saliva CMV DNA level of at least 3-log and/or an average urine CMV DNA level of at least 0.5-log was considered the primary CMV infection. The collection date of the saliva and/or urine sample with first detectable CMV DNA was considered the time of primary infection.

Statistical Analyses

Kaplan-Meier survival analysis was used to assess the probability and timing of primary CMV infection among infants. Time at risk was defined as months between birth and the time of primary infection or the collection date of the last sample with CV DNA PCR results, if the infant never acquired CMV. Incidence was calculated as the number of infections per 100 person-months at risk. Cox proportional hazards regression was used to identify risk factors of primary CMV infection.

Potential risk factors were selected *a priori* based on infant, sociodemographic, and maternal characteristics previously shown or hypothesized to be associated with CMV infection. Risk factors included: infant sex assigned at birth, infant HIV exposure, visit-level feeding mode (exclusively breastfeeding, defined as infant fed only breast milk and prescribed medication, versus not exclusively breastfeeding, defined as infant fed anything other than breast milk and prescribed medication, with or without breastfeeding), living with ≥1 sibling under age five,

household crowding (>3 people/room in house), having a toilet outside the house, sharing a toilet with other households, log_{10}-CMV DNA levels in maternal breast milk and saliva, maternal CD4 count (cells/μl) and ART regimen at enrollment, visit-level maternal postpartum ART regimen, and infant cotrimoxazole prophylaxis exposure. Models were both unadjusted and adjusted for infant sex, HIV exposure, feeding mode, and household crowding, determined to be confounders *a priori*. Descriptive plots of maternal saliva and breast milk CMV levels over time were created in R (version 4.3.3). All other analyses were conducted in Stata (version 17) with a significance level of 0.05.

RESULTS

Participant Characteristics

Seventy-two (34%) of the 211 enrolled mothers elected to receive weekly home visits between delivery and 12 months postpartum, of which 28 (29%) living with HIV and 44 (61%) were not living with HIV. Mother-infant pairs had a median of 41 home and clinic visits (interquartile range [IQR] 31-44) in the infant's first year of life; follow-up was similar CHEU (42 visits, IQR 35-44) and CHUU (40 visits, IQR 30-44). Median follow-up time in the first year was 12 months (IQR 10-12) for both CHEU and CHUU. CMV DNA levels were measured in 1,910 infant urine, 3,285 infant saliva, 2,223 maternal breast milk, and 3,393 maternal saliva samples.

As shown in Table 1, fewer mothers living with HIV than without HIV had other children <5 years old (32% vs 61). Two thirds of mothers experienced household crowding, which was more prevalent among mothers living with HIV than mothers without HIV (75% vs 61%). Most mothers shared a toilet with other households (90%). Most infants were delivered vaginally (96%), and adverse birth outcomes were rare—one CHEU was born preterm (<37 weeks gestation) and one CHEU and one CHUU were born with low birthweight (<2.5kg). More CHUU than CHEU were assigned male sex at birth (64% vs 39%). A substantially greater proportion of CHEU than CHUU

were exclusively breastfed (EBF) for 6 months (82% vs 55%), though duration of any breastfeeding was longer among CHUU (median 18 vs 12 months).

At enrollment, mothers living with HIV had received a median 3.5 years (IQR 1.5-6) of ART and 89% reported receiving tenofovir (TDF) + lamivudine (3TC) + efavirenz (EFV); among three mothers receiving other regimens, two reported zidovudine (AZT)+3TC+EFV and one reported AZT+3TC+ritonavir-boosted atazanavir (ATV/r). Most (89%) mothers living with HIV were diagnosed prior to the enrollment pregnancy, all of whom initiated ART before conception. All but one mother living with HIV (95%) had an enrollment CD4 count ≥200 cells/μl (median 562, IQR 416-680) and just over three-quarters (79%) reported receiving cotrimoxazole preventive therapy (CPT) during pregnancy. All CHEU received antiretrovirals for HIV prophylaxis and CPT and no infants acquired HIV.

Table 1. Selected participant characteristics.

		All		HIV-unexposed uninfected		HIV-exposed uninfected
	N	**Median (IQR) or n (%)**	**N**	**Median (IQR) or n (%)**	**N**	**Median (IQR) or n (%)**
Maternal Characteristics						
Age (years) at enrollment	72	26 (24-30)	44	25 (23-30)	28	27 (24-31)
Married or steady partner	72	68 (94.4)	44	43 (97.7)	28	25 (89.3)
Highest education level	72		44		28	
Secondary or higher		42 (58.3)		27 (61.4)		15 (53.6)
Primary or lower		30 (41.7)		17 (38.6)		13 (46.4)
Employed	72	24 (33.3)	44	14 (31.8)	28	10 (35.7)
Weekly income (USD)[1]	16	15.00 (8.75-27.00)	11	15.00 (5.00-30.00)	5	12.50 (10.00-20.00)
Lifetime number of pregnancies	72	2 (2-3)	44	2 (2-3)	28	2 (2-4)
Number of living children[2]	60	2 (1-2)	35	1 (1-2)	25	2 (1-3)
Sociodemographics						
Home roofing material	72		44		28	
Metal		54 (75.0)		32 (72.7)		22 (78.6)
Concrete		17 (23.6)		12 (27.3)		5 (17.9)
Other		1 (1.4)		0 (0.0)		1 (3.6)
Experienced crowding (>3 people/room) in home	72	48 (66.7)	44	27 (61.4)	28	21 (75.0)
Any siblings <5 years[3]	72	36 (50.0)	44	27 (61.4)	28	9 (32.1)
Number of siblings <5 years[3]	36	1 (1-1)	27	1 (1-1)	9	1 (1-1)
Toilet is outside the home	72	66 (91.7)	44	40 (90.9)	28	26 (92.9)
Toilet is shared with other households	72	65 (90.3)	44	39 (88.6)	28	26 (92.9)
Home has running water	72	19 (26.4)	44	10 (22.7)	28	9 (32.1)
Home has electricity	72	70 (97.2)	44	43 (97.7)	28	27 (96.4)
Infant Characteristics						
Male sex assigned at birth	72	39 (54.2)	44	28 (63.6)	28	11 (39.3)
Vaginal delivery	69	66 (95.7)	43	41 (95.4)	28	25 (96.2)
Gestational age at birth (weeks)	68	38 (38-40)	43	38 (38-40)	25	38 (38-39)

Birthweight (kg)	68	3.3 (3.0-3.6)	42	3.4 (3.2-3.7)	26	3.2 (3.0-3.5)
Ever breastfed	72	72 (100.0)	44	44 (100.0)	28	28 (100.0)
Exclusively breastfed for 6 months[4]	65	43 (66.2)	38	21 (55.3)	27	22 (81.5)
Months of exclusive breastfeeding[4]	65	6 (5-6)	38	6 (5-6)	27	6 (6-6)
Months of any breastfeeding[5]	49	15 (12-19)	29	18 (15-20)	20	12 (12-14)

IQR = Interquartile range. USD = US dollars. [1] If employed. [2] If ≥1 pregnancy prior to pregnancy at enrollment. Includes enrolled infant. [3] If mother reporting having other children <5 years at time of enrollment. [4] Among mother-infant pairs followed for ≥6 months in the parent cohort. [5] Among mother-infant pairs followed for 24 months in the parent cohort.

CMV Acquisition in CHEU and CHUU

Overall, 53/72 (74%) infants acquired CMV during the first year of follow-up. One CHUU had detectable CMV DNA the first urine sample collected four days after birth, followed by persistent shedding of CMV DNA in both urine and saliva at all subsequent time points (Figure S1), and was considered to have congenital CMV infection; this infant was excluded from further analyses. The cumulative probability of primary CMV infection in the first 12 months of life among the remaining 71 infants was 79% overall and was similar between CHEU and CHUU (log-rank p=0.7; Figure 1). Median time to CMV infection was similar between CHEU (2.6, IQR 1.7-4.7) and CHUU (2.2, IQR 2.0-6.0; overall 2.4, IQR 2.0-4.7), as was median age at primary infection (CHEU: 1.9 months, IQR 1.5-3.4; CHUU: 2.0, IQR 1.4-3.2; overall: 2.0, IQR 1.5-3.2).

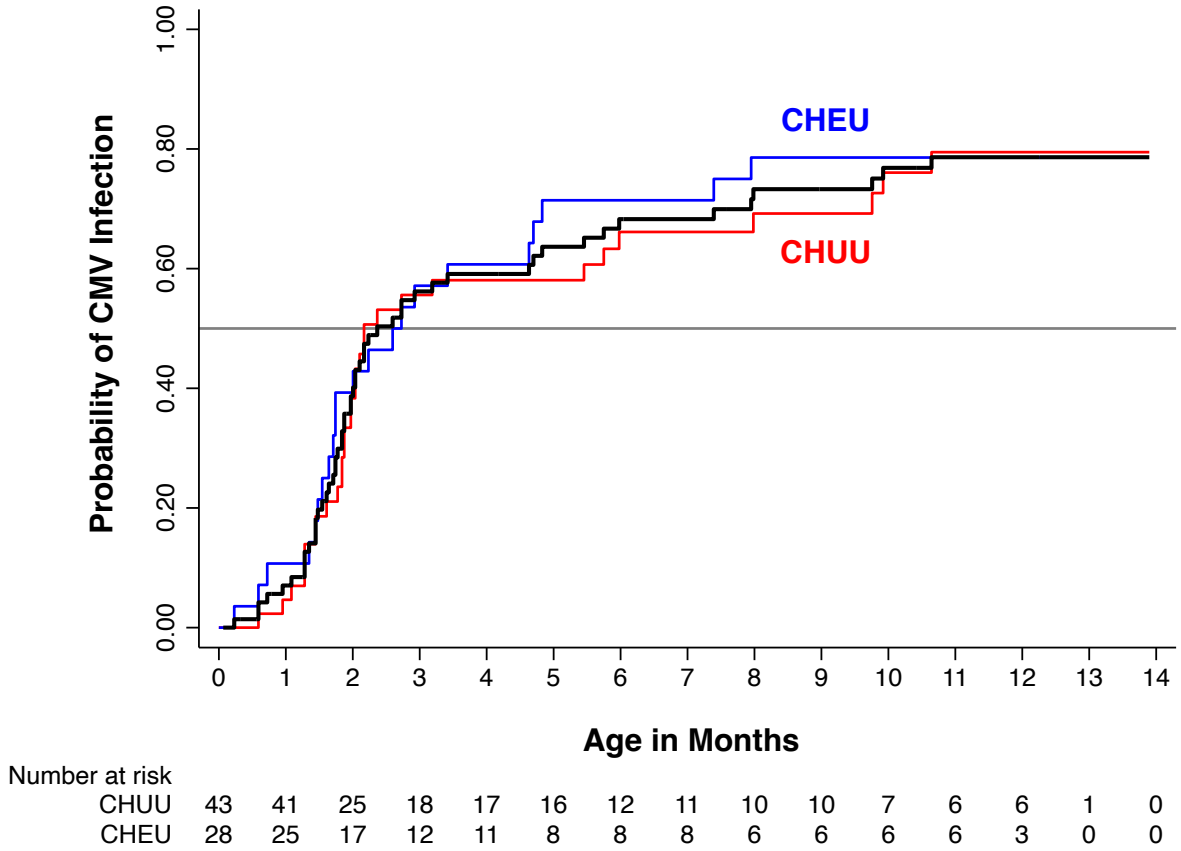

Figure 1. Kaplan-Meier curve for the probability of primary CMV infection. The black line indicates the survival probability among all infants. The horizontal line shows the median survival probability (0.5).

Correlates of CMV Acquisition

Among all infants, the incidence of primary CMV infection was 15.9 per 100 person-months (95% CI: 12.1-20.8) and did not differ by most infant, social, and environmental characteristics (Table 2), including between CHEU (15.4, 95% CI: 10.8-21.9 per 100 person-months) and CHUU (16.6, 95% CI: 11.0-25.3 per 100 person-months). Incidence was higher when infants were EBF (22.1, 95% CI: 16.5-29.5 per 100 person-months) than when they received mixed feeds or were weaned (5.7, 95% CI: 2.7-12.0 per 100 person-months). However, because EBF was collinear with age—most infants were introduced new foods and liquids other than breast milk at approximately 6 months old (Table 1)—models adjusting for feeding mode violated the proportional hazards assumption. Consequently, we excluded feeding mode as a correlate in the survival analysis and stratified the adjusted models by visit-level EBF versus not EBF to account for confounding by feeding mode.

The hazard of primary CMV infection did not differ in either the unadjusted or adjusted analysis by infant sex, having any siblings <5 years old, experiencing household crowding, having a toilet outside the home, or sharing a toilet with other households (Table 2). HIV exposure also was not significantly associated with CMV acquisition either before adjusting for infant sex and household crowding (HR=1.10, 95% CI: 0.64-1.89, p=0.737) or after adjusting, regardless of whether infants were EBF (aHR=1.24, 95% CI: 0.69-2.23, p=0.471) or not EBF (aHR=0.52, 95% CI: 0.10-2.68, p=0.438). However, there was an approximately two times greater hazard of CMV acquisition for every $\log_{10}$ increase in breast milk CMV DNA, both when infants were EBF (aHR=2.09, 95% CI: 1.48-2.95, p<0.001) and not EBF (aHR=1.98, 95% CI: 1.26-3.10, p=0.003). All mothers had detectable CMV DNA in ≥1 breast milk sample, even if their infant did not acquire CMV. There was a weak, but statistically significant, correlation between CMV DNA levels in breast milk and maternal saliva (Person correlation coefficient 0.2, p<0.001) and in the adjusted analysis, the hazard of CMV was 33% higher for every $\log_{10}$ increase of CMV DNA in maternal

saliva when infants were EBF (aHR=1.33, 95% CI: 1.07-1.66, p=0.011); however, there was no association between maternal saliva CMV DNA level and infection when infants were not EBF (Table 2).

Correlates of CMV Acquisition, by HIV Exposure Status

To evaluate whether risk factors for primary CMV infection differed by HIV exposure, we repeated the correlates analysis for CHEU and CHUU separately. To account for confounding by feeding mode, we again stratified by visit-level EBF; however, because most infections occurred before most infants received foods other than breast milk, there was insufficient data to evaluate risk factors at time points when infants were not EBF and results are restricted to visits at which infants were EBF.

As in the combined analysis, having any siblings <5 years old, experiencing household crowding, having a toilet outside the home, or sharing a toilet with other households were not associated with primary CMV infection among either CHEU or CHUU that were EBF (Table S1). Unlike the combined analysis, though, there was a significant association between infant sex and CMV acquisition that was modified by HIV exposure. Among CHEU that were EBF, the hazard of CMV acquisition was 2.71 (95% CI: 1.05-6.95, p=0.038) times greater among female infants after adjusting for household crowding, while among CHUU, the hazard was 65% lower among female infants (aHR=0.35, 95% CI: 0.14-0.92, p=0.028).

The hazard of primary CMV infection remained about two times greater for each $\log_{10}$ increase in breast milk CMV DNA level among both CHEU (aHR=1.80, 95% CI: 1.16-2.79, p=0.009) and CHUU (aHR=1.97, 95% CI: 1.06-3.67, p=0.033) despite higher mean $\log_{10}$-CMV DNA levels in breast milk from mothers living with HIV throughout most of follow-up (Figure 2). CMV DNA levels in maternal saliva were not significantly associated with infection among CHUU (aHR=1.42, 95% CI: 0.83-2.45, p=0.203) and though there was a 29% higher risk of infection

among CHEU in the unadjusted analysis (HR=1.29, 95% CI: 1.03-1.61, p=0.027), the association was reduced to a trend after adjusting for infant sex and household crowding (aHR=1.25, 95% CI: 0.96-1.62, p=0.100).

Table 2. Incidence and hazard ratios (HR) for hypothesized correlates of primary CMV infection among all infants.

	Person-months	CMV Incidence (cases/100 person-months, 95% CI)	Unadjusted HR (95% CI)	p-value	Exclusively Breasted		Not Exclusively Breastfed	
					Adjusted[1] HR (95% CI)	p-value	Adjusted[1] HR (95% CI)	p-value
Infant characteristics								
Sex assigned at birth								
Female	165.5	13.9 (9.2-20.9)	0.87 (0.50-1.50)	0.612	0.95 (0.53-1.70)	0.860	0.95 (0.17-5.23)	0.956
Male	168.1	17.9 (12.5-25.5)	*Ref*	--	*Ref*	--	*Ref*	--
HIV exposure								
CHEU	132.2	15.4 (10.8-21.9)	1.10 (0.64-1.89)	0.737	1.24 (0.69-2.23)	0.471	0.52 (0.10-2.68)	0.438
CHUU	201.3	16.6 (11.0-25.3)	*Ref*	--	*Ref*	--	*Ref*	--
Social & environmental characteristics								
Siblings <5 years								
Yes	185.6	13.5 (9.1-19.9)	0.72 (0.42-1.24)	0.234	0.68 (0.36-1.29)	0.237	5.48 (0.17-171.98)	0.333
No	147.9	18.9 (13.1-27.4)	*Ref*	--	*Ref*	--	*Ref*	--
Household crowding								
Yes	235.9	14.4 (10.3-20.2)	0.73 (0.41-1.33)	0.309	0.60 (0.32-1.11)	0.103	n/a[2]	n/a[2]
No	97.6	19.5 (12.4-30.5)	*Ref*	--	*Ref*	--	*Ref*	--
Toilet outside of home								
Yes	311.1	15.8 (11.9-20.8)	0.84 (0.27-2.63)	0.766	0.97 (0.33-2.85)	0.962	n/a[2]	n/a[2]
No	22.5	17.8 (6.7-47.4)	*Ref*	--	*Ref*	--	*Ref*	--
Toilet shared with other households								
Yes	298.9	16.4 (12.4-21.7)	1.25 (0.40-3.97)	0.701	1.14 (0.41-3.16)	0.803	n/a[2]	n/a[2]
No	34.6	11.6 (4.3-30.8)	*Ref*	--	*Ref*	--	*Ref*	--
Maternal characteristics								
Log_{10}-CMV DNA level in breast milk	261.2	n/a	**1.72 (1.33-2.23)**	**<0.001**	**2.09 (1.48-2.95)**	**<0.001**	**1.98 (1.26-3.10)**	**0.003**
Log_{10}-CMV DNA level in maternal saliva	331.8	n/a	1.19 (0.94-1.50)	0.143	**1.33 (1.07-1.66)**	**0.011**	1.07 (0.48-2.42)	0.863

CI = Confidence interval. CHEU = Children that were HIV-exposed, uninfected. CHUU = Children that were HIV-unexposed, uninfected. Significant associations (p<0.05) are bolded.
[1] Adjusted models include the correlate, if applicable, HIV exposure, infant sex, and household crowding. [2] Insufficient sample size to calculate hazard ratio.

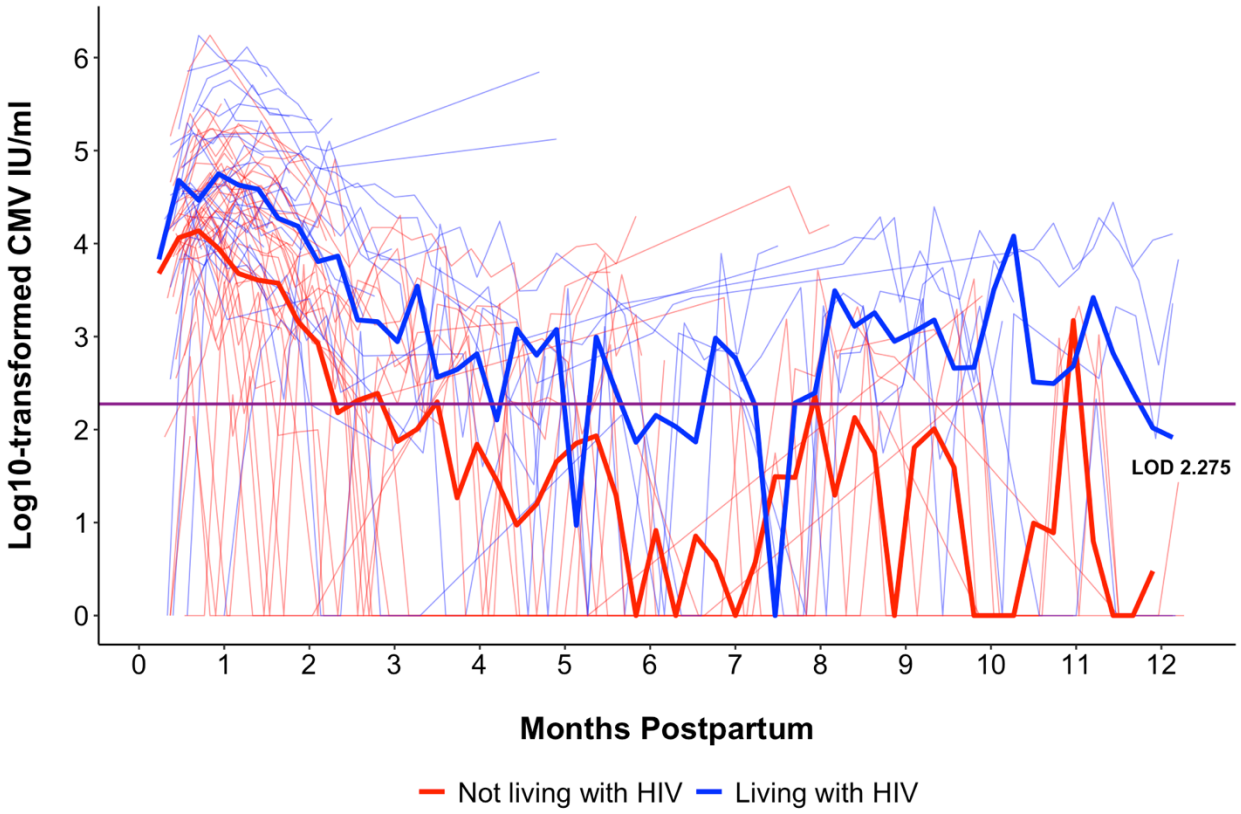

Figure 2. Log_{10}-CMV DNA levels in breast milk, by maternal HIV status. The bold lines represent overall mean log_{10}-CMV DNA levels for each time point. The background lines represent individual patterns of log_{10}-CMV DNA levels. The quantitative PCR limit of detection (LOD) was 2.275 log, as indicated by the horizontal line.

Correlates of CMV Acquisition among CHEU

Finally, we assessed HIV-associated correlates of primary CMV infection among CHEU. Maternal CD4 count at enrollment in the third trimester of pregnancy was not associated with CMV acquisition regardless of whether the infant was EBF (Table 3). However, infants that were EBF whose mothers received TDF+3TC+EFV for ART at enrollment had a 62% lower risk of infection (aHR=0.38, 95% CI: 0.15-0.94, p=0.035) than infants whose mothers received AZT+3TC+EFV or AZT+3TC+ATV/r. Similarly, infants whose mothers received TDF+3TC+EFV or TDF+3TC+dolutegravir (DTG) versus another regimen (see Table 3 footnote) postpartum had a 71% lower risk of infection when EBF (aHR=0.29, 95% CI: 0.10-0.85, p=0.024). There was also a trend for lower risk of CMV infection at time points when CHEU that were EBF received cotrimoxazole prophylaxis (aHR=0.32, 95% CI: 0.090-1.12, p=0.075).

The sample size was insufficient to assess the relationship between maternal ART or infant cotrimoxazole prophylaxis and primary CMV infection at time points when infants were not EBF. Additionally, nearly all infants received antiretroviral (ARV) prophylaxis for prevention of mother-to-child HIV transmission throughout the first year of life, so we did not have enough data to assess ARV exposure as a correlate of CMV.

Table 3. Incidence and hazard ratios (HR) for hypothesized HIV-associated correlates of primary CMV infection among CHEU.

	Person-months	CMV Incidence (cases/100 person-months, 95% CI)	Unadjusted HR (95% CI)	p-value	Exclusively Breasted		Not Exclusively Breastfed	
					Adjusted[1] HR (95% CI)	p-value	Adjusted[1] HR (95% CI)	p-value
Maternal CD4 count at enrollment (cells/μl)	132.2	n/a	1.00 (0.999-1.00)	0.653	1.00 (0.999-1.00)	0.550	0.998 (0.99-1.01)	0.649
Maternal ART regimen at enrollment								
TDF+3TC+EFV	126.9	15.0 (9.6-23.5)	**0.34 (0.16-0.73)**	**0.005**	**0.38 (0.15-0.94)**	**0.035**	n/a[3]	n/a[3]
Other regimen[2]	5.4	56.0 (18.1-173.7)	*Ref*	--	*Ref*	--	*Ref*	--
Maternal postpartum ART regimen								
TDF+3TC+EFV or TDF+3TC+DTG	125.7	15.9 (10.3-24.7)	**0.30 (0.11-0.88)**	**0.027**	**0.29 (0.10-0.85)**	**0.024**	n/a[3]	n/a[3]
Other regimen[4]	4.9	40.6 (10.1-162.3)	*Ref*	--	*Ref*	--	*Ref*	--
Cotrimoxazole prophylaxis exposure[5]								
Yes	96.1	14.6 (8.6-24.6)	0.53 (0.18-1.62)	0.266	0.32 (0.090-1.12)	0.075	n/a[3]	n/a[3]
No	35.6	22.5 (11.2-44.9)	*Ref*	--	*Ref*	--	*Ref*	--

CI = Confidence interval. Significant associations (p<0.05) are bolded. [1] Adjusted models included the correlate and household crowding. [2] AZT+3TC+EFV (n=1) and AZT+3TC+ATV/r (n=2). No mothers received TDF+3TC+DTG at enrollment. [3] Insufficient sample size to calculate hazard ratio. [4] AZT+3TC+EFV (n=1), TDF+3TC+NVP (n=4), TDF+FTC+EFV, TDF+FTC+DTG, and AZT+3TC+ATV/r. [5] Visit-level, time-varying correlate.

DISCUSSION

In this birth cohort of healthy women and infants, infant CMV acquisition was associated with higher breast milk CMV DNA levels among all infants. While mothers living with HIV had higher breast milk CMV DNA levels than mothers without HIV, there was no difference in the timing or incidence of primary CMV infection between CHEU and CHUU. These findings emphasize the role of breast milk as the primary source of mother-to-child CMV transmission, but suggest that CMV acquisition among CHEU is similar to that of CHUU when mothers initiate optimized ART before pregnancy.

The overall probability of infant CMV infection in the first year of life was high (79%) and over half of infants acquired CMV by age 3 months. Though we did not confirm the mothers' CMV serostatus, all had ≥1 breast milk sample with detectable CMV DNA, indicating they all had a new or reactivated CMV infection. Few studies have compared postnatal risk of CMV acquisition between infants who are HEU and HUU. We observed a higher probability of infant CMV infection than a similar study of mother-infant pairs in Uganda followed weekly for one year between 2008-2009, which found a 59% overall probability of CMV infection among CHEU and CHUU (26). Probability of CMV infection was also lower in a study from Zimbabwe conducted in the Option B+ era, in which 74% of CHEU and 48% of CHUU acquired CMV by 3 months (27).

Vertical CMV transmission declines with improved maternal immune reconstitution and HIV viral suppression on ART. In Kenya, we previously compared CHEU and CHUU during the pre-ART and early ART eras, and observed higher rates of transmission in this context. Among mothers receiving short-course zidovudine during pregnancy. Ninety percent of CHEU acquired CMV by 3 months (28). In a subsequent cohort comparing HIV-exposed infants born to mothers receiving highly active ART versus short-course zidovudine, probability of CMV acquisition by 12 months was 75% and 94%, respectively (29). The probability of CMV transmission in the current Linda Kizazi cohort is similar to the ART arm of our previous study, underscoring the role of ART in decreasing vertical CMV transmission.

We identified only one case of congenital CMV (cCMV) infection in an infant who was HUU and thus could not assess and compare risk factors of cCMV. While no CHEU had cCMV, our cohort size was likely too small to compare cCMV prevalence by HIV exposure. Recent studies suggest CHEU continue to have higher risk of cCMV in the era of universal ART—researchers in Spain identified more cCMV cases among CHEU than CHUU, though noted the prevalence of cCMV among CHEU had declined from the pre-universal ART era (30). Similarly, data from a cohort in South Africa showed the odds of cCMV were 20 times greater among CHEU born to women on long-term ART than among CHUU (31).

Breastmilk is the major route of vertical CMV transmission (32). Breast milk CMV DNA level was significantly associated with primary CMV infection among all infants, consistent with other studies of both CHEU (29,33–35) and CHUU (9,36–38), though most research among CHUU has focused on children born at low birthweight or preterm. Surprisingly, the risk of CMV infection did not differ between CHEU and CHUU despite higher mean breast milk CMV DNA levels among mothers living with HIV. We were underpowered to demonstrate differences in risk of CMV infection in CHEU versus CHUU given our small sample of 28 CHEU. Higher HIV viral load and lower CD4 count are associated with higher cervicovaginal and breast milk CMV DNA levels, as well as earlier and higher risk of CMV infection among HIV-exposed infants (29,33–35,39). We found that despite initiation of optimized ART before pregnancy and normal CD4 count, mothers living with HIV still had higher breast milk CMV DNA levels than mothers without HIV.

Similarly, we did not observe an association between third trimester CD4 count and CMV acquisition among CHEU, likely due to low heterogeneity of CD4 in our cohort. Our results are similar to a study in Malawi among women who started optimized ART during pregnancy, which also observed persistently high breast milk CMV DNA levels throughout the first year postpartum and high probability of CMV acquisition among CHEU despite maternal CD4 recovery (40). Breast milk CMV DNA levels among women living with HIV may not be representative of how much

infectious versus latent virus is present, or may not be correlated with immune factors, such as antibodies, that could confer protection against CMV to breastfed infants.

Among both CHEU and CHUU, there was no association between having any siblings <5 years old, experiencing household crowding, having a toilet outside the home, or sharing a toilet with other households and CMV acquisition. These findings differ from other studies that found higher CMV risk among individuals exposed to young children, who experienced lower socioeconomic status, or that had frequent contact with body fluids (26,41–43). This is likely due to the small sample size of our comparison. It is also possible we observed less heterogeneity in these characteristics between infants with and without CMV infection because all participants were recruited the same small, densely populated neighborhood. Alternatively, risk may have differed by other unmeasured factors known to be associated with CMV, such as daycare attendance or underlying health conditions.

Among CHEU, hazard of CMV was significantly lower when their mothers received a recommended first line ART regimen than a different regimen, though we had limited statistical power to explore this relationship. CHEU morbidity in infancy is associated with higher maternal HIV viral load, lower CD4 count, and higher inflammation, which can lead to immune dysfunction and inflammation in the infant themselves (44). Compared to older regimens, current optimized ART regimens—TDF+3TC+EFV with or without a switch to TDF+3TC+DTG, as directed by the Kenya Ministry of Health during study follow-up (45)—more effectively suppress viral replication, restore immune function, and reduce inflammation with good adherence, which in turn could minimize these risk factors for infection among CHEU. However, we did not have data on why mothers received alternative ART regimens, nor for postpartum HIV viral load or CD4 count, so could not assess whether this difference was independently associated with ART regimen or was confounded by underlying maternal health characteristics, such as drug resistance, stage of HIV disease, or immune impairment. Additional research in larger cohorts is needed to determine whether current ART regimens influence mother-to-child CMV transmission.

There was also a trend for lower hazard of CMV infection when CHEU were receiving cotrimoxazole. To our knowledge, no study has assessed the relationship between cotrimoxazole prophylaxis and CMV incidence among CHEU. As an antibiotic, cotrimoxazole does not directly affect CMV, but by reducing other infectious morbidity, but it could lower infants' susceptibility to CMV by reducing burden on the developing immune system and minimizing systemic inflammation. However, recent studies do not show an association between cotrimoxazole prophylaxis and lower morbidity or mortality among CHEU, except for malaria (46). Additionally, we did not find an association between cotrimoxazole and diarrhea or any respiratory infection among all CHEU in the full Linda Kizazi cohort (25).

Finally, we found evidence of effect modification by HIV exposure on the association between infant sex assigned at birth and CMV infection. In the analysis stratified by HIV exposure, female CHEU had nearly three times greater hazard of CMV than male CHEU, while female CHUU had 65% lower hazard than male CHUU. Male children have higher levels of inflammation and greater susceptibility to infectious pathogens in childhood, which may explain why we observed a lower risk of CMV among female CHUU (47). In a cohort of infants that were HEU in Kenya, female infants were more likely to have HIV-specific T-cell responses at 3 months of age, which suggests there may be sex-specific immune responses to HIV exposure that could explain the higher risk of CMV among female CHEU (48). However, this study was conducted before availability of ART and further investigation is needed to describe and compare immune responses to well-treated maternal HIV among female and male infants.

There were several limitations to this study. The small sample size and relatively high proportion of infants that were EBF for 6 months limited our ability to quantify the association between several correlates and CMV infection when infants were not EBF. However, our findings from time points at which infants were EBF are likely most relevant to understanding risk of CMV infection since most infants acquired CMV within the first three months of life. Our statistical power

was also improved by weekly, longitudinal sampling for one year postpartum, which is a unique strength of this study.

CONCLUSION

This study assessed the timing and incidence of CMV between CHEU versus CHUU in the era of universal, optimized ART. We found that infants had high probability of CMV acquisition in the first year of life regardless of HIV exposure. Mothers with HIV had higher breast milk CMV levels and larger studies in the optimized ART era are necessary to determine whether CMV risk is elevated in CHEU.

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SUPPLEMENTARY INFORMATION

Oral Swab Preservative

The study laboratory prepared a premixed solution of 18.65g KCl, 500g ACS Reagent, 25ml Tris HCl 1M (pH 8.0), 125ml EDTA 0.5M, 25ml IGEPAL CA_630, and 325ml sterile water, diluted in a 1:4 ratio with additional sterile water. One milliliter of solution was aliquoted into a 2ml cryovial that was sealed and stored at room temperature until use for sample collection.

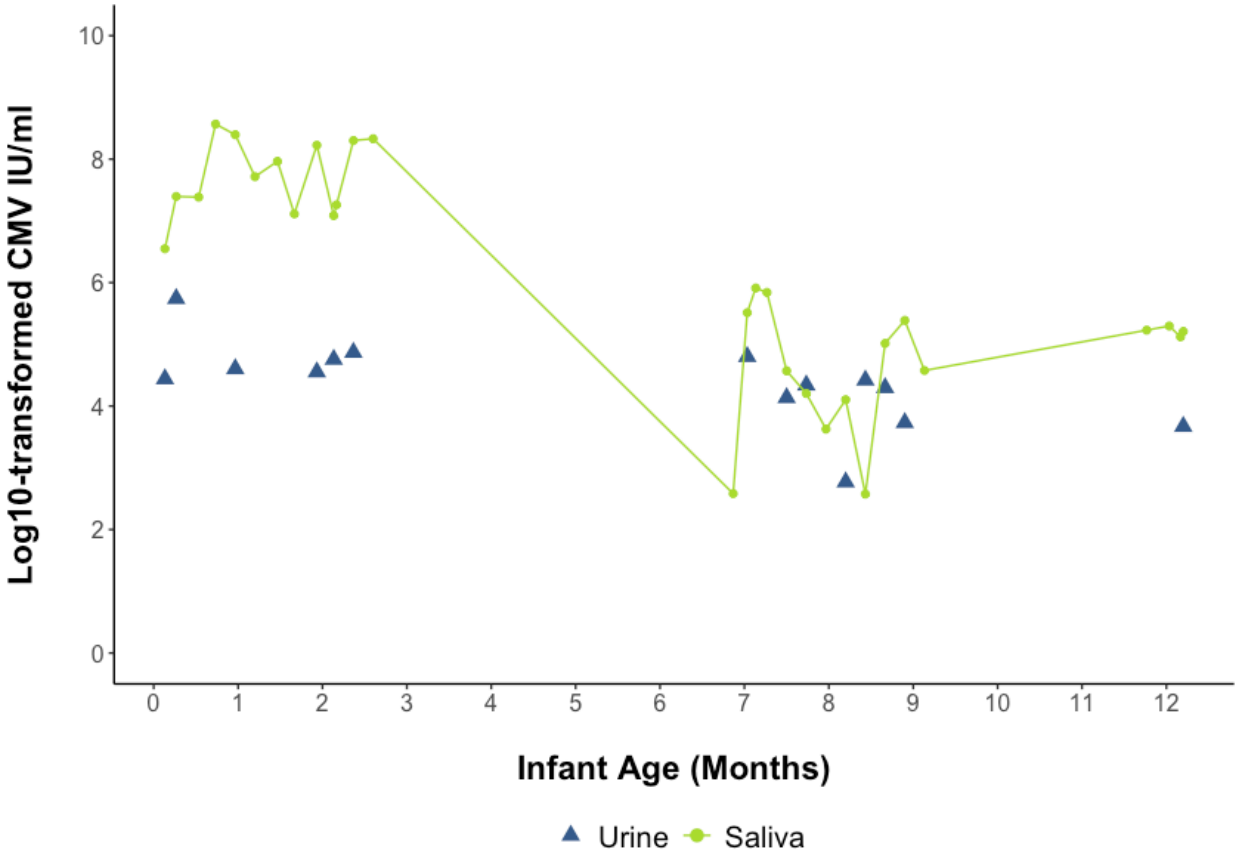

Figure S1. Urine and saliva CMV DNA levels over time for the HIV-unexposed infant with congenital CMV infection.

Table S1. Hazard ratios (HR) for hypothesized correlates of primary CMV infection among exclusively breastfed CHEU versus CHUU.

	HIV-unexposed, uninfected				HIV-exposed, uninfected			
	Unadjusted HR (95% CI)	**p-value**	**Adjusted[1] HR (95% CI)**	**p-value**	**Unadjusted HR (95% CI)**	**p-value**	**Adjusted[1] HR (95% CI)**	**p-value**
Infant characteristics								
Sex assigned at birth								
Female	**0.34 (0.14-0.84)**	**0.020**	**0.35 (0.14-0.92)**	**0.028**	**2.64 (1.04-6.70)**	**0.040**	**2.71 (1.05-6.95)**	**0.038**
Male	*Ref*	--	*Ref*	--	*Ref*	--	*Ref*	--
Social & environmental characteristics								
Siblings <5 years								
Yes	0.66 (0.31-1.42)	0.288	0.63 (0.32-1.23)	0.175	0.56 (0.21-1.49)	0.248	0.64 (0.22-1.87)	0.411
No	*Ref*	--	*Ref*	--	*Ref*	--	*Ref*	--
Household crowding								
Yes	0.69 (0.32-1.46)	0.328	0.80 (0.36-1.77)	0.584	0.51 (0.20-1.31)	0.161	0.49 (0.17-1.40)	0.184
No	*Ref*	--	*Ref*	--	*Ref*	--	*Ref*	--
Toilet outside of home								
Yes	0.56 (0.23-1.35)	0.196	0.80 (0.27-2.38)	0.688	0.96 (0.062-14.83)	0.977	3.87 (0.20-74.40)	0.370
No	*Ref*	--	*Ref*	--	*Ref*	--	*Ref*	--
Toilet shared with other households								
Yes	0.82 (0.31-2.16)	0.690	1.18 (0.43-3.23)	0.748	0.96 (0.062-14.83)	0.977	3.87 (0.20-74.40)	0.370
No	*Ref*	--	*Ref*	--	*Ref*	--	*Ref*	--
Maternal characteristics								
Log_{10}-transformed CMV viral load in breast milk	**2.07 (1.16-3.70)**	**0.014**	**1.97 (1.06-3.67)**	**0.033**	**1.88 (1.34-2.65)**	**<0.001**	**1.80 (1.16-2.79)**	**0.009**
Log_{10}-transformed CMV viral load in saliva	1.44 (0.87-2.41)	0.160	1.42 (0.83-2.45)	0.203	**1.29 (1.03-1.61)**	**0.027**	1.25 (0.96-1.62)	0.100

CI = Confidence interval. Significant associations (p<0.05) are bolded. [1] Adjusted models included the correlate, as applicable, infant sex, and household crowding.

CHAPTER 4. The gut virome of HIV-exposed, uninfected versus HIV-unexposed Kenyan infants

The gut virome of HIV-exposed, uninfected versus HIV-unexposed Kenyan infants

ABSTRACT

Background: The gut virome is seeded at birth and rapidly develops in the first years of life, influenced by numerous health, behavioral, and environmental factors. HIV exposure increases infant morbidity and mortality and could alter virome development. However, no study has characterized the gut virome of children who are HIV-exposed, uninfected (CHEU). To address this gap, we described and compared the gut virome of Kenyan CHEU and children who are HIV-unexposed, uninfected (CHUU) from birth-21 months.

Methods: We performed metagenomic sequencing of the DNA virome on stool samples collected between January 2019-December 2020 from a birth cohort of healthy infants in Nairobi, Kenya. Data from 37 CHUU and 32 CHEU whose mothers received optimized ART during pregnancy and breastfeeding were included. To compare gut virome composition by HIV exposure we used generalized estimating equations models for virome richness and Shannon (alpha) diversity, principal coordinates analysis and PERMANOVA for Bray-Curtis distances (beta diversity), and MaAsLin2 to identify discriminating viral taxa.

Results: Among all infants, viral richness increased over time and was higher after infants were introduced to foods other than breast milk. Adjusting for maternal age, feeding mode, and socioeconomic status, virome richness was lower among CHEU than CHUU, but there were no differences in Shannon diversity or Bray-Curtis distances by HIV exposure. Twenty-eight unique viral contigs were associated with HIV exposure, of which 17 (61%) were of the family *Anelloviridae*.

Conclusions: Infant age and feeding mode significantly altered gut virome richness and Shannon diversity in both CHEU and CHUU. However, despite some differences in the presence versus absence of specific viral taxa between CHEU and CHUU, we did not observe a large effect of HIV exposure on virome composition. These results suggest that CHEU born to women on optimized ART have similar gut virome ecology and development as their CHUU peers.

INTRODUCTION

The human microbiome consists of bacteria, viruses, fungi, and other microbes that colonize the body. The viral component of the microbiome, or the "virome", includes eukaryotic viruses, some of which infect human cells, and bacteriophages, the prokaryotic viruses that infect our microbiome's bacteria (1). The gut contains the highest concentration of microbiota (1,2) and a growing body of research suggests the gut microbiome is an essential component of immune system development and regulation throughout life (3,4).

Studies have linked the gut bacterial microbiome to a wide range of health outcomes including inflammatory bowel disease, metabolic disorders such as diabetes, allergies and auto-immune diseases, and mental health (2,5), but substantially less is known about the virome's role in health and disease. The virome is difficult to study, in part because viruses do not have a common, conserved genetic region like the 16S ribosomal RNA gene in bacteria and also because they evolve rapidly, are more difficult to isolate from specimens, and cannot be cultured easily (6). Much of the virome remains uncharacterized, sometimes referred to as "viral dark matter" (7). However, despite these challenges, recent advancements in sequencing technology and bioinformatics methods have helped us better describe the gut virome's composition and development.

The gut virome is thought to be seeded at birth (8–10), rapidly expanding and undergoing a series of developmental changes in the first years of life until a stable, highly individual-specific virome is established (1,11). Mother-to-child transmission of viruses during birth and after delivery through breastfeeding and close contact is thought to be a primary source of the infant virome, though there is less evidence that *in utero* transmission of viruses contributes substantially to virome seeding (1,12). Caesarean section is associated with lower virome diversity than vaginal delivery since infants are not directly exposed to the mother's virome in the birth canal (13,14). Breastfeeding facilitates mother-to-infant transmission of viruses in breast milk and from the breast skin (15–18). Breast milk also is a primary source of colonizing bacteria that contain viral

prophages, which are induced to replicate and comprise a large proportion of the early gut virome (9,10). Compared to formula feeding, breastfeeding is associated with lower virome alpha diversity, lower richness of temperate bacteriophages, and fewer eukaryotic viruses (9,10). Diet continues to shape the gut virome through infancy—plant viruses appear after the introduction of foods other than breast milk (19) and malnutrition can be associated with altered virome development (20,21). Additionally, studies have shown that virome varies with cohabitation and by geographic location, suggesting that environmental exposures are a key source of our virome (22–24).

Children who are HIV-exposed, uninfected (CHEU) have greater risk of morbidity and mortality than children who are HIV-unexposed, uninfected (CHUU), especially in the first months of life, and the mechanisms for this difference remain unclear (25,26). Because infancy is a critical time for gut immune development, understanding the gut virome composition of CHEU in this critical window may help clarify mechanisms of morbidity and identify new opportunities for intervention. HIV infection is known to alter the gut bacterial microbiome and virome of individuals living with HIV (27), which could affect which viruses are transmitted from mother to infant to seed the virome. Additionally, numerous factors associated infant morbidity and mortality, such feeding practices, socioeconomic and environmental factors, and exposure to antimicrobial medications, often differ between CHEU and CHUU (25,26) and could alter gut virome composition both directly and through interactions with the bacterial microbiome and immune system. However, there are limited data comparing the gut microbiome of CHEU and CHUU, and to our knowledge, no study has described the gut virome of CHEU.

To address this gap, we leveraged a prospective cohort of mother-infant pairs in Nairobi, Kenya to describe the gut virome in CHEU born to healthy women living with HIV on lifelong, optimized ART, and to compare gut virome ecology between these CHEU and their CHUU peers.

METHODS

Study Population

This analysis uses data and specimens collected in the Linda Kizazi Study, a prospective cohort of healthy mother-infant pairs followed from the third trimester of pregnancy (28-42 weeks gestation) through two years postpartum. Detailed study methods were described previously (28). Briefly, pregnant women living with HIV and women without HIV from the same neighborhood were recruited at Mathare North Health Centre, an urban maternal and child health clinic that provides both pre- and post-natal care. Eligible women were 18-40 years old, planned to breastfeed, did not have a serious medical condition, and if living with HIV, had received at least 6 months of ART. Infants that had ≥3 stool samples collected between birth-12 months or ≥1 stool sample between 12-24 months were eligible for this nested study.

Data & Specimen Collection

Participants received a home visit approximately 4 days postpartum and attended clinic-based visits at postpartum week 6, week 10, and every 3 months from month 6 to month 24. Study clinicians collected self-reported data on participants' health, medication use, and infant feeding. Infant stool samples for virome sequencing were collected in a sterile plastic collection container. If the infant produced stool during the visit, a study clinician collected the sample from the infant's diaper using the container's built-in scoop. If the infant did not produce stool during the visit, mothers collected the sample themselves following the same procedures (in-person demonstration and picture instructions were provided prior to sample collection) and returned the sample to the study clinic for processing within 4 hours of collection. Stool samples were aliquoted into 2ml vials and stored at -80°C without preservatives until sequenced.

Virome sequencing

After thawing, 200mg of stool was diluted at a 1:6 ratio with SM Buffer and fully homogenized by vortexing at maximum speed for three minutes. Samples were then centrifuged at 4°C for five minutes at 20,000g. The resulting pellet was processed using a DNeasy PowerSoil Pro Kit (Qiagen) per protocol recommendations then prepared for sequencing using the Illumina DNA Prep workflow. Viral supernatant was passed through a 0.20µm filter. To enrich virus-like particles (VLPs), samples were combined with Benzonase (2µl), Baseline Zero 10x Buffer (100µ), and Baseline Zero DNASE (4µl) then heated at 37°C for one hour. Total nucleic acid was extracted from 1ml of the heat-treated sample using the BioMérieux EMAG nucleic acid extraction system then amplified using a GenomiPhi V2 DNA amplification kit (GE Healthcare). The processed samples were then sequenced using the NextSeq 2000 platform (Illumina) with 2x150 base pair output. Identically processed controls of SM Buffer with and without lambdavirus DNA were used to assess contamination during amplification and sequencing steps.

Virome analysis

The paired-end Illumina sequencing reads were filtered for quality and assembled into 3,036,160 DNA contigs (set of reads with overlapping genetic sequences) using metaSPAdes (29). Human contigs were removed with bowtie2 (30) and the remaining 1,513,561 contigs were clustered into potential genomes using CD-HIT (31) and filtered to a minimum length of 1000 base pairs using bbduk (32). Viral contig candidates were identified using cenoteTaker2 (33), VirSorter2 (34), and National Center for Biotechnology Information (NCBI) blastx/blastn. Results from each database tool were filtered using CheckV (35) then combined and duplicates were removed. We retained contigs with at least medium quality and that had more viral than host genes, as well as all identified as proviruses, resulting in 5,657 viral DNA contigs. NCBI blastx was used to query the viral contigs against the viral RefSeq and neighboring sequences database (downloaded January 2023) and taxonomy was assigned to the family level using taxonomizr

(36). Contaminants were identified and removed using decontam (37) at the default threshold of 0.1; 68 contigs were removed and 5,589 contigs were included in the final analysis. Viral contig length was normalized using reads per kilobase million to account for differences in sequencing depth across samples.

Statistical analysis

To describe and compare the gut virome ecology of CHEU and CHUU, we calculated the relative abundance of viral families, richness (number of unique viruses), alpha diversity measured by the Shannon diversity index (a metric accounting for both richness and relative abundance), and beta diversity measured by Bray-Curtis distances (a metric of how dissimilar samples are from each other). Richness, Shannon diversity index, and unweighted and weighted Bray-Curtis distances were calculated using the vegan package (38). The Microbiome Multivariable Association with Linear Models (MaAsLin2) package (39) was used to identify viral contigs that differentiated between groups using the default false discovery rate threshold of 0.25.

Mann-Whitney U and Kruskall-Wallis tests were used to assess overall differences in median richness and Shannon diversity between groups defined by infant HIV exposure (CHEU vs CHUU), study visit, and visit-level infant feeding mode (exclusively breastfeeding, mixed feeding of breast milk and other foods and liquids, or no breastfeeding). Comparisons between each study visit and feeding mode were made using pairwise Wilcoxon rank-sum tests adjusted for multiple comparisons with the Benjamini-Hochberg correction. The associations between infants' HIV exposure (versus no HIV exposure), age in months, and current exclusive breastfeeding (versus mixed feeding or no breastfeeding) and gut virome richness and Shannon diversity index were evaluated using generalized estimating equations models with the independence working correlation. Models were both unadjusted and adjusted for maternal partnership status and education (proxy measures of socioeconomic status), identified *a priori* as confounders.

Principal coordinates analysis (PCoA) was used to visualize beta diversity and differences in weighted Bray-Curtis distances between HIV exposure, feeding mode, and study time points were assessed using a PERMANOVA model (adonis function from vegan). The model included HIV exposure, infant age (grouped as <6 months, 6-12 months, and >12 months), and feeding mode. The Benjamini-Hochberg correction was used to account for multiple comparisons. All statistical analyses were conducted in R (version 4.3.3).

RESULTS

Analysis Sample & Population Characteristics

We identified 219 stool samples collected between January 2019-December 2020 from 69 infants, of which 102 (47%) were from 32 CHEU and 117 (53%) were from 37 CHUU. Sixteen samples did not pass quality controls and were dropped from the analysis; 12 samples excluded before contig assembly had insufficient read depth (<200K reads) and 4 samples failed to produce contigs. These exclusions resulted in the omission of two infants (one CHEU, one CHUU) from the final analysis, which included 98 samples (48%) from 31 CHEU and 105 samples (52%) from 36 CHUU. Both CHEU and CHUU contributed a median of 3 samples (range 1-7) from study visits between day 4 and month 21. Samples contained an average of 3,188,760 $\pm$ 2,598,239 viral metagenomic reads.

Mothers living with HIV were older than mothers without HIV (median 30 vs 26 years at enrollment) and a greater proportion had secondary or higher education (68% vs 36%, respectively). A few HIV-exposed mother-infant pairs (n=3, 9.7%) were exposed to cigarette smoking in their household versus no HIV-unexposed pairs. More CHUU than CHEU were assigned male sex at birth (64% vs 52%). Additionally, a substantially greater proportion of CHEU than CHUU were exclusively breastfed for 6 months (90% vs 53%), although median duration of any breastfeeding was longer among CHUU than CHEU (18 vs 12 months; Table 1).

Overall, in both mothers with and without HIV, most had a partner (96%), a median of 2 other living children in addition to the enrolled infant, and 45% were employed, earning a median 15 US dollars per week. A majority (61%) of mother-infant pairs experienced household crowding, few (10%) had a toilet inside the home, and most (88%) shared a toilet with other households. Though only 21% of households had running water, most (97%) had electricity. All infants, except one CHEU, were born by vaginal delivery. Consistent with the parent cohort, there were few adverse birth outcomes—no infants had low birthweight (<2.5 kg) and two CHEU were born preterm (<37 weeks gestation; 6%). Median gestational age at delivery was 38 weeks (IQR 38-39) for all infants and median birthweight was similar for CHEU (3.2 kg) and CHUU (3.3 kg).

Because eligibility criteria included requiring being on ≥6 months of ART, all but two mothers living with HIV (n=29, 94%) were diagnosed before pregnancy, all of whom initiated ART before pregnancy. Median years of ART at enrollment was 5 (IQR 3-6) and most mothers living with HIV (87%) received combination tenofovir/lamivudine/efavirenz (TDF+3TC+EFV) as ART at enrollment. None reported receiving tenofovir/lamivudine/dolutegravir (TDF+3TC+DTG) during pregnancy, though 21/31 (68%) switched to this regimen by the end of follow-up per changes to Kenya Ministry of Health guidelines. All mothers living with HIV had a CD4 count >200 cells/μl at enrollment (median 596, IQR 467-686). All CHEU and about three-quarters (74%) of mothers living with HIV received cotrimoxazole preventive therapy and all CHEU received antiretroviral prophylaxis.

Table 1. Selected participant characteristics

		All		HIV-unexposed uninfected		HIV-exposed uninfected
	N	Median (IQR) or n (%)	N	Median (IQR) or n (%)	N	Median (IQR) or n (%)
Maternal Characteristics						
Age (years) at enrollment	67	27 (24-32)	36	26 (23-30)	31	30 (26-32)
Married or steady partner	67	64 (96)	36	35 (97)	31	29 (94)
Highest education level	67		36		31	
Secondary or more		34 (51)		13 (36)		21 (68)
Primary or less		33 (49)		23 (64)		10 (32)
Employed	67	30 (45)	36	17 (47)	31	13 (42)
Weekly income (USD)[1]	22	15 (10-30)	13	15 (15-30)	9	13 (8-20)
Lifetime number of pregnancies	67	3 (2-3)	36	2 (2-3)	31	3 (2-4)
Number of other living children[2]	57	2 (1-2)	27	2 (1-2)	30	2 (1-2)
Sociodemographics						
Experienced crowding (>3 people/room) in home	67	41 (61)	36	22 (61)	31	19 (61)
Home roofing material	67		36		31	
Metal		50 (75)		25 (69)		25 (81)
Concrete		17 (25)		11 (31)		6 (19)
Toilet is outside the home	67	60 (90)	36	32 (89)	31	28 (90)
Toilet is shared with other households	67	59 (88)	36	31 (86)	31	28 (90)
Home has running water	67	14 (21)	36	7 (19)	31	7 (23)
Home has electricity	67	65 (97)	36	36 (100)	31	29 (94)
Someone smokes cigarettes inside home	67	3 (4.5)	36	0 (0)	31	3 (9.7)
Infant Characteristics						
Male sex assigned at birth	67	39 (58)	36	23 (64)	31	16 (52)
Gestational age at birth (weeks)	61	38 (38-39)	33	38 (38-39)	28	38 (38-39)
Birthweight (kg)	60	3.3 (3.0-3.5)	32	3.3 (3.1-3.5)	28	3.2 (3.0-3.5)
Ever breastfed	67	67 (100)	36	36 (100)	31	31 (100)

Exclusively breastfed for 6 months[3]	67	47 (70)	36	19 (53)	31	28 (90)
Months of exclusive breastfeeding[3]	67	6 (5-6)	36	6 (4-6)	31	6 (6-6)
Months of any breastfeeding	67	13 (12-19)	36	18 (15-23)	31	12 (11-12)

IQR = Interquartile range. USD = US dollars. [1] If employed. [2] In addition to the enrolled infant, if ≥1 pregnancy prior to pregnancy at enrollment. [3] All mother-infant pairs were followed for ≥6 months.

Virome Composition

First, we assessed which viral taxa were present in the gut virome of infants in our cohort. Generally, the gut DNA virome was dominated by bacteriophages in most samples and at most time points. Phages primarily were from the class *Caudoviricetes* and family *Microviridae* within the class *Malgrandaviricetes*. Of the 5,589 unique viral contigs, 38% (n=2111) were *Caudoviricetes* viruses without a family level classification and 1.6% (n=93) could not be classified at either the class or family level. *Caudoviricetes* with a family-level taxonomic assignment (607/2718, 22%) belonged to families *Salasmaviridae*, *Winoviridae*, and the crAssphages *Suoliviridae*, *Steigviridae*, and *Intestiviridae*. The most abundant eukaryotic virus families were *Anelloviridae* (overall mean relative abundance 17%) and *Genomoviridae* (6%), though we also detected *Circoviridae* (0.4%), *Adenoviridae* (0.4%), *Geminiviridae* (0.3%), and *Parvoviridae* in our samples (0.2%; Table S1).

Next, we evaluated whether the relative abundance of these taxa differed between groups defined by HIV exposure. Without accounting for differences in age or feeding mode, the overall pattern of relative abundance was similar between CHEU and CHUU (Figure 1A). Mean relative abundance of *Caudoviricetes* was similar between CHEU and CHUU (56% vs 57%, respectively), though CHEU had slightly higher relative abundance of *Microviridae* (20% vs 15%) and slightly lower relative abundance of eukaryotic viruses (21% vs 28%).

Given the similar distribution of viral taxa between CHEU and CHUU, we next assessed changes over time among all infants, regardless of HIV exposure. The relative abundance of any *Caudoviricetes* generally was highest before month 6 and decreased over time (Figure 1B) from a mean relative abundance of 72% at Day 4 to 43% at month 18. Conversely, the relative abundance of *Microviridae* increased over time, from a mean of 13% at Day 4 to 41% at month 18 (Figure S1). *Caudoviricetes* were more abundant when infants were exclusively breastfed, which primarily occurred in the first 6 months of life (Table 1), than when they received both breast milk and other foods (mixed feeding) or were not breastfed (Figure 1C; mean relative abundance

65% vs 50% and 47%, respectively). Similarly, *Microviridae* were more common when infants no longer breastfed after 12-18 months (Table 1) than when exclusively breastfeeding or receiving mixed feeding (mean relative abundance 33% vs 13% and 17%, respectively). Eukaryotic viruses and were prevalent at all time points with variable relative abundances but no clear temporal trend, though the relative abundance was highest at month 6 (mean 44% vs ≤30% at other time points; Figure S1). The relative abundance of eukaryotic viruses was higher with mixed feeding than exclusive breastfeeding (mean 31% vs 19%) but did not differ between no breastfeeding and exclusive breastfeeding (mean 19% each).

We also compared the prevalence of viral contigs across samples to assess whether the infant virome was shared versus unique. Of the 5,589 contigs, 94 (1.7%) were each found in only one sample, 28% of which were of the class *Caudoviricetes*. Only one unclassified *Caudoviricetes* contig was found in all samples and only 126 contigs (2.3%) were found in >50% of samples, suggesting most contigs were unique to individual infants and time-points.

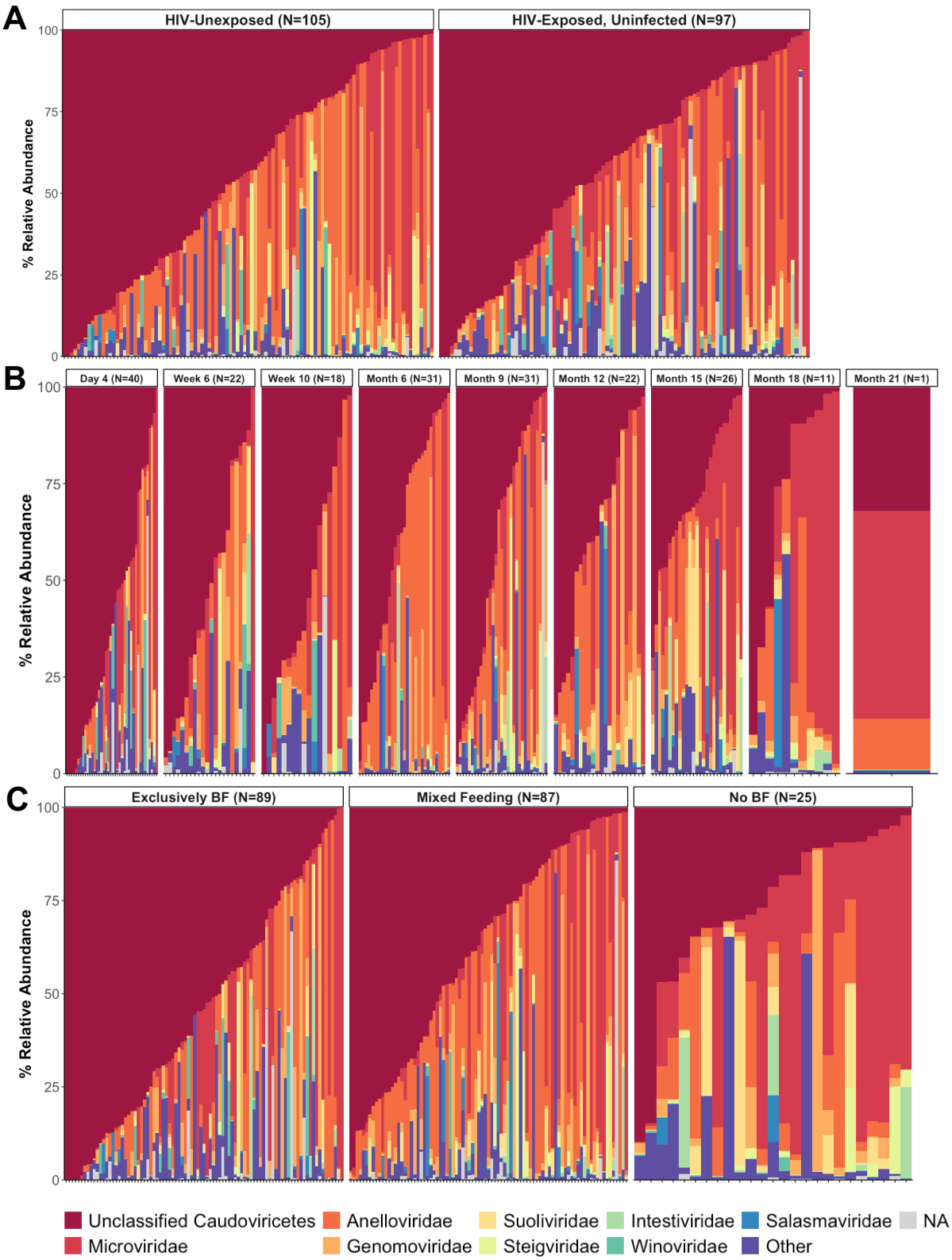

Figure 1. Relative abundance of viral families in stool samples by A) HIV exposure, B) study visit, and C) infant feeding. Families with <1% mean relative abundance were grouped into Other (see Table S1 for all families). Contigs that did not match any known viral families and that were not of the class *Caudoviricetes* were categorized as NA. All families are bacteriophages except eukaryotic viral families *Anelloviridae* and *Genomoviridae*. All bacteriophage families belong to class *Caudoviricetes* except for *Microviridae*. Families *Suoliviridae*, *Steigviridae*, and *Intestiviridae* are crAssphages.

Diversity Analyses

To more comprehensively describe and compare gut virome ecology by infant age and feeding mode, we next evaluated virome alpha and beta diversity, which combine information about viral prevalence and abundance within samples (alpha diversity; richness, Shannon Index) and between samples (beta diversity; Bray-Curtis distances). Viral contig richness varied significantly by study visit ($p<0.0001$) and increased over time (Figure 2A). Richness was lower in the first three months of life than after 6 months (median 464 [IQR 312-676] vs 1100 [IQR 871-1386]), increasing significantly between the week 10 and month 6 study visits (median 534 [IQR 462-742] vs 1147 [IQR 854-1583]; $p=0.0009$) when many infants were introduced to foods other than breast milk (Table 1). Compared to exclusive breastfeeding (median 605, IQR 322-715), richness was also significantly higher during mixed feeding (median 1121, IQR 850-1448) and no breastfeeding (median 1098, IQR 926-1357; $p<0.0001$ for both).

Shannon diversity also varied significantly by study visit ($p=0.007$) indicating a change in alpha diversity over time, though unlike for richness, there were no statistically significant changes between consecutive time points (Figure 2B). Infants that received both breast milk and other foods had higher Shannon diversity than exclusively breastfed infants (Figure 3B; median 3.3 [IQR 2.4-3.8] vs 2.9 [IQR 2.2-3.3]; $p=0.01$) but did not differ significantly between exclusive breastfeeding and no breastfeeding (median 2.9, IQR 2.4-3.3; $p=0.8$). However, there were fewer time points at which infants were not receiving any breast milk ($n=25$ vs $n=89$ and $n=87$ for exclusive breastfeeding and mixed feeding, respectively), which limited the statistical power of the latter comparison.

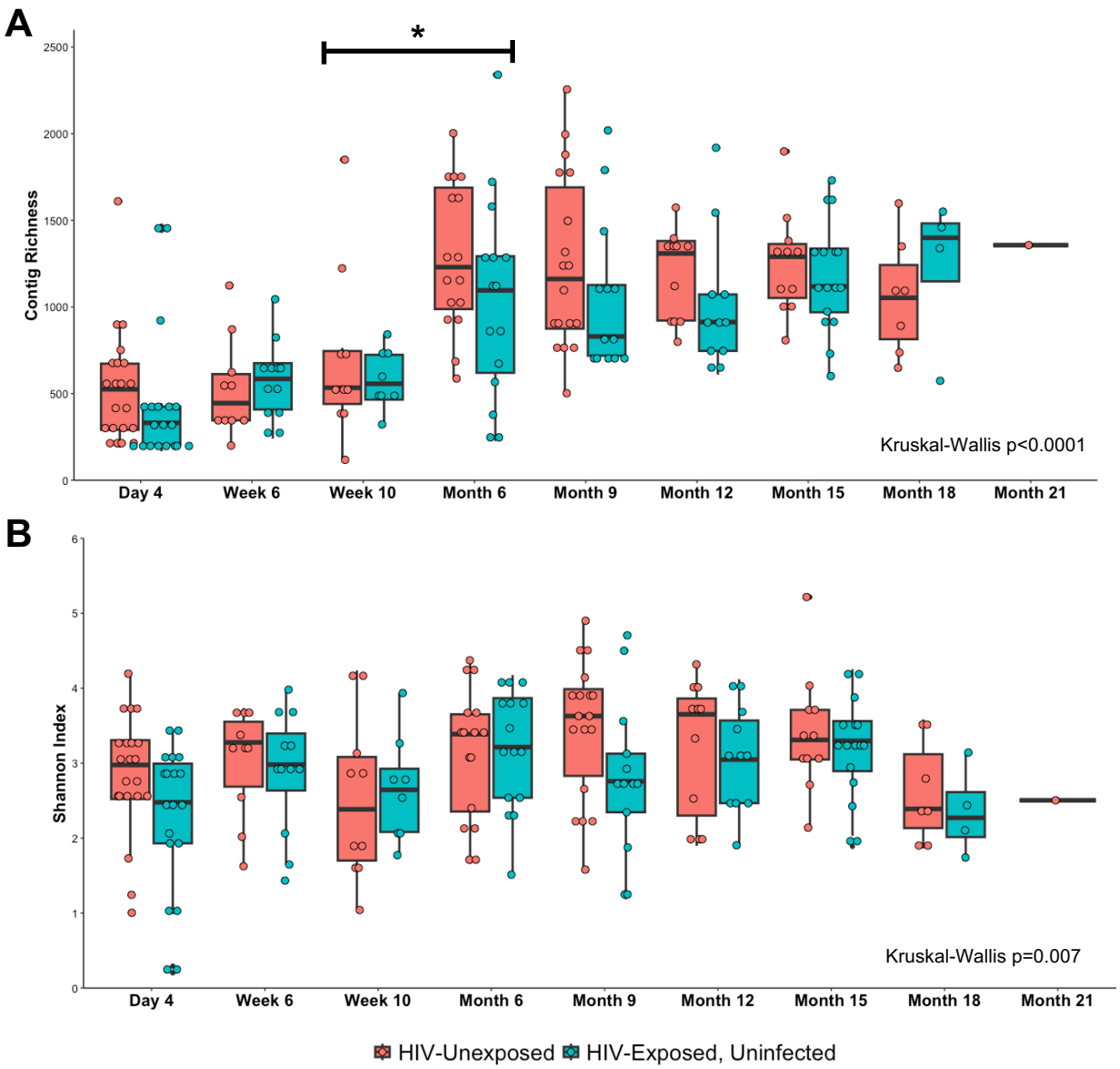

Figure 2. A) Contig richness and B) Shannon diversity index of stool samples by study visit and HIV exposure. Statistical tests included all infants regardless of HIV exposure. Kruskal-Wallis p-values assess differences over time. *A pairwise Wilcoxon rank-sum test comparing richness between week 10 and month 6 was significant (p=0.0009). No other comparisons between consecutive study visits had significantly different richness or Shannon diversity; see Table S2 for all pairwise p-values.

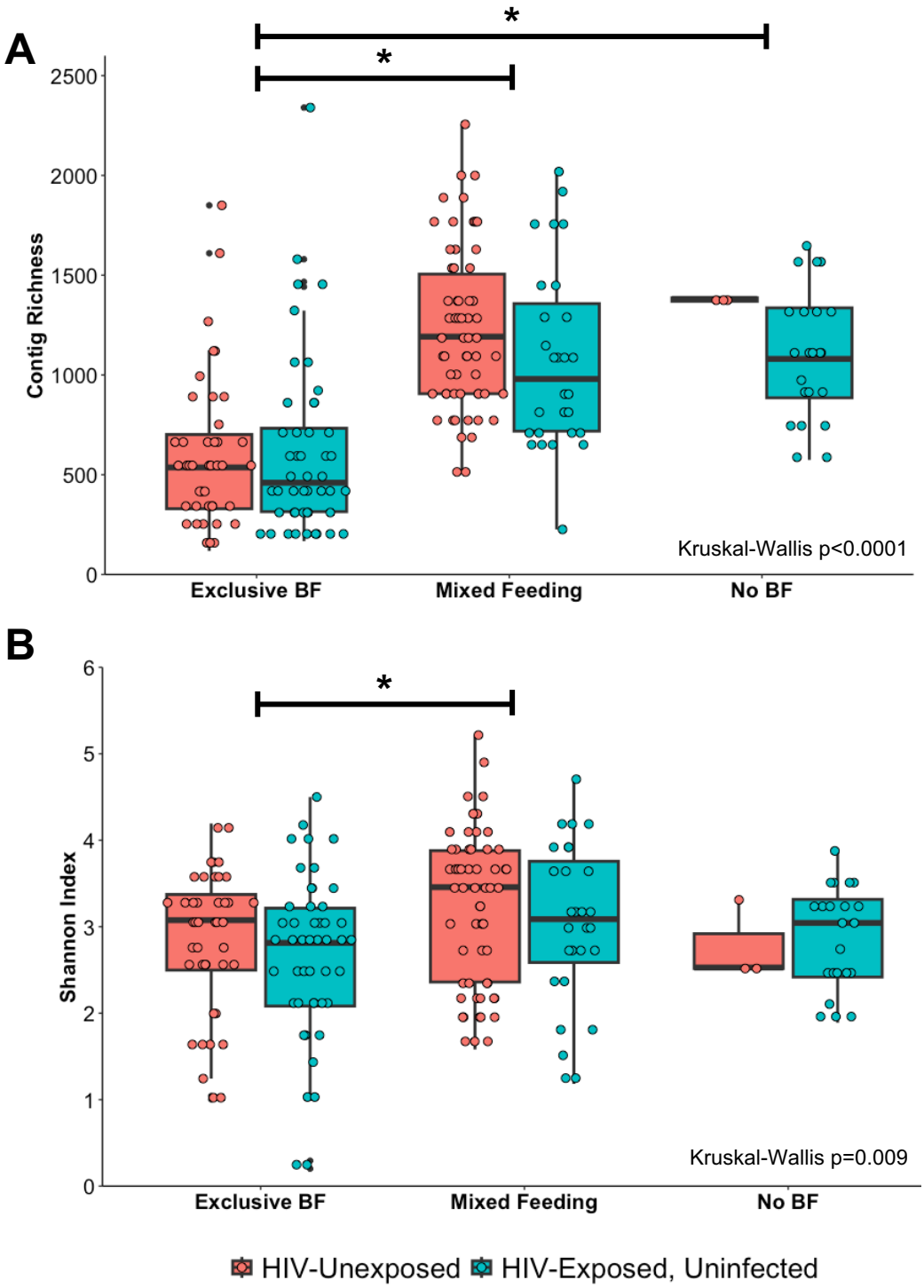

Figure 3. A) Contig richness and B) Shannon diversity index of stool samples by infant feeding and HIV exposure. Kruskal-Wallis tests included all infants regardless of HIV exposure. *Pairwise Wilcoxon rank-sum tests were significant when comparing richness between exclusive breastfeeding (BF) and both mixed feeding and no breastfeeding ($p<0.0001$ for both) and when comparing Shannon diversity between exclusive breastfeeding and mixed feeding ($p=0.01$). All other pairwise tests had $p>0.05$.

Although we did not see differences in the relative abundance of specific viral taxa between CHEU and CHUU, we wanted to assess whether HIV exposure was associated with richness or Shannon diversity. We used generalized estimating equations models, unadjusted and adjusted for infant age, feeding mode, and socioeconomic status (SES) measured by maternal partnership status and education level, identified *a priori* as confounders (Table 2). Independently, HIV exposure was not associated with richness, although there was a trend for lower Shannon diversity. After adjusting for age and feeding, HIV exposure was associated with lower richness and there remained no significant association with Shannon diversity.

Table 2. Unadjusted and adjusted associations between infant characteristics and contig richness and Shannon diversity.

Richness				
	Unadjusted Coefficient (95% CI)	**p-value**	**Adjusted[1] Coefficient (95% CI)**	**p-value**
HIV-exposed, uninfected	-115 (-253 – 22.8)	0.102	**-130 (-250 – -9.72)**	**0.0341**
Infant age (months)	**45.1 (36.6 – 53.6)**	**<0.0001**	**24.1 (4.22 – 43.9)**	**0.0175**
Feeding				
Exclusive breastfeeding	*Ref*	--	*Ref*	--
Mixed feeding	**574 (454 – 694)**	**<0.0001**	**343 (112 – 573)**	**0.00355**
No breastfeeding	**560 (371 – 689)**	**<0.0001**	180 (-155 – 515)	0.293
Shannon Diversity				
	Unadjusted		**Adjusted[1]**	
HIV-exposed, uninfected	-0.229 (-0.467 – 0.00946)	0.06	-0.229 (-0.504 – 0.0454)	0.102
Infant age (months)	**0.0267 (0.00718 – 0.0463)**	**0.00736**	0.0216 (-0.0222 – 0.0654)	0.334
Feeding				
Exclusive breastfeeding	*Ref*	--	*Ref*	--
Mixed feeding	**0.426 (0.163 – 0.684)**	**0.00143**	0.198 (-0.256 – 0.653)	0.392
No breastfeeding	0.102 (-0.172 – 0.375)	0.466	-0.188 (-0.884 – 0.507)	0.596

[1]Adjusted model included HIV exposure, visit-level mode of feeding, infant age in months, and maternal partnership status and education level at enrollment.

The results of the unadjusted analysis also were consistent with the tests comparing richness and Shannon diversity across study visits (Kruskal-Wallis tests; Figure 2) and between modes of feeding (pairwise Wilcoxon rank-sum tests; Figure 3). After accounting for other factors in the adjusted analysis, infant age and mixed feeding remained significantly associated with

higher richness but not with Shannon diversity. No breastfeeding was also not significantly associated with Shannon diversity in the adjusted analysis.

There was no statistically significant difference in beta diversity between CHEU and CHUU (PERMANOVA p=0.85); infants who were <6 months, 6-12 months, or >12 months (p=0.93); or by feeding mode (p=0.67) as shown by the considerable overlap of weighted Bray-Curtis distances between groups in the PCoA analysis (Figure 4). Results were similarly not significant when measuring beta diversity using unweighted versus weighted Bray-Curtis distances (PERMANOVA p=0.68 for HIV exposure, p=0.79 for age group, and p=0.87 for feeding mode).

Discriminating Viral Taxa

We then used MaAsLin2 to explore whether any specific viral contigs had a significantly different relative abundance between CHEU and CHUU after accounting for potential confounding factors. After adjusting for infant age, feeding mode, and socioeconomic status, 28 (0.5%) of the 5,589 unique viral contigs were associated with HIV exposure (Figure 5). Of these, 17 (61%) were eukaryotic Anelloviruses, all of which had lower relative abundance among CHEU than CHUU. Ten contigs (36%) were of unassigned families within the class *Caudoviricetes* with half found in higher relative abundance and half found in lower relative abundance among CHEU. The remaining contig was of the family *Intestiviridae*, a crAssphage in the class *Caudoviricetes*, and had higher relative abundance among CHEU than CHUU.

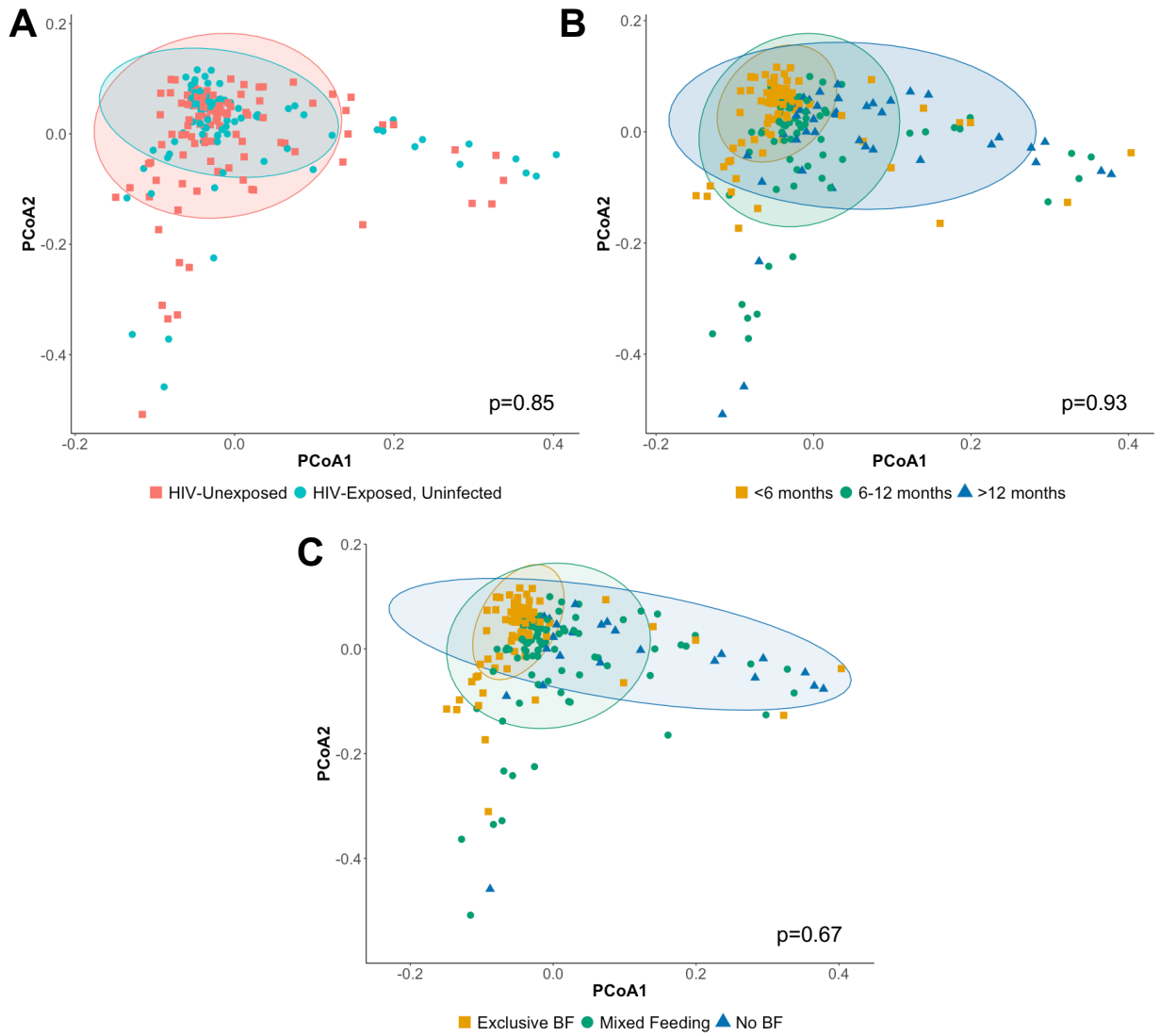

Figure 4. PCoA plots of weighted Bray-Curtis dissimilarity by A) HIV exposure, B) infant age, and C) feeding. P-values are from a PERMANOVA model including HIV exposure, infant age group, and feeding as defined in the plots. BF = breastfeeding.

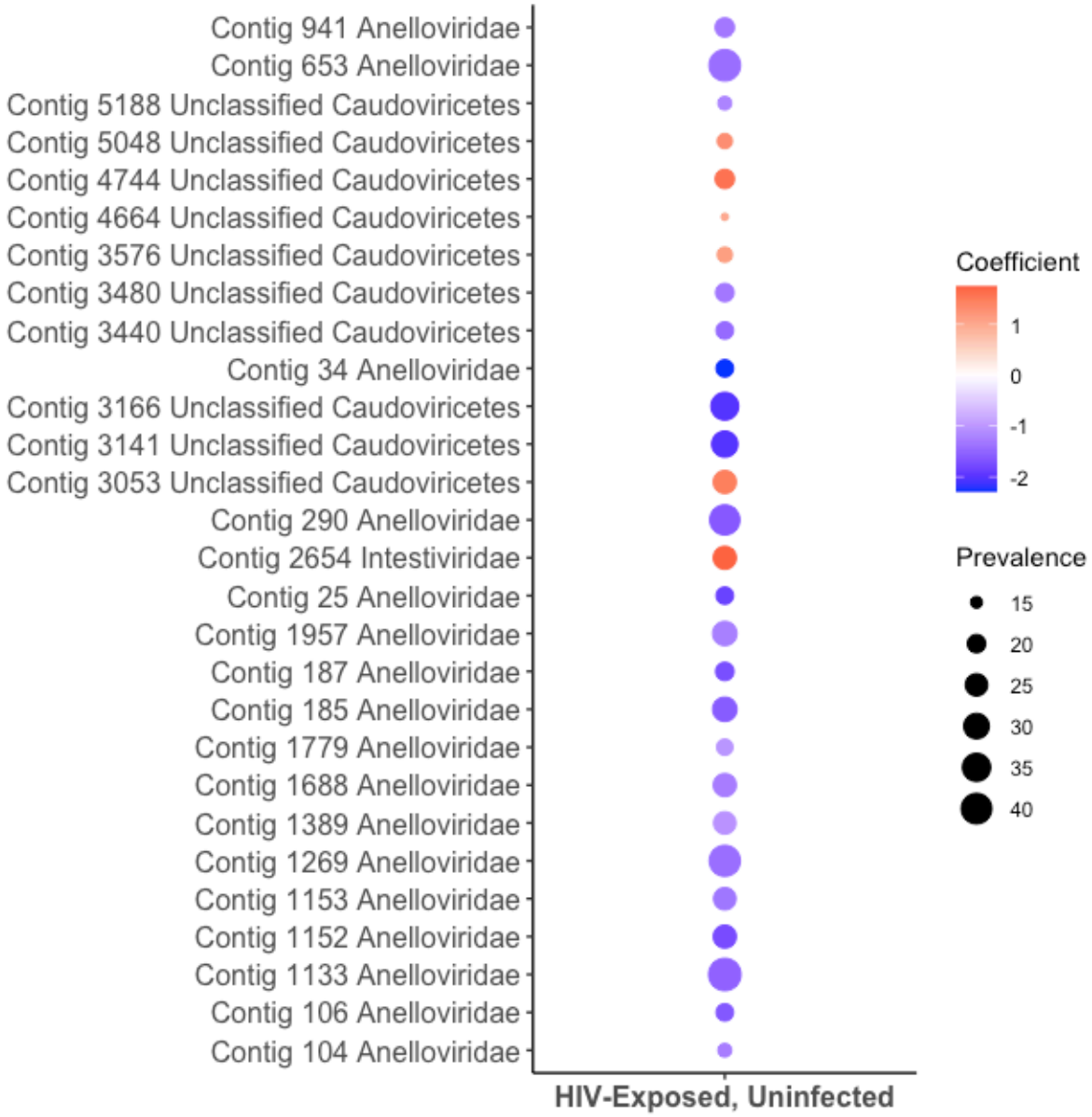

Figure 5. Contigs with differential relative abundance between CHEU and CHUU. Contigs are identified at the family level. Circle color corresponds to the coefficient for association between HIV exposure and the relative abundance of each contig from generalized linear mixed effects models adjusted for infant age, feeding and socioeconomic status; red indicates a positive association and blue indicates a negative association. Circle size indicates the proportion of samples in which the contig was identified. The Benjamani-Hochbeg method with a false discovery rate threshold of 0.25 was used to correct for multiple testing.

DISCUSSION

In this cohort of healthy infants, the gut virome was dominated by bacteriophages. We observed that age and feeding mode significantly altered gut virome richness and Shannon diversity in both CHEU and CHUU. Generally, however, we did not observe a large effect of HIV exposure on virome ecology—though CHEU had lower virome richness than CHUU, the Shannon index and beta diversity did not differ between groups after adjusting for maternal age, feeding mode, and socioeconomic status.

Consistent with previous studies of infants in North America (9,13,18,19,22,40,41), Europe (10,16), Asia (20), and other African countries (9,21), we observed bacteriophages of the class *Caudoviricetes* and family *Microviridae* to be the most abundant viruses at all study time points regardless of HIV exposure and feeding mode. Most contigs assigned to *Caudoviricetes* could not be classified at lower taxonomic ranks, highlighting the ongoing need to characterize the "viral dark matter" that comprises most of the human virome. The relative abundance of *Caudoviricetes* decreased with age while the abundance of *Microviridae* increased, following a commonly observed trajectory of gut virome development (9,13,14,19,21,22). This shift is thought to be influenced by concurrent changes to developing bacterial host populations (1), though the direction of the relationship between viruses and bacteria is unclear and further research is needed to better understand the ecological dynamics of trans-kingdom interactions in the gut. Generally, findings from our cohort suggest that despite high inter-individual variability in specific viral genetic sequences, the infant gut virome is colonized by similar viral taxa regardless of geographic location and other population characteristics.

We also observed higher relative abundance of *Caudoviricetes* at time points with exclusive breastfeeding versus mixed feeding or no breastfeeding, and relative abundance of *Microviridae* was higher with no breastfeeding. Breastfeeding has been shown to alter gut bacteriophage populations, possibly due to the transfer of host bacteria in breast milk—particularly *Bifidobacterium* and *Lactobacillus* species—compared to other modes of feeding (9),

so it is possible this change is directly related to feeding. However, studies have shown this shift in *Caudoviricetes* to *Microviridae*-dominance occurs regardless of feeding mode (9,19,22) and in our cohort, all mothers initiated breastfeeding and many followed recommendations exclusively breastfeed their infants for ≥6 months, reducing heterogeneity in feeding mode over time. Therefore, it is possible we observed this pattern due to collinearity of infant feeding and age.

Eukaryotic viruses were identified at all study time points in both CHEU and CHUU and regardless of feeding mode, and their relative abundance was highest at month 6 when many infants were introduced to new foods. Consistent with this finding, mean relative abundance of eukaryotic viruses was lower during exclusive breastfeeding than during mixed feeding, suggesting that the introduction of new foods increases their abundance in the virome. This pattern also has been observed in other cohorts(9,19), though children may also acquire eukaryotic viruses from other environmental exposures, which diversify with age (22). *Anelloviridae* and *Genomoviridae* were the most abundant eukaryotic virus taxa, consistent with other studies of both adults and children (9,11,21,22,41). Current evidence suggests Anelloviruses are a core component of the human virome in all biological compartments. Though Anelloviruses infect human cells, they appear to be commensal with no known adverse effects on health (42,43). However, the relative abundance of Anelloviruses appears to be higher in individuals who are immunocompromised (27,44) or are experiencing disease (45,46), which suggests interactions between these viruses, the bacterial microbiome, and the immune system influence their ecology.

Interestingly, HIV exposure was associated with lower relative abundance of several *Anelloviridae* contigs after accounting for age, feeding, and socioeconomic status. Previous studies have observed higher abundance of Anelloviruses in adults with respiratory disease (45,46). Because we observed lower incidence of respiratory tract infections among CHEU than CHUU in this cohort (28), it is possible this finding is related to differences in respiratory morbidity between groups. However, additional research is needed to characterize the specific

Anelloviruses that differ between CHEU and CHUU and evaluate whether these viruses are associated with other characteristics that differ between HIV exposure groups.

HIV exposure was also associated with lower virome richness, but not Shannon diversity, which suggests the presence and/or absence of specific taxa among CHEU versus CHUU did not substantially shift overall community structure. No previous studies have compared the virome of CHEU and CHUU. However, findings are similar to those from a recent study of the gut bacterial microbiome in a South African cohort of CHUU and CHEU born to women receiving ART during pregnancy. Jackson et al. also found no association between HIV exposure and longitudinal Shannon diversity when accounting for exclusive breastfeeding; however, unlike our study, there was no association with HIV exposure and bacterial richness (47). Further study is needed to identify other factors that affect virome development and to assess whether those factors differ by infant HIV exposure.

Richness, but not Shannon diversity, also increased with age among both CHEU and CHUU, with a marked increase at month 6 likely due to the introduction of new foods. Our results are consistent with several longitudinal studies of the infant gut virome that showed an increase in richness, but not Shannon diversity, with age (9,10,14,16,19,21). However, we did not observe a decrease in richness or diversity of either bacteriophages or eukaryotic viruses at later ages as some studies did (22,40,41), possibly because we had fewer samples from the second year of life, limiting statistical power.

The observed lower richness among CHEU could be due to residual confounding associated with feeding mode since a significantly greater proportion of CHEU than CHUU were exclusively breastfed for 6 months. Previous work has shown infants who were breastfed had lower gut virome richness and diversity than infants who were not breastfed (9,10,14,16,19), though it is unclear whether differences in bacteriophage populations, eukaryotic virus populations, or both drive this difference. It is possible the breast milk virome differs between mothers living with and without HIV, though a prior study by our group showed HIV-associated

immunosuppression was not associated with breast milk virome composition in a cohort of Kenyan women without ART (48). Given that mothers with HIV in our cohort were healthy and on lifelong ART, we expect minimal changes in their breast milk virome composition; however, research comparing the breast milk virome of women living with versus without HIV is needed to test this hypothesis.

Finally, we did not find an association between beta diversity and HIV exposure, feeding mode, or age. Some studies (10,14,22), but not others (16,19,40,41,49), have shown beta diversity to change with age and more research is needed to understand the reasons for these mixed findings, although differences in study population and methodology likely contribute. There is limited data on the relationship between feeding and beta diversity. A study by Zeng et al. found an association between exclusive breastfeeding and beta diversity, but only at 3 and 12 months of age (14), suggesting different factors influence beta diversity over time. Future work should consider how infant age modifies the relationships between different exposures and gut virome composition.

This analysis had several limitations. We did not have data for the infant gut bacterial microbiome and thus could not compare the composition of or assess the interactions between these microbial communities. Similarly, we did not have data for the maternal gut virome or breast milk virome to assess whether there were similarities with the infant gut virome. Like many studies, we could only describe the DNA virome and more research is needed to understand the composition and development of the RNA virome. Because this was a healthy cohort, very few infants received antimicrobial medications other than antiretroviral and cotrimoxazole prophylaxis if HIV-exposed. Since antimicrobial use was collinear with HIV exposure, we did not explore the effects of these medications on the virome, and further investigation is needed to understand how they affect the infant gut virome. Finally, as intended by the parent cohort design, CHEU had similar exposures to maternal ART regimen, maternal CD4 count, and antiretroviral and cotrimoxazole prophylaxis, which did not allow us to assess whether these characteristics of HIV

exposure are associated with the infant gut virome and may limit the generalizability of our findings to other populations of CHEU. However, these similarities limited confounding by both measured and unmeasured factors, allowing for a more accurate comparison of CHEU and CHUUS.

CONCLUSION

In summary, HIV exposure was not a major determinant of gut virome composition or development in children from the Linda Kizazi cohort. While HIV exposure was independently associated with lower richness and lower relative abundance of several Anellovirus contigs, virome diversity was similar between CHEU and CHUU, suggesting other differences between these groups may have influenced the presence versus absence of specific viral taxa. In contrast, age and feeding mode were associated with major shifts in virome richness and diversity. Our data suggest that the gut virome of CHEU born to women on optimized, pre-conception ART is similar to that of CHUU.

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SUPPLEMENTARY INFORMATION

Table S1. Overall mean relative abundance of viral families identified in analysis samples.

Family	Overall Mean Relative Abundance[1] (%)	Type
Unclassified *Caudoviricetes*	43.4778228572	Bacteriophage – class *Caudoviricetes*
Microviridae	17.0647977503	Bacteriophage – class *Malgrandaviricetes*
Anelloviridae	16.5566975886	Eukaryotic virus
Genomoviridae	6.3167222309	Eukaryotic virus
Suoliviridae	2.3077821380	Bacteriophage – crAssphage[2]
Unclassified (NA)	1.8494084299	n/a
Steigviridae	1.6330767548	Bacteriophage – crAssphage[2]
Intestiviridae	1.2409598119	Bacteriophage – crAssphage[2]
Winoviridae	1.2323542952	Bacteriophage – class *Caudoviricetes*
Salasmaviridae	1.1694466125	Bacteriophage – class *Caudoviricetes*
Autographiviridae	0.9923485844	Bacteriophage – class *Caudoviricetes*
Aliceevansviridae	0.9472889278	Bacteriophage – class *Caudoviricetes*
Peduoviridae	0.8755354424	Bacteriophage – class *Caudoviricetes*
Herelleviridae	0.4477716832	Bacteriophage – class *Caudoviricetes*
Circoviridae	0.4477248819	Eukaryotic virus
Adenoviridae	0.4060580141	Eukaryotic virus
Helgolandviridae	0.3833195477	Bacteriophage – class *Caudoviricetes*
Inoviridae	0.3718295827	Bacteriophage – class *Faserviricetes*
Chaseviridae	0.3441483244	Bacteriophage – class *Caudoviricetes*
Geminiviridae	0.2540788082	Eukaryotic virus
Duneviridae	0.2519254917	Bacteriophage – class *Caudoviricetes*
Drexlerviridae	0.2370905141	Bacteriophage – class *Caudoviricetes*
Straboviridae	0.1946454220	Bacteriophage – class *Caudoviricetes*
Parvoviridae	0.1862552316	Eukaryotic virus
Schitoviridae	0.1457853309	Bacteriophage – class *Caudoviricetes*
Mesyanzhinovviridae	0.1248461642	Bacteriophage – class *Caudoviricetes*
Pachyviridae	0.1120142240	Bacteriophage – class *Caudoviricetes*
Demerecviridae	0.0867689707	Bacteriophage – class *Caudoviricetes*
Grimontviridae	0.0762489005	Bacteriophage – class *Caudoviricetes*
Rountreeviridae	0.0644118487	Bacteriophage – class *Caudoviricetes*
Vilyaviridae	0.0514531652	Eukaryotic virus
Vilmaviridae	0.0456396947	Bacteriophage – class *Caudoviricetes*
Forsetiviridae	0.0335272608	Bacteriophage – class *Caudoviricetes*
Assiduviridae	0.0239434689	Bacteriophage – class *Caudoviricetes*
Plasmaviridae	0.0145331273	Bacteriophage – unclassified
Crevaviridae	0.0110003914	Bacteriophage – crAssphage[2]
Guelinviridae	0.0060703013	Bacteriophage – class *Caudoviricetes*
Stanwilliamsviridae	0.0037403694	Bacteriophage – class *Caudoviricetes*
Kyanoviridae	0.0026036058	Bacteriophage – class *Caudoviricetes*
Smacoviridae	0.0022795198	Eukaryotic virus
Paulinoviridae	0.0021338203	Bacteriophage – class *Faserviricetes*
Hafunaviridae	0.0015789440	Archaeal virus – class *Caudoviricetes*
Nenyaviridae	0.0008661652	Eukaryotic virus
Polyomaviridae	0.0006308246	Eukaryotic virus
Leisingerviridae	0.0005257098	Archaeal virus – class *Caudoviricetes*
Redondoviridae	0.0001721971	Eukaryotic virus
Ackermannviridae	0.0001370700	Bacteriophage – class *Caudoviricetes*

Virus type data sources: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi, https://ictv.global/taxonomy.
[1] Families with relative abundance <1% were grouped as Other in Figure 1. [2] CrAssphages are of the class Caudoviricetes.

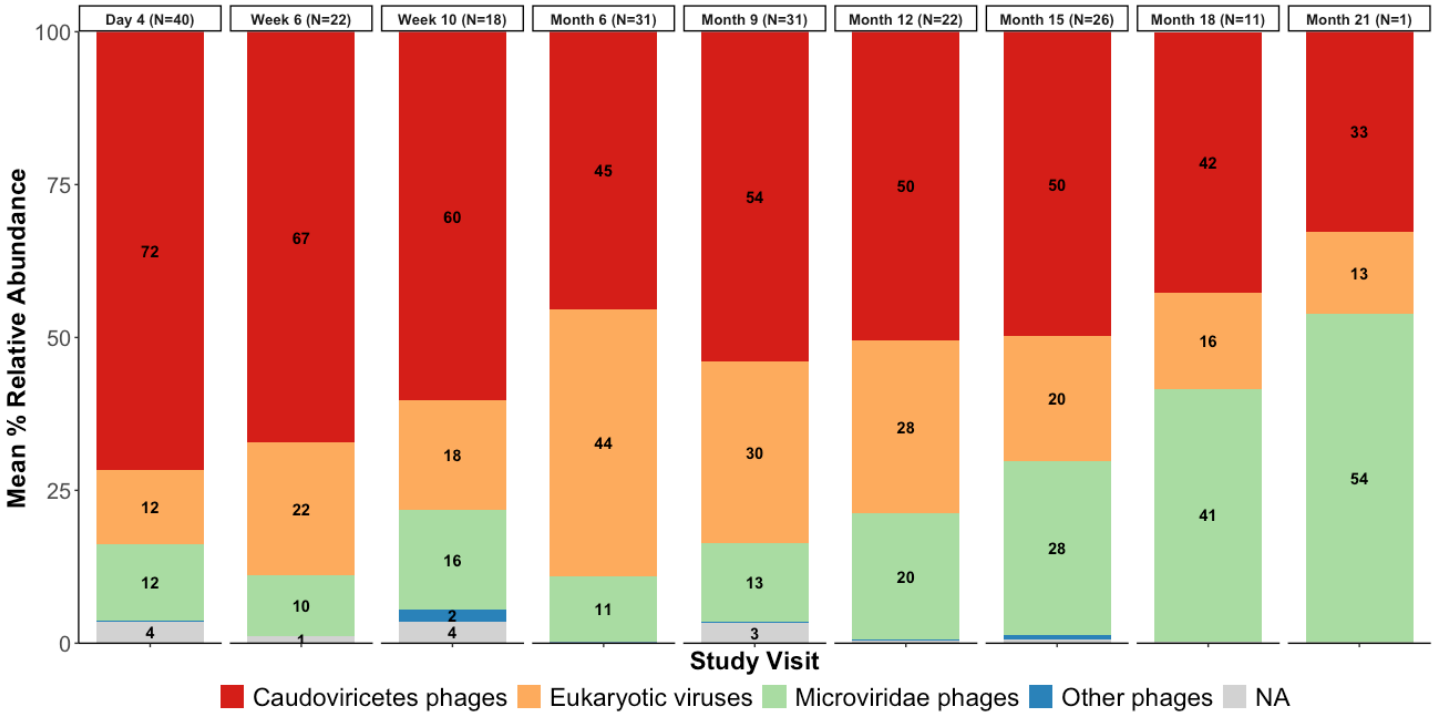

Figure S1. Mean relative abundance of bacteriophages and eukaryotic viruses by study visit. Viruses that could not be classified at the class or family level are categorized as NA. If no group label, mean relative abundance was <1%.

Table S2. Pairwise Wilcoxon rank-sum p-values for between-visit richness.

	Day 4	Week 6	Week 10	Month 6	Month 9	Month 12	Month 15	Month 18
Week 6	0.199							
Week 10	0.0649	0.688						
Month 6	**<0.0001**	**<0.0001**	**0.0009**					
Month 9	**<0.0001**	**<0.0001**	**<0.0001**	0.948				
Month 12	**<0.0001**	**<0.0001**	**0.0001**	0.808	0.992			
Month 15	**<0.0001**	**<0.0001**	**<0.0001**	0.808	0.485	0.439		
Month 18	**0.0003**	**0.0002**	**0.0037**	0.948	1.0	0.948	0.808	
Month 21	0.309	0.184	0.379	0.808	0.808	0.808	0.604	0.808

Bold if statistically significant ($p<0.05$).

CHAPTER 5. SUMMARY OF KEY FINDINGS

This dissertation provided contemporary data comparing the health of children who are HIV-exposed, uninfected (CHEU) and children who are HIV-unexposed, uninfected (CHUU) in the current era of universal antiretroviral therapy (ART). Our work leveraged data from a unique cohort of healthy mother-infant pairs in Nairobi, Kenya that aimed to overcome limitations of earlier studies. Participants were enrolled from a small catchment area to minimize confounding by both measured and unmeasured socioeconomic and environmental characteristics. Additionally, mothers living with HIV and their children had good uptake of contemporary recommendations for the prevention of mother-to-child HIV transmission, including maternal initiation of lifelong, optimized ART before or during early pregnancy, infant antiretroviral prophylaxis, and maternal and infant cotrimoxazole preventive therapy, and there was little heterogeneity in these characteristics within the HIV-affected groups.

In Chapter 2, we found that among all children in the first two years of life, acute diarrhea and respiratory tract infection were common, but there were few cases of pneumonia, malaria, hospitalization, and death. CHEU and children that were exclusively breastfed for 6 months had lower incidence of respiratory tract infections, including pneumonia, than CHUU or children not exclusively breastfed for 6 months. Interestingly, exclusive breastfeeding was more common among CHEU than CHUU (88% vs 57%), which together with early maternal ART initiation and cotrimoxazole preventive therapy, may have contributed to the lower incidence of respiratory morbidity among CHEU. Overall, our findings suggest that universal optimized ART can successfully reduce morbidity and mortality among otherwise healthy CHEU, resulting in outcomes similar to their CHUU peers. Because our sample size was relatively small, further study of the relationships between HIV exposure, breastfeeding, cotrimoxazole preventive therapy, and morbidity is needed in larger, contemporary cohorts with greater heterogeneity of children's HIV exposure characteristics.

In Chapter 3, we observed that primary cytomegalovirus (CMV) infection was high among all infants (cumulative probability 79%), but HIV exposure was not associated with higher hazard of CMV acquisition or earlier age at primary CMV infection as observed in cohorts from the pre-ART era. Consistent with other studies, though, the hazard of CMV acquisition was two times greater for each $\log_{10}$ increase in breast milk CMV DNA level, emphasizing the role of breast milk as the primary source of mother-to-child CMV transmission. Interestingly, mothers living with HIV had higher CMV DNA levels in breast milk than mothers without HIV, which is consistent with studies conducted before the availability of optimized, long-term ART. However, this difference did not translate into a difference in risk of CMV acquisition between CHEU and CHUU. Additionally, we found evidence of effect modification by HIV exposure on the association between infant sex assigned at birth and primary CMV infection, with female CHEU at higher risk and female CHUU at lower risk of CMV acquisition. Together, our results support previous findings that CMV transmission declines with improved maternal immune reconstitution and HIV viral suppression on ART, but remains common with breastfeeding. However, larger studies in the current era of optimized, universal ART are needed to understand the relationship between breast milk CMV levels, HIV exposure, and CMV acquisition among CHEU and to explore mechanisms by which infant sex assigned at birth may affect infants' risk of CMV.

In Chapter 4, we showed that CHEU and CHUU had similar gut virome ecology in the first years of life. Among all infants, the gut virome was dominated by bacteriophages in most samples and at most time points. To our knowledge, this was one of the first studies of the gut virome among individuals from sub-Saharan Africa; in our study, the relative abundance of different viral families followed the same developmental trajectory observed in earlier studies of the infant gut virome, which suggests that the same viral taxa colonize the gut regardless of geographic location and other population characteristics. After accounting for age, feeding mode, and socioeconomic status, CHEU had lower virome richness and a lower relative abundance of several different Anelloviruses, a type of commensal virus found in most human virome samples. However, there

was no difference in virome alpha or beta diversity between CHEU and CHUU, which suggests that differences in the presence versus absence of specific viruses due to HIV exposure (and/or associated characteristics) does not substantially alter infant gut virome ecology. Because, to our knowledge, this was the first study of the virome in CHEU, additional study of the virome in CHEU and comparable populations of CHUU is needed to contextualize these findings, explore reasons for the observed difference in richness, and determine whether gut virome composition is associated with infant health outcomes.

Overall, findings from this dissertation provide encouraging evidence that current optimized interventions for the prevention of mother-to-child HIV transmission may minimize differences in morbidity and mortality between CHEU and CHUU, especially compared to earlier eras of the HIV pandemic. In contrast, exclusive breastfeeding was a much more significant determinant of morbidity and infant virome than was HIV exposure. We acknowledge that the results of this study are not generalizable to all populations of CHEU since most mothers in our cohort initiated optimized ART before pregnancy with evidence of good adherence and most infants received recommended HIV prophylaxis medications and were exclusively breastfed for 6 months. These interventions are not implemented uniformly or equitably in all global regions affected by HIV, and our findings underscore the importance of ensuring all women living with HIV and their children have access to effective HIV treatment and prophylaxis with support for exclusive breastfeeding. Nonetheless, our results offer several new directions of investigation to better understand why CHEU historically have had higher morbidity and mortality than CHUU, which in turn will inform new interventions to support the health of all populations of CHEU.

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