**Longitudinal analysis of mature breastmilk and serum immune
composition among mixed HIV-status mothers and their infants**

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24 Abbreviations: BMI, body mass index; IFN, interferon; Ig, immunoglobulin; IL,
25 interleukin; IQR, interquartile range; LOD, limit of detection; SD, standard deviation;
26 TNF, tumor necrosis factor; WHO, World Health Organization

Abstract

Background & aims: Understanding mature breastmilk immunology may benefit infants chronically exposed to infectious pathogens in resource-limited regions.

Methods: This prospective rural/semi-rural Tanzanian cohort of women (n=102 at delivery; 38% HIV-positive) and their infants (n=102) investigated breastmilk, maternal and infant serum immunoglobulins (IgA/IgG₁₋₄/IgM) and cytokines (IL-1 β /IL-2/IL-6/IL-10/IL-12p70/IL-13/IL-15/TNF- α /IFN- γ) at 1, 2, 3, 6-months postpartum.

Results: Milk immunoglobulins followed an inverse U-shaped pattern, while cytokine patterns were mixed. Exclusive breastfeeding duration and feeding intensity were associated with greater breastmilk total immunoglobulin and IgA, IgG₁₋₃ and IL-12p70 concentrations. Maternal mastitis, fever or cough was associated with higher breastmilk total cytokine concentrations, while infant fever was associated with lower milk immunoglobulins or cytokines. Strong ($r \geq 0.40$) to weak ($r = 0.20$ to 0.29) positive correlations between maternal serum-breastmilk or breastmilk-infant serum immunoglobulins were evident. Breastmilk cytokines were moderate to weakly negatively correlated with infant serum. Breastmilk immunology did not differ by maternal malnutrition or HIV-seropositivity.

Conclusions: Mature breastmilk is a dynamic source of many specific and non-specific immune factors associated with maternal and infant health and infant nutrition. Breastfeeding practices are associated with differential breast milk immunological composition providing immunological support for universal recommendations to exclusively breastfeed for 6-months.

50   Keywords: Africa; Breastfeeding; Cytokine; Immunoglobulin; Immunology; Stunting

1. Introduction

Early infancy infectious disease vulnerability is attributed to neonatal immune system immaturity; although this justification is being challenged as developmental immunology understanding advances. Regardless of precise immune mechanisms, considerable early infancy morbidity and mortality is due to infectious diseases worldwide. Fortunately, breastfeeding is linked to reduced incidence, severity and mortality from infectious diseases, particularly diarrhea and pneumonia (1), with >800,000 deaths attributed to infectious diseases and/or malnutrition in children <5 years possibly avoided through optimal breastfeeding practices among infants up to 23 months in 2011 alone (2). While understanding the practice of breastfeeding and associated protection against infectious diseases is a necessary focus, until correlates of breastmilk immune protection are identified (3-6) our understanding will remain incomplete despite major global public health implications.

Immunoglobulin (Ig) A is the most abundant immunoglobulin in human milk, providing protection by neutralizing and eliminating pathogens (7, 8). Although breastmilk IgA is often studied, other immunoglobulins are also needed against infections including HIV (9). Cell-mediated (e.g. lymphocytes) and many non-specific immune factors including cells (e.g. macrophages, stem cells), molecules (e.g. cytokines, lactoferrin, human milk oligosaccharides) and commensal microbes together contribute to breastmilk-mediated immunity (10-14). While fundamental immune composition similarities exist, numerous individual-level and dynamic factors influence milk composition throughout lactation including maternal health, immunity/infection and nutritional status (15-19).

For the most part, the maternal-infant immunological bridge has been considered a unidirectional pathway with infants perceived as passive recipients of maternal-directed immune factors, however, preliminary observations hint at a paradigm-shift suggesting infant infection status may independently influence breastmilk immune composition of otherwise healthy mothers (20). Hypothesized infant-directed influence could arise from maternal psychological stress and accompanying non-specific immune consequences associated with caring for an unhealthy infant, but also through differential infant oral microbiome transfer to breastmilk ducts or through unknown mechanisms. Overall, this suggests that breast milk immune composition is likely a function of the shared environment and health status of both the mother and infant.

In developing countries, diarrheal and respiratory pathogen exposure is often chronic necessitating an understanding of mature breastmilk immunology that extends beyond early and transitional milk phases. Additionally, while breastfeeding and transmission of HIV have been well-studied (21), HIV-exposed and antiretroviral-exposed breastmilk immune composition data remains limited. Consequently, this prospective cohort was designed to: 1) characterize mature breastmilk immune composition from women with and without HIV between one and 6-months post-partum; 2) investigate correlations between circulating immunoglobulin and cytokine concentrations in biological compartments of the maternal-infant dyad including maternal serum, breastmilk and infant serum; and 3) compare breastmilk immunoglobulin and cytokine profiles in relation to maternal and infant health and nutritional status.

2. Materials and methods

2.1 Study setting and cohort participants

This prospective cohort enrolled HIV-positive and HIV-negative mothers and their HIV-exposed and HIV-unexposed infants living in rural and semi-rural northwestern Tanzania (Figure 1). Recruitment was restricted to a 9-month period (March 27 and December 19, 2012) during which time 125 pregnant women seeking antenatal care at the semi-rural based Kisesa Health Centre or at more remote health care dispensaries throughout Magu District were recruited. Feasibility assessment suggested that HIV-negative women may be more likely to withdraw, so women were recruited in a 1 HIV-positive (n=50) to 1.5 HIV-negative (n=75) ratio. Eligibility included gestation <37 weeks, singleton birth, confirmed HIV-serostatus and stated intention to reside within the clinic catchment through 6-months post-partum. In addition to mother and child health services, Kisesa Health Centre includes a HIV clinic where women were offered antiretroviral therapy for their own HIV care and/or for prevention-of-mother-to-child-transmission in accordance with Tanzanian national HIV/AIDS guidelines. HIV-exposed infants received antiretroviral prophylaxis and HIV testing at 3-months; none were HIV-positive. Participants with signs of malnutrition or reporting infection-related symptoms during the study follow-up were referred for follow-up care at Kisesa Health Centre.

2.2 Ethics

This study was ethically approved by the Tanzania National Health Research Ethics Review Committee and Cornell University. Written informed consent was obtained from mothers for themselves and on behalf of their infants.

2.3 Data collection

A research nurse-administered questionnaire elicited information on maternal and infant demographics, morbidity, nutritional status and infant feeding practices at each follow-up visit. Exclusive breastfeeding was defined according to the World Health Organization (WHO) whereby only “breastmilk...oral rehydration solution, drops, syrups (vitamins, minerals, medicines), but nothing else...” was given. Breastfeeding intensity was defined as the number of breastfeeding episodes within the previous 24 hours. Household food security was assessed using five questions from the Household Food Insecurity Access Scale previously validated in rural Tanzania (22). Maternal height and weight were measured (Health-O-Meter, Inc., Bridgeview, IL) to the nearest 0.2 kg and nearest 0.1 cm, respectively. Infant weight was measured (Seca 334 Digital Baby Scale) to the nearest 0.01 kg; and infant length by an infant length board to the nearest 0.1cm. Maternal body mass index (BMI) was calculated, as were infant weight-for-age and height-for-age z-scores using WHO growth references.

2.4 Laboratory analyses of breastmilk immunoglobulin and cytokine concentrations

Hand-expressed breastmilk, maternal and infant blood were collected at each follow-up. Milk was stored in polypropylene containers and blood drawn in EDTA tubes with temporary storage at Kisesa Health Centre at 4°C for ≤5 hours before transport (travel time <1.5 hours) in ice-chilled coolers to the National Institute for Medical Research laboratory in Mwanza, Tanzania. Serum separation and removal of the milk fatty layer was performed before storage at -20°C prior to batch analyses. A priori selection of target analytes included: IgA, IgG₁, IgG₂, IgG₃, IgG₄, IgM, as well as cytokines with pro-inflammatory, anti-inflammatory or regulatory roles including interleukin (IL)-1 β , IL-2, IL-6, IL-10, IL-12p70, IL-13, IL-15, tumor necrosis factor

(TNF)- α and interferon (IFN)- γ . MAGPIX® instrumentation with xMAP® technology (Luminex Corporation, Texas, USA) and MILLPLEX® MAG magnetic bead-based multianalyte assays (HGAMMAG-301K, HCYTOMAG-60K, EMD Millipore, Massachusetts, USA) were used. Breastmilk was assayed by adapting the manufacturer's protocol using a commercial serum matrix provided by Millipore on undiluted samples (23). Samples with concentrations reported below the assay limit-of-detection (LOD) were imputed with the LOD/ $\sqrt{2}$.

2.5 Statistical analyses

Data were analyzed in STATA10 (STATA Corporation, Texas, USA). Student's t -test, χ^2 test, Pearson's or Spearman's correlation, linear and multilevel mixed-effects linear regression was used. To investigate total immunoglobulins, a unit-less combined variable was calculated: IgA and IgM were converted to 10^{-7} g/ml, IgG₁ and IgG₂ to μ g/ml and IgG₃ and IgG₄ to 10^{-8} g/ml. Breastmilk IL-1 β , IL-2, IL-10 and serum IL-13 were <LOD for at least one time point among all participants and therefore excluded from total cytokine concentration analyses. Inclusion of milk type (fore/hind) or time of day of breastmilk collection did not alter interpretations; therefore, these variables were not included in these analyses. Results were considered statistically significant at $p < 0.05$ (two-sided). Given the limited published data in this context, results are presented without adjustments for multiple statistical testing.

3. Results

3.1 Maternal health characteristics did not differ according to HIV-seropositivity

Maternal characteristics are summarized in Table 1. Average maternal BMI was within a healthy range (defined as 18.5 to 24.9 kg/m²) throughout the 6-month post-

partum period. Although households with HIV-positive mothers experienced more food insecurity relative to households with HIV-negative mothers, post-partum nutritional status estimated by BMI did not differ based on HIV status. All HIV-positive women received antiretrovirals during lactation, and most did not have severe immunosuppression: 45% had CD4 cell counts above the lower limit of normal (defined as >500 cells/ μ L) with only 9% had CD4 counts <200 cells/ μ L. Symptoms of maternal infection were common amongst all women throughout the study (fever=24 to 43%; cough=17 to 30%), although maternal morbidity did not significantly differ based on maternal HIV-status. Mastitis was uncommon (4 to 8% among all women) and diarrhea, skin rash, vomiting, abdominal pain, anorexia, nausea, fatigue and difficulty breathing were rare (data not shown).

3.2 Early infancy stunting and symptoms of infection were common

Birth and early infancy growth anthropometrics indicated early and ongoing infant health challenges (Table 1). Thirty-five percent of HIV-exposed and one quarter of HIV-unexposed infants were considered pre-term, although neither mean gestational age at birth nor the proportion classified as preterm significantly differed by infant HIV-exposure status. Nonetheless, HIV-exposed infants had lower mean birth weights and weight-for-age z-scores throughout early infancy, and a higher proportion were considered to have low birth weight. Infant stunting (height-for-age z-score <-2) was common and increased over the first 6-months for all infants. Indeed, by 6-months of age, 56% of HIV-exposed and 45% of HIV-unexposed infants were stunted. Infant fever was common throughout the study, and increased in frequency from 26 to 66% by 6-months among all infants. Infant diarrhea, cough, breathing problems, convulsions,

vomiting, skin rash and anorexia were less commonly reported, and did not significantly differ between HIV-exposed and HIV-unexposed infants (data not shown).

3.3 Early infancy feeding practices were suboptimal, especially for HIV-unexposed infants

Infant feeding practices differed according to maternal HIV status, with HIV-positive mothers exclusively breastfeeding for an average of 1.5 months compared to only 3.4 weeks among HIV-negative mothers ($p < 0.05$), however, these periods are much shorter than WHO guidelines recommending exclusive breastfeed for 6-months in similar settings, regardless of HIV status or antiretroviral access (24). HIV-exposed infants were consistently fed a greater proportion of breastmilk in their diets compared to HIV-unexposed infants (*Month 2* Exclusive breastfeeding: HIV-exposed=25 vs. HIV-unexposed=11%, predominant: HIV-exposed=45 vs. HIV-unexposed=40%;, partial: HIV-exposed=28 vs. HIV-unexposed=48%, $p = 0.048$; *Month 3*: Exclusive breastfeeding=23 vs. 10%, predominant=48 vs. 26%, partial=35 vs. 65%, $p = 0.012$; *Month 6*: Exclusive=10 vs. 2%, predominant=30 vs. 8%; partial=53 vs. 90%, $p < 0.001$), although differences in breastfeeding intensity (e.g. 24-hour maternal recall of the number of times an infant breastfed) based on HIV-exposure were not observed.

3.4 Evolution of mature breastmilk immunoglobulin and cytokine concentrations to 6-months post-partum

For each breastmilk immunoglobulin, an inverted U-shaped pattern was observed during the post-partum period, with statistically significant differences in monthly mean concentrations for IgA, IgG₃, IgG₄ and IgM (Figure 2A). Breast milk cytokine evolution patterns were less consistent (Figure 2B). TNF- α following an inverted U-shaped

distribution, while IL-15 exhibited a linear decline. Monthly differences were not detected among IL-6, IFN γ , IL-12p70 and IL-13. IL-1 β , IL-2 and IL-10 remained undetected. Maternal HIV-seropositivity was not associated with differences in breastmilk cytokine or immunoglobulin concentrations (data not shown), although IgG₁ remained consistently higher throughout lactation in HIV-exposed breastmilk (HIV-exposed vs HIV-unexposed breastmilk IgG₁: Month 1=1033 vs. 668; Month 2=1061 vs. 975; Month 3=1114 vs. 834; Month 6=858 vs. 584 ng/ml).

3.5 Mature breastmilk immune composition was associated with breastfeeding practices

While breastmilk immunoglobulin and cytokine concentrations did not differ according to maternal malnutrition (defined as BMI<18.5 kg/m²), breastmilk immune composition was associated with breastfeeding practices. Breastmilk from mothers who were exclusively breastfeeding contained higher overall IgA (263 vs 192 ng/ml, p=0.048), IgG₁ (1638 vs. 702 ng/ml, p=0.003), IgG₂ (6918 vs 3300 ng/ml, p=0.033) and IgG₃ (40 vs 18 ng/ml, p <0.001) concentrations compared to milk from mothers who were predominantly or partially breastfeeding. Breastmilk from women who were exclusively breastfeeding also had higher IL-12p70 at 2- and 3-months (2.58 vs. <LOD, p=0.006; 3.31 vs. 2.42 pg/ml, p=0.017, respectively).

3.6 Maternal serum with breastmilk and breastmilk with infant serum immunoglobulin concentrations were positively correlated, while cytokine correlations were complex

The biological compartment correlation matrices (e.g. maternal serum with breastmilk and breastmilk with infant serum) are shown in Table 2, with correlations

ranging from strong to negligible. Maternal serum with breastmilk associations were moderately to weakly positively correlated for IgA, IgG₂, IgG₄ and IgM for at least one lactation time-point (Table 2A, upper left), while the only maternal serum with breastmilk cytokine correlation was TNF α at 2-months post-partum that was moderately positively correlated (Table 2B, lower left). Like maternal serum with breastmilk, breastmilk with infant serum immunoglobulins revealed a pattern of positive correlations. In this instance, strong to weak correlations were observed in early infancy (e.g. 1- to 2-months) for IgA, IgG₁, IgG₂, IgG₄, and IgM, but demonstrated limited correlation in later infancy (Table 2A, upper right). Cytokine correlations between breastmilk and infant serum revealed a more complex pattern exhibiting moderate to weak associations in both positive (TNF α , IL-6) and negative (IL-15, TNF α) directions throughout infancy (Table 2B, lower right).

3.7 Mature breastmilk immune composition is associated with infant feeding practices, maternal and infant health

Breast milk immune composition was associated with multiple infant health, nutrition and feeding factors in regression models summarized in Table 3. Exclusive breastfeeding and breastfeeding intensity were associated with higher total immunoglobulins and lower total cytokines at multiple months throughout infancy. Increasing birth weight, gestational age at birth and weight-for-age were associated with lower total immunoglobulins or cytokines only in early infancy (e.g. month 1). Mothers reporting symptoms of infection including mastitis, fever or cough had greater breastmilk total cytokines for at one time point during lactation, while total immunoglobulins were not associated with maternal health status. In contrast, infant fever was associated with

lower total breastmilk immunoglobulins and cytokines. Maternal nutritional status and household food security were not associated with breastmilk total immunoglobulin or total cytokine concentrations in regression analyses.

4. Discussion

In a rural/semi-rural African setting typified by chronic pathogen exposure and immune activation, longitudinal evaluation of mature breastmilk demonstrates immune composition is dynamic and differs according to multiple modifiable factors, including breastfeeding practices. This study provides unique insight into complex time-varying correlations at interfaces of biological compartments of the maternal-infant immunological bridge. Given the small cohort size in relation to the number and complexity of associations examined, future studies are needed to build upon these findings. This study links immunological evidence and WHO infant feeding guidelines recommending exclusive breastfeeding for 6-months, and in resource-restricted regions, regardless of maternal HIV status or antiretroviral access (24). At the same time, this study confirms global reports (25) that very few women are exclusively breastfeeding for this duration in this setting, indicating significant effort will be needed to meet WHO goal of optimal breastfeeding among 50% by 2025 (25).

Immune factors decline from colostrum to transitional milk, and further to mature milk, with stabilization thereafter for some, but not all (8, 11, 12, 17, 26). While most evidence comes from developed countries, ethnicity or country of birth was associated with differential milk immunoglobulin, pro- and anti-inflammatory cytokine profiles (6, 14, 17). However, current type and frequency of pathogen exposure is likely important, and women living in rural/semi-rural African environments may produce breastmilk with

immune compositions different to ethnically-similar urban-based counterparts or migrants. To our knowledge, this is the first investigation of the 6-month evolution of a number of mature milk immunoglobulin and cytokine profiles in a mixed HIV-status population. In this setting, it appears mature breastmilk immunoglobulin profiles followed an inverted U-shaped pattern while breastmilk cytokine patterns were mixed and more difficult to generalize.

Breastfeeding practices and HIV transmission has been well-studied (21) without comprehensive investigation of breastmilk immunology. One of the first studies reported higher breastmilk IgA, IgG and IgM concentrations among HIV-positive versus HIV-negative Botswanan women, but no differences in IL-8 or monocyte chemoattractant protein-1; and IL-2, IL-4, IL-10, IL-12p70, IFN- γ and TNF- α remained undetected (4). Although these non-specific immune factors differed, pathogen-specific IgG or IgA did not. Direct comparison with the current study is difficult given only single milk samples were included from women with greater immunosuppression who were not receiving antiretrovirals. In a subsequent Zambian study investigating a single transitional milk sample (27), no differences were observed for IL-8 concentrations, but breastmilk IL-15 was lower among mothers whose infants HIV-seroconverted compared to non-transmitting or HIV-negative mothers. Direct comparisons are again restricted given that study investigated different lactation periods among women with more advanced immunosuppression who were not using antiretrovirals and since infants in the current study remained HIV-negative. Without additional data from developing countries, generalizations of breastmilk immunology based upon HIV-seropositivity alone do not appear warranted. In this study, symptoms suggestive of localized (mastitis) or systemic

(fever, cough) inflammation were associated with greater breastmilk cytokine concentrations among all women as anticipated, and further understanding of infection mitigation of breastmilk immune composition and clinical implications for the infant are needed (12).

Maternal malnutrition has been shown to be variably associated with milk immunology. Colostrum IgA among 60 healthy Taiwanese mothers was twice that of malnourished mothers at two weeks post-partum, but differences disappeared at 8-weeks (19). Higher colostrum IgG and IgA, but not IgM concentrations, were observed in 10 healthy Columbian mothers compared to seven malnourished mothers (26), but when maternal nutritional status later equalized, differences disappeared in mature milk. In 127 Congolese women, malnutrition status alone was not associated with differential IgA concentrations up to 18-months post-partum (17). Interestingly, rural-based women were more malnourished and had higher absolute milk IgA, but more frequent breastfeeding among urban-based women provided higher infant breastmilk IgA doses. In a later study, no differences were observed in milk IgA at 1-month between 17 malnourished versus 14 healthy Congolese women, but malnutrition was associated with lower IgA at 4-months (5). In the current study, maternal nutritional status was not associated with mature breastmilk immunology, but lactation stage differences and unconfirmed HIV-status in previous studies make inter-study comparisons difficult.

To our knowledge, this is the first study to longitudinally examine immune factors in multiple biological compartments interfacing the immunological bridge of the maternal-infant dyad. Breastmilk immunoglobulins appeared positively correlated with maternal and infant serum, although infant correlations were restricted to early infancy.

Given the complexity of anti-inflammatory, pro-inflammatory and regulatory cytokine functions and implications in developmental immunology, it is not surprising that both positive and negative cytokine correlations and only single month associations were observed. Cytokine correlations appeared more closely associated with breastmilk and infant serum than with maternal serum. In healthy American women investigating asthma-like infant symptoms, gestational serum was compared to breastmilk immunology at 3-weeks post-partum with an inverse correlation reported for IgA and positive correlations for IL-5, IL-6, IL-13 and IFN γ reported (28). While not directly comparing compartments simultaneously like in the current study, infant serum exhibited a less inflammatory profile throughout the first year of life if exclusively breastfed compared to formula-fed infants (29). Additional research is needed with larger sample sizes to further understand longitudinal inter-compartment relationships and identify both determinants and clinical relevance.

Findings from this study suggesting that breastfeeding practices are linked to breastmilk immunology suggest that unraveling determinants of milk immunology requires a broad consideration of factors. A large European study of rural-based healthy women reported an inverse relationship between breastfeeding duration and milk IgA concentration (30), and the direction was attributed to subclinical breast tissue inflammation with short durations. While the current study found no associations between exclusive breastfeeding duration or mastitis and milk IgA concentrations, direct comparisons are limited since the European study used different breastfeeding definitions and feeding practice questions did not correspond to timing of milk collection.

While offering a rarely studied context and unique approach, without quantifying actual breastmilk volume consumed, true infant immunoglobulin and cytokine milk dose remains unknown. Additionally, only non-specific immune factors were measured and pathogen-specific protection is also of interest. Multiplexing breastmilk is novel, but more data are needed to confirm collection time and storage influences. Despite successful cohort retention, missing data (e.g. mothers often refused infant blood sampling) was present. This caveat should be considered for this study, but also for designing future studies in similar settings. While longitudinal studies are important given the dynamic nature of breastmilk and maternal-infant dyad changes, findings are restricted to the 6-month postpartum period. Lastly, single-month statistically significant associations require follow-up to determine whether due to chance or important but temporary developmental relevance.

5. Conclusions

The immunological bridge provided by breastmilk is complex and dynamic during early infancy, and differs according to modifiable factors including exclusive breastfeeding. Understanding mature breastmilk immunology in chronic pathogen exposure settings is needed to identify correlates of immune protection. While symptoms of maternal and infant infection were associated with milk immunology differences, HIV-seropositivity or maternal malnutrition were not. Despite well-established WHO infant feeding guidelines, exclusively breastfeeding for 6-months was rare, in stark contrast to the high vaccine uptake in this same region. This juxtaposition necessitates further consideration, and perhaps integrating ongoing breastfeeding support at vaccination visits with

370   messaging that indicates “breastmilk is a temporary vaccine you offer” may be helpful
371   towards meeting the WHO optimal breastfeeding goals.

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Statement of authorship

SHP and JMM conceived and designed the study, with contributions from all co-authors. SHP and ALW collected the clinical data and biological samples, with medical oversight provided by DM. SHP performed laboratory analyses, with laboratory oversight provided by AA and JC. SHP conducted the statistical analyses, and all authors participated in data interpretation. SHP and JMM drafted the article, and all authors contributed to revisions and final approval of the submitted manuscript. JMM is the guarantor for the study.

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Figures and tables legend

Figure 1 Flow diagram of maternal and infant cohort recruitment, follow-up and data/biological sample collection

Figure 2 Mean mature breastmilk A) immunoglobulin and B) cytokine

concentrations from women with and without HIV infection between one to 6-months post-partum

Data are presented as mean analyte concentration; error bars represent standard error of the mean.

The y-axes are as follows: A) Original units of measure differed between immunoglobulins and mean values were magnitudes of order different, therefore for presentation purposes, IgA and IgM were converted to 10^{-7} g/ml; IgG₁ and IgG₂ to μ g/ml; IgG₃ and IgG₄ to 10^{-8} g/ml; and B) Cytokines were measured in pg/ml.

The number of unique sample data points contributing to group means at each time point is as follows: A) *IgA* at Month 1=87; Month 2=85; Month 3=89; Month 6=82; *IgG₁* 1=87; 2=85; 3=89; 6=82; *IgG₂* 1=87; 2=83; 3=0; 6=0.; *IgG₃* 1=0; 2=85; 3=89; 6=82; *IgG₄*=87; 2=85; 3=89; 6=83; *IgM* 1=87; 2=85; 3=89; 6=82; and B) All cytokines at Month 1=85; Month 2=81; Month 3=89; Month 6=82.

Different letters above histogram bars indicate concentrations were significantly different from other months, whereas shared letters indicate no significant differences between months; comparisons using Student's *t*-test, (two-sided) $p < 0.05$.

Table 1 Characteristics of HIV-positive and HIV-negative mothers and their infants from birth through 6-months post-partum

522   **Table 2 Pair-wise correlation matrix of (A) immunoglobulin and (B) cytokine**
523   **concentrations in mixed HIV-status maternal serum with breastmilk (left panel),**
524   **and breastmilk with infant serum (right panel)**
525   **Table 3 Unadjusted and adjusted linear regression of total breastmilk**
526   **immunoglobulin (left) and total breastmilk cytokine (right) concentrations**
527

Table 1 Characteristics of HIV-positive and HIV-negative mothers and their infants from birth through 6-months post-partum

Maternal Characteristics				
		All	HIV-positive	HIV-negative
<i>Age, years, Mean (SD)</i>		28.4 (5.9)	29.4 (6.0)	27.7 (5.8)
<i>Parity, Mean (SD)</i>		2.6 (1.7)	2.7 (1.9)	2.5 (1.6)
<i>Primiparous, % (n)</i>		9 (9)	8 (3)	10 (6)
Maternal Health Status				
<i>CD4 Cell Count, cells/μL, Median (IQR)</i>			459 (330 to 774)	
<i>Mastitis, % (n)</i>	Month 1	4 (4)	5 (2)	4 (2)
	2	4 (4)	3 (1)	5 (3)
	3	5 (5)	3 (1)	7 (4)
	6	8 (7)	9 (3)	7 (4)
<i>Fever, % (n)</i>	Month 1	24 (22)	21 (8)	26 (14)
	2	29 (26)	24 (8)	32 (18)
	3	29 (28)	30 (11)	29 (17)
	6	43 (40)	46 (16)	42 (24)
<i>Cough, % (n)</i>	Month 1	20 (19)	23 (9)	19 (10)
	2	17 (15)	15 (5)	18 (10)
	3	19 (18)	14 (5)	22 (13)
	6	30 (28)	40 (14)	25 (14)
Maternal Nutrition Status				
<i>Body Mass Index, Mean (SD)</i>	Month 1	22.0 (2.6)	22.2 (2.7)	21.8 (2.5)

	2	21.9 (2.6)	22.0 (2.6)	21.8 (2.6)
	3	21.9 (2.8)	21.9 (2.9)	21.8 (2.7)
	6	21.6 (2.7)	21.8 (2.7)	21.6 (2.8)
<i>Food Security Index</i> , Mean (SD) Month 1		13.0 (2.1)	12.2 (2.5)	13.6 (1.6) ^a
	2	12.7 (2.1)	12.3 (2.5)	13.0 (1.9)
	3	12.5 (2.1)	12.2 (2.2)	12.7 (2.0)
	6	12.1 (2.4)	11.5 (2.5)	12.5 (2.2) ^a
Infant Characteristics	All	HIV-exposed	HIV-unexposed	
Birth and Growth Anthropometry				
<i>Gestational Age at Birth</i> , weeks, Mean (SD)		38.2 (3.5)	37.7 (3.0)	38.5 (3.7)
<i>Preterm</i> (<37 weeks), % (n)		29 (28)	35 (13)	25 (15)
<i>Infant Birth Weight</i> , kg, Mean (SD)		3.2 (0.44)	3.1 (0.48)	3.3 (0.39) ^a
<i>Low Birth Weight</i> , <2.5kg, % (n)		8 (8)	15 (6)	3 (2) ^a
<i>Weight-for-Age Z-Score</i> , Mean (SD) Month 1		-0.19 (0.95)	-0.50 (1.13)	0.03 (0.73) ^a
	2	-0.13 (1.01)	-0.55 (1.17)	0.13 (0.81) ^a
	3	-0.27 (1.12)	-0.69 (1.12)	0.01 (1.03) ^a
	6	-0.37 (1.04)	-0.79 (1.13)	-0.11 (0.88) ^a
<i>Height-for-Age Z-Score</i> , Mean (SD) Month 1		-1.73 (1.44)	-1.95 (1.58)	-1.58 (1.32)
	2	-1.78 (1.36)	-2.17 (1.43)	-1.54 (1.26) ^a
	3	-1.87 (1.34)	-2.03 (1.31)	-1.77 (1.36)
	6	-2.33 (1.44)	-2.67 (1.20)	-2.12 (1.54)
Infant Feeding Practices				

<i>Exclusive Breastfeeding</i> , weeks duration, Mean (SD)		4.4 (6.5)	6.0 (7.5)	3.4 (5.6)
<i>Breastfeeding Intensity</i> , feeds/day, Mean (SD)	Month 1	11.8 (4.7)	11.8 (4.3)	11.9 (5.0)
	2	10.9 (4.6)	11.4 (4.7)	10.6 (4.5)
	3	10.7 (3.9)	11.8 (3.8)	10.1 (3.8) ^a
	6	9.2 (4.4)	9.0 (4.5)	9.4 (4.4)
Infant Health Status				
<i>Fever</i> , % (n)	Month 1	26 (24)	33 (13)	20 (11)
	2	29 (26)	31 (11)	27 (15)
	3	42 (41)	45 (17)	41 (24)
	6	66 (61)	56 (20)	72 (41)

IQR=interquartile range; n=number with attribute; SD=standard deviation

^a p<0.05 for comparisons between HIV-positive and HIV-negative women or HIV-exposed and HIV-unexposed infants.

Table 2. Pair-wise correlation matrix of (A) immunoglobulin and (B) cytokine concentrations in mixed HIV-status maternal serum with breast milk (left panel), and breast milk with infant serum (right panel)

	Maternal Serum with Breast Milk				Breast Milk with Infant Serum			
	Month 1	Month 2	Month 3	Month 6	Month 1	Month 2	Month 3	Month 6
A: Immunoglobulins								
IgA	0.19	0.24	0.24	0.14	0.49	0.35	-0.19	-0.28
IgG₁	0.13	0.15	0.11	-	-0.18	0.20	-0.15	0.04
IgG₂	0.03	0.24	-	-	0.24	-0.08	-	-
IgG₃	-	0.09	0.11	-0.03	-	0.15	-0.12	-0.11
IgG₄	0.20	0.18	0.07	0.01	0.20	0.43	-0.14	-0.01
IgM	0.09	0.34	0.14	-	-0.01	0.41	-0.13	0.17
B: Cytokines								
IFN-γ	-0.09	-0.12	-0.13	0.16	ND	ND	-0.08	ND
TNF-α	0.10	0.34	0.16	-0.02	0.00	-0.23	0.25	-0.11
IL-6	-0.07	0.03	-0.07	-0.05	0.33	ND	-0.11	ND
IL-12p70	-0.09	ND	0.08	-0.06	ND	ND	-0.14	ND
IL-15	0.09	-0.06	0.01	0.12	-0.08	ND	ND	-0.26
Strength of Correlation ^a Legend					ND = not detected in breast milk; - = unavailable due to insufficient pairwise data. ^a There are no established criteria for interpreting strength of correlation coefficients in nutritional sciences, therefore this legend represents one such “rule of thumb”.			
Very strong positive = >0.69								
Strong positive = 0.40 to 0.69								
Moderate positive = 0.30 to 0.39								
Weak positive = 0.20 to 0.29								
None to negligible = -0.19 to 0.19								
Weak negative = -0.20 to -0.29								
Moderate negative = -0.30 to -0.39								
Strong negative = -0.40 to -0.69								
Very strong negative = <-0.69								

Table 3

Table 3 Unadjusted and adjusted linear regression of total breast milk immunoglobulin (left) and total breast milk cytokine (right) concentrations

	Total Breast Milk Immunoglobulins								Total Breast Milk Cytokines							
	Month 1		Month 2		Month 3		Month 6		Month 1		Month 2		Month 3		Month 6	
Maternal Health																
HIV-status Unadjusted	1.10	-4.85 to 7.06	-0.53	-7.47 to 6.41	-1.14	-6.22 to 3.94	1.26	-1.50 to 4.01	9.87	-11.96 to 31.71	-15.04	-48.04 to 17.96	-1.06	-18.95 to 16.83	3.60	-13.42 to 20.61
Adjusted	-2.02	-7.75 to 3.71	-3.90	-10.80 to 3.00	-2.39	-7.85 to 3.07	-0.16	-3.11 to 2.79	-6.51	-31.04 to 18.03	-9.31	-49.15 to 30.53	2.98	-18.21 to 24.16	2.80	-15.61 to 21.22
Mastitis Unadjusted	3.04	-10.83 to 16.90	-2.86	-20.84 to 15.11	-6.39	-20.21 to 7.43	-1.79	-7.30 to 3.72	65.45	16.74 to 114.16	40.68	-43.66 to 125.02	8.58	-40.26 to 57.42	15.97	-18.01 to 49.95
Adjusted									76.42	23.25 to 129.59	6.30	-116.20 to 128.80	7.60	-48.45 to 63.66	13.44	-20.07 to 46.94
Fever Unadjusted	-3.78	-10.53 to 2.96	-2.53	-9.91 to 4.85	-3.46	-8.86 to 1.94	-0.58	-3.24 to 2.08	19.78	-5.19 to 44.76	33.50	-0.36 to 67.36	-4.73	-23.89 to 14.43	17.69	1.70 to 33.69
Adjusted									9.18	-18.84 to 37.19	41.40	-1.17 to 83.97	4.34	-22.60 to 31.28	18.55	-1.35 to 38.44
Cough Unadjusted	-4.51	-11.48 to 2.46	-4.62	-14.11 to 4.87	-3.52	-9.85 to 2.81	-1.65	-4.44 to 1.15	9.56	-16.67 to 35.79	-23.02	-65.08 to 19.03	6.30	-16.10 to 28.70	25.50	9.02 to 41.98
Adjusted									9.03	-18.61 to 36.67	-38.65	-93.94 to 16.66	17.20	-9.72 to 44.13	21.43	1.20 to 41.67
Maternal Nutrition																
Body Mass Index Unadjusted	0.85	-0.26 to 1.96	0.51	-0.78 to 1.80	0.67	-0.21 to 1.55	0.32	-0.17 to 0.82	-0.69	-4.86 to 3.48	3.95	-2.01 to 9.92	2.78	-0.31 to 5.86	0.56	-2.51 to 3.64
Adjusted	0.67	-0.35 to 1.68	0.38	-0.86 to 1.62	0.67	-0.24 to 1.58	0.32	-0.18 to 0.82	0.50	-3.87 to 4.87	3.02	-4.55 to 10.59	2.48	-1.08 to 6.04	0.57	-2.64 to 3.79
Food Security Unadjusted	-1.17	-2.55 to 0.21	-0.41	-1.95 to 1.12	-0.47	-1.69 to 0.75	-0.40	-0.96 to 0.16	2.12	-3.02 to 7.26	2.96	-4.41 to 10.34	4.10	-0.12 to 8.31	1.53	-1.97 to 5.03
Adjusted	-1.09	-2.43 to 0.26	-0.32	-1.82 to 1.18	-0.35	-1.62 to 0.92	-0.29	-0.85 to 0.28	4.03	-1.75 to 9.81	3.30	-5.22 to 11.82	3.65	-1.15 to 8.45	2.78	-0.75 to 6.32
Infant Health																
Fever Unadjusted	1.60	-4.90 to 8.10	1.25	-6.15 to 8.65	-2.24	-7.31 to 2.82	-4.16	-6.96 to -1.36	19.03	-4.49 to 42.54	8.48	-26.56 to 43.51	-13.28	-30.95 to 4.39	-16.59	-34.44 to 1.26
Adjusted									3.13	-24.61 to 30.88	16.75	-33.17 to 66.67	-18.06	-41.61 to 5.50	-33.03	-53.78 to -12.28
Infant Nutrition																
Exclusive Breastfeeding Unadjusted	0.50	0.11 to 0.89	0.81	0.33 to 1.30	0.27	-0.09 to 0.62	0.31	0.12 to 0.49	-0.14	-1.63 to 1.36	0.79	-1.66 to 3.24	0.43	-0.84 to 1.70	0.33	-0.83 to 1.50
Adjusted	0.55	0.18 to 0.92	0.88	0.38 to 1.38	0.28	-0.09 to 0.66	0.29	0.09 to 0.49								
Breastfeeding Intensity Unadjusted	0.61	0.02 to 1.21	0.22	-0.57 to 1.02	0.06	-0.61 to 0.74	0.61	0.31 to 0.90	-1.89	-4.10 to 0.32	-1.89	-5.65 to 1.87	-0.17	-2.55 to 2.21	-2.12	-4.06 to -0.18
Adjusted									-0.90	-3.19 to 1.39	-2.51	-7.01 to 1.98	-0.08	-2.87 to 2.70	-2.53	-4.43 to -0.63
Birth Weight (kg) Unadjusted	-3.19	-10.09 to 3.71	-3.17	-11.62 to 5.28	-4.18	-10.00 to 1.64	-2.04	-5.21 to 1.12	-36.78	-61.84 to -11.72	1.41	-39.40 to 42.21	1.98	-18.62 to 22.59	-4.91	-24.49 to 14.66
Adjusted									-11.39	-52.58 to 29.70	-15.51	-72.69 to 41.66	-9.39	-36.68 to 17.89	11.81	-10.93 to 34.55
Weight-for-Age Unadjusted	0.17	-2.88 to 3.23	-0.33	-3.77 to 3.12	-0.35	-2.72 to 2.02	0.57	-0.87 to 2.01	-16.23	-26.81 to -5.66	4.94	-11.75 to 21.63	5.30	-2.96 to 13.57	5.24	-3.43 to 13.91
Adjusted									-16.22	-35.56 to 3.12	-6.93	-38.29 to 24.42	4.70	-8.76 to 18.16	3.52	-9.52 to 16.56
Height-for-Age Unadjusted	-0.09	-2.10 to 1.92	-1.63	-4.07 to 0.81	0.90	-1.00 to 2.81	0.23	-0.70 to 1.16	-6.69	-13.90 to 0.52	3.12	-8.87 to 15.10	4.54	-2.12 to 11.19	2.09	-3.58 to 7.75
Adjusted									-1.67	-11.35 to 8.01	10.42	-8.56 to 29.41	2.88	-6.99 to 12.75	0.42	-6.87 to 7.71
Gestational Age at Birth Unadjusted	-0.88	-1.69 to -0.07	0.19	-0.77 to 1.15	-0.63	-1.37 to 0.12	-0.31	-0.71 to 0.10	-1.08	-3.95 to 1.79	-0.09	-4.81 to 4.63	1.06	-1.61 to 3.73	-0.24	-2.79 to 2.31
Adjusted	-0.85	-1.65 to -0.05	0.17	-0.77 to 1.11	-0.67	-1.44 to 0.09	-0.18	-0.59 to 0.23								
Immunoglobulin Models									Cytokine Models							
Model, Adjusted ^{a,b} , P-value	Month 1	0.003	Month 2	0.041	Month 3	0.145	Month 6	0.019	Month 1	0.007	Month 2	0.511	Month 3	0.446	Month 6	0.002

Data are presented as linear regression coefficient estimator (β) and the corresponding coefficient 95% confidence interval. Bold font indicates evidence of an association (e.g. 95% confidence interval does not include 0) and p-value <0.05 (not shown, except for model p-values).

^aTotal breast milk immunoglobulin model=β(maternal HIV-status)+β(maternal body mass index)+β(household food security)+β(weeks exclusive breastfeeding)+β(gestational age at birth)+μ

^bTotal breast milk cytokine model= β (maternal HIV-status)+ β (mastitis)+ β (maternal fever)+ β (maternal cough)+ β (maternal body mass index)+ β (household food security)+ β (infant fever)+ β (breastfeeding intensity)+ β (infant birth weight)+ β (weight-for-age)+ β (height-for-age)+ μ

Variables coding: Maternal HIV-status: 0=HIV-negative, 1=HIV-positive; mastitis: 0=not experiencing mastitis, 1=experiencing mastitis; maternal fever: 0=not experiencing fever, 1=experiencing fever; maternal cough: 0=not experiencing cough, 1=experiencing cough; infant fever: 0=maternal report that infant was not experiencing fever, 1=maternal report infant was experiencing fever

Figure 1

Figure 2A

Figure 2B