Maternal HIV infection influences the microbiome of HIV-uninfected infants

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More than 1 million HIV-exposed, uninfected infants are born annually to HIV-positive mothers worldwide. This growing population of infants experiences twice the mortality of HIV-unexposed infants. We found that although there were very few differences seen in the microbiomes of mothers with and without HIV infection, maternal HIV infection was associated with changes in the microbiome of HIV-exposed, uninfected infants. Furthermore, we observed that human breast milk oligosaccharides were associated with bacterial species in the infant microbiome. The disruption of the infant’s microbiome associated with maternal HIV infection may contribute to the increased morbidity and mortality of HIV-exposed, uninfected infants.

INTRODUCTION

More than 1 million HIV-exposed, uninfected infants are born annually to HIV-positive mothers worldwide (1). This growing population of infants experience higher morbidity and twice the mortality of HIV-unexposed, uninfected infants in the same community (2–9). Furthermore, many of these HIV-exposed, uninfected infants go on to experience immunological derangements that may persist into adolescence (10). With the growing number of young women infected with HIV worldwide and the persistently high HIV seroprevalence among childbearing women in sub-Saharan Africa, HIV-exposed, uninfected infants are rapidly becoming another significant medical casualty in the HIV pandemic.

It remains unclear why HIV-exposed, uninfected infants experience higher morbidity and mortality compared to matched HIV-unexposed, uninfected infant controls. The establishment of a normal microbiome in infants greatly influences the development of a healthy infant metabolism and immunity (11). Many studies have now shown that the infant acquires much of its microbiome from the mother and continues to be conditioned with commensal bacteria found in breast milk (12, 13). Studies have further shown that HIV-infected adults experience dysbiosis in their microbiome (14–17). Because HIV preferentially targets CD4+ T cells within the gut lamina propria (18), the immune system is less well equipped to interact with the microbes that make up the intestinal microbiome. Culture-based methods looking at the microbiome of mother and infant pairs have shown some associations with HIV infection (19), but how this dysbiosis is established in the HIV-exposed, uninfected infant is poorly understood.

Breastfeeding conveys health benefits in both HIV-infected and HIV-exposed, uninfected infants (20–24). For this reason, breastfeeding in combination with maternal antiretroviral therapy is the recommended form of feeding for HIV-infected women in resource-limited settings (25). Milk is a complex fluid providing not only nutrition to the infant but also many immune-active components. How these contribute to infant health remains poorly defined. Human milk oligosaccharides (HMOs) are the third largest constituent of human milk, but they are not digested by humans (26). HMOs are multifunctional, but their major purpose appears to be as a prebiotic, providing nutrition to the infant microbiome (26). This in turn conditions the developing immune system (27, 28). We have previously shown that changes in HMO composition influence postnatal transmission and survival of HIV-exposed, uninfected infants (20). We hypothesized that perturbation of both the mother’s microbiome and the mother’s breast milk HMO composition by HIV infection alters the microbiome in HIV-exposed, uninfected infants and may account for some of the immunological and survival differences seen in HIV-exposed, uninfected infants compared to HIV-unexposed, uninfected infants.

RESULTS

Characteristics of mother-infant pairs were similar regardless of mother’s HIV status

We enrolled 50 mother and infant pairs from Port-au-Prince, Haiti, evenly split between HIV-positive and HIV-negative mothers (Table 1). HIV-positive mothers had more antibiotic exposure during pregnancy and during the postpartum period (P = 0.02) and higher body mass indices (BMIs) compared to HIV-negative mothers (P = 0.023), but otherwise, the groups were generally comparable. All HIV-positive mothers were on antiretroviral therapy at the time of presentation and throughout their pregnancy. These mothers had low levels of plasma HIV RNA (68% undetectable) and had correspondingly high CD4 T cell counts [median, 567 cells/mm3; interquartile range (IQR), 369 to 681], suggesting preserved immune function (Table 2). All infants were breastfed (Table 3). None of the mothers were on antibiotics at the time of the study, and none of the infants received antibiotics at any point, including trimethoprim-sulfamethoxazole prophylaxis. Samples from six different body sites were collected from each pair (mother: areolar skin, breast milk, and vagina; infant: mouth, skin, and stool). A single infant was found to be HIV-infected through vertical transmission, and the corresponding mother-infant dyad was excluded from all further analyses.

Microbiomes were related on the basis of individual, site, and mother-infant pairs

We first looked broadly at the microbiomes at each sample site. DNA sequencing of the variable region 4 of the 16S ribosomal RNA gene characterized the bacterial composition of the samples. A total of 77 million reads...
were classified into 698 operational taxonomic units (OTUs) using standard approaches (29) and mapped to the Greengenes database at ≥97% identity (30). Principal coordinates analysis (PCoA) and multivariate analysis of variance (ANOVA) revealed site- and individual-dependent effects on bacterial composition (Fig. 1A). Overall, $\alpha$-diversity within sample diversity was relatively similar between sample sites (Fig. 1B); only vagi-
nal samples demonstrated significantly lower diversity than did the other five sample sites (Shannon index, $P = 0.021$). Bacterial composition varied by sample site (Fig. 1, C and D). For example, at the family taxo-
nomical level, areolar skin swab samples had more Pseudomonadaceae compared to breast milk samples. Vaginal samples and infant stool samples had a relatively greater abundance of Bifidobacteriaceae.

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and had very high CD4 T cell counts (median, 567 cells/mm³), and their stool was not tested. Notably, when we further analyzed our data based on the history of maternal antibiotic use and BMI, no differences were identified (table S3).

**Mother’s HIV infection influences the infant microbiome**

In contrast to the mostly consistent microbial communities identified in the mothers, the microbiomes of HIV-exposed, uninfected and HIV-unexposed, uninfected infants showed striking differences based on the mother’s HIV status. Although PCoA demonstrated some separation based on sample site, there was no clear separation between HIV-exposed, uninfected and HIV-unexposed, uninfected infants when each site was analyzed separately (Fig. 3A). We observed lower a-diversity in the stool of HIV-exposed, uninfected infants compared to HIV-unexposed, uninfected infants (Shannon index, \( P = 0.018 \); observed OTU, \( P = 0.005 \); Chao, \( P = 0.017 \)) (Fig. 3B and table S1). Further stratification of the infant gut bacterial diversity by maternal CD4 T cell count (1 to 350 versus ≥350) (fig. S2A) demonstrated a trend toward loss of diversity with lower maternal CD4 T cell counts. When we looked at maternal viral load (undetectable versus detectable), we identified a dose-response relationship with HIV-exposed, uninfected infants born to mothers with detectable viral loads (\( P = 0.017 \); fig. S2B). After identifying the differences outlined above in the microbial communities of infants born to HIV-positive mothers, we looked for further evidence of dysbiosis to better understand its origins. We applied a random effects model to account for the slight variation in age between infants, Preventing biased results by other factors. We performed a permutational ANOVA (PERMANOVA) based on distance matrices to identify contributions to the differences seen in infant stool microbiome composition. We found that maternal HIV status had the only statistically significant influence accounting for about 8% of the differences (\( P = 0.004 \) (table S3)). Age was associated with about 15% of the variation but did not reach statistical significance (\( P = 0.09 \)). When these identified specific microbes were analyzed in relation to the child’s age- and sex-adjusted weight and length, no associations were found. Adjustments for a child’s anthropometric status did not change maternal HIV status associations. When the analysis was further run to account for the slight variation in age between infants, Prevotellaceae and Pseudomonadaceae remained significantly different between the two groups.

**Further evidence for dysbiosis in HIV-exposed, uninfected infants**

After identifying the differences outlined above in the microbial communities of infants born to HIV-positive mothers, we looked for further evidence of dysbiosis to better understand its origins. We applied a random effects model to account for the slight variation in age between infants, Preventing biased results by other factors. We performed a permutational ANOVA (PERMANOVA) based on distance matrices to identify contributions to the differences seen in infant stool microbiome composition. We found that maternal HIV status had the only statistically significant influence accounting for about 8% of the differences (\( P = 0.004 \) (table S3)). Age was associated with about 15% of the variation but did not reach statistical significance (\( P = 0.09 \)). When these identified specific microbes were analyzed in relation to the child’s age- and sex-adjusted weight and length, no associations were found. Adjustments for a child’s anthropometric status did not change maternal HIV status associations. When the analysis was further run to account for the slight variation in age between infants, Prevotellaceae and Pseudomonadaceae remained significantly different between the two groups.
We then applied a Bayesian approach (31) to estimate the proportion of microbes in the infant samples that came from the mother. The mouth and skin microbiomes of infants were represented similarly in both the HIV-unexposed, uninfected and HIV-exposed, uninfected in-

Fig. 1. Microbiomes of 50 mother-infant dyads. There were differences in the microbiomes based on the site of sample collection. (A) PCoA of un-

a previously published model using a random forest regression algorithm to compare our infants against a cohort of healthy Bangladeshi infants (32). A relative maturity index was calculated for each experimental sample based on the relative stool microbiome age compared to the age of the subject at collection. This was then plotted along with the normalized midline of the previously published reference cohort of healthy controls (Fig. 4C). HIV-exposed, uninfected infants had relatively less mature gut microbial communities compared to HIV-unexposed, uninfected infants (Wilcoxon rank sum, \( P = 0.03 \)). When we specifically looked at the 33 age-discriminatory taxa, we found that a single species (Bacteroides fragilis) was more abundant in HIV-unexposed, uninfected infants (Wilcoxon rank sum, \( P = 0.025 \)) (table S4).

Our next step was to look at the predicted metagenomic functional content of the studied bacterial communities using PICRUSt analysis (33). The mother’s HIV status led to significant differences in infant stool and skin microbial pathways (table S5). There were no significant differences in any of the mother’s sample sites or the infant’s mouth based on the mother’s HIV status. Glycan biosynthesis and metabolism of cofactors/vitamins were both up-regulated in the HIV-unexposed, uninfected infants but not in

forest classification scheme (14) to identify taxa associated with HIV status. We found that at the family level, Prevotellaceae, Alcaligenaceae, Desulfovibrionaceae, and Pseudomonadaceae in the infant stool were most predictive of the mother’s HIV status (Fig. 4A). These are the same taxa identified earlier as significantly different in the stool microbiomes of the infants based on the mother’s HIV status using the FDR-corrected \( P \) values based on relative abundances.

We further looked at the infant microbial gut community by examining the relative maturity of the stool microbiome. To do this, we applied

We then applied a Bayesian approach (31) to estimate the proportion of microbes in the infant samples that came from the mother. The mouth and skin microbiomes of infants were represented similarly in both the HIV-unexposed, uninfected and HIV-exposed, uninfected infants with strong influences from breast milk and areolar skin sites (Fig. 4B). However, among HIV-exposed, uninfected infants, it appeared that a greater proportion of the infant stool traced back to the mother’s breast milk and areolar skin, although this finding was not statistically significant. This may suggest that direct seeding of the infant gut via the mother’s microbiome was increased in the context of HIV exposure.

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HIV-exposed infants. In total, the results of our random forest analysis, source tracker evaluation, relative maturity index calculations, and PICRUSt analyses provided further evidence that HIV infection in mothers was associated with robust changes in the HIV-exposed, uninfected infant microbiome.

Disruptions in HMO composition correlate with the infant microbiome

We asked why the HIV-exposed, uninfected infants in our study had significantly different and potentially dysbiotic gut bacterial communities compared to the unexposed infants. We observed that the bacterial communities of mothers with and without HIV infection in our cohort were relatively similar. The disruptions seen in their infants were thus not completely explained by the maternal-to-infant transfer. The normal succession of the infant microbiome is further driven by exposure to food and the environment. All of the infants in our study were breastfed. Given this, we next examined the breast milk from our cohort of mothers in more detail. Here, we saw differences between HIV-infected and uninfected mothers in their milk oligosaccharide composition. HMOs are not digestible by infants but are primarily thought to provide metabolic substrate for specific commensal bacterial communities. Changes in the HMO content of a mother’s milk may thus have profound downstream effects on the establishment of the infant microbiome.

We used high-performance liquid chromatography (HPLC) to characterize the milk oligosaccharides associated with our study samples. The
Fig. 3. Effects of maternal HIV infection on the infant microbiome. (A) PCoA of unweighted UniFrac distances based on the mother's HIV status at each infant sample site (mouth, skin, and stool). (B) α-Diversity (Shannon index) at each infant sample site based on the mother's HIV status. (C) Bacterial compositions across infant sample sites at the taxonomic level of family. (D) Heat map representation of individual bacterial compositions at the family level. Families with an overall relative abundance of less than 0.5% are omitted for the sake of clarity. (E) List of all taxa at any family level or infant sample site that were significantly different based on the mother's HIV status using corrected P values (FDR P < 0.1).
HMO composition of breast milk was altered in HIV-positive compared to HIV-negative women (Fig. 5A). The small sample size meant that these differences did not reach statistical significance; however, they were consistent with differences our group has previously reported from larger samples of HIV-positive and HIV-negative women in Zambia (20, 23). The relative abundance of 3′-sialyllactose (Wilcoxon rank sum, \( P = 0.067 \)), 3′-fucosyllactose (\( P = 0.10 \)), and 2′-fucosyllactose (\( P = 0.077 \)) trended toward being increased in breast milk from HIV-positive mothers compared to HIV-negative mothers. In contrast, human milk lacto-N-tetraose (\( P = 0.064 \)) and lacto-N-neotetraose (\( P = 0.080 \)) trended toward being increased in HIV-negative mothers. All other HMO comparisons, including the total, the total fucosylated, and the total sialylated HMOs, were similar when compared between HIV-positive and HIV-negative mothers.

Surprisingly, we saw few distinct differences in the breast milk bacterial communities between HIV-positive and HIV-negative mothers (Fig. 2). However, when we used Pearson correlations to define the associations between specific HMOs and bacteria, we did see significant direct associations with the infant stool bacteria (Fig. 5B). These correlations were distinct and dependent on the mother’s HIV status. For example, in HIV-negative mothers, increases in 3′-sialyllactose were correlated with relative increases in Enterococcaceae and Fusobacteriaceae in the infant’s stool. In contrast, when we looked at HIV-positive mothers, we found that an increase in lacto-N-fucopentaose 1 in breast milk led to an increase in Bifidobacteriaceae in the infant’s stool.

**DISCUSSION**

In 2013, an estimated 1.5 million women living with HIV gave birth, accounting for nearly 20% of all births in sub-Saharan Africa (1). Although mother-to-child transmission rates are decreasing due to improved availability of antiretrovirals (1), there is now a large and rapidly growing cohort of HIV-exposed, uninfected infants. These children experience nearly twice the mortality rates compared to matched controls (7). The results of our study show that maternal HIV infection is associated with changes in the microbiome of HIV-exposed, uninfected infants. Furthermore, the HMO content trended toward being different based on maternal HIV status. Specific oligosaccharides appeared to be directly linked with specific bacteria within the infant’s microbiome. These data suggest that maternal HIV infection disrupts the normal development of the infant microbiome. This relatively immature and dysbiotic microbiome potentially could compromise development of the infant’s immune system. This may help to explain the higher morbidity and mortality of HIV-exposed, uninfected infants compared to HIV-unexposed infants in the same communities.

We identified differences in the microbiomes of our cohort of Haitian mother and infant pairs based on the mother’s HIV status. Most importantly, we found significant differences in the microbiome of HIV-exposed, uninfected infants compared to the HIV-unexposed, uninfected infants. This was most clearly demonstrated in the stool composition of the HIV-exposed, uninfected infants but was also observed in the mouth
and skin samples as well. *Prevotella* and *Pseudomonas* species have both been associated with HIV infection in previous microbiome studies ([14, 15, 17, 34]) and were significantly different in our HIV-exposed, uninfected infant cohort as well. The reason for this relationship in HIV infection remains unclear. *Prevotella*-rich bacterial communities have previously been associated with proinflammatory states including bacterial vaginosis associated with HIV transmission ([35–37]). The gut mucosa of HIV-infected individuals is inflamed. Recent studies suggest that the chronic gut inflammatory states seen in HIV-positive individuals may be related to dendritic cell activation and association with *Prevotella* species ([17, 38]).

Although previous studies have shown differences in the stool microbiomes of infected versus uninfected individuals ([14, 15, 17]), the microbiomes in the body sites we investigated (vagina, breast milk, and areolar skin) were relatively similar in HIV-infected versus HIV-uninfected women. Initial studies in macaques looking at the stool microbiomes based on the presence or absence of simian immunodeficiency virus infection did not show significant differences in the microbial communities between the two groups ([39]). Similarity in the microbiomes of HIV-uninfected and HIV-infected women in our study is likely explained by the fact that the HIV-infected women in our study had relatively intact immune systems, were virologically suppressed with antiretroviral drug therapy, were well nourished, and remained relatively healthy on antiretroviral therapy with good follow-up and health care. We also did not study the mother’s stool microbiome; previous studies examining the microbiomes of HIV-infected individuals have identified dysbiosis of the gut microbiome.

How did the HIV-exposed, uninfected infants develop such clearly different microbiomes compared to those uninfected infants in the
same community? We propose that it may be the combination of the slight perturbations in the mother’s microbiome and differences in the HMO composition of the mother’s breast milk that may explain these differences. Although there were only minor differences in the mothers’ microbiomes, when we traced back to see the origins of the infants’ microbiome taxa, there was a clear influence from the mother. This was especially true in the HIV-positive mothers and their exposed infants. Treatment of HIV-infected mothers with antiretroviral drugs may further biome taxa, there was a clear influence from the mother. This was especially true in the HIV-positive mothers and their exposed infants. Treatment of HIV-infected mothers with antiretroviral drugs may further contribute to the exposed infant’s disrupted microbiome. The reason for differences in the HMO breast milk composition observed remains unclear but also may be associated with HIV infection itself or the antiretroviral medications.

With only very small differences in the breast milk microbial communities, we believe that the HMOs produced in breast milk are not directed toward the growth of specific bacteria within breast milk itself. Rather, we propose that the changes in the HMO composition may have downstream probiotic effects on the growth and colonization of various bacterial species in the infant microbiome. In this way, HMOs in breast milk may be responsible for the dysbiosis seen in HIV-exposed uninfected infants. We identified clear relationships between specific HMOs altered by HIV infection that were associated with differences seen in the infant’s stool microbiome. Many of these same HMOs have been identified as key components of breast milk in previous HIV studies (20, 23). Unfortunately, little has been done to understand the relationships between HMOs and the infant microbiome in humans. Correlations linking mother’s breast milk HMOs to specific bacteria have recently been identified in a study of 14 mother and premature infant dyads (40). Furthermore, links between specific prebiotics and Prevotella communities in the gut microbiome have been established previously in mice (41). It has also been shown that Bifidobacterium species, thought to be beneficial, preferentially predominate in the presence of lacto-N-neotetraose in germ-free mice (42).

Identifying these differences in the microbiome of HIV–exposed, uninfected infants and their origins is just the beginning of our understanding of this complex system. We found that the relative microbial maturity of the HIV–exposed, uninfected infants’ stool was significantly lower than that observed in the infants born to HIV-negative women. This immaturity of the infant microbiome has been associated with a decreased nutritional state (32). The next step will be to understand the mechanisms by which the microbiome and HMOs work together to affect the health of these at-risk HIV–exposed, uninfected infants. This knowledge can then be applied toward developing interventions to prevent the increased morbidity and mortality seen in HIV–exposed, uninfected infants. Providing infants with important beneficial bacteria (probiotics) or potentially specific milk oligosaccharides (prebiotics) may potentially improve long-term outcomes, although this remains to be thoroughly investigated and tested.

In-depth studies looking at the impact of prebiotics and probiotics in HIV–exposed, uninfected infants are needed. Already, studies in simian immunodeficiency virus–infected macaques have shown modest benefit in preventing long-term gastrointestinal complications with the administration of probiotics in combination with prebiotics (43). A study looking at treating children in Malawi with high HIV prevalence for severe acute gastroenteritis did not show improvement in outcomes with a combined prebiotic and probiotic therapy (44). Probiotics alone appear to be useful in decreasing the severity of necrotizing enterocolitis in premature HIV–exposed, uninfected infants but do not work as well as they did in the HIV–unexposed, uninfected cohort (45). With better understanding, we may be able to target bacteria and HMOs that are most needed for normal development and protection against morbidity and mortality in HIV–exposed, uninfected infants.

Our study has several limitations. We looked at a cross-sectional sampling of an at-risk population at a single site. The women in our study had relatively high CD4 T cell counts, and we have previously shown that infant morbidity and mortality among HIV–exposed, uninfected infants are inversely related to maternal CD4 T cell count (8). Prospective, long-term, multicenter enrollment may better identify differences in microbiomes and maternal breast milk HMO composition over time. That being said, this is a relatively large microbiome study cohort with 50 mother and infant pairs including multiple sample sites. Furthermore, many of the findings, such as some of the differences seen in bacterial taxa and HMOs, have been identified in previous HIV studies from different geographic locations. HIV-infected mothers in our cohort had higher BMIs and received more antibiotics during pregnancy or the peripartum period than did HIV-uninfected mothers. However, these parameters did not influence the microbiome of the infant and adjustment for these and other covariates did not change associations of the infant microbiome with maternal HIV status. Selection bias in this impoverished community may have selected a cohort of HIV-negative mothers at higher risk of microbiome dysbiosis. This might explain why clear differences were not observed in the microbiomes of HIV–infected and uninfected mothers. Nevertheless, this bias is toward the null, suggesting that the differences between HIV–exposed and HIV–unexposed infants that we observed may be more marked than reported here.

We found that maternal HIV infection was associated with changes in the microbiome of HIV–exposed, uninfected infants and further observed that human breast milk oligosaccharides were associated with specific bacterial species in these high-risk infants. This disruption of the infant’s microbiome may contribute to the increased morbidity and mortality observed for HIV–exposed, uninfected infants.

MATERIALS AND METHODS

Study design

We conducted a cross-sectional study of 50 mother and infant pairs attending the Haitian Group for the Study of Kaposi’s Sarcoma and Opportunistic Infections (GHESKIO) clinics in Port-au-Prince, Haiti: 25 women were HIV–infected and 25 were HIV–uninfected. Women more than 15 years of age who had delivered a baby in the past 6 months and who were breastfeeding were eligible to participate in this study. Participants were excluded if they were unable to provide informed consent or if their child did not primarily feed via the breast. This study was approved by the Institutional Review Boards of Children’s Hospital Los Angeles, Columbia University, and GHESKIO.

At the time of the study visits, mothers were interviewed by study personnel to collect clinical data. This included sociodemographic data, current medications, pregnancy and delivery history, and infant feeding details. All clinical data were entered into a database on site.

Sample collection

We prospectively collected demographic and clinical data and six samples from each pair (mother: areolar skin, breast milk, and vagina; infant: mouth, skin, and stool). Stool and breast milk samples were collected in sterile containers and frozen at –20°C. Areolar skin, vaginal, skin, and mouth mucosa swabs were placed in PSP DNA stabilization
buffer (Stratec) before being frozen. A separate control swab was exposed to the air in the room at the time of collection and was placed in the buffer as well.

**Extraction**

DNA was extracted from all sample types using the NucliSENS easyMag following the manufacturer’s protocol. We homogenized the frozen stool and breast milk samples in PSP stool stabilization buffer first. Then, we used the PSP Spin Stool kit for extraction following the manufacturer’s protocol, substituting Lysing Matrix E tubes (MP Biomedicals) for the provided zirconia beads. Extracted DNA was stored in elution buffer at -80°C.

**Library preparation**

We conducted library preparation on the extracted DNA from the six samples above for each mother-infant pair. The 16S ribosomal DNA was amplified in triplicate and barcoded using a previously published protocol (46). Briefly, this protocol uses the V4 region of the 16S ribosomal RNA gene. Illumina flow cell adapter sequences and a 12–base pair (bp) barcode region were incorporated into the polymerase chain reaction primers. DNA amplicon concentrations were then quantified on a 2200 TapeStation (Agilent Technologies).

**Sequencing**

We followed the detailed, massively parallel sequencing protocol as presented previously by Caporaso et al. (46). Briefly, we pooled the amplicons and diluted to 2 nM. The amplicons were then denatured and loaded on a MiSeq desktop sequencer (Illumina) using 2 × 150-bp v2 chemistry. After cluster formation, the amplicons were sequenced with specific primers designed to be complimentary to the V4 amplification primers. The barcode was read using a third sequencing primer in an additional cycle.

**HMO analysis**

HPLC was used to characterize HMOs in breast milk as previously described (23). Briefly, 20 μl of human milk was spiked with the non-HMO raffinoside as an internal standard to allow for absolute quantification. Oligosaccharides were extracted by high-throughput, solid-phase extraction over C18 and Carbograph microcolumns and fluorescently labeled with 2-aminobenzamide. Labeled oligosaccharides were analyzed by HPLC on an amide-80 column (4.6-mm inner diameter, 25-cm length, and 5-μm particle size; Tosoh Biosciences) with a 50 mM ammonium formate–acetonitrile buffer system. Separation was performed at 25°C and monitored with a fluorescence detector at 360-nm excitation and 425-nm emission. Peak annotation was based on standard retention times and mass spectrometric analysis on a Thermo LCQ Duo Ion trap mass spectrometer equipped with a nano-ESI source. The total concentration of HMOs was calculated as the sum of the most common oligosaccharides. The proportion of each HMO per total HMO concentration was calculated.

**Statistics**

Analysis of the sequencing data was carried out using QIIME 1.8.0 (29). Briefly, reads were merged using fastq-joint and then demultiplexed. Open reference OTU picking was performed using UCLUST at 97% similarity using the Greengenes 13.8 database (30, 47). Chimeras were removed using the mothur implementation of ChimeraSlayer (48). The resulting OTU table was filtered at a minimum abundance threshold of 0.005%, giving a total of 698 OTUs. A rarefaction depth of 16,737 reads was selected because most samples were sufficiently saturated. α- and β-diversity statistics and taxonomic compositions were calculated as previously described (29).

Additional statistical analyses were performed using R (version 3.0.3). For comparisons of demographic data, either Fisher’s exact test or Wilcoxon rank-sum tests were used as appropriate. Comparisons of α- and β-diversity distances were performed using a nonparametric t test with 1000 permutations. Comparisons of bacterial abundances were performed using a bootstrapped Kruskal-Wallis test with 1000 permutations. Comparisons of HMO composition were performed using a Wilcoxon rank-sum test. All P values were adjusted for multiple comparisons using the Benjamini-Hochberg method, except where indicated otherwise. FDR-corrected P values of <0.1 were considered significant. We assessed the genetic effects on microbiome composition by comparing distances between true mother-infant pairs versus 1000 permutations of randomly drawn pairs of mothers and infants.

Random forest analysis was performed as previously described (15), using HIV status as the outcome and family-level taxa abundances as the covariates. Source tracking analysis was performed using SourceTracker v0.9.5 (31). Relative maturity distributions between HIV-exposed, uninfected and HIV-unexposed, uninfected infants were compared using a Wilcoxon rank-sum test. Analysis of the predicted metagenome was performed using the PICRUSt software package (33). To help identify truly important relationships, significant differences were accepted only for FDR-corrected P values of <0.05 in this metagenome analysis.

To identify relationships between HMO levels and selected bacterial abundances, Spearman correlation coefficients were calculated for all pairs of HMO levels and bacterial abundances where at least 10 samples had nonzero abundance. P values were calculated using 1 million permutations. Because the purpose of this analysis was primarily hypothesis generation, all relationships with a FDR-corrected P value of <0.2 were accepted as significant. We further evaluated HMOs compared to the relative maturity index using Spearman correlations. FDR-corrected P values of <0.1 were considered significant.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Sample analysis based on individual, site, and mother-infant pairs.

Fig. S2. Further stratification of the infant gut bacterial diversity by maternal CD4 T cell count and maternal HIV viral load.

Table S1. α-Diversity values based on the mother's HIV status.

Table S2. Unadjusted relative abundance percentages.

Table S3. PERMANOVA based on distance matrices.

Table S4. Comparison of the 33 age-discriminatory taxa based on maternal HIV status.

Table S5. Evaluation of infant stool and skin microbial pathways based on maternal HIV status using PICRUSt analysis.

**REFERENCES AND NOTES**


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Editor's Summary

Influencing the infant microbiome

Annually, there are more than 1 million children born to HIV-infected women. Most of these children do not acquire HIV infection, but they experience twice the mortality of children born to HIV-negative women. Bender et al. now report that maternal HIV infection was associated with changes in the microbiome of these HIV-exposed, uninfected infants. Furthermore, they observed that human breast milk oligosaccharides were associated with specific bacterial species in the infant microbiome. The disruption of the HIV-exposed infant’s microbiome may contribute to the increased morbidity and mortality of these infants.

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