Longitudinal gut virome analysis identifies specific viral signatures that precede necrotizing enterocolitis onset in preterm infants

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Necrotizing enterocolitis (NEC) is a serious and sudden necroinflammatory complication of preterm birth. NEC incidence in infants born at <32 weeks’ gestation ranges from 2 to 7% in high-income countries, with case mortality ranging from 22 to 38%. NEC survivors face lifelong sequelae, including short bowel syndrome and neurodevelopmental disabilities. The aetiology of NEC is unclear but risk factors in addition to prematurity include formula feeding and prolonged use of antibiotics early in life. Numerous studies have shown that gut microbiome alterations contribute to the development of NEC, with several recent large studies converging on a risk community state consisting of over-representation of Gram-negative facultative bacteria (for example, Gammaproteobacteria, Proteobacteria) and relative under-representation of obligate anaerobic bacteria. Notably, however, no single bacterial genus, species, serotype or sequence type has reproducibly been implicated as the cause of NEC. How the microbiome contributes to NEC pathogenesis is unclear but proposed mechanisms include stimulation of Toll-like receptor 4 by lipopolysaccharide from Gram-negative bacteria, leading to poorly controlled inflammatory responses in the preterm gut. Some reports have associated eukaryotic viruses with NEC and a recent next-generation sequencing (NGS) study described a limited spectrum of bacteriophages, including enrichment of Staphylococcus phage 363_30, before NEC in preterm infants, but these studies did not address overall virome composition and dynamics. Factors such as breastfeeding, delivery route, antibiotics and the environment influence gut bacterial community composition and microbiome maturation. Time series studies of the preterm gut microbiome identified choreographed patterns of microbiome acquisition. For example, stool samples from preterm infants in the first days of life are characterized by high proportions of Bacilli, giving way over time to Gammaproteobacteria and then Clostridia.

In contrast to the relatively stable adult virome, a small number of studies have been performed, which suggest that temporal changes are common in the gut viromes of infants and young children, including changes in bacteriophage diversity and increases in prevalence and richness of eukaryotic viruses over time. Bacteriophages are believed to influence gut bacterial communities. Recent experimental evidence demonstrated that bacteriophages influence microbiome composition in mice and affect the microbiome and intestinal health after fecal microbiota transfer. Moreover, virome alterations have been associated with inflammatory bowel disease and colitis, suggesting that the virome plays a role in digestive disorders. Given the importance of microbiome acquisition and of the interacting role played by the virome in health and disease, it is logical to study the development of the preterm virome over time to understand factors that may influence health and disease.

In this study, we present a longitudinal, metagenomic NGS study of the gut viromes of 23 preterm infants. This cohort includes 9 infants who subsequently developed NEC and 14 controls matched for gestational age at birth and birthweight. We found substantial interpersonal variation in gut viromes across both phage and eukaryotic viruses at various ages in infants at risk for NEC. However, the viromes of infants who developed NEC converged towards a reduced level of beta diversity before NEC ensued and this convergence was characterized by specific viral signatures.

Results
Preterm virome varies within and between infants over time. We analysed 138 stool samples collected over time from 23 preterm infants in the neonatal intensive care unit (NICU) at St. Louis Children’s Hospital (Supplementary Tables 1 and 2). Nine of the infants (cases) developed NEC and 14 infants matched for weight
Circoviridae (1 sample, 20.7%) and of eukaryotic virus families Tectiviridae (Fig. 1b and Supplementary Table 2). After controlling for repeated variation at each time point (Extended Data Fig. 2a). Contig richness within individuals over time. Grouping samples by week of life, varied between control infants in each week of the study, spanning samples, IQR P

Dissimilarity (Extended Data Fig. 2b and Supplementary Tables 7 and 8). Principal coordinates analysis (PCoA) on weighted Bray–Curtis dissimilarity showed substantial overlap of samples obtained at different postmenstrual ages, while permutational multivariate analysis of variance (PERMANOVA) testing showed a significant association with PMA (P = 0.05) (Fig. 1d and Supplementary Table 9). Taken together, these results demonstrate high inter- and intra-individual variation in the preterm infant gut virome. However, gut viromes in individual infants were more similar to self than to non-self (other infants) over time, indicating some degree of intra-host stability.

Preterm NEC case infant viromes vary when compared by age. We next examined the viromes of the nine case infants who subsequently developed NEC. Median age at NEC onset was 31.1 weeks' PMA (IQR = 30.0–32.8 weeks); 4 case infants died. As with the control preterm infants, a substantial proportion of case infant viromes consisted of viruses that could not be assigned family-level taxonomy and were categorized as unclassified viruses (median relative abundance = 84.5%; IQR = 75.8–88.2%). Classifiable viral contigs in the case viromes included the bacteriophage families Myoviridae, Podoviridae and Siphoviridae, with median relative abundances of 5.6% (IQR = 1.6–7.4%), 2.7% (1.8–4.1%) and 2.9% (1.7–5.4%), respectively (Fig. 2a and Supplementary Table 5). As in the controls, Microviridae were present at high relative abundance (55.4%) in only 1 sample and at lower relative abundances in other samples (median relative abundance = 0.1%; IQR = 0.01–0.2%). Other low relative abundance bacteriophages include Gokushovirinae, Herelleviridae and Tectiviridae. Some control samples had high relative abundance of eukaryotic virus families Anelloviridae (1 sample, 20.7%) and Circoviridae (2 samples, 10.4 and 15.0%), while other control samples had considerably lower relative abundances (Anelloviridae, 8 samples, IQR = 0.1–0.9%; Circoviridae, 31 samples, IQR = 0.01–0.3%). Relative abundances of bacteriophage and eukaryotic virus families varied between control infants in each week of the study, spanning the 26th to the 37th week PMA. Family relative abundance also varied within individuals over time. Grouping samples by week of life, rather than the PMA at which they were obtained, yielded similar variation at each time point (Extended Data Fig. 2a). Contig richness and Shannon diversity varied within and between individuals (Fig. 1b and Supplementary Table 2). After controlling for repeated sampling of individuals by linear mixed modelling, neither richness nor Shannon diversity changed significantly over time (P = 0.47 and P = 0.61, respectively). Finally, we compared viromes between control preterm infants by examining beta diversity. Median weighted Bray–Curtis dissimilarity, which accounts both for virus presence–absence and virus abundance, was significantly lower within than between infants (Mann–Whitney U-test, P < 0.0001) (Fig. 1c and Supplementary Table 6). We observed similar results for Sorensen dissimilarity (P < 0.0001) and Hellinger distance (P < 0.0001) (Extended Data Fig. 2b and Supplementary Tables 7 and 8). Principal coordinates analysis (PCoA) on weighted Bray–Curtis dissimilarity showed substantial overlap of samples obtained at different postmenstrual ages, while permutational multivariate analysis of variance (PERMANOVA) testing showed a significant association with PMA (P = 0.05) (Fig. 1d and Supplementary Table 9). Taken together, these results demonstrate high inter- and intra-individual variation in the preterm infant gut virome. However, gut viromes in individual infants were more similar to self than to non-self (other infants) over time, indicating some degree of intra-host stability.

### Table 1 | Cohort characteristics

| Variable | Controls n = 14 | Cases n = 9 | Statistical significance |
|----------|----------------|-------------|-------------------------|
| Gestational age at birth, weeks | 25.5 (24.9–26.0) | 25.0 (23.1–25.4) | NS, P = 0.19 |
| Birth weight, g | 810 (670–920) | 780 (570–955) | NS, P = 0.82 |
| Vaginal delivery | 2 (14%) | 4 (44%) | NS, P = 0.16 |
| Male | 5 (36%) | 6 (67%) | NS, P = 0.21 |
| 1 and 5 min Apgar scores | 2 (1–6) and 5.5 (2.8–6.3) | 3 (1.5–5) and 5 (4.5–6) | NS, P = 0.93/NS, P = 0.96 |
| Exposed to human milk during sampling period (yes) | 13 (93%) | 8 (89%) | NS, P = 0.99 |
| Percentage of days of antibiotic exposure during the sampling period | 25.3% (61.3–33.3%) | 10.5% (0–29.4%) | NS, P = 0.25 |
| Stool samples analysed per infant | 7 (6–8) | 4 (4–6) | P = 0.01 |

Statistical significance assessed by two-sided Mann–Whitney U-test for continuous variables and two-sided Fisher’s exact test for categorical variables. NS, not significant. Data are expressed as the median (IQR) or number (percentage) as appropriate.
Virome convergence precedes NEC onset. We next considered the possibility that virome community dynamics might influence NEC development by examining virome progression in cases relative to the time of NEC onset. We used a sliding 7 d window with steps of 2 d between windows, counting backwards from the day NEC occurred. Sorensen dissimilarity between case infants, which considers virus presence–absence, decreased in sliding windows spanning the 10 d immediately before NEC onset (Fig. 3a, red and Supplementary Table 10). Specifically, viral populations more closely resembled each other in this interval. In contrast, dissimilarity between matched samples among controls was stable during this immediate pre-NEC interval (Fig. 3a, blue and Supplementary Table 10). Between-case dissimilarity was less than between-control dissimilarity at windows spanning 11–4 d, 9–2 d and 7–0 d before NEC (Mann–Whitney U-test; \( P = 0.002, P = 0.003 \) and \( P = 0.05 \), respectively). This suggests that the beta diversity of the gut viromes of case infants begins to converge about 10 d before NEC onset. We then used linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify the viral contigs associated with the 10 d period immediately before NEC onset compared to the immediate antecedent period (that is, 11–46 d before NEC onset). We identified 137 contigs associated with the time period 0–10 d before NEC (NEC-associated contigs), whereas only 11 contigs were associated with the earlier period, that is, 11–46 d before NEC onset (Fig. 3b). Most of these contigs could not be assigned family-level taxonomy, although some belonged to Myoviridae, Podoviridae and Siphoviridae (Supplementary Table 11).
abundance increased significantly in the 10 d before this event (Fig. 3c and Supplementary Table 12; Friedman test with Dunn’s multiple comparisons, see Supplementary Table 13 for the P values). The overall relative abundance of the NEC-associated contigs was low (<20%, data not shown), suggesting that changes in low-abundant viruses are implicated in NEC virome risk convergence. This is consistent with the Sorensen dissimilarity data, which do not consider species abundance. We reasoned that a NEC-associated signature should be progressively enriched closer to NEC onset. Moreover, enrichment should be specific to case infants but not gestational age-matched control infants. Indeed, prevalence and abundance of NEC-associated contigs increased in case infants in relation to...
Fig. 3 | Virome convergence before NEC onset. a, Sorensen dissimilarity between cases (red) and between controls (blue) in sliding windows before NEC onset (7 d windows with 2 d steps). Medians with 95% confidence intervals are shown. Statistical significance at each window was assessed by two-sided Mann–Whitney U-test. b, LEfSe of contigs in case samples. Purple indicates features associated with 0–10 d before NEC. Green indicates features associated with 11–46 d before NEC. c, Prevalence and abundance of NEC-associated contigs in cases, in 5 d intervals before NEC. Statistical significance was assessed by Friedman test with Dunn’s multiple comparisons (P values in Supplementary Table 13). d, For each discriminant contig, linear regression was performed on prevalence and average abundance in 5 d intervals before NEC in cases (red) and controls (blue). Regression coefficients for abundance and prevalence are shown on the x and y axes, respectively.
controls (Fig. 3d (compare red to blue) and Supplementary Table 12). By contrast, prevalence and abundance of NEC-associated contigs decreased in controls in proximity to case NEC onset (Extended Data Fig. 3a and Supplementary Table 14). To determine if the large number of contigs associated with NEC onset was characteristic of longitudinal virome development, we conducted a similar LEfSe analysis using control samples. Specific contigs were associated with late and early time points in control infants (< or >10 d before their respective case infant’s time of NEC onset (Extended Data Fig. 3b and Supplementary Table 15). Prevalence and abundance of the control late-associated contigs increased significantly over time in controls, whereas in case infants prevalence of these contigs varied and abundance rose slightly (Extended Data Fig. 3c,d and Supplementary Tables 16–18). Notably, the NEC-associated contigs in case infants were different from the late-associated contigs in controls. Of the NEC-associated contigs with at least 5 open reading frames (ORFs), 31.7% were predicted to have temperate lifestyles and 68.3% were predicted to be lytic (Extended Data Fig. 3e). The proportions of predicted lytic and temperate viruses did not differ significantly between NEC-associated contigs, control late-associated contigs and the dataset as a whole (chi-squared test, \( P > 0.9 \)). Taken together, these results indicate that the gut viromes of preterm infants who developed NEC converged in beta diversity before the event and this convergence was driven by enrichment of specific viruses and loss of others. While control infants also gained and lost viral contigs over time, the specific viruses gained and lost differed from those gained and lost in case infants. Furthermore, this turnover was insufficient to drive a substantial change in beta diversity in control infants.

**Bacterial-viral interactions before NEC onset.** We next considered the possibility that virome convergence might mirror changes in the bacterial microbiome before NEC onset. Therefore, we used a similar approach to examine bacterial sequencing data from these samples (that is, in reference to when NEC occurred). Major classes of bacteria found in cases and controls included *Gammaproteobacteria*, *Clostridia* and *Bacilli* (Fig. 4a and Supplementary Table 19). *Enterococccaeae* abundance was significantly different between case and control infants (Extended Data Fig. 4a; ANCOM-II, adjusted for repeat sampling). Differences in *Gammaproteobacteria*, *Bacilli*, *Enterobacteriaceae* and *Veillonellaceae* abundances were not significant when adjusted for repeated sampling (Extended Data Fig. 4a,b). Interestingly, unlike virome beta diversity, bacterial beta diversity in case infants was stable in windows spanning the 25 d before NEC (Fig. 4b and Supplementary Tables 20 and 21). Weighted UniFrac distance during this time was significantly less in case infants than control infants, while unweighted UniFrac distance was not (Fig. 4b, Extended Data Fig. 4c and Supplementary Tables 22 and 23). We did not observe a convergence of the bacterial microbiome in case infants, possibly because case bacterial beta diversity was already low 18–25 d before NEC.

Finally, we investigated the interactions between the virome and bacterial microbiome using linear mixed modelling to identify correlations between viral and bacterial abundance. We found that the NEC-associated contigs were correlated with specific bacterial genera in case infants but did not follow the same correlation pattern in control infants (Fig. 4c, left and Supplementary Table 24). For example, several NEC-associated contigs were positively correlated with *Escherichia* and *Streptococcus*, while many of the contigs associated with >10 d before NEC were negatively correlated with these genera. Correlations between NEC-associated contigs and *Proteus* and *Bifidobacterium* were generally negative. On the other hand, correlations with *Acinetobacter*, *Clostridium*, *Lactobacillus* and *Haemophilus* were generally positive. These specific interactions were absent in control infants (Fig. 4c, right and Supplementary Table 25). We also observed interactions between control late-associated contigs and bacterial genera in control samples (Extended Data Fig. 4d, left and Supplementary Table 26). For example, specific contigs were positively correlated with *Enterococccaeae*, *Escherichia*, *Staphylococcus*, *Enterobacteriaceae*, *Clostridium*, *Veillonella*, *Haemophilus*, *Streptococcus* and *Enterococcus* in control samples. We found relatively few associations between control time-associated contigs and bacterial genera in case samples, except for some positive correlations with *Diabister*, *Bifidobacterium*, *Haemophilus* and *Streptococcus*, and some negative correlations with *Corynebacterium*, *Proteus* and *Propionibacterium* (Extended Data Fig. 4d, right and Supplementary Table 27). Overall, these results indicate that virus-bacteria interactions in case infants who developed NEC differed substantially from control preterm infants who did not develop NEC.

**Discussion**

We identified convergence of viral communities and specific viral contigs in the days before NEC onset. The viral signatures of NEC were observed immediately before NEC (beginning at 10 d preceding onset), compared to the bacterial shift observed 25 d before NEC occurred. Detecting patterns of change in the virome before NEC onset could enable early identification of preterm infants at excessive risk of developing NEC.

We found substantial interindividual variation in preterm infant gut viromes (Figs. 1 and 2). Viral family relative abundance, richness and alpha diversity varied between and within individuals over time. We found that within-individual Bray–Curtis dissimilarity was significantly lower than between individuals, suggesting that over time the viromes of individual infants were more similar to self than non-self. This is consistent with previous studies that found substantial interindividual variation in the adult virome.

The high proportion of *Mimiviridae*, *Anelloviridae* and *Circoviridae* in some samples could reflect our use of Φ29 DNA polymerase for viral DNA amplification, which biases towards small circular single-strand DNA viruses.

Interestingly, the gut viromes of preterm infants who developed NEC converged before NEC ensued (Fig. 3). While viruses were associated with specific times before NEC onset in both cases and controls, the specific viruses in each group differed. This indicates that accrual of NEC-associated viruses may be a distinctive feature of the pre-NEC state. For example, virome convergence and shift may alter mucosal immunity. Indeed, bacteriophages have been implicated in mucosal immunity and pathobiology.

For example, *Escherichia coli*, *Lactobacillus plantarum* and *Bacteroides thetaiotaomicron* bacteriophages stimulate interferon-γ production through Toll-like receptor 9 signalling independent of bacteria and *E. coli* bacteriophages worsen colitis in mice. *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteriophages stimulate both pro- and anti-inflammatory gene expression and cytokine production. Given the ability of diverse bacteriophages to directly influence mucosal immunity, it is possible that the viruses we identified trigger a cascade that stimulates inflammatory mucosal responses and contributes to NEC pathogenesis. It is also possible that increases in NEC-associated viruses may be a result of bacterial microbiome alterations and mucosal inflammation occurring in the context of bacterial community metabolism before NEC onset. Current data do not permit us to speculate about the mechanistic underpinnings of bacteriophage kinetics, bacterial interactions and the imminent development of NEC. In addition to direct action of bacteriophages on the host, several possibilities are worthy of consideration. These include lysis-independent effects of bacteriophages on bacterial metabolism and expression of effector molecules, lysis-dependent release of host-injurious bacterial molecules and emergence in the infant host of bacterial resistance to bacteriophages.

We found that in the 25 d before NEC onset, the abundance of *Gammaproteobacteria*, *Bacilli*, *Enterococccaeae*, *Enterobacteriaceae*...
**Fig. 4** | Bacterial microbiome stability and virus-bacteria interactions before NEC onset. (a) Average relative abundance of bacterial orders in case and control samples in the 25 d preceding case NEC onset. (b) Weighted UniFrac distance between case samples (red) and between matched control samples (blue) in sliding windows before time of case NEC onset (7 d windows with 2 d steps). Medians with 95% confidence intervals are shown. Statistical significance at each window was assessed by two-sided Mann–Whitney U-test. (c) Significant correlations between case discriminant contigs (NEC-associated and associated with earlier period) and bacterial genera in case (left) and control (right) infants. Dendrograms were ordered based on row and column means. Coefficient refers to the linear regression coefficient (that is, slope).
and Veillonellaceae differed between case and control samples, as reported previously⁷ (Fig. 4). Note that while that study focused on the class Negativicutes, the preponderance of genera in that class were Veillonella. However, only the Enterococcaceae family was significantly different when adjusted for repeated sampling. These results might be explained by differences in sample size (subset of cohort), analyses pipelines (QIME 2) and statistical methodologies (ANCOM-II, adjusting for repeated sampling). Unlike virome beta diversity, bacterial beta diversity did not converge. Rather, in the 25d before NEC onset, weighted UniFrac distance between case samples was low, that is, the bacterial community was highly similar among case infants. During the same time, interactions between bacterial genera and viral contigs differed in the case and control groups. Interestingly, several NEC-associated contigs were positively correlated with Escherichia and Streptococcus, while several contigs associated with earlier periods were negatively correlated with these genera. The differential correlation of NEC-associated contigs and contigs associated with earlier periods with clinically relevant bacterial genera may indicate a role for these viruses in NEC development. Although bacteriophage predation on bacterial communities has been implicated in community modulation in experimental systems in mice, soil and bacteria isolated from the fecal samples of young children, whether the same mechanism contributes to diseases such as NEC will need to be addressed in relevant bacterial genera may indicate a role for these viruses in NEC development. Furthermore, beta diversity was used to assess whether the virome changed over time in NEC. The convergence of beta diversity was used to assess whether the virome changed over time in NEC. The convergence of beta diversity was used to assess whether the virome changed over time in NEC. The convergence of beta diversity was used to assess whether the virome changed over time in NEC. The convergence of beta diversity was used to assess whether the virome changed over time in NEC.

Virome sequencing. Stools were collected at ~80°C until TNA extraction. Stools (approximately 200 mg) were shipped from the stool samples were stored at −80°C until RNA extraction. The TNA was extracted from stool filtrates using the COBAS Amplicon Instrument (Roche Diagnostics). DNA was amplified using the Qiagen primer set (Enterobacteriaceae V2 Kit; GE Healthcare), libraries were constructed using the Nextera DNA library preparation kit and sequenced on the Illumina MiSeq platform (v2, 2 × 250 base pairs per sample) as described elsewhere. Pbs spiked with Orsay virus RNA generated by in vitro transcription was used as a positive sequencing control. Samples were randomized for sample processing and NGS using a random number generator. Sample processing and NGS were carried out blind to the experimental groups. Subsequent analyses of sequencing data were not performed blind because sample metadata such as infant ID, age and case/control status were essential for statistical analysis.

Virome analysis. Sequencing reads were quality-filtered with BBTools (v37.64) and phiX sequences removed, reads mapping to the human genome removed, paired reads merged and reads deduplicated. Contigs were assembled from the reads as described elsewhere. A total of 778,612 contigs were assembled from the infant stool samples. Sample and Orsay control contigs were deduplicated separately using CD-HIT-EST v4.8.1 at minimum 95% identity and 95% overlap. Overlapping contigs were merged using minimum2 (as implemented in AMOS v3.1.0, https://sourceforge.net/ projects/amos/files/amos/) (overlapping 95% identity). Sample and control contigs were then combined into a 1 file and filtered by length (minimum length 800 nucleotides). After deduplication and length filtering, 81,873 sample contigs (median length = 1,379 bases; IQR = 998–2,369 bp) remained for analysis. Length-filtered contigs were queried against the Gut Phage⁴⁸ and the Gut Virome databases using Blastx (minimum e-value x 10⁻⁵), resulting in 55,002 candidate viral contigs. The quality-filtered, deduplicated reads from the samples and Orsay controls were mapped to the resulting contig database. Contig counts for each sample were normalized by RPK (as follows: (79,000/total quality control reads of sample) × (number of reads mapping to each contig)). The resulting read counts for each contig were divided by the contig length in kilobases. After normalization, counts smaller than 0.5 were removed to reduce noise. Circular contigs were identified using VirSorter v1.0.5 (ref. ⁴⁹). We used the decontam package v1.4.0 in R v3.6.1 (refs. ⁵⁰,⁵¹) to identify sequencing contaminants by comparing samples to Orsay controls (threshold = 0.1). Contigs identified as contaminants were removed. Candidate viral contigs were matched to the National Center for Biotechnology Information (NCBI) NT database (downloaded February 2018) using megablast; contigs with high percentage identity and query coverage to the human genome were removed (percentage identity and query coverage both ≥90%; either percentage identity or query coverage ≥95%). Two papillomavirus contigs that were traced to contamination during the sequencing run were removed. After decontamination, 40,210 viral contigs remained (median length = 1,362 bp; IQR = 1,054–3,000 bp), of which 692 were circular.

Taxonomy for the viral contigs was assigned based on the taxonomy of the viruses in the Gut Phage and Gut Virome databases. Contig ORFs were predicted using Prodigal v2.6.3 (ref. ⁵²). Phage lifestyles were predicted using PHACTS v0.3 (ref. ⁵³). Lifestyle predictions were only performed for contigs with at least five ORFs and which were not classified as euukaryotic viruses.

Ecological analysis. Alpha (Shannon index) and beta (Sorensen dissimilarity) diversities were calculated with the vegan package v2.5-6 in R⁵⁴, using RPK counts and contig presence/absence. Hellinger distance was calculated with the adespatial package⁵⁵ v0.3.14 in R using log(Fisher-transformed RPK counts). PCA was conducted with this package v1.28.0 in R using weighted Bray–Curtis distance on RPK counts. Samples were binned by week (postmenstrual age, PMA) for representation of virus family relative abundance. Family relative abundance and Bray–Curtis dissimilarity were plotted using prism v9.1.0 (GraphPad Software). Alpha diversity, richness and community profiles were generated with ggplo2 v3.3.3–3.3.5 in R. Locally estimated scatterplot smoothing (LOESS) regression was used to obtain trend lines and 95% confidence bands. Matched case and control samples were used to compare between-case and between-control Sorensen dissimilarity as a function of time preceding NEC onset (sliding windows with a window size of 7 d, with 2-d steps between windows). We ordered LEfSe to identify a LEfSe with a difference of 1 between NEC and case onset. A prevalence threshold of 10% was set for contigs being tested. If LEfSe was selected, that is, contigs were only included in the LEfSe analysis if they were found in at
least 10% of the samples being analysed. Prevalence and average abundance of selected contigs were compared in cases and controls in 5-d intervals preceding NEC onset, up to 25 d before NEC. All case and control samples in the 25 d before NEC were included. Prevalence was calculated as the analysis of microbiome composition dropped because of insufficient reads, resulting in 79 samples being used in the SRA (for all 40 case samples and the 41 control samples collected in the 25 d preceding case NEC onset). Quality trimming was performed using bbduk (BBDuk37.64) followed by denoising using the dad2 plugin in QIIME 2 v.2019.1 (ref. 12). Samples were rarefied to a depth of 2,500 reads. Two samples were dropped because of insufficient reads, resulting in 79 samples being used in the final analysis. Alpha (Shannon index) and beta (weighted and unweighted UniFrac distance) diversity were calculated in QIIME 2. Differentially abundant bacteria in cases and controls were identified using the analysis of microbiome composition (ANCOM-II) in R. Correlations between contig and bacterial abundance were determined by linear mixed modelling as implemented in MaAsLin 2 (ref. 13). A prevalence threshold of 10% was set for contigs and bacterial genera being analysed with MaAsLin 2, that is, contigs and bacterial genera had to be present in at least 10% of the samples being analysed to be included. Correlations were considered significant if they had a P < 0.05 and q < 0.25.

Statistical analyses. Metadata variables. Statistical significance for continuous and categorical variables was assessed using the Mann–Whitney U-test or Fisher’s exact test, respectively.

Virome analysis. Statistical significance of changes in alpha diversity and richness over time were assessed by linear mixed modelling with postmenstrual age as a fixed effect and infant ID as a random effect. Statistical significance for Bray–Curtis dissimilarity (within-individual dissimilarity compared to between-individual dissimilarity) was determined using the Mann–Whitney U-test. Statistical significance for PCoA was determined using PERMANOVA, with FMA as a continuous variable and case or control status as a categorical variable. To analyse the time preceding NEC, differences in case or control Sorenson dissimilarity across multiple time windows were assessed using a Kruskal–Wallis test with Dunn’s multiple comparisons. Differences between case and control dissimilarity at each specific window were assessed by the Mann–Whitney U-test. Differences in prevalence and abundance of selected contigs in case samples across different time points were compared using a Friedman test with Dunn’s multiple comparisons.

Bacterial analysis. Statistical significance of differences in case or control beta diversity across multiple time windows was assessed using a Kruskal–Wallis test with Dunn’s multiple comparisons. Friedman tests with Dunn’s multiple comparisons, Fisher’s exact tests and chi-squared tests were performed in Prism. PERMANOVA was performed using the vega package in R. Mixed linear modelling for virome alpha diversity and richness was performed using the nlme package v.3.1-149 in R (ref. 14). Where appropriate, we chose non-parametric tests that do not assume data to be normally distributed (for example, Mann–Whitney U-test, Kruskal–Wallis test). P ≤ 0.05 was considered statistically significant. NS, P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001, ****P ≤ 0.0001.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The sequencing data have been deposited with the NCBI SRA (BioProject ID: PRJNA682649). Readings mapping to the human genome were removed from the submitted sequence data.

Code availability

The code used for the analyses in this study is available at https://github.com/ASU-Lim-Lab/NEC-Virome.

Received: 7 January 2021; Accepted: 2 March 2022; Published online: 21 April 2022

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Acknowledgements
We thank the study participants and their families. This research was funded by National Institutes of Health grant nos. R01HD092311 (E.S.L.), R00DK107923 (E.S.L.), R01DK122029 (L.R.H.), R01HD092414 (P.I.T.), UH3AI1083265 (P.I.T.) and P30DK052574 (Biobank Core), as well as funding from the Children’s Discovery Institute of Washington University.

Author contributions
P.I.T., L.R.H., E.S.L., B.B.W. conceptualized the study. E.A.K. curated the data. E.A.K. carried out the formal analysis. P.I.T., B.B.W., J.A.H., L.A.L. and I.M.N. recruited the study participants and managed the metadata. E.A.K., C.R. and C.H-M. carried out the investigation. B.B.W. and P.I.T. managed the resources. E.S.L. and L.R.H. supervised the study. E.A.K. and E.S.L. wrote the original draft. All authors contributed to, reviewed and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41564-022-01096-x.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41564-022-01096-x.
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Peer review information *Nature Microbiology* thanks Corinne Maurice and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.
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Extended Data Fig. 1 | Sample timeline. a: All samples by postmenstrual age at sample collection. b: Samples used for analysis of time relative to NEC onset (Figs. 3 and 4). Matched case and control infants are grouped together. Paired samples used for beta diversity analysis are indicated in red and blue (Figs. 3a and 4b).
Extended Data Fig. 2 | Relative abundance by week of life, Sorensen dissimilarity and Hellinger distance within and between infants. a: Virus family relative abundance in control samples from Fig. 1a, grouped by week of life rather than postmenstrual age. Multiple pie charts in the same square indicate multiple samples for an infant in the same week. b: Sorensen dissimilarity and Hellinger distance within and between control infants, n = 14 infants. Center line = median; box limits = 25th and 75th percentiles; whiskers = 2.5 and 97.5 percentiles. Statistical significance assessed by two-sided Mann-Whitney test, p < 0.0001. c: Virus family relative abundance in case samples from Fig. 2a, grouped by week of life rather than PMA. Multiple pie charts in the same square indicate multiple samples for an infant in the same week. The same colors are used to indicate virus families in A and C. d: Sorensen dissimilarity and Hellinger distance within and between case infants, n = 9 infants. Center line = median; box limits = 25th and 75th percentiles; whiskers = 2.5 and 97.5 percentiles. Statistical significance assessed by two-sided Mann-Whitney test, p = 0.04 and p = 0.002, respectively.
Extended Data Fig. 3 | Control infant discriminant contigs and phage lifestyle predictions. 

**a**: Prevalence and abundance of NEC-associated contigs in control infants, in 5-day intervals prior to respective case NEC onset. Statistical significance assessed by Friedman test with Dunn’s multiple comparisons (p-values in Supplementary Table 14). 

**b**: Linear discriminant analysis effect size (LEfSe) of contigs in control samples. Purple indicates features associated with 0–10 days prior to case NEC onset. Green indicates features associated with 10–46 days prior to case NEC onset.

**c**: Prevalence and abundance of control early- and late-associated contigs in control infants (left) and case infants (right), in 5-day intervals prior to case NEC onset. Statistical significance assessed by Friedman test with Dunn’s multiple comparisons (p-values in Supplementary Tables 17,18).

**d**: For each control discriminant contig, linear regression was performed on prevalence and average abundance values in 5-day intervals prior to NEC in cases (red) and controls (blue). Regression coefficients for abundance and prevalence were plotted on the x- and y-axes, respectively.

**E**: Lifestyle predictions (temperate or lytic) for case and control late-associated viral contigs.
Extended Data Fig. 4 | ANCOM-II, unweighted bacterial beta diversity, and bacterial-viral interactions in controls. a: ANCOM-II comparing bacterial family abundance in case and control samples, unadjusted (left) and adjusted (right) for repeated sampling. W statistic is the number of significant comparisons between a taxon and the other taxa being tested. CLR mean difference corresponds to effect size. Points in red represent taxa that are differentially abundant when W statistic threshold is set at the 70th percentile. Points in orange represent taxa that are differentially abundant when W statistic threshold is set at the 60th percentile. Points in blue represent taxa that are not differentially abundant. b: ANCOM-II comparing bacterial class abundance in case and control samples, unadjusted (left) and adjusted (right) for repeated sampling. W statistic is the number of significant comparisons between a taxon and the other taxa being tested. CLR mean difference corresponds to effect size. Points in red represent taxa that are differentially abundant when W statistic threshold is set at the 70th percentile. Points in blue represent taxa that are not differentially abundant. c: Unweighted UniFrac distance between case samples (red) and between matched control samples (blue) in sliding windows prior to NEC onset (7-day windows with 2-day steps). Medians with 95% confidence intervals are shown. Statistical significance at each window assessed by two-sided Mann-Whitney U test. d: Significant correlations between control discriminant contigs (late- and early-associated) and bacterial genera in control infants (left) and case infants (right). Dendrograms were ordered based on row and column means.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  REDCap software (7.3.5) was used to collect and manage metadata associated with study participants.

Data analysis  Code used for the analyses in this study is available at https://github.com/ASU-Lim-Lab/NEC-Virome.

We used the following software and packages: BBTools (37.64); BLAST+ (2.7.1); R (3.6.1); (decontam (1.4.0); vegan (2.5-6); phylseq (1.28.0); ggplot2 (3.1.1); ggplot2 (3.3.3 -- 3.3.5); GraphPad Prism (9.1.0); SPADEs (3.14.0); CD-HT EST (4.8.1); minisum2; BWA (0.7.17-r1188); VirSorter (1.0.5); leise (1.0.0); MaAsLine (1.0.0); SAMtools (1.7); bowtie2 (2.3.5); adespatial (0.3-14); taxonomizr (0.5.3); rime (3.1-149); QiIME2 (2019.1); Prodigal (2.6.3); PHACSI (0.3); Microsoft Excel (16.45); tidyverse (1.3.1); compositions (2.0-4); ANCOM v2.1

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Sequencing data for this study has been deposited to the NCBI Sequence Read Archive under accession number PRJNA682649. Reads mapping to the human genome have been removed from the submitted sequence data.

The Gut Phage Database (Camarillo-Guerrero et al., 2021) used for virome analysis is available at http://ftp.ebi.ac.uk/pub/databases/metagenomics-genome_sets/gut_phage_database/
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Life sciences study design

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Sample size

We selected available stool samples from the study participants, collected during the first three months of life. In total, 138 samples were sequenced. No statistical analysis was used to predetermine sample size.

Data exclusions

We excluded two samples from the virome analysis and two samples from the bacterial microbiome analysis because of insufficient sequencing reads. One sample was excluded from analysis because the infant’s age at sample collection was substantially older than any other sample. These criteria were not predetermined.

Replication

We repeated the analysis with multiple databases (initial analysis with NCBI RefSeq; final analysis with Gut Phage Database and Gut Virome Database). We observed a similar virome convergence signature with both analyses.

Randomization

Samples were randomized during sample processing and NGS using a random number generator. Allocation to experimental groups (cases and controls) was based on whether infants did or did not develop NEC, and therefore randomization was not applicable.

Blinding

Blinding was used during sample processing and NGS. Blinding was not applicable for the subsequent analysis because metadata such as infant ID, age, and case/control status were necessary for statistical analysis.

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Population characteristics

The participants in this study were preterm infants who developed necrotizing enterocolitis (n=9; 6 male and 3 female) and preterm control infants who did not develop NEC [n=14; 5 male and 9 female]. The case and control infants were matched based on gestational age at birth (+/- 2 weeks) and birthweight (+/- 200 grams). All infants were born at <27 weeks gestation. Samples were collected longitudinally during the first 3 months of life. Additional cohort characteristics recorded include delivery route, sex, Apgar scores, exposure to human milk during the sampling period and antibiotic exposure during the sampling period.

Recruitment

All premature infants admitted to the neonatal intensive care unit at St. Louis Children’s Hospital were considered for study eligibility, and infants who met the eligibility criteria were enrolled if their family provided informed consent. Infants were eligible if they weighed 1500 grams or less at birth and were expected to survive past the first week of life. Infants with congenital heart disease or spontaneous intestinal perforation without radiographic evidence of NEC were excluded.

Ethics oversight

This research was approved by the Human Research Protection Office, Washington University in St. Louis School of Medicine and the Arizona State University Institutional Review Board.
Note that full information on the approval of the study protocol must also be provided in the manuscript.