Title

Intestinal Virome in Patients With Alcoholic Hepatitis

Journal

Hepatology, 72(6)

ISSN

0270-9139

Authors

Jiang, Lu
Lang, Sonja
Duan, Yi
et al.

Publication Date

2020-12-01

DOI

10.1002/hep.31459

Peer reviewed

HHS Public Access

Author manuscript

Hepatology. Author manuscript; available in PMC 2021 May 28.

Published in final edited form as:

Hepatology. 2020 December ; 72(6): 2182–2196. doi:10.1002/hep.31459.

Intestinal virome in patients with alcoholic hepatitis

Lu Jiang[#1,2], **Sonja Lang**[#1], **Yi Duan**[1,2], **Xinlian Zhang**[3], **Bei Gao**[1], **Jessica Chopyk**[4], **Leila K. Schwanemann**[4], **Meritxell Ventura-Cots**[5], **Ramon Bataller**[5], **Francisco Bosques-Padilla**[6], **Elizabeth C. Verna**[7], **Juan G. Abraldes**[8], **Robert S. Brown Jr**[9], **Victor Vargas**[10,11], **Jose Altamirano**[10], **Juan Caballería**[11,12], **Debbie L. Shawcross**[13], **Samuel B. Ho**[1,2], **Alexandre Louvet**[14], **Michael R. Lucey**[15], **Philippe Mathurin**[14], **Guadalupe Garcia-Tsao**[16], **Tatiana Kisseleva**[1], **David A. Brenner**[1], **Xin M. Tu**[3], **Peter Stärkel**[17], **David Pride**[1,4,18], **Derrick E. Fouts**[19], **Bernd Schnabl**[1,2,18]

[1]Department of Medicine, University of California San Diego, La Jolla, CA, USA

[2]Department of Medicine, VA San Diego Healthcare System, San Diego, CA, USA

[3]Division of Biostatistics and Bioinformatics, Department of Family Medicine and Public Health, University of California San Diego, La Jolla, CA, USA

[4]Department of Pathology, University of California San Diego, La Jolla, CA, USA

[5]Division of Gastroenterology, Hepatology and Nutrition, Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh Liver Research Center, Pittsburgh, PA, USA

[6]Hospital Universitario, Departamento de Gastroenterología, Universidad Autonoma de Nuevo Leon, Monterrey, México

[7]Division of Digestive and Liver Diseases, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY, USA

[8]Division of Gastroenterology (Liver Unit). Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

[9]Division of Gastroenterology and Hepatology, Weill Cornell Medical College, New York, NY, USA

[10]Liver Unit, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain

[11]Centro de Investigación en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Barcelona, Spain

[12]Liver Unit, Hospital Clinic, Barcelona, Spain

Correspondence: Bernd Schnabl, M.D., Department of Medicine, University of California San Diego, MC0063, 9500 Gilman Drive, La Jolla, CA 92093, Phone 858-822-5311, Fax 858-822-5370, beschnabl@ucsd.edu.

Author contributions: L.J. was responsible for acquisition and interpretation of data, and drafting of the manuscript; S. L. was responsible for analysis and interpretation of the data and editing the manuscript; Y.D. provided assistance with acquisition and interpretation of data, and editing of the manuscript; B.G. provided assistance with data interpretation; X.Z. and X.M.T. provided assistance with statistical analysis; J.P., L.K.S., and D.P. provided assistance with data acquisition; M.V.-C., F.B.-P., E.C.V., J.G.A., R.S.B. Jr, V.V., J.A., J.C., D.L.S., S.B.H., A.L., M.R.L., P.M., G.G.-T., R.B. and P.S. were responsible for collection of human samples; D.E.F. was responsible for bioinformatical analysis and interpretation of data; B.S. was responsible for study concept and design, interpretation of data, editing the manuscript, and study supervision.

[13]Institute of Liver Studies, King's College London School of Medicine at King's College Hospital, King's College Hospital, London, UK

[14]Service des Maladies de L'appareil Digestif et Unité INSERM, Hôpital Huriez, Lille, France

[15]Division of Gastroenterology and Hepatology, Department of Medicine, University of Wisconsin School of Medicine and Public Health, WI, USA

[16]Section of Digestive Diseases, Yale University School of Medicine, New Haven, CT, USA, and Section of Digestive Diseases, VA-CT Healthcare System, West Haven, CT, USA

[17]St. Luc University Hospital, Université Catholique de Louvain, Brussels, Belgium

[18]Center for Innovative Phage Applications and Therapeutics, University of California San Diego, La Jolla, CA, USA

[19]J. Craig Venter Institute, Rockville, MD, USA

[#] These authors contributed equally to this work.

Abstract

Alcoholic hepatitis is a severe manifestation of alcohol-associated liver disease with high mortality. Although gut bacteria and fungi modulate disease severity, little is known about the effects of the viral microbiome (virome) in patients with alcohol-associated liver disease. We extracted virus-like particles from 89 patients with alcoholic hepatitis who were enrolled in a multicenter observational study, 36 with alcohol use disorder, and 17 individuals without alcohol use disorder (controls). Virus-like particles from fecal samples were fractionated using differential filtration techniques and metagenomic sequencing was performed to characterize intestinal viromes. We observed an increased viral diversity in fecal samples from patients with alcohol-associated liver disease, with the most significant changes in samples from patients with alcoholic hepatitis. *Escherichia-, Enterobacteria-,* and *Enterococcus* phages were overrepresented in fecal samples from patients with alcoholic hepatitis, along with significant increases in mammalian viruses such as *Parvoviridae* and *Herpesviridae*. Antibiotic treatment was associated with higher viral diversity. Specific viral taxa, such as *Staphylococcus* phages and *Herpesviridae*, were associated with increased disease severity, indicated by a higher median model for end-stage liver disease (MELD) score, and associated with increased 90-day mortality. In conclusion, intestinal viral taxa are altered in fecal samples from patients with alcoholic hepatitis and associated with disease severity and mortality. Our study, for the first time, described an intestinal virome signature associated with alcoholic hepatitis.

Keywords

bacteriophages; microbiome; microbiota; alcohol-associated liver disease

INTRODUCTION

Alcohol-associated liver disease is the main cause of liver-related mortality worldwide, and targeted therapies are urgently needed (1). Alcoholic hepatitis is a severe form of this disease, with 90-day mortality as high as 50% (2). Treatment options are limited for patients

with alcoholic hepatitis—corticosteroids are only marginally effective (3). Early liver transplantation, a curative treatment, is available to only select patients and is not accepted at most transplant centers (2–4).

Alcohol-associated liver disease is associated with changes in the intestinal microbiota (5). Susceptibility to ethanol-induced liver disease is transmissible via the gut microbiota in mice; transplantation of feces from patients with alcoholic hepatitis to mice exacerbates ethanol-induced liver disease (6). In a pilot study, transplantation of stool from a healthy donor to patients with alcoholic hepatitis increased their survival times (7). These findings indicate that the gut microbiota can contribute to the severity of alcoholic hepatitis. Patients with alcoholic hepatitis have compositional changes in gut bacteria and fungi and decreased bacterial and fungal diversity compared with alcohol use disorder patients and non-alcoholic controls (6–10).

Although most studies of the roles of the gut microbiome in alcohol-associated liver disease have focused on bacteria and fungi, the enteric virome has not been studied in any liver disease. The human intestinal virome is specific to each individual and contains mostly bacteriophages (phages) (11). Phages are viruses that infect bacteria. Some phages are highly specific to one bacterial strain, whereas others have broader host ranges (12). Upon infection, virulent phages cause cell lysis (lytic cycle) (13) whereas temperate phages either lyse the host cell or keep it alive and reproduce normally (lysogenic cycle) (14). It is estimated that in many environments, phages outnumber their prokaryotic hosts by a factor of 10 (15). The human gastrointestinal tract contains 10^{15} phages, among which the order *Caudovirales* (with the families *Siphoviridae, Myoviridae, and Podoviridae* in particular) is overwhelmingly predominant (16). Other less-abundant components of the virome include eukaryotic viruses (animal and plant viruses), archaeal viruses, and viral-derived elements that can alter gene expression in cells (17, 18). Mammalian viruses are associated with human diseases that range from mild to fatal (19).

Alterations in the gut virome composition have been reported in patients with inflammatory bowel disease, yet different conclusions were drawn based on differences in analysis methods (20–23). A database-dependent analysis revealed that patients with inflammatory bowel diseases have an increased overall viral diversity with a significant expansion in *Caudovirales* phage taxa compared with healthy individuals (controls). *Caudovirales* was inversely correlated with the *Bacteroidaceae* family of bacteria in patients with Crohn's disease (20). In contrast, a database-independent analysis method revealed no changes in viral richness and found increased numbers of temperate phage sequences in individuals with Crohn's disease (23). Virome alterations were also observed in patients with colorectal cancer; fecal samples from patients had increased viral diversity and altered interactions between phages and oral bacterial commensals, compared with controls (24). However, unaltered viral diversity and richness were reported in patients with colorectal cancer compared to heathy controls in another study (25). These results indicate the presence of disease-specific viromes in patients and that interactions between bacteria and viruses might associate with disease activity.

To characterize the intestinal virome in patients with alcohol-associated liver disease, we isolated virus-like particles from fecal samples using differential filtration techniques and performed metagenomic sequencing. We aimed to investigate disease-specific virome signatures associated with disease severity and mortality, focusing on patients with alcoholic hepatitis enrolled in a prospective, multicenter study.

MATERIAL AND METHODS

Patient Cohorts

Patient cohorts have been described before (8, 9, 26). In brief, our study comprised a total of 142 subjects: 89 patients with alcoholic hepatitis, 36 patients with alcohol use disorder, and 17 individuals who do not abuse alcohol (controls). Patients with alcoholic hepatitis were hospitalized in 12 centers from 7 regions (France, n=19; Mexico, n=6; Spain, n=8; United Kingdom, n=10; US East, n=13; US Midwest, n=16; US West, n=17) that were part of the InTeam Consortium (ClinicalTrials.gov identifier number: NCT02075918) between June 2014 and May 2018. Lipopolysaccharide-binding protein (LPS-BP) was measured as previously described (9). Patients with alcohol use disorder were recruited from an alcohol treatment program in San Diego, US (n=7) and Brussels, Belgium (n=29). Patients with alcohol use disorder fulfilled the Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV criteria) (27) of alcohol dependence with active alcohol consumption (self-reported > 60 g/day for more than one year). Patients from Brussels, Belgium (n=29) were recruited from an alcohol withdrawal unit, followed a highly standardized and controlled 3-week detoxification and rehabilitation program consisting of first week inpatient detoxification. Fecal samples were collected at the time point of the admission (28). Patients from San Diego (n=7) were recruited in an outpatient setting. Non-alcoholic controls were enrolled in Brussels, Belgium. Patients with alcohol use disorder and non-alcoholic controls (social drinkers who consumed alcohol less than 20 g/day as one inclusion criteria) did not take antibiotics or immunosuppressive medications in the 2 months preceding enrollment. A complete medication and medical history were collected at admission. Bio-specimens, basic demographic data (such as age, gender, weight, and height) were collected and patients underwent a physical examination. Other exclusion criteria were diabetes, inflammatory bowel diseases, liver diseases of any other etiology, and clinically significant cardiovascular, pulmonary, or renal comorbidities. The baseline characteristics of the cohort with alcoholic hepatitis patients are summarized in Table 1 and Table 2. Inclusion criteria for alcoholic hepatitis patients were active alcohol abuse (>50 g/day for men and >40 g/day for women) in the past 3 months; level of aspartate aminotransferase (AST) greater than level of alanine aminotransferase (ALT), and total bilirubin > 3 mg/dl in the past 3 months; liver biopsy and/or clinical picture consistent with alcoholic hepatitis. Exclusion criteria were autoimmune liver disease (ANA > 1/320), chronic viral hepatitis, hepatocellular carcinoma, complete portal vein thrombosis, extra-hepatic terminal disease, pregnancy, and lack of signed informed consent (29). MELD scores were calculated for all patients with available required data. In all patients, the clinical picture was consistent with alcoholic hepatitis and in patients who underwent liver biopsy, histology findings supported the diagnosis of alcoholic hepatitis. Liver biopsies were only collected if indicated, as part of routine clinical care for diagnostic purposes of alcoholic hepatitis. Biospecimens were collected during

patients' admission to the hospital. The protocol was approved by the ethics committee of each participating center, and patients were enrolled after written informed consent obtained.

Bacterial DNA extraction and 16S rRNA sequencing

16S rRNA sequencing of human stool samples was previously described (8). Raw 16S sequence reads are available for download in the NCBI Sequence Read Archive (SRA) associated with Bioproject PRJNA525701.

Virome preparation and metagenomic sequencing

Viromes were prepared using the NetoVIR protocol with minor modifications (30). Briefly, human stool samples were resuspended in phosphate-buffered saline and sequentially filtered using 0.8 μm (PES) filter (Sartorius). Any remaining DNA that was not encapsidated was degraded by treating with a mixture of benzonase (EMD Millipore) and micrococcal nuclease (New England Biolabs) followed by EDTA inactivation of DNases. The remaining supernatant was subjected to lysis and virome DNA and RNA were extracted using the QIAamp Viral RNA mini kit without carrier RNA (Qiagen). Amplification was performed using a modified WTA2 (Complete Transcriptome Amplification Kit) protocol from Sigma Aldrich. Library preparation was performed using an adjusted protocol for the Nextera XT DNA Library Preparation kit from Illumina. The size of amplified viral products was determined using a High Sensitivity DNA Kit on a Bioanalyzer (Agilent Technologies), and concentration was measured by High Sensitivity Double Stranded DNA kit on a Qubit Fluorometer (Thermo Fisher Scientific). The sterile water control contained no detectable DNA, indicating no contamination of exogenous DNA during the analysis. Viral DNA from each sample was pooled into equimolar proportions and sequenced on the Illumina platform at the UCSD IGM Genomics Center.

Virome analysis

We obtained 3,071,815 reads per sample on average. Raw sequence reads were deduplicated using Clumpify (https://sourceforge.net/projects/bbmap/) followed by trimming and filtered for low-quality and contaminating human reads using Kneaddata (31) with the GRCh38_v25 human genome reference. Reads were aligned and assigned taxonomy using the PathSeq pipeline (distributed in GATK v4.1.3.0) (32, 33). Default settings were used, including --min-score-identity and --identity-margin, which were 0.90 and 0.02, respectively. Also, by default, PathSeq will discard alignments if both read pairs do not match the same organism (https://gatk.broadinstitute.org/hc/en-us/articles/360036717051-PathSeqScoreSpark). An inhouse Perl script was made (pathseq2taxsummary.pl) to convert PathSeq concatenated scores.txt files into a MOTHUR (34) style .taxsummary file. Unambiguous read counts for selected viral taxa were imported into R, data were normalized, and richness and diversity were calculated.

Real-time qPCR

To exclude the possibility of viral contamination from the QIAamp Viral RNA mini kit (Qiagen), we extracted fecal DNA from selected alcoholic hepatitis patients using a different DNA extraction kit, the FastDNA SPIN Kit for Soil (MP Biomedicals). Sterile water was

used as negative control for extraction. Primer sequences targeting the highly selective region of EBV, Epstein-Barr nuclear antigen-1 (EBNA-1), were GCC GGT GTG TTC GTA TAT GG (forward) and CAA AAC CTC AGC AAA TAT ATG AG (reverse) (35). Amplification of EBNA-1 gene was determined with Sybr Green (Bio-Rad Laboratories) using ABI StepOnePlus real-time PCR system as described previously (8). qPCR products were confirmed on a 2% agarose gel by 213bp band (36) (Supplementary Figure 7), which was subsequently purified using QIAquick Gel Extraction Kit (Qiagen) and sequenced (Eurofins). Sequences were analyzed by BLAST program (37) and aligned to the EBNA-1 gene at 98% coverage (data not shown).

Statistical analysis

Results are expressed as median and range unless stated otherwise. Two groups were compared using the Student's t-test or Mann-Whitney-Wilcoxon rank-sum test or the Fisher's exact test for categorical variables, as appropriate. Three or more groups were compared using one-way ANOVA with Tukey's post-hoc test or the Kruskal-Wallis test with Dunn's post-hoc test, as appropriate, or the Fisher's exact test for categorical variables, each followed by false discovery rate (FDR) procedures to correct for multiple comparisons. All statistical tests were two-sided. Viral diversity and richness were calculated by including all detected viruses (phages, mammalian viruses and other viruses, including plant/food derived viruses) and using the "phyloseq" package in R. The proportion of phages, mammalian viruses and other viruses, including plant/food derived viruses in between groups, was calculated at the family level. Relative abundances for further analyses were calculated within each virus category (phages vs mammalian viruses) for family, genus, and species level. Only virus families that could be assigned to known viral taxa were included when calculating the relative abundance. Single phages were analyzed at the species level and summarized according to their hosts. For mammalian viruses, we further determined not only the relative abundance but also the proportion of fecal samples positive or negative for a specific mammalian virus. The non-invasive fibrosis-4 index (FIB-4) was calculated, and alcohol use disorder patients were stratified in two groups using published cut-off values (38). We assigned alcoholic hepatitis patients according to MELD scores into tertiles, with cut points of 22.1 and 26.3. LEfSe was performed (39) to determine the features most likely to account for differences between groups. We included relative abundances of phages at family, genus, and species levels and mammalian viruses at family and genus levels. For survival analysis, we performed a multivariate Cox regression, to investigate associations of intestinal viruses with 90-day mortality. Patients were censored at the timepoint they were last seen alive. For the only patient who underwent liver transplantation, the transplantation date was considered as date of death. Viral taxa that were present in at least 10% of the patients were included in a Cox regression, adjusted for antibiotic and steroid treatment, as well as the geographic region where the patient was enrolled. All viral taxa with a P value below 0.05 were included into a multivariate model to calculate a virome-associated risk model associated with 90-day mortality. The maximally selected rank statistic was used to determine the model cutoff value that represents the maximum difference of two groups regarding 90-day survival (40). Kaplan-Meier curves were used to compare 90-day survival between these groups. To investigate associations between the abundance of intestinal bacteria, determined by 16S rRNA gene sequencing, we performed a heat map analysis,

which produced color-coded Spearman correlations. Bacterial diversity and richness were correlated with viral diversity and richness. We further correlated the relative abundances of the 30 most-abundant phages, with the 30 most abundant bacteria, at the genus level. For all analyses, *P* values of 0.05 or less were considered to be statistically significant. Statistical analysis was performed using R statistical software, R version 3.5.1, 2018 the R Foundation for Statistical Computing.

RESULTS

Altered virome composition in patients with alcohol-associated liver disease

To identify virome alterations in patients with alcohol-associated liver disease, we isolated virus-like particles and performed metagenomic sequencing using fecal samples from 89 patients with alcoholic hepatitis, 36 patients with alcohol use disorder, and 17 patients without alcohol use disorders (controls). Most of the study subjects (75%) were male. Compared with patients with alcohol use disorder, patients with alcoholic hepatitis had significantly higher levels of bilirubin and international normalized ratio (INR) and lower levels of albumin, indicating decreased liver synthesis (Table 1). Among patients with alcoholic hepatitis, 25% had an infection, and the median model for end-stage liver disease (MELD) score was 24 (Table 2).

We first explored the proportion of phages, mammalian viruses, and other viruses (including plant/food derived viruses) among the groups. Phages were the most abundant viruses (Figure 1a). Remarkably, fecal samples from patients with alcoholic hepatitis contained 3-fold more mammalian viruses than samples from controls (9% mean relative abundance in alcoholic hepatitis, 5% in alcohol use disorder, and 3% in controls; Figure 1a, $P<0.001$).

We compared the overall viral diversity and richness among groups by calculating the Shannon index, inverse Simpson index, and Chao1 richness. In both groups of patients with alcohol dependence, we observed significant increases in viral diversity and richness compared with controls; the largest changes were in fecal samples from patients with alcoholic hepatitis (Figure 1b). Heatmap analysis, which shows the log10 relative abundance of the 100 most abundant phages, demonstrated a wide spread of different phages in patients with alcoholic hepatitis compared with alcohol use disorder and controls (Figure 1c).

We summarized the most abundant phages at the species level that parasitized the same putative bacterial host species to determine whether there were observable trends in alcoholic hepatitis compared with controls. We observed decreased abundances of *Lactococcus* phages and *Parabacteroides* phages in alcoholic hepatitis patients, whereas abundances of some Proteobacteria and Firmicutes phages, such as *Lactobacillus* phages, *Escherichia* phages, *Enterobacteria* phages, and *Enterococcus* phages, were increased (Supplementary Figure 1a).

We performed linear discriminant effect size analysis (LEfSe) (39) to identify features most likely to account for differences among groups. Our analysis included relative abundance of phages at family, genus, and species levels, and mammalian viruses at family and genus levels. *Myoviridae*, *Lactobacillus* phages, *Streptococcus* phages, *Podoviridae*, *Geobacillus*

phages, *Escherichia* phages, and *Herpesviridae* were the most discriminating factors for alcoholic hepatitis. Alcohol use disorder was mainly characterized by increased *Parvoviridae* and *Lactococcus* phages, whereas controls were mainly characterized by *Sk1virus* and *Siphoviridae* (Supplementary Figure 1b, Supplementary Table 1).

We further characterized the taxonomic composition of mammalian viruses at the family level (Figure 1d). The *Parvoviridae* family was detected in all cohorts, but *Parvoviridae* were detected in a significantly higher proportion of patients with alcoholic hepatitis and alcohol use disorder than controls (P=0.0001). The family of *Herpesviridae* was only present in fecal samples from patients with alcoholic hepatitis (16% of patients were positive); *Herpesviridae* was not detected in any patients with alcohol use disorder or controls (Figure 1d). Within the *Herpesviridae* family, 80% of sequence reads could be assigned to human gammaherpesvirus 4 (Epstein-Barr virus, EBV), 10% to human betaherpesvirus 7, 8% to human betaherpesvirus 6B, and 2% remained unassigned. These results indicate that alcohol-associated liver disease is accompanied by alterations in the diversity and composition of intestinal viruses, with the largest changes in patients with alcoholic hepatitis.

Disease severity associates with alterations in the gut virome in patients with alcohol use disorder and alcoholic hepatitis

Alcoholic hepatitis is associated with high mortality. MELD scores, calculated based on levels of bilirubin, creatinine, and INR, are used to determine disease severity and patient prognosis for patients with alcoholic hepatitis (41). To investigate if alterations in the intestinal virome are associated with disease severity, we grouped patients with alcoholic hepatitis into tertiles based on MELD scores; tertile cut points were 22.1 and 26.3. Each group included 29 patients with alcoholic hepatitis. Overall, we observed a 14% relative abundance of mammalian viruses in the high-score tertile compared with 6% in the low- and intermediate-score tertiles (P=0.31, Figure 2a).

Analysis of alpha diversity revealed a trend towards increased viral diversity in patients with increased risk of death (Figure 2b). When we compared the phages grouped by their bacterial host, we observed an increase of *Staphylococcus* phage abundance in the high-score tertile compared with the lower-score tertiles among the groups (Figure 2c). *T4virus*, *Staphylococcus* phages, *Phietavirus,* and *Citrobacter* phages were the most discriminating factors for high MELD-score tertile group compared with the lower MELD-score tertiles (Figure 2d, Supplementary Table 1).

When we analyzed mammalian viruses at the family level in alcoholic hepatitis, we found *Parvoviridae* to be the most prevalent virus family, followed by *Circociridae* and *Herpesviridae*. *Parvoviridae* and *Herpesviridae* were more often present in fecal samples from patients with alcoholic hepatitis in the highest MELD-score tertile (Figure 2e). Patients with alcoholic hepatitis and *Herpesviridae* in fecal samples had a significantly higher MELD score than patients with alcoholic hepatitis without *Herpesviridae* (Figure 2f, P=0.041). Further, alcoholic hepatitis patients positive for *Herpesviridae* in fecal samples had significantly higher white blood cell counts, even after adjustment for steroid treatment (P=0.046).

To investigate differences in the virome composition among patients with alcohol use disorder with different disease stages, we stratified patients into two groups according to the noninvasive FIB-4 index. Patients with high FIB-4 (n=5) showed a significantly increased alpha diversity compared with the low FIB-4 group (n=27). We observed increased abundances of *Lactobacillus* and *Streptococcus* phages in the high FIB-4 group whereas *Lactococcus* phages were decreased (Supplementary Figure 2).

Overall, these data indicate that compositional alterations in the intestinal virome are more pronounced in patients with alcohol use disorder and alcoholic hepatitis with a more severe disease stage.

Clinical features in alcoholic hepatitis are associated with compositional changes in the virome

Next, we investigated associations between the gut virome and the presence of cirrhosis (Supplementary Figure 3), infections (Supplementary Figure 4), and antibiotic treatment (Supplementary Figure 5). Whereas fecal samples from patients with alcoholic hepatitis and cirrhosis (n=35) did not differ in viral diversity or richness from patients without cirrhosis (Supplementary Figure 3b), fecal samples from patients with cirrhosis had more *Klebsiella*-, *Bacteroides*-, and *Parabacteroides* phages (Supplementary Figure 3c). Some viral taxa, identified by LEfSe analysis, were associated with the absence of cirrhosis (n=13) (Supplementary Figure 3d, Supplementary Table 1).

Fecal samples from patients with alcoholic hepatitis and infections (n=19) contained 14% of mammalian viruses, compared to 8% in patients without infections (n=57) (Supplementary Figure 4a and 4e). In the LEfSe analysis, Proteobacteria phages (e.g. *Salmonella* phages and *Enterobacter* phages) and Firmicutes phages (e.g. *Lactococcus* phages, *Staphylococcus* phages, and *Enterococcus* phages) significantly discriminated patients with alcoholic hepatitis with infections vs without infections (Supplementary Figure 4d and Supplementary Table 1).

Fecal samples from patients with alcoholic hepatitis treated with antibiotics (n=40), including prophylactic treatment, had a significant increase in viral diversity compared with samples from untreated patients (Supplementary Figure 5b; Shannon index, P=0.035, inverse Simpson index, P=0.037). Several viral taxa differed between the two groups (Supplementary Figure 5c–e and Supplementary Table 1).

To investigate associations with endotoxemia, we used LPS-BP as a surrogate marker for circulating endotoxin levels. We did not find significant differences in the proportion of phages/eukaryotic viruses among alcoholic hepatitis patients with high or low LPS-BP (based on the median LPS-BP value in the cohort) and no associations between the viral diversity or richness and LPS-BP (Supplementary Figure 6).

Virome alterations associate with mortality in patients with alcoholic hepatitis

After describing compositional characteristics in fecal viromes of patients with alcoholic hepatitis of different stages, we performed multivariate Cox regression analyses to investigate whether intestinal viral taxa are associated with 90-day mortality. Viral taxa (at

the family, genus, and species level) that were present in at least 10% of fecal samples from patients with alcoholic hepatitis were included in a Cox regression analysis, adjusted for antibiotic and steroid treatment as well as the geographic region where the patients were enrolled. Eleven viral taxa were associated with 90-day survival, independently of antibiotics, steroids, and geographic origin (Figure 3a). *Herpesviridae* was the only mammalian virus family among these 11 taxa. Based on the identified taxa, we calculated a virome-associated risk model by including these 11 taxa into a multivariate Cox regression model (see Material and Methods).

We determined a cut point using maximally selected rank statistics (40). We found that patients with alcoholic hepatitis with a high virome-associated risk score (n=12) had significantly greater risk of death within 90 days compared to patients with low virome-associated risk score (n=70) (hazard ratio, 35.8; 95% CI, 10.8–119; $P<0.001$) (Figure 3b). This association remained significant even after adjustment for levels of creatinine, bilirubin and INR (components of the MELD score), platelet counts (indicator of advanced fibrosis and cirrhosis), antibiotics or steroid use, and geographic origin of the patients (hazard ratio, 125.5; 95% CI, 31.1–506; $P<0.001$).

Correlation between phages and bacteria in fecal samples from patients with alcoholic hepatitis

To characterize the bacterial communities in fecal samples from patients with alcoholic hepatitis, we previously performed bacterial 16S rRNA sequencing in this cohort. We observed a significant decrease in bacterial diversity in patients with alcoholic hepatitis compared with alcohol use disorder and controls (8). To determine associations between the viral diversity and richness and bacterial diversity and richness in alcoholic hepatitis, we performed a heatmap analysis, representing color-coded Spearman correlations between those indices. Overall, a higher viral diversity correlated with a lower bacterial diversity (Figure 4a).

We examined relationships between the 30 most-abundant phages after combining individual phages by their hosts and the 30 most-abundant bacterial genera by calculating Spearman correlations (Figure 4b). We found positive correlations between a variety of phages and their hosts, such as *Lactobacillus* and *Lactobacillus* phages, *Staphylococcus* and *Staphylococcus* phages, *Pseudomonas* and *Pseudomonas* phages, and *Clostridium_XI* and *Clostridium* phages. In contrast, we found inverse correlations between *Rhodoferax* phages and *Bacteroides*, *Faecalibacterium*, *Clostridium sensu stricto, Clostridium XlVa, Parabacteroides,* and *Blautia* (Figure 4b).

DISCUSSION

We studied fecal viromes of patients with alcohol-associated liver disease. Compared with fecal samples from patients with alcohol use disorder and controls, samples from patients with alcoholic hepatitis had increased intestinal viral diversity along with decreased bacterial diversity (8). Beside alterations in the abundance of several phages, gut viromes of patients with alcoholic hepatitis were characterized by increases in mammalian viruses that were

found in a few samples from only controls. Moreover, we identified several viral taxa associated with disease severity and mortality in alcoholic hepatitis patients.

Using metagenomic sequencing, we identified enteric eukaryotic viruses that might contribute to pathogenesis of alcoholic hepatitis. *Herpesviridae* was detected in fecal samples from only alcoholic hepatitis patients. It is unclear which factors are responsible for detectable levels of mammalian viruses in stool samples; immune dysfunction, medication use, or other factors associated with alcoholic hepatitis might have caused increased carriage of eukaryotic viruses. The relative abundance of *Herpesviridae* in alcoholic hepatitis patients was associated with 90-day mortality after adjustment for antibiotic and steroid use. Previous studies have associated the seroprevalence of human herpesvirus 6 (42) and human herpesvirus 8 (43, 44) with severity of chronic liver diseases—in patients with alcohol use disorder, in particular.

In our study, most detected *Herpesviriadae* reads in fecal samples could be assigned to EBV. EBV is a member of the *Gammaherpesvirinae,* and hepatitis is a common feature of EBV infection, although the pathogenesis of EBV-associated liver injury is poorly understood (45). EBV has been identified as potential trigger for autoimmune hepatitis (46) and post-transplant lymphoproliferative disease in transplant recipients (47, 48). In a recent retrospective study of 97 patients with liver cirrhosis, blood samples from 37% were positive for EBV DNA. These patients had lower level of albumin and a higher mean Child-Pugh score, indicating more advanced liver disease. Most importantly, the EBV-positive patients had a higher rate of acute-on-chronic liver failure than the EBV-negative patients (49). In a separate study, fecal samples from 43.9% of immunocompromised patients had detectable EBV DNA as compared with 25% of immunocompetent patients (50). Since 90–95% of adults are seropositive for EBV-IgG, the presence of EBV DNA in fecal samples in our alcoholic hepatitis cohort might be due to their immune dysfunction and increased susceptibility to EBV reactivation (51). These findings, together, raise the interesting question of whether EBV-reactivation might trigger or contribute to development of hepatitis in alcoholic patients.

Our observation of increased viral diversity in alcohol-associated disease is consistent with findings from some studies of patients with inflammatory bowel diseases or colorectal cancer (20, 24) although results among studies are in part conflicting (23, 25). Although viral diversity in alcoholic hepatitis patients correlated inversely with bacterial diversity, we observed positive correlations between many phages and their hosts, consistent with findings from other studies (21, 24). This observation can be explained by models of bacteria–phage dynamics (52, 53). The positive correlation could also be due to the expansion of phages integrated in the bacterial genomes or new viruses that enter the community. Further, antibiotic treatment was associated with higher viral diversity.

Several studies have demonstrated that patients with alcohol-associated liver disease have a reduced bacterial diversity and compositional changes in the gut bacterial microbiota (6, 8, 54). There are various potential mechanisms of how alterations in the gut bacterial microbiota can modulate disease in these patients. Gut barrier dysfunction can lead to the translocation of microbial products to the liver where they can induce an inflammatory

cascade. In addition, intestinal bacteria deconjugate bile acids and convert primary bile acids into secondary bile acids. Primary and secondary bile acids bind to host receptors and modulate metabolic functions. Certain intestinal bacteria produce short-chain fatty acids, a reduced abundance of these bacteria might mediate colonic inflammation and gut barrier dysfunction (55). Further, specific virulence factors expressed by bacteria, could induce direct liver damage (8). In this interplay, the gut virome might play a role by either affecting the host directly through causing host immune responses or as a source of mammalian viruses, or, it could mediate disease by influencing the bacterial microbiota. It is conceivable that depletion of specific phages results in a quantitative increase of specific bacterial pathobionts that modulate disease, while increased phage numbers might deplete specific beneficial bacteria. On the other hand, alterations in the gut virome might also mirror the taxonomic composition of the bacterial microbiota or could be primarily driven by the host disease stage. Longitudinal studies in humans are needed to further characterize the interaction between bacteria and phages in patients with alcohol-associated liver disease and preclinical studies are required to determine the contribution of phages to disease progression. Increasing our understanding of correlations between intestinal phages and other host microbes might eventually lead to new treatment strategies for alcoholic hepatitis.

The strength of our study is the inclusion of patients from a multicenter study including Europe, the United States (US), and Mexico, with different environmental and dietary backgrounds. Although we acquired more than 3 million reads per sample, only a small proportion of reads could be assigned to known viral taxa. Therefore, the identity of a substantial fraction of intestinal viruses remains unknown. For phages, the limitation was the lack of depth in the available database, so we were only able to assign sequences to the closely related host genera. As the database expands, more sequences will be assigned to either phages or eukaryotic viruses.

In summary, this is the first characterization of intestinal viromes of patients with liver disease. We report a large increase in the viral diversity of fecal samples from patients with alcoholic hepatitis and identified specific viral taxa associated with disease severity and 90-day mortality. Although this study provides novel insights into associations between the gut microbiome and alcohol-associated liver disease, our findings require validation in an independent cohort of patients with alcoholic hepatitis. Longitudinal studies will provide a better understanding of phage-bacteria interactions and variations in the gut virome associated with disease activity. Furthermore, studies are required to identify mechanisms by which specific viral taxa might increase liver disease severity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

S.L. was supported by a DFG fellowship (LA 4286/1-1). This study was supported in part by a Biocodex Microbiota Foundation Grant, NIH grants R01 AA24726, R01AA020703, U01 AA026939, by Award Number BX004594 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development (to B.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999.

Conflicts of interest:

B.S. has been consulting for Ferring Research Institute, Intercept Pharmaceuticals, HOST Therabiomics and Patara Pharmaceuticals. B.S.'s institution UC San Diego has received grant support from BiomX, NGM Biopharmaceuticals, CymaBay Therapeutics, Synlogic Operating Company and Axial Biotherapeutics.

Abbreviations:

AH	alcoholic hepatitis
AUD	alcohol use disorder
ALT	alanine aminotransferase
AST	aspartate aminotransferase
HR	hazard ratios
CI	confidence interval
MELD	Model for End-stage Liver Disease
INR	international normalized ratio
FDR	false discovery rate
LEfSe	linear discriminant effect size analysis
LDA	linear discriminant analysis
LPS-BP	Lipopolisaccharide-binding protein
SRA	Sequence Read Archive
EBV	Epstein-Barr virus
EBNA-1	Epstein-Barr nuclear antigen-1
BMI	body mass index
GGT	gamma-glutamyl transferase
FIB-4	fibrosis-4 index

REFERENCES

1. Blachier M, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. The burden of liver disease in Europe: a review of available epidemiological data. J Hepatol 2013;58:593–608. [PubMed: 23419824]
2. Lucey MR, Mathurin P, Morgan TR. Alcoholic hepatitis. N Engl J Med 2009;360:2758–2769. [PubMed: 19553649]
3. Thursz MR, Richardson P, Allison M, Austin A, Bowers M, Day CP, Downs N, et al. Prednisolone or pentoxifylline for alcoholic hepatitis. N Engl J Med 2015;372:1619–1628. [PubMed: 25901427]
4. Mathurin P, Lucey MR. Management of alcoholic hepatitis. J Hepatol 2012;56 Suppl 1:S39–45. [PubMed: 22300464]

5. Leclercq S, Matamoros S, Cani PD, Neyrinck AM, Jamar F, Starkel P, Windey K, et al. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. Proc Natl Acad Sci U S A 2014;111:E4485–4493. [PubMed: 25288760]
6. Llopis M, Cassard AM, Wrzosek L, Boschat L, Bruneau A, Ferrere G, Puchois V, et al. Intestinal microbiota contributes to individual susceptibility to alcoholic liver disease. Gut 2016;65:830–839. [PubMed: 26642859]
7. Philips CA, Pande A, Shasthry SM, Jamwal KD, Khillan V, Chandel SS, Kumar G, et al. Healthy Donor Fecal Microbiota Transplantation in Steroid-Ineligible Severe Alcoholic Hepatitis: A Pilot Study. Clin Gastroenterol Hepatol 2017;15:600–602. [PubMed: 27816755]
8. Duan Y, Llorente C, Lang S, Brandl K, Chu H, Jiang L, White RC, et al. Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. Nature 2019;575(7783):505–511. [PubMed: 31723265]
9. Lang S, Duan Y, Liu J, Torralba MG, Kuelbs C, Ventura-Cots M, Abraldes JG, et al. Intestinal Fungal Dysbiosis and Systemic Immune Response to Fungi in Patients With Alcoholic Hepatitis. Hepatology 2020;71:522–538. [PubMed: 31228214]
10. Chu H, Duan Y, Lang S, Jiang L, Wang Y, Llorente C, Liu J, et al. The Candida albicans exotoxin candidalysin promotes alcohol-associated liver disease. J Hepatol 2020;72:391–400. [PubMed: 31606552]
11. Shkoporov AN, Clooney AG, Sutton TDS, Ryan FJ, Daly KM, Nolan JA, McDonnell SA, et al. The Human Gut Virome Is Highly Diverse, Stable, and Individual Specific. Cell Host & Microbe 2019;26:527–541.e525. [PubMed: 31600503]
12. Ross A, Ward S, Hyman P. More Is Better: Selecting for Broad Host Range Bacteriophages. Front Microbiol 2016;7:1352. [PubMed: 27660623]
13. Cahill J, Young R. Phage Lysis: Multiple Genes for Multiple Barriers. Adv Virus Res 2019;103:33–70. [PubMed: 30635077]
14. Howard-Varona C, Hargreaves KR, Abedon ST, Sullivan MB. Lysogeny in nature: mechanisms, impact and ecology of temperate phages. ISME J 2017;11:1511–1520. [PubMed: 28291233]
15. Wommack KE, Colwell RR. Virioplankton: viruses in aquatic ecosystems. Microbiol Mol Biol Rev 2000;64:69–114. [PubMed: 10704475]
16. Dalmasso M, Hill C, Ross RP. Exploiting gut bacteriophages for human health. Trends Microbiol 2014;22:399–405. [PubMed: 24656964]
17. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. Cell 2009;138:30–50. [PubMed: 19596234]
18. Virgin HW. The virome in mammalian physiology and disease. Cell 2014;157:142–150. [PubMed: 24679532]
19. Scarpellini E, Ianiro G, Attili F, Bassanelli C, De Santis A, Gasbarrini A. The human gut microbiota and virome: Potential therapeutic implications. Dig Liver Dis 2015;47:1007–1012. [PubMed: 26257129]
20. Norman JM, Handley SA, Baldridge MT, Droit L, Liu CY, Keller BC, Kambal A, et al. Disease-Specific Alterations in the Enteric Virome in Inflammatory Bowel Disease. Cell 2015;160:447–460. [PubMed: 25619688]
21. Zuo T, Lu XJ, Zhang Y, Cheung CP, Lam S, Zhang F, Tang W, et al. Gut mucosal virome alterations in ulcerative colitis. Gut 2019;68:1169–1179. [PubMed: 30842211]
22. Fernandes MA, Verstraete SG, Phan TG, Deng X, Stekol E, LaMere B, Lynch SV, et al. Enteric Virome and Bacterial Microbiota in Children With Ulcerative Colitis and Crohn Disease. J Pediatr Gastroenterol Nutr 2019;68:30–36. [PubMed: 30169455]
23. Clooney AG, Sutton TDS, Shkoporov AN, Holohan RK, Daly KM, O'Regan O, Ryan FJ, et al. Whole-Virome Analysis Sheds Light on Viral Dark Matter in Inflammatory Bowel Disease. Cell Host Microbe 2019;26:764–778 e765. [PubMed: 31757768]
24. Nakatsu G, Zhou H, Wu WKK, Wong SH, Coker OO, Dai Z, Li X, et al. Alterations in Enteric Virome Are Associated With Colorectal Cancer and Survival Outcomes. Gastroenterology 2018;155:529–541 e525. [PubMed: 29689266]
25. Hannigan GD, Duhaime MB, Ruffin MTt, Koumpouras CC, Schloss PD. Diagnostic Potential and Interactive Dynamics of the Colorectal Cancer Virome. mBio 2018;9.

26. Gao B, Lang S, Duan Y, Wang Y, Shawcross DL, Louvet A, Mathurin P, et al. Serum and Fecal Oxylipins in Patients with Alcohol-Related Liver Disease. Dig Dis Sci 2019;64:1878–1892. [PubMed: 31076986]
27. Ball SA, Tennen H, Poling JC, Kranzler HR, Rounsaville BJ. Personality, temperament, and character dimensions and the DSM-IV personality disorders in substance abusers. J Abnorm Psychol 1997;106:545–553. [PubMed: 9358685]
28. Gao B, Emami A, Zhou R, Lang S, Duan Y, Wang Y, Jiang L, et al. Functional Microbial Responses to Alcohol Abstinence in Patients With Alcohol Use Disorder. Frontiers in Physiology 2020;11.
29. Brandl K, Hartmann P, Jih LJ, Pizzo DP, Argemi J, Ventura-Cots M, Coulter S, et al. Dysregulation of serum bile acids and FGF19 in alcoholic hepatitis. J Hepatol 2018;69:396–405. [PubMed: 29654817]
30. Conceicao-Neto N, Yinda KC, Van Ranst M, Matthijnssens J. NetoVIR: Modular Approach to Customize Sample Preparation Procedures for Viral Metagenomics. Methods Mol Biol 2018;1838:85–95. [PubMed: 30128991]
31. McIver LJ, Abu-Ali G, Franzosa EA, Schwager R, Morgan XC, Waldron L, Segata N, et al. bioBakery: a meta'omic analysis environment. Bioinformatics 2018;34:1235–1237. [PubMed: 29194469]
32. Kostic AD, Ojesina AI, Pedamallu CS, Jung J, Verhaak RG, Getz G, Meyerson M. PathSeq: software to identify or discover microbes by deep sequencing of human tissue. Nat Biotechnol 2011;29:393–396. [PubMed: 21552235]
33. Walker MA, Pedamallu CS, Ojesina AI, Bullman S, Sharpe T, Whelan CW, Meyerson M. GATK PathSeq: a customizable computational tool for the discovery and identification of microbial sequences in libraries from eukaryotic hosts. Bioinformatics 2018;34:4287–4289. [PubMed: 29982281]
34. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75:7537–7541. [PubMed: 19801464]
35. Stevens SJ, Vervoort MB, van den Brule AJ, Meenhorst PL, Meijer CJ, Middeldorp JM. Monitoring of epstein-barr virus DNA load in peripheral blood by quantitative competitive PCR. J Clin Microbiol 1999;37:2852–2857. [PubMed: 10449464]
36. Lay MLJ, Lucas RM, Ratnamohan M, Taylor J, Ponsonby AL, Dwyer DE, AIG AIG. Measurement of Epstein-Barr virus DNA load using a novel quantification standard containing two EBV DNA targets and SYBR Green I dye. Virology Journal 2010;7.
37. Kent WJ. BLAT - The BLAST-like alignment tool. Genome Research 2002;12:656–664. [PubMed: 11932250]
38. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, SS M, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology 2006;43:1317–1325. [PubMed: 16729309]
39. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60. [PubMed: 21702898]
40. Hothorn T, Lausen B. On the exact distribution of maximally selected rank statistics. Computational Statistics & Data Analysis 2003;43:121–137.
41. Forrest EH, Atkinson SR, Richardson P, Masson S, Ryder S, Thursz MR, Allison M. Application of prognostic scores in the STOPAH trial: Discriminant function is no longer the optimal scoring system in alcoholic hepatitis. J Hepatol 2018;68:511–518. [PubMed: 29175535]
42. Rojo J, Simoes P, Krueger GR, Humberto CO, Ramon AM. Human herpesvirus-6 has no apparent influence on course of HCV hepatitis, but may complicate HBV hepatitis and alcoholic liver disease. A pilot study. In Vivo 2003;17:29–33. [PubMed: 12655786]
43. Tsai JP, Tseng KC, Lin MN, Su CC. A high seroprevalence of human herpesvirus type 8 already present in patients with chronic hepatitis before the development of cirrhosis. Pathology 2019;51:86–90. [PubMed: 30497802]

44. Chou AL, Huang WW, Tsao SM, Li CT, Su CC. Human herpesvirus type 8 in patients with cirrhosis: correlation with sex, alcoholism, hepatitis B virus, disease severity, and thrombocytopenia. Am J Clin Pathol 2008;130:231–237. [PubMed: 18628092]
45. Markin RS. Manifestations of Epstein-Barr virus-associated disorders in liver. Liver 1994;14:1–13. [PubMed: 8177024]
46. Vento S, Cainelli F. Is there a role for viruses in triggering autoimmune hepatitis? Autoimmunity Reviews 2004;3:61–69.
47. Randhawa PS, Markin RS, Starzl TE, Demetris AJ. Epstein-Barr virus-associated syndromes in immunosuppressed liver transplant recipients. Clinical profile and recognition on routine allograft biopsy. Am J Surg Pathol 1990;14:538–547. [PubMed: 2159731]
48. Holmes RD, Sokol RJ. Epstein-Barr virus and post-transplant lymphoproliferative disease. Pediatr Transplant 2002;6:456–464. [PubMed: 12453197]
49. Hu J, Zhang X, Yu G, Cai H, Gu J, Hu M, Xiang D, et al. Epstein-Barr virus infection is associated with a higher Child-Pugh score and may predict poor prognoses for patients with liver cirrhosis. BMC Gastroenterol 2019;19:94. [PubMed: 31215410]
50. Nahar S, Iraha A, Hokama A, Uehara A, Parrott G, Ohira T, Kaida M, et al. Evaluation of a multiplex PCR assay for detection of cytomegalovirus in stool samples from patients with ulcerative colitis. World J Gastroenterol 2015;21:12667–12675. [PubMed: 26640344]
51. Dhanda AD, Collins PL. Immune dysfunction in acute alcoholic hepatitis. World J Gastroenterol 2015;21:11904–11913. [PubMed: 26576079]
52. Maslov S, Sneppen K. Population cycles and species diversity in dynamic Kill-the-Winner model of microbial ecosystems. Sci Rep 2017;7:39642. [PubMed: 28051127]
53. Silveira CB, Rohwer FL. Piggyback-the-Winner in host-associated microbial communities. NPJ Biofilms Microbiomes 2016;2:16010. [PubMed: 28721247]
54. Smirnova E, Puri P, Muthiah MD, Daitya K, Brown R, Chalasani N, Liangpunsakul S, et al. Fecal microbiome distinguishes alcohol consumption from alcoholic hepatitis but does not discriminate disease severity. Hepatology 2020:10.1002/hep.31178.
55. Cresci GA, Bush K, Nagy LE. Tributyrin supplementation protects mice from acute ethanol-induced gut injury. Alcohol Clin Exp Res 2014;38:1489–1501. [PubMed: 24890666]

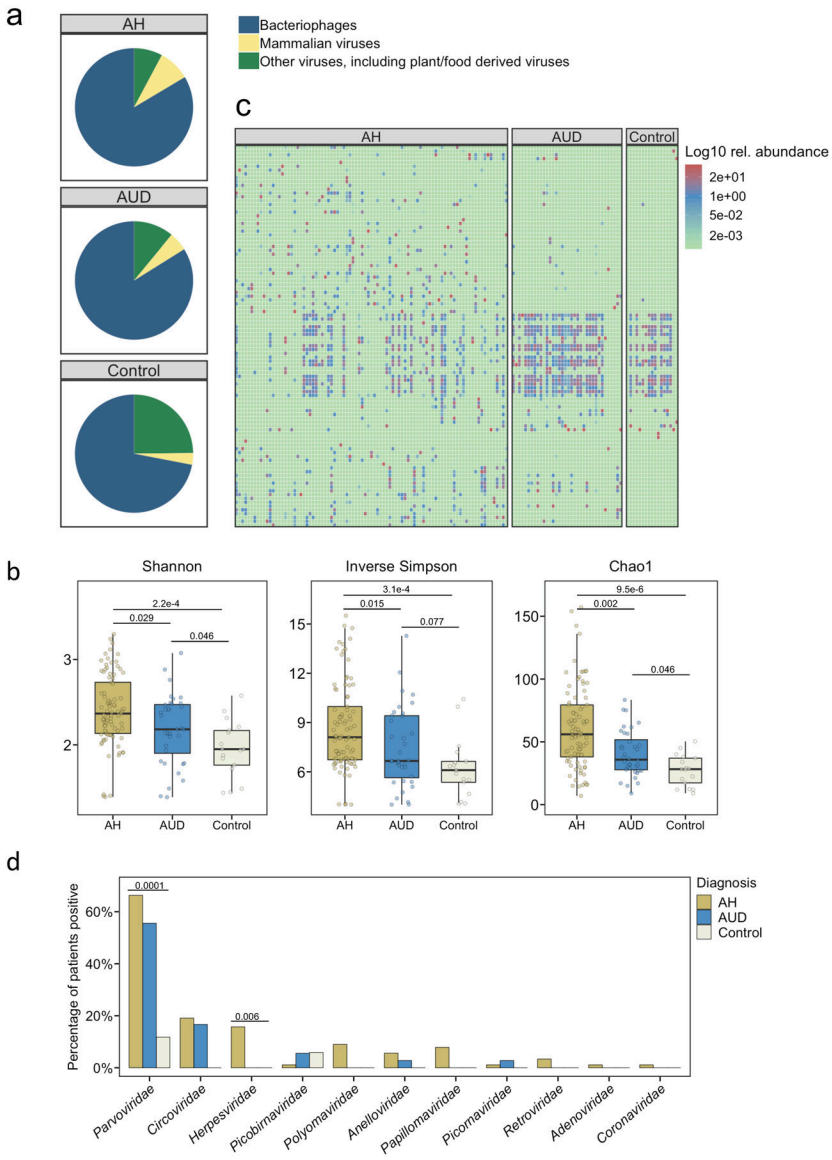

Figure 1. Altered virome composition in alcohol-associated liver disease

(a) Mean relative abundance of intestinal bacteriophages (phages), mammalian viruses, and other viruses, calculated at the family level, in fecal samples from patients with alcoholic hepatitis (AH), patients with alcohol use disorder (AUD), or controls. (b) Viral diversity, based on the Shannon and inverse Simpson indices, and viral richness, based on the Chao1 index. (c) Log10 relative abundance of the 100 most-abundant phages, at the species level. Each column indicates one study subject and each row indicates one single phage. (d) Percentage of subjects in each group positive for mammalian viruses at family level; only *P* values below 0.05 are shown. Post-hoc *P* values for *Parvoviridae* are: AH vs AUD, *P*=0.308; AH vs control, *P*=0.0001; and AUD vs control, *P*=0.004. Post-hoc *P* values for *Herpesviridae* are: AH vs AUD, *P*=0.030; AH vs control, *P*=0.179; and AUD vs control, *P*=1.0. Panels a–d present data from 89 alcoholic hepatitis patients, 36 alcohol use disorder patients, and 17 controls. *P* values were determined by two-tailed Kruskal-Wallis test with

Dunn's post-hoc test (b) and Fisher's exact test (d), each followed by false discovery rate (FDR) procedures to correct for multiple comparisons. For the box and whisker plots in panel b, the box extends from the 25th to 75th percentile, with the center line indicating the median; the bottom whiskers indicate the minimum values and the top whiskers indicate the 75th percentile plus 1.5-fold the inter-quartile distance (the distance between the 25th and 75th percentiles). Values greater than this were plotted as individual dots. AH, alcoholic hepatitis; AUD, alcohol use disorder.

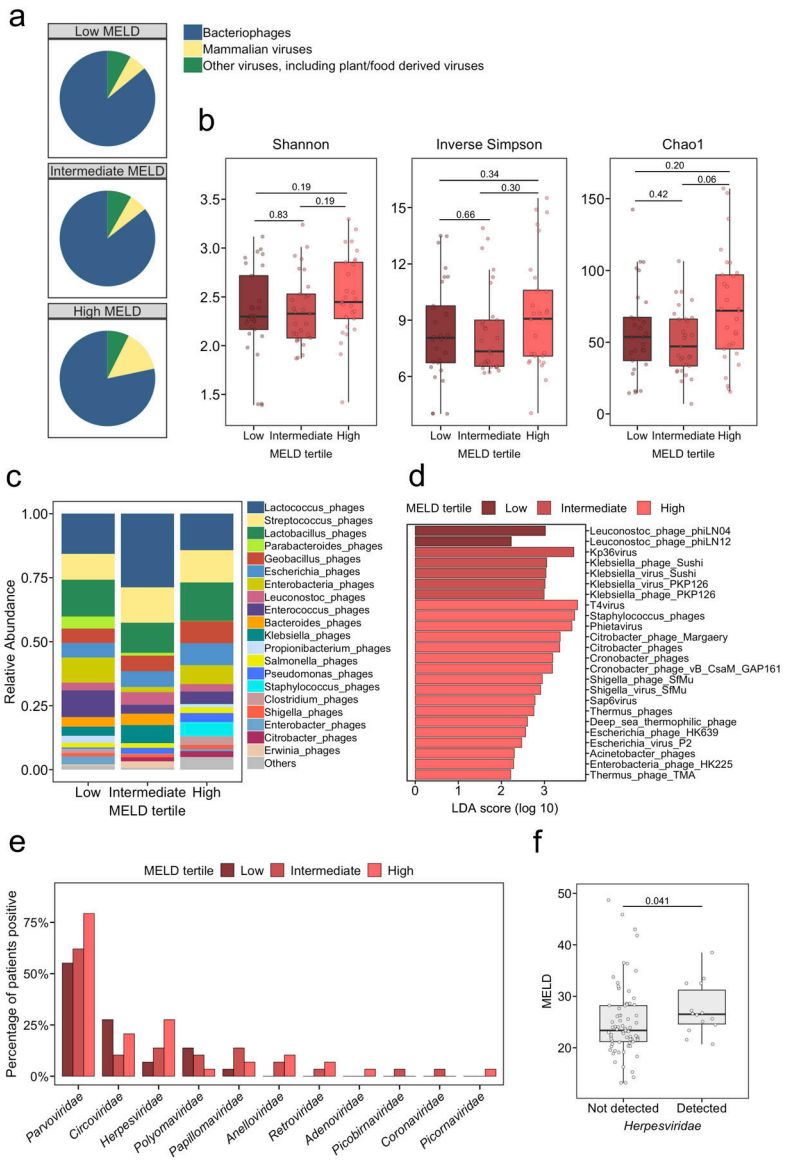

Figure 2. Severity of alcoholic hepatitis is associated with alterations in fecal viromes

Patients with alcoholic hepatitis (n=87) were assigned to tertiles based on MELD scores (cut points of 22.1 and 26.3), with 29 patients in each tertile. (a) Relative abundance of intestinal phages, mammalian viruses, and other viruses, calculated at the family level, in each tertile. (b) Viral diversity, based on the Shannon and inverse Simpson indices, and viral richness, based on the Chao1 index. (c) Mean relative abundance of phages in each tertile, according to their bacterial host, at the species level for each group; 0–1 corresponds to 0–100% abundance. (d) Linear discriminant effect size analysis (LEfSe) identification of features most likely to account for differences between tertiles: relative abundances of phages at family, genus, and species levels and mammalian viruses at family and genus levels. (e) Percentage of subjects in each group positive for mammalian viruses, at the family level. In all comparisons, *P* values were higher than 0.05, determined by the two-sided Fisher's exact test followed by false discovery rate (FDR) procedures. (f) MELD scores of 87 alcoholic

hepatitis patients with fecal samples either positive or negative for *Herpesviridae*. *P* values were determined by two-tailed Kruskal-Wallis test with Dunn's post-hoc test followed by false discovery rate (FDR) procedures (b), or by the two-sided Mann-Whitney-Wilcoxon rank-sum test (f). For the box and whisker plots in panel b, the box extends from the 25th to 75th percentile, with the center line indicating the median; the bottom whiskers indicate the minimum values and the top whiskers indicate the 75th percentile plus 1.5-fold the inter-quartile distance (the distance between the 25th and 75th percentiles). Values greater than this were plotted as individual dots. LDA, Linear discriminant analysis; MELD, model for end-stage liver disease.

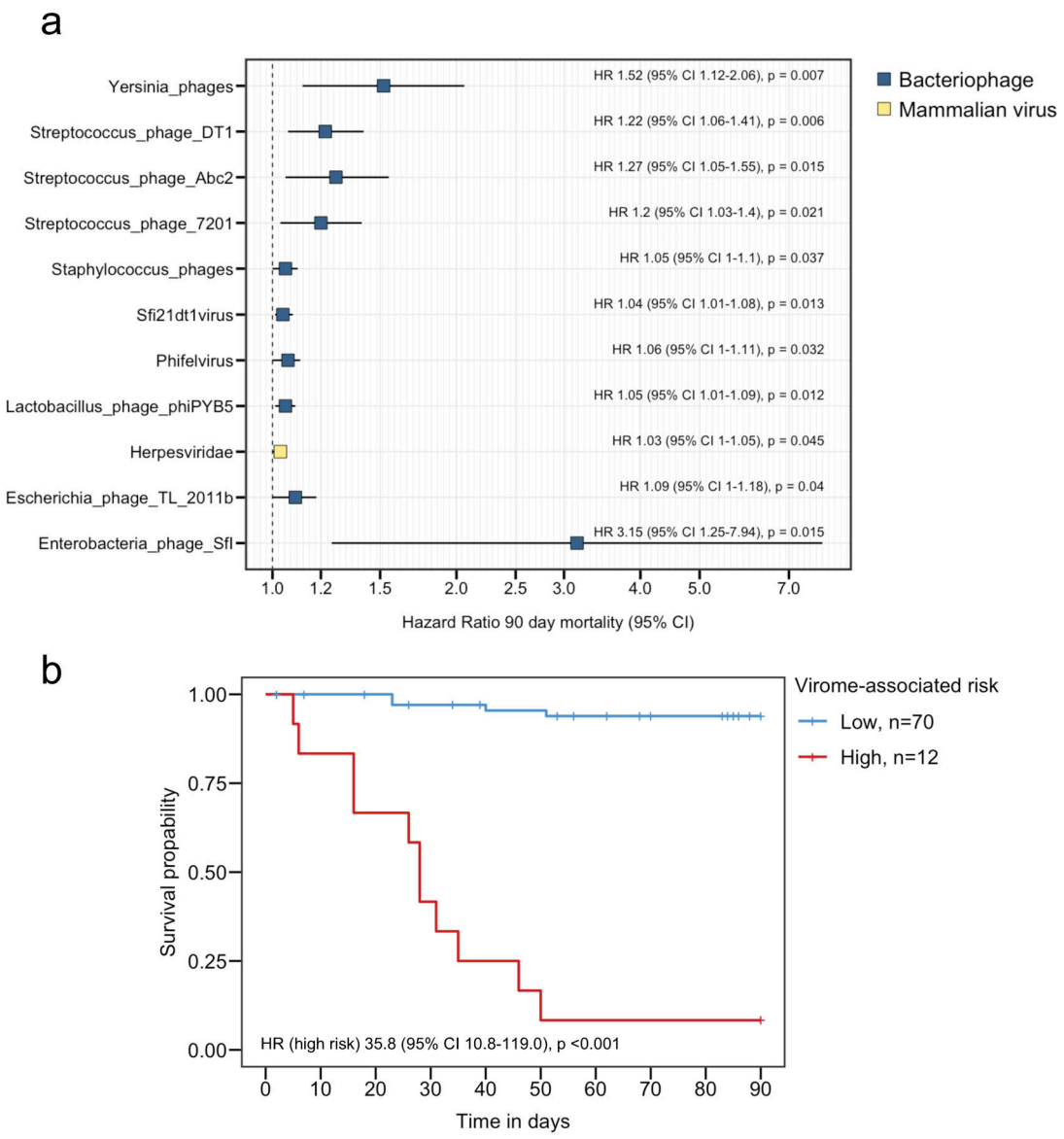

Figure 3. Intestinal viruses associated with mortality in alcoholic hepatitis patients

(a) Cox regression model identifies associations of intestinal viruses with 90-day mortality. Hazard ratios (HRs) were determined using Cox regression analysis of viral taxa detected in at least 10% of fecal samples from alcoholic hepatitis patients, adjusted for antibiotic and steroid treatment and geographic regions of patient enrollment. Patients were censored at the timepoint they were last seen alive. For the only patient who underwent liver transplantation, the transplantation date was considered as date of death. Squares identify hazard ratios (HRs), with lower and upper 95% confidence intervals (CI) values shown as lines. Colors indicates origins (phage vs mammalian virus) of viral taxa. (b) A virus-associated risk model was calculated by performing a multivariate analysis including taxa shown in (a). Patients were categorized according to the model calculated for each patient using a cut-off value determined by the maximally selected rank statistic into a low- or high virus-associated risk group. Kaplan-Meier curve of survival of alcoholic hepatitis patients according to their

intestinal virus-associated risk. Data in both panels collected from 82 patients with alcoholic hepatitis. HR, hazard ratio, CI, confidence interval.

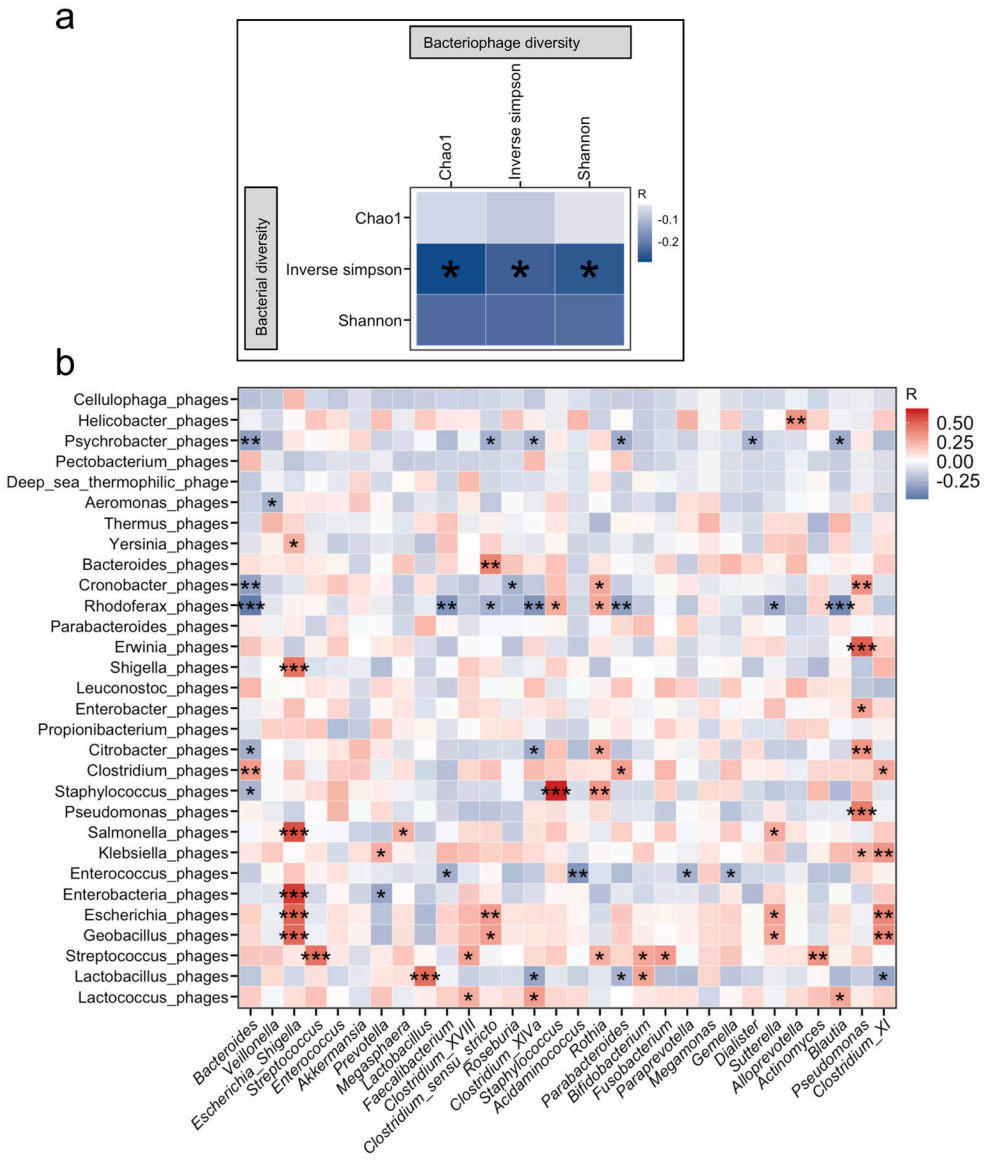

Figure 4. Correlation between phages and bacteria in fecal samples from patients with alcoholic hepatitis

Heatmaps show color-coded Spearman correlations of (a) bacterial diversity and richness with phage diversity and richness and (b) the 30 most abundant phages with the 30 most abundant bacteria at genus level, determined from 16S rRNA gene sequencing. Red color indicates positive correlation and blue indicates negative correlation. **P*<0.05 *P*>0.01, ***P*<0.01 *P*>0.001, ****P*<0.001. Data obtained from 65 patients with alcoholic hepatitis and available 16S rRNA gene sequencing and viral metagenomic sequence data.

Table 1.

Demographic and laboratory parameters of the study cohort

Variables	Controls (n=17)	Alcohol use disorder (n=36)	Alcoholic hepatitis (n=89)	*P* Value
Sex (% male), n (%), n=141	13 (76.5)	29 (80.6)	60 (67.4)	0.370
Age (years), n=141	39.0 (27.0–71.0)	42.0 (27.0–67.0)	51.3 (30.1–74.8)	<0.001 *
BMI (kg/m^2), n=128	22.8 (18.8–29.6)	23.6 (17.9–31.4)	27.4 (16.3–48.3)	<0.001 *
Creatinine (mg/dL), n=124		0.8 (0.5–1.3)	0.8 (0.3–8.1)	0.635
Bilirubin (mg/dL), n=124		0.5 (0.2–1.5)	15.8 (2.5–41.1)	<0.001
AST (IU/L), n=124		43.0 (15.0–283.0)	130.0 (41.0–394.0)	<0.001
ALT (IU/L), n=124		37.0 (9.0–184.0)	44.0 (15.0–216.0)	0.148
Albumin (g/dL), n=113		4.5 (2.2–5.2)	2.4 (1.3–3.9)	<0.001
INR, n=119		0.9 (0.8–1.3)	1.9 (1.0–4.4)	<0.001
GGT (IU/L), n=76		42.0 (4.0–1131.0)	166.5 (33.0–3632.0)	<0.001
Platelet count (10^9/L), n=118		222.0 (80.0–434.0)	124.0 (13.0–447.0)	<0.001
FIB-4, n=118		1.4 (0.4–9.1)	8.0 (1.4–79.7)	<0.001
FIB-4 > 3.25 (F3-F4), n (%)		5 (16.1)	78 (90.1)	<0.001

Post-hoc *P* values for age: AH vs AUD, *P*=0.003; AH vs controls, *P*=0.008; AUD vs controls, *P*=0.859. Post-hoc *P* values for BMI: AH vs AUD, *P*=0.001; AH vs controls, *P*=0.025; AUD vs controls, *P*=0.999.

*Values presented are median with range in parentheses for continuous variables or number and percentage in parentheses for categorical variables. Percentages were calculated based on the actual number of patients in each group, when data were available. The number of subjects for which data were available is indicated in the first column.

AH, alcoholic hepatitis; AUD, alcohol use disorder; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio; GGT, gamma-glutamyl transferase; FIB-4, fibrosis-4 index.

Table 2.

Characteristics of patients with alcoholic hepatitis (n=89)

Variable	
Steroids, n (%), n=87	33 (37.9)
Pentoxifylline, n (%), n=66	6 (9.1)
Antibiotics, n (%), n=87	40 (46.0)
Proton pump inhibitors, n (%), n=44	5 (11.3)
Infections, n (%), n=76	19 (25)
Abstinent at day 30, n=47	
Abstinent, n (%)	42 (89.4)
Reduced alcohol consumption, n (%)	3 (6.4)
Continued alcohol abuse, n (%)	2 (4.3)
Stage of Fibrosis, n (%), n=48 0 / 1 / 2 / 3 / 4	2 (4.1) / 0 (0.0) / 6 (12.5) / 5 (10.4) / 35 (72.9)
MELD, median (range), n=87	23.9 (13.2–48.7)
Child-Pugh stage, n (%), n=86 A / B / C	1 (1.2) / 23 (26.7) / 62 (72.1)
30-day survival rate	88.7% (95% CI 81.9–95.9%)
90-day survival rate	80.8% (95% CI 72.5–90.0%)

Antibiotics include prophylactic antibiotics. Values are presented as median (range) for continuous variables or number (percentage) for categorical variables. Percentages are calculated based on the actual number of patients in each group where the respective data was available. The number of subjects for which data were available is indicated in the first column. Survival rates were calculated using Kaplan-Meier curves. Patients were censored at the timepoint they were last seen alive.

MELD, model for end-stage liver disease; CI, confidence interval.