Research Article
Cirrhosis and Liver Failure

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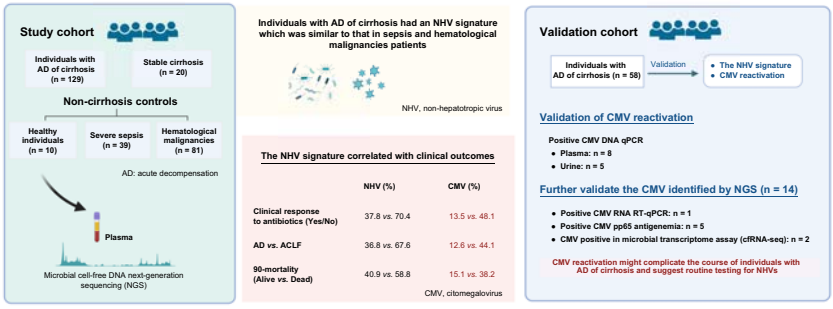
Graphical abstract



Highlights

- A non-hepatotropic viral signature was identified in individuals with AD of cirrhosis.
- This viral signature correlated with clinical outcomes.
- CMV reactivation might play a pathogenic role in AD and progression toward ACLF.
- Further refinement and validation are needed to define the clinical relevance of our results.

Impact and implications

A non-hepatotropic virus (NHV) signature, which was similar to that in individuals with sepsis and hematological malignancies, was identified in individuals with acute decompensation of cirrhosis. The detected viral signature had clinical correlates, including clinical efficacy of empirical antibiotic treatment, progression to acute-on-chronic liver failure and short-term mortality. Cytomegalovirus reactivation, which is treatable, may adversely affect clinical outcomes in some individuals with decompensated cirrhosis. Routine screening for NHVs, especially cytomegalovirus, may be useful for the management of individuals with acute decompensation of cirrhosis.

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Prognostic and therapeutic significance of microbial cell-free DNA in plasma of people with acute decompensation of cirrhosis

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Background & Aims: Although the effect of bacterial infection on cirrhosis has been well-described, the effect of non-hepatotropic virus (NHV) infection is unknown. This study evaluated the genome fragments of circulating microorganisms using metagenomic next-generation sequencing (mNGS) in individuals with acute decompensation (AD) of cirrhosis, focusing on NHVs, and related the findings to clinical outcomes.

Methods: Plasma mNGS was performed in 129 individuals with AD of cirrhosis in the study cohort. Ten healthy volunteers and 20, 39, and 81 individuals with stable cirrhosis, severe sepsis and hematological malignancies, respectively, were enrolled as controls. Validation assays for human cytomegalovirus (CMV) reactivation were performed in a validation cohort (n = 58) and exploratory treatment was instituted.

Results: In the study cohort, 188 microorganisms were detected in 74.4% (96/129) of patients, including viruses (58.0%), bacteria (34.1%), fungi (7.4%) and chlamydia (0.5%). A NHV signature was identified in individuals with AD, and CMV was the most frequent NHV, which correlated with the clinical effect of empirical antibiotic treatment, progression to acute-on-chronic liver failure, and 90-day mortality. The NHV signature in individuals with acute-on-chronic liver failure was similar to that in those with sepsis and hematological malignancies. CMV was detected in 24.1% (14/58) of patients in the validation cohort. Of the 14 cases with detectable CMV by mNGS, nine were further validated by real-time PCR or pp65 antigenemia testing. Three patients with CMV reactivation received ganciclovir therapy in an exploratory manner and experienced clinical resolutions.

Conclusions: The results of this study suggest that NHVs may play a pathogenic role in complicating the course of AD. Further validation is needed to define whether this should be incorporated into the routine management of individuals with AD of cirrhosis.

Introduction

Cirrhosis-associated immune dysfunction (CAID) is related to the progression of acute decompensation (AD), which can affect the risk of bacterial infection and mortality.[1] The mechanisms underlying CAID are unclear but indicate widespread disturbances affecting both the innate and adaptive immune systems. The severity of CAID increases with the severity of cirrhosis and can manifest with immune paralysis in individuals with advanced grades of acute-on-chronic liver failure (ACLF), a syndrome that is characterized by precipitating events, multi-organ failure and systemic inflammation.[2] Bacterial infection is the most common precipitating event in individuals with AD.[3] In about 30% of individuals with AD, no precipitating illness is found.[4] Given that cirrhosis is associated with CAID,[1,5] it is possible that infections other than those caused by bacteria, such as viral infections, may be involved as precipitants.

Previous studies have focused on the potential role of hepatotropic viruses as potential precipitants of AD.[6,7] However, the role of infections with non-hepatotropic viruses (NHVs) such as herpes simplex virus (HSV), human cytomegalovirus (CMV), human parvovirus B19, and Epstein-Barr virus (EBV), which are known to cause acute liver failure, is unknown.[8,9]

Metagenomic next-generation sequencing (mNGS) is emerging as an important culture-independent technique that can detect nearly all known pathogens simultaneously from a clinical sample.[10] Sequencing of microbial cell-free DNA (cfDNA) can enable diagnosis of several infections.[11–13] Few relevant studies have focused on the clinical application of

Keywords: Cirrhosis; metagenomic next-generation sequencing; non-hepatotropic virus; cytomegalovirus.
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mNGS in individuals with cirrhosis.[14] The primary aim of this study was to comprehensively evaluate the circulating microbial genome fragments in individuals with AD of cirrhosis (by sequencing plasma microbial cfDNA) and relate this to clinical outcomes. The secondary aim was to validate the potential role of CMV reactivation, a known NHV for which antiviral drugs are available, in determining the prognosis of individuals with decompensated cirrhosis.

Patients and methods

This study was approved by the local ethics committee of Nanfang Hospital (NFEC-2017-097 and NFEC-2020-255) and was performed in compliance with the Declaration of Helsinki. As shown in Fig. 1, this study consists of a study cohort, stable cirrhosis and non-cirrhosis controls, and an independent validation cohort.

Study cohort

The study cohort was extracted from Chinese Acute-on-Chronic Liver Failure (CATCH-LIFE) study, a multicenter cohort study which consists of an investigation cohort (NCT02457637) and a validation cohort (NCT03641872). Individuals with AD of cirrhosis (ascites, hepatic encephalopathy, infection and gastrointestinal bleeding) enrolled at Nanfang Hospital, one of the participating centers of CATCH-LIFE study, were included. The flow chart of study cohort is presented in Fig. S1A. Demographic, clinical, laboratory and microbiological data as well as the treatments administered were analyzed. Details of the study are presented in the supplementary materials and methods.

Cirrhosis and non-cirrhosis controls

Individuals with stable cirrhosis were included as controls. Non-cirrhosis controls: 1) healthy volunteers: healthy individuals who were free of chronic/acute disease were considered as healthy controls; 2) two groups of patients were included as non-cirrhosis controls: individuals with severe sepsis and those with hematological malignancies who were hospitalized in Nanfang Hospital during 2017-2020 and had undergone plasma mNGS (BGI Clinical Laboratories, Shenzhen China) for clinical infection were retrospectively included. Sex and age were matched in healthy individuals and stable cirrhosis subgroups. No matching was performed between the severe sepsis/hematological malignancy subgroups and the AD groups. Details of these controls are presented in the supplementary materials and methods.

Validation cohort

The 'validation cohort' prospectively included individuals with AD to validate the NHV signature observed in the study cohort. The inclusion and exclusion criteria were identical to those of the study cohort (Fig. S1B). Plasma samples were transferred to BGI Clinical Laboratories (Shenzhen, China) for mNGS detection immediately when blood samples were collected. The turnaround time was about 48-72 h for mNGS reports, which were sent to the treating clinicians. They were free to decide whether they treated (or not) the patients based on the detection of NHV using mNGS. Simultaneously, to further validate the presence of CMV, plasma and urine quantitative CMV DNA were measured using real-time PCR (qPCR) and white blood cells were also collected for CMV pp65 antigenemia assay. CMV real-time reverse-transcription PCR (RT-qPCR) and microbial transcriptome assays were performed in patients with detectable CMV using mNGS.

The microbial cfDNA sequencing (PM-seq®) and microbial cfRNA sequencing detection were performed by BGI Clinical Laboratories (Shenzhen, China) and Goodwill Clinical Laboratories (Guangzhou, China), respectively.

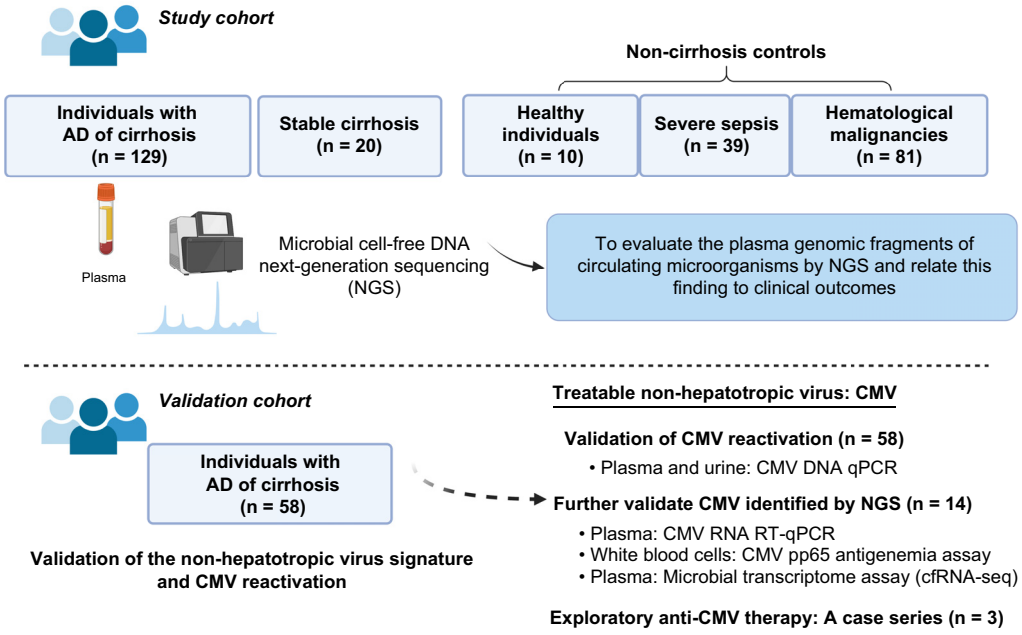

Fig. 1. Study design. AD, acute decompensation; CMV, human cytomegalovirus; qPCR, quantitative PCR; RT-qPCR, reverse-transcription quantitative PCR.

For further details regarding definitions and methods, please refer to the supplementary materials and methods.

Results

Clinical characteristics

Study cohort

Demographic and clinical characteristics of individuals with AD included in the study cohort are presented in Table 1. Most patients were men (86%) and the mean age was 52 ± 11 years. The most frequent AD event was ascites (96.1%), followed by infection (49.6%), gastrointestinal bleeding (10.9%) and hepatic encephalopathy (8.5%). Hepatitis B virus (HBV) was the most common etiology of cirrhosis followed by alcohol consumption. The most common infections were pneumonia (21.7%) and spontaneous bacterial peritonitis (10.1%). Fever was found in 25.6% of patients. Microbiological cultures were positive in 12.4% of patients, and 16 organisms were isolated.

Control groups

Twenty patients with stable cirrhosis (Table S1) and 10 healthy individuals (Table S2) were included as controls. None of these

Table 1. Demographic, clinical and laboratory characteristic of the study and validation cohorts.

Baseline characteristics	Study cohort (n = 129)	Validation cohort (n = 58)
Age (years), mean (SD)	52 (11)	52 (11)
Male, n (%)	111 (86.0)	43 (74.1)
Etiology of cirrhosis, n (%) - (HBV-related/alcohol/HCV-related/other)	95/17/0/17 (73.6/13.2/0/13.2)	43/8/1/6 (74.1/13.8/1.7/10.4)
Ascites at admission, n (%)	124 (96.1)	44 (75.9)
Gastrointestinal bleeding at admission, n (%)	14 (10.9)	4 (6.9)
Hepatic encephalopathy, n (%)	11 (8.5)	7 (12.1)
Broad-spectrum antibiotic treatment in the previous 2 weeks, n (%)	22 (17.1)	15 (25.9)
Under anti-HBV treatment, n (%)	51/95 (53.7)	31/43 (72.1)
HBV DNA undetectable – n (%)	40/95 (42.1)	6/43 (14.0)
HBV DNA (log_{10} IU/ml) - median (IQR)	4.7 (3.3-6.3)	3.5 (1.9-5.1)
CMV IgM positive[#], n (%)	2/124 (1.6)	2/58 (3.4)
CMV IgG positive, n (%)	126/127 (99.2)	58/58 (100)
EBV IgM positive, n (%)	0/124 (0)	1/58 (1.7)
EBV IgG positive, n (%)	32/126 (25.4)	6/58 (10.3)
HSV-1 IgM positive, n (%)	2/124 (1.6)	0/58 (0)
HSV-1 IgG positive, n (%)	125/126 (99.2)	55/58 (94.8)
Heart rate (bpm), mean (SD)	87 (14)	95 (16)
Body temperature (°C), mean (SD)	37.1 (0.8)	37.0 (0.9)
Respiratory rate (breath/min), mean (SD)	19 (3)	18 (3)
Leukocytes count ($\times 10^9$/L), median (IQR)	5.2 (3.5-9.4)	7.09 (4.0-10.2)
Bilirubin (μmol/L), median (IQR)	72.4 (28.3-324.8)	265.4 (129.7-401.1)
INR, median (IQR)	1.7 (1.4-2.2)	1.8 (1.2-2.5)
Serum creatinine (μmol/L), median (IQR)	77.0 (61.0-101.5)	70.0 (54.4-85.6)
C-reactive protein (mg/L), median (IQR)	11.2 (6.7-29.8)	14.48 (1.8-27.1)
Fever at admission, n (%)	33 (25.6)	14 (24.1)
Infection, n (%)	64 (49.6)	45 (77.6)
Sites of infection, n (%)		
Pneumonia	28 (21.7)	28 (48.3)
Spontaneous bacterial peritonitis	13 (10.1)	10 (17.2)
Spontaneous bacteremia[*]	5 (3.9)	1 (1.7)
Urinary tract infection	4 (3.1)	1 (1.7)
Skin and soft tissue infection	3 (2.3)	1 (1.7)
Unproven infection	18 (14.0)	11 (19.0)
Others[&]	7 (5.4)	2 (3.4)
Type of strains isolated, n (%)		
Gram-positive bacteria	4 (25.0)	0/11 (0)
Gram-negative bacteria	11 (68.7)	9/11 (81.2)
Fungi	1 (6.3)	2/11 (18.2)
ACLF at baseline, n (%)	28 (21.7)	18 (31.0)
ACLF grade, n (%)		
Grade 1	4 (3.1)	2 (3.4)
Grade 2	22 (17.1)	12 (20.7)
Grade 3	2 (1.6)	4 (6.9)
MELD score, mean (SD)	21 (10)	24 (9)
Child-Pugh score, mean (SD)	9 (2)	10 (2)
CLIF-C-AD score, mean (SD)	49 (9)	51 (10)
28-day transplant-free mortality, n (%)	20/127 (15.7)	19/58 (32.8)
90-day transplant-free mortality, n (%)	34/127 (26.8)	24/56 (42.9)

ACLF, acute-on-chronic liver failure; AD, acute decompensation; CLIF, chronic liver failure; CMV, human cytomegalovirus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HSV, herpes simplex virus; MELD, model of end-stage liver disease.

[#]5 patients had insufficient volume of plasma for serology assay.

[*]25 patients underwent blood culture.

[&]Cholangitis (2), acute pancreatitis (1), bacterascites (3), the spleen infection after surgery (1), infectious diarrhea (1), and secondary bacterial peritonitis (1).

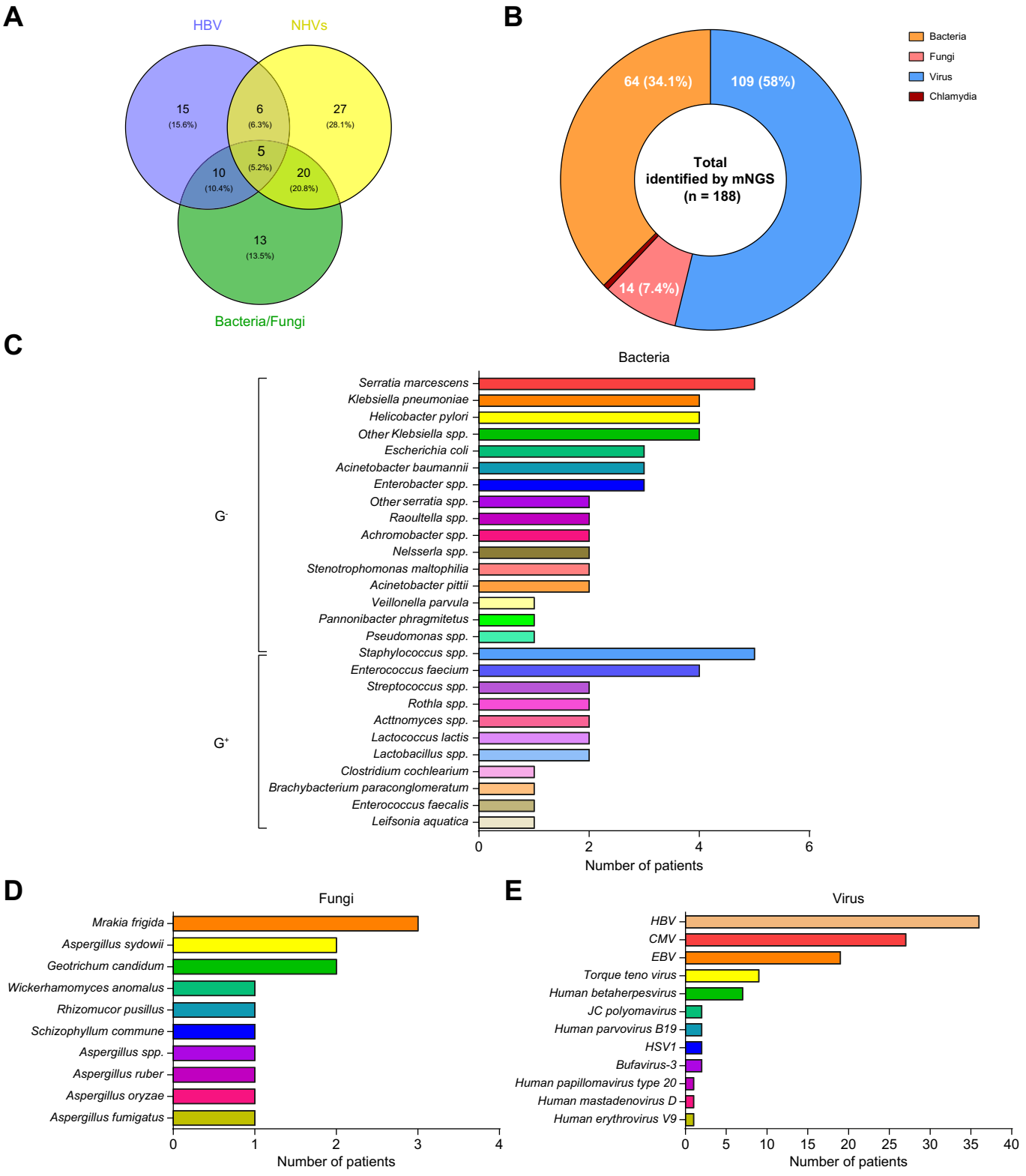

Fig. 2. A NHV signature identified in individuals with AD of cirrhosis. (A) Distribution of the four types of microorganisms detected by NGS (n = 129). (B) Numbers and categories of microorganisms identified using NGS. (C-E) Details of bacteria (C), fungi (D) and viruses (E) identified by NGS. AAV, adeno-associated virus; AD, acute decompensation; CMV, human cytomegalovirus; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HHV, human herpes virus; HSV-1, herpes simplex virus type 1; NGS, next-generation sequencing; NHV, non-hepatotropic virus; TTV, torque teno virus; VZV, varicella-zoster virus.

participants had any evidence of proven or unproven bacterial infections.

For non-cirrhosis immunocompromised controls, we included 39 individuals in the intensive care unit (ICU) with severe sepsis (Fig. S2). The most common primary disorder in these individuals was severe pneumonia, followed by malignant tumor and cerebrovascular disease. Individuals with hematological malignancies (n = 81) were retrospectively analyzed in this study (Fig. S2 and Table S3). Acute myelocytic leukemia and acute lymphoblastic leukemia were the most prevalent underlying diseases.

Non-hepatotropic viral signature in the different cohorts

Individuals with AD: Of the 129 individuals with AD of cirrhosis, genome fragments of microorganisms were detectable in 96 (74.4%) patients using plasma mNGS (15 with only HBV, 58 with NHVs and 23 with bacteria or fungus in the absence of NHVs) (Fig. 2A). In total, 188 microorganisms were identified, including viruses (58.0%), bacteria (34.1%), fungi (7.4%) and chlamydia (0.5%) (Fig. 2B). Gram-negative bacteria were the predominant bacteria identified by mNGS, and *Klebsiella spp.* and *Serratia marcescens* were the most prevalent. *Staphylococcus spp.* and *Enterococcus faecium* were the most frequent gram-positive bacteria (Fig. 2C). Fungi were also found (Fig. 2D). A variety of NHVs were detected, including CMV, EBV, torque teno virus (TTV), human betaherpesvirus and human parvovirus B19 (Fig. 2E). CMV was detected in 20.9% of patients and was the most common NHV in the study cohort.

Immunocompromised individuals without cirrhosis: To determine whether the viral signature was related to immune dysfunction, we evaluated the NHV signature in individuals in the ICU with sepsis and those with hematological malignancies without cirrhosis (Fig. S3A-F).

The high prevalence of NHVs detected in individuals with AD of cirrhosis with overt infections (51.6%) was similar to that in those with sepsis requiring organ support in the ICU (59.0%) and those with hematological malignancies (59.3%) (Fig. 3A). CMV was also the most common detectable NHV in individuals with severe sepsis and those with hematological malignancies

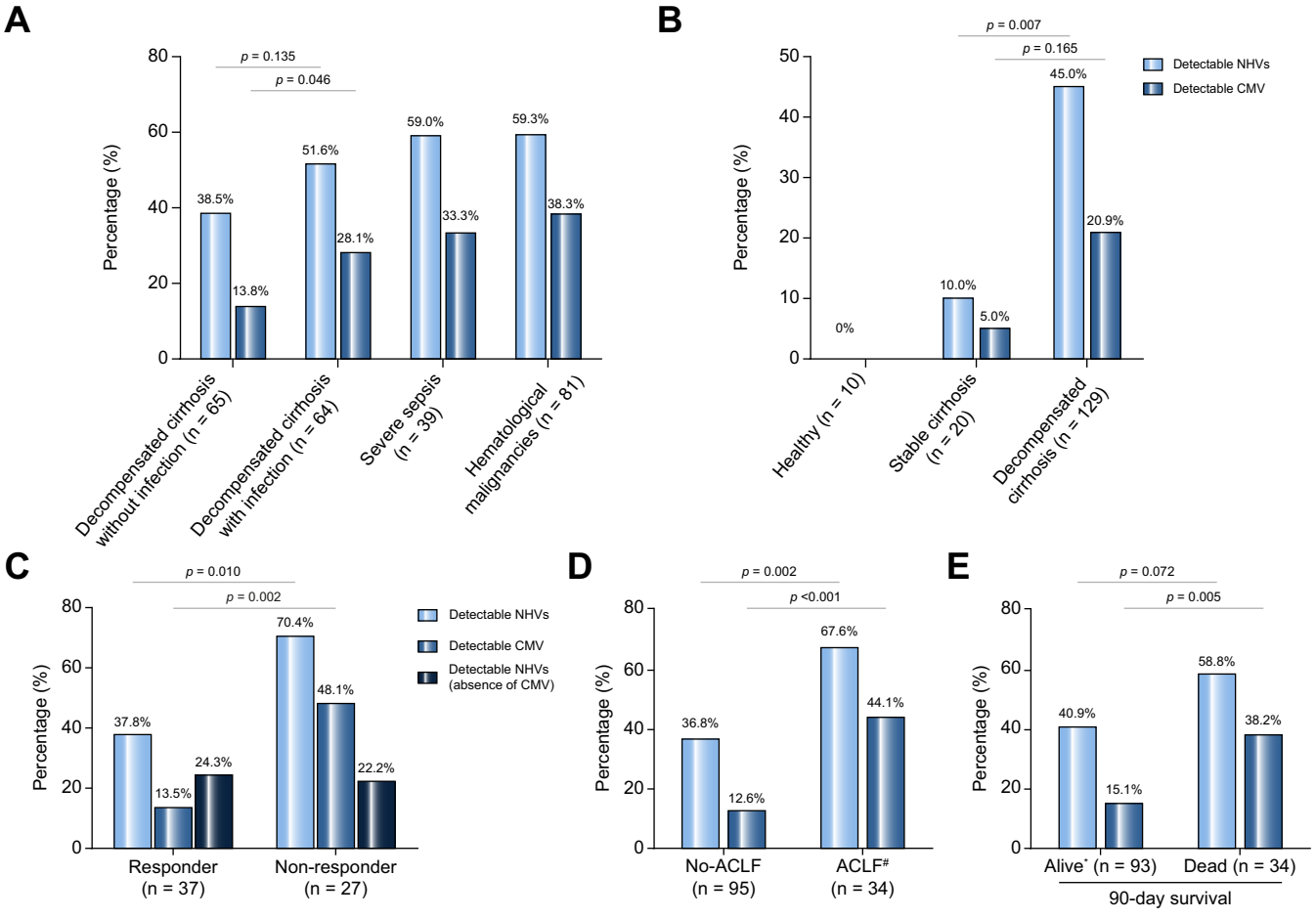

Fig. 3. Clinical correlates of the viral signature in AD of cirrhosis. (A) Proportions of detectable NHVs and CMV identified by NGS in individuals with decompensated cirrhosis and non-cirrhosis controls. Level of significance: $p = 0.135$; $p = 0.046$ (Chi-Square test). (B) Proportions of detectable NHVs and CMV in healthy individuals, those with stable cirrhosis, and those with AD of cirrhosis. Level of significance: $p = 0.007$; $p = 0.165$ (Chi-Square test). (C) Detectable NHVs and CMV in patients with and without a clinical response to empirical treatment. Levels of significance: $p = 0.010$; $p = 0.002$ (Chi-Square test). (D) Comparison of detectable NHVs and CMV between individuals with ACLF and without. Levels of significance: $p = 0.002$; $p < 0.001$ (Chi-Square test). (E) Proportions of detectable NHVs and CMV based on 90-day mortality. *2 patients were censored for liver plantation. Level of significance: $p = 0.072$; $p = 0.005$ (Chi-Square test). ACLF, acute-on-chronic liver failure; AD, acute decompensation; CMV, human cytomegalovirus; NHV, non-hepatotropic virus.

Table 2. Case series of individuals with AD of cirrhosis who progressed from AD to ACLF within 28 days.

Case ID	Microorganism identified by metagenomic NGS	Blood culture	Sites of infection at baseline	Initially antibiotic treatment (duration, days)	Clinical response to initial antibiotic treatment	28-day mortality	90-day mortality
2[a]	*Aspergillus fumigatus*, CMV, HBV	Negative	Pneumonia	Carbapenem (3)	No	Alive	Dead
37[b]	CMV	–	Pneumonia	Classical beta-lactams plus β-lactamases inhibitor (10)	No	Alive	Dead
49[c]	*Klebsiella pneumoniae*	–	Pneumonia, cholangitis	Classical beta-lactams plus β-lactamases inhibitor (4)	No	Dead	–
96[d]	CMV, HBV	–	Bacterascites	Classical beta-lactams plus β-lactamases inhibitor (5)	No	Alive	Alive
99[e]	CMV, HBV	Negative	Pneumonia	Classical beta-lactams plus β-lactamases inhibitor (3)	No	Dead	–
120[f]	*Acinetobacter baumannii*, CMV	–	Pneumonia	Classical beta-lactams plus β-lactamases inhibitor (3)	No	Alive	Dead

ACLF, acute-on-chronic liver failure; AD, acute decompensation; CMV, cytomegalovirus; HBV, hepatitis B virus.
[a]The patient was admitted with pneumonia and treated with meropenem for 3 days. Based on the chest radiography results, the patient was considered to have fungal lung infection, and the treatment was changed to an antifungal agent (fluconazole). He died within 90 days.
[b]The patient was admitted with pneumonia and treated with cefoperazone-tazobactam for 10 days, which was escalated to meropenem. The infection did not resolve and he developed ACLF-2 on day 15. The patient died on day 34.
[c]The patient was admitted with pneumonia and cholangitis. He was treated with cefoperazone-tazobactam for 4 days empirically with poor clinical response. He developed ACLF-1 on day 5 and he died on day 21 because of septic shock and acute kidney failure.
[d]The patient was admitted with suspected spontaneous bacterial peritonitis and treated with cefoperazone-tazobactam empirically. He progressed to ACLF-2 on day 4. He was then treated with imipenem and linezolid based on the positive ascites culture results. His infection resolved and he was discharged.
[e]The patient was admitted with pneumonia and treated with cefoperazone-sulbactam. Antibiotic therapy was escalated to meropenem plus teicoplanin because of poor clinical response. He developed ACLF-2 on day 6 and he died on day 16.
[f]The patient was empirically treated with cefoperazone-sulbactam for 3 days but did not respond. He progressed to ACLF-1 and the antibiotic treatment was changed to imipenem plus teicoplanin empirically. The patient was discharged without ACLF but died within 90 days.

(Fig. S3C and 3F). CMV was much more prevalent in individuals with cirrhosis with evidence of infection than in those without (28.1% *vs.* 13.8%, $p = 0.046$; Fig. 3A).

Stable cirrhosis and healthy individuals: The proportion of detectable NHVs in individuals with AD of cirrhosis was much higher than that in individuals with stable cirrhosis (45.0% *vs.* 10.0%, $p = 0.007$; Fig. 3B). All 10 healthy individuals showed a complete absence of the NHV signature.

Clinical correlates of the viral signature in AD of cirrhosis

Unproven infection: Of the 64 individuals with evidence of overt infections, 18 had unproven infections. The details of plasma mNGS detection in these patients are reported in Table S4. Genome fragments of microorganisms were detected in 15 (83.3%) of these patients, and NHVs were found in eight (four with CMV; two with EBV; one with CMV, TTV, and human parvovirus B19; and one with TTV). Empirical antibiotic therapy was administered to all 18 patients, despite there being no pathogen isolated. Of the nine patients who did not respond to the initial empirical antibiotic therapy, eight had genome fragments of circulating microorganisms on mNGS testing. NHVs coexisted with bacteria in four patients (CMV, n = 2; EBV, n = 2) and NHV(s) alone were identified in three patients (CMV, n = 1; TTV, n = 1; CMV, human parvovirus B19, and TTV, n = 1).

Clinical response to antibiotics: Empirical antibiotics were initiated for all patients diagnosed with overt infections; antifungal therapy was initiated in five patients. Clinical response was achieved in 37/64 (57.8%) patients with overt infection; the remaining 27 (42.2%) patients were non-responders. The prevalence of detectable NHVs was higher in non-responders than in responders (70.4% *vs.* 37.8%, $p = 0.010$; Fig. 3C). NHVs were found in two of four patients with positive blood cultures that did not resolve despite targeted anti-bacterial therapy (Table S5). CMV was more frequent in non-responders (13/27, 48.1%) than in responders (5/37, 13.5%, $p = 0.002$; Fig. 3C). No significant difference in the prevalence of detectable NHVs other than CMV was observed between responders and non-responders (Fig. 3C).

Progression from AD to ACLF: Among the 101 individuals with AD without ACLF on admission, 17 (16.8%) had detectable CMV. Of the six patients who progressed to ACLF within 28 days, 5 had detectable CMV DNA on mNGS. As shown in Table 2, these 6 patients showed evidence of infection on admission and were treated empirically with antibiotics. However, none achieved clinical resolution of infection. According to the plasma mNGS detection, one patient had *Klebsiella pneumoniae* infection, and CMV was detected in the remaining 5 patients. Of these 6 patients, 5 died within 90 days.

AD *vs.* ACLF: At admission, 28 patients were diagnosed as having ACLF and 10 had detectable CMV using plasma mNGS. Six patients progressed to ACLF during hospitalization. These 34 individuals with ACLF had a higher proportion of detectable NHVs than those without ACLF (67.6% *vs.* 36.8%, $p = 0.002$; Fig. 3D). The proportions of NHVs were similar among individuals with ACLF, regardless of grade (grade 1, 4/6 [66.7%], grade 2 and 3, 19/28 [67.9%]). Individuals with ACLF had a higher prevalence of detectable CMV than those without ACLF (44.1% *vs.* 12.6%, $p < 0.001$; Fig. 3D), and CMV prevalence increased as the grade of ACLF increased (grade 1, 2/6 [33.3%]; grade 2 and 3, 13/28 [46.4%]).

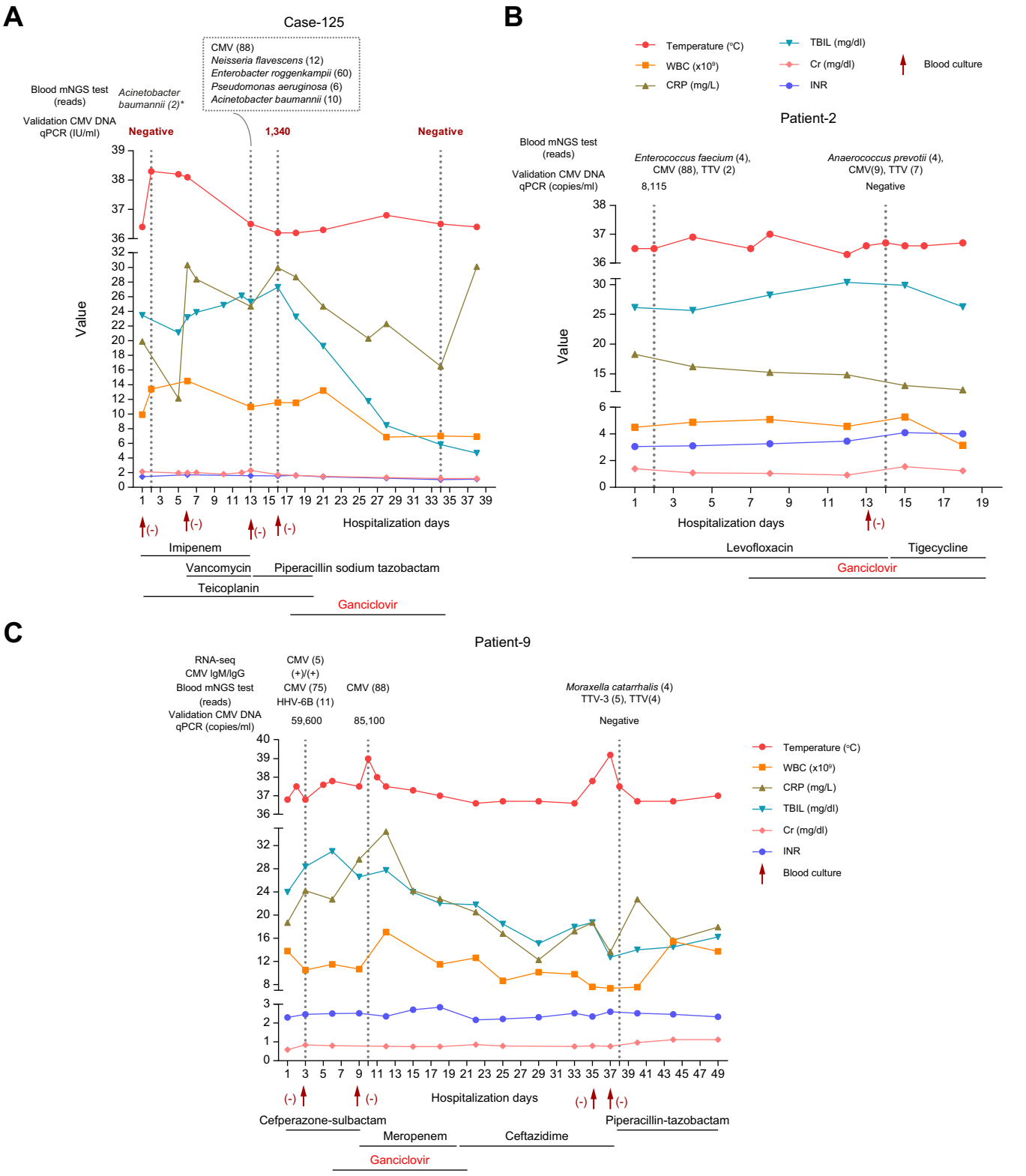

Fig. 4. CMV reactivation cases treated with ganciclovir. Trend of laboratory values and antibiotics administered during the hospitalization of (A) Case-125 in the first series and (B) Patient-2 and (C) Patient-9 in the validation series. *This plasma mNGS detection was performed retrospectively. CMV, human cytomegalovirus; Cr, creatinine; CRP, C-reactive protein; INR, international normalized ratio; mNGS, metagenomic next-generation sequencing; TBIL, total bilirubin; WBC, white blood cell.

Table 3. CMV reactivation diagnosed using plasma cfDNA NGS in the validation cohort.

Patient	ACLF diagnosis	Plasma cfDNA NGS result	Plasma CMV DNA (log_{10} copies/ml)	Urine CMV DNA (log_{10} copies/ml)	CMV pp65 antigenemia	Microbial cfRNA-seq* (reads)	Blood culture	Treatment for CMV	90-day mortality
P1	ACLF-2	TTV, **CMV**	(–)	(–)	Undetected	–	(–)	No	Alive
P2	ACLF-2	*Enterococcus faecium*, **CMV**, TTV	3.91	(–)	(+)	–	(–)	**Yes**	Dead
P3	No-ACLF	**CMV**	4.60	(–)	(–)	–	(–)	No	Alive
P4	ACLF-3	*Citrobacter sedlakii*, **CMV**	5.14	(–)	(+)	–	(–)	No	Dead
P5	ACLF-2	**CMV**	2.74	5.67	(–)	–	(–)	No	Dead
P6	ACLF-2	**CMV**	4.24	2.5	(+)	–	(–)	No	**LT**
P7	No-ACLF	**CMV**	(–)	(–)	(–)	–	(–)	No	Dead
P8	ACLF-2	**CMV,** TTV	2.90	2.82	(–)	CMV (1)	(–)	No	Dead
P9	ACLF-2	**CMV**, HHV-6B	4.78[#]	4.35	(–)	CMV (5)	(–)	**Yes**	Alive
P10	No-ACLF	**CMV**	(–)	(–)	Undetected	–	(–)	No	Alive
P11	No-ACLF	**CMV**	(–)	(–)	(–)	–	(–)	No	Dead
P12	ACLF-2	*Bacteroides*, **CMV**, TTV	(–)	(–)	(+)	–	*Escherichia coli*	No	Alive
P13	ACLF-1	*Acinetobacter baumannii*, *Pneumocystis jirovecii*, **CMV**	3.66	3.90	(+)	–	(–)	No	Dead
P14	No-ACLF	**CMV**	(–)	(–)	(–)	–	n.a.	No	Alive

ACLF, acute-on-chronic liver failure; cfDNA, cell-free DNA; CMV, human Cytomegalovirus; HPyV-6, Human polyomavirus 6; HHV-6B, human beta-herpesvirus 6 B; LT, liver transplantation; n.a., not applicable; TTV, torque teno virus.
*12 samples failed in library construction because of RNA degradation.
[#]This patient had positive CMV IgM and IgG, with positive CMV RNA qPCR.

Viral signature and mortality: No significant differences were observed in the 28-day transplant-free mortality between individuals with detectable NHVs (20.7% *vs.* 11.6%, $p = 0.161$) or CMV (22.2% *vs.* 14.0%, $p = 0.298$) and those without. However, patients that died within 90 days had a higher proportion of detectable CMV than those who survived (38.2% *vs.* 15.1%, $p = 0.005$; Fig. 3E).

Validation of the viral signature and CMV reactivation in individuals with AD of cirrhosis

In the validation cohort, 58 individuals with AD of cirrhosis were prospectively included for plasma mNGS testing. Their baseline characteristics are described in Table 1. All patients in the validation cohort were positive for CMV IgG and 2 (3.4%) patients were positive for CMV IgM. Genome fragments of bacteria, fungi and NHVs were identified in 34 (58.6%) patients. In total, 73 microorganisms were identified; 25 were bacteria, 4 were fungi and 44 were viruses (Fig. S4A-D). There were 22 (37.9%) patients with detectable NHVs and 14 had CMV DNA. NHV (51.5% *vs.* 20.0%, $p = 0.014$) and CMV (36.4% *vs.* 8.0%, $p = 0.012$) were more prevalent in those with ACLF than in those without ACLF, which was consistent with the findings from study cohort (Fig. S4E-F).

All the CMV negative observations using mNGS were further confirmed by plasma qPCR. Of the 14 individuals with detectable CMV using plasma mNGS, 9 (64.2%) cases were further validated by qPCR or CMV pp65 antigenemia testing; eight patients had positive plasma qPCR (log_{10} CMV DNA: 4.1 [IQR 3.1-4.7]), 5 patients had urine qPCR positivity (log_{10} CMV DNA: 3.9 [IQR 2.7-5.0]) and CMV pp65 antigenemia was positive in five patients. One patient was positive for plasma CMV RNA on RT-qPCR. Microbial transcriptome assays were performed in the 14 patients with CMV detectable on NGS. Two patients were CMV positive by RNA-seq while library construction failed in the remaining 12 patients due to RNA degradation of the stored samples. All the CMV negative observations using mNGS were further confirmed by qPCR. Details of the patients with detectable CMV by mNGS test in this validation set are shown in Table 3.

CMV reactivation and ganciclovir therapy

To better understand the clinical relevance of CMV reactivation in individuals with AD of cirrhosis, the clinical outcomes of 3 patients (one from the study cohort and two from the validation cohort) who received exploratory antiviral therapy are described below.

Case-125

A 48-year-old man with cirrhosis attributed to HBV was admitted with jaundice and abdominal distention. At admission, he had ACLF Grade-2. Diagnostic paracentesis revealed ascites and a white cell count of 125 cells/mm^3 (15% neutrophils). Because of the presence of fever and leukocytosis, a diagnosis of unproven infection was considered and imipenem and teicoplanin were initiated empirically, although blood and ascites cultures were negative (Fig. 4A). On day 6, vancomycin was added as fever recurred and liver and renal function deteriorated. He had blood mNGS testing on day 13 and CMV reactivation was identified, which was subsequently validated by qPCR. Ganciclovir was finally started on day 17 and the

patient completed an 18-day course. His clinical condition improved markedly and the repeated qPCR for blood CMV was negative. The patient was discharged and remained well during follow-up.

Patient-2

A 54-year-old man with HBV-related cirrhosis was hospitalized with a history of jaundice for 2 months. The patient had mild ascites and ACLF Grade-2 at admission. Levofloxacin was initiated for pneumonia. As presented in Fig. 4B, his blood mNGS test on day 2 identified *Enterococcus faecium* and CMV, which was validated by qPCR and ganciclovir was started on day 7. The patient completed a 13-day course. Repeated mNGS assay on day 14 suggested the persistence of CMV reactivation, although CMV was undetectable with routine qPCR after a 13-day antiviral therapy prior to his self-discharge.

Patient-9

A 62-year-old man with HBV-related cirrhosis was hospitalized and had ACLF Grade-2 at admission. He was empirically treated with cephalosporins-sulbactam for pneumonia (Fig. 4C). Because the fever was unresolved, treatment was escalated to meropenem on day 9. During his hospitalization, blood cultures on day 3, day 9, day 35 and day 37 were all negative. The blood mNGS test on day 3 identified CMV reactivation, which was confirmed by qPCR. CMV IgM and IgG were both positive. Ganciclovir was started on day 6. After completing a 16-day course of anti-CMV treatment, CMV DNA was negative on qPCR and confirmed with repeat mNGS testing on day 38. The patient showed improvement in his clinical condition with resolution of ACLF, reduction in total bilirubin and model for end-stage liver disease score at the time of his discharge. On 90-day follow-up, the patient remains well.

Discussion

This study detected circulating microorganism genome fragments by plasma NGS in individuals with AD of cirrhosis and observed that NHVs may be involved as precipitants in the occurrence of AD, complicate its course and be potential therapeutic targets. The presence of CMV fragments correlated with the clinical response to empirical antibiotic treatment, progression from AD to ACLF, and 90-day mortality.

Individuals with cirrhosis have immune dysfunction, which is most marked in those with ACLF, reducing their ability to generate adequate immune responses and explaining why infection(s) remain the most important cause of death.[5] Conventional culture-dependent techniques have low capability of identifying pathogens and are therefore unable to provide guidance regarding targeted anti-microbial treatment. Our study suggests that mNGS testing may provide circulating microbiological profiles of patients with poor clinical response to empirical therapy. In our study, three of four patients with positive blood cultures who responded poorly to targeted antibiotics had superinfections identified by plasma mNGS. Fungal infections in individuals with cirrhosis are rare but a strong independent predictor of 30-day mortality (~70%), and delayed diagnosis is common.[15] Plasma mNGS might be able to identify fungal infections that were missed using current microbiological techniques.

One important finding of this study was that the NHV signature observed in individuals with ACLF was similar to that in those with sepsis or hematological malignancies and the NHV signature correlated with clinical outcomes. Plasma mNGS identified various genome fragments of NHVs known to cause chronic infection in humans, such as CMV, EBV, TTV, and human parvovirus B19. In this study, CMV viremia was the most common and was associated with progression of AD. CMV can lead to cholestasis, worsening of liver injury, portal vein thrombosis and pneumonia, which can increase all-cause mortality.[16,17] Whether CMV infection is associated with CMV disease cannot be confirmed in the current study due to the lack of data on specific tissue damage, such as liver or gut histology. As individuals with cirrhosis have defective antiviral immunity,[18] which worsens with the severity of liver disease, it was not surprising that the NHV signature was more marked in those with ACLF than in those without ACLF. The data presented in this study suggest that earlier diagnosis and treatment of CMV may prevent the progression to ACLF, as exhibited in the validation cohort. Poor clinical response to empirical antibiotic treatment and higher 90-day mortality were observed in individuals with detectable CMV fragments, further consolidating the clinical relevance of CMV viremia in individuals with AD of cirrhosis.

To validate the potential role of CMV reactivation in the progression of AD in cirrhosis, we studied a second cohort of patients. In this validation series, 64.2% of individuals with CMV fragments identified by plasma mNGS were validated using traditional CMV qPCR assays in plasma or urine samples, and a small number of patients also had positive CMV pp65 antigenemia results, which confirmed reactivation of CMV infection. Whether the presence of CMV viremia in individuals with AD was clinically relevant or simply a bystander is difficult to confirm from the current study. However, the observed clinical response to anti-CMV therapy with ganciclovir in three patients suggests a potential causal association between CMV viremia and disease progression in individuals with AD. Nevertheless, a much denser sampling and testing study is needed to provide more compelling evidence that disappearance of the virus was linked to anti-CMV therapy.

Other NHVs were identified by plasma mNGS in individuals with AD, including EBV and HSV, which has previously been shown to be associated with severity of cirrhosis and hepatitis.[19,20] It is reasonable to hypothesize that NHVs act as potential pathogens that complicate the course of AD. CMV, for which there are several approved antiviral drugs, was the most common NHV in our study.[21] Therefore, screening and surveillance for the reactivation of CMV infection might be valuable for individuals with AD. As ganciclovir can lead to neutropenia, further studies will be needed to define which patients would benefit from ganciclovir treatment.

The data from our study suggested that CMV fragments observed in our study are likely due to reactivation, indicated by the detection of CMV antibodies in most patients. However, genomic DNA fragment translocation from the gut cannot be excluded. Individuals with cirrhosis have markedly increased intestinal permeability, which worsens disease severity and leads to microorganism translocation. Recent descriptions of disturbed intestinal fungome and virome argue that translocation of these microorganisms may contribute to the NHV signature observed in this study.[22] It is also interesting to note

that there is a bidirectional relationship between bacterial infection and infection with NHVs.[23] In this study, the prevalence of NHVs was higher in those with proven bacterial infection.

The most prevalent infection in individuals with AD is spontaneous bacterial peritonitis, followed by pneumonia and urinary tract infections.[24] Herein, we detected the microbiological signature in plasma samples using mNGS but other body fluids, such as ascitic fluid and sputum, were not tested. Therefore, plasma mNGS may miss pathogens, which could be the reason why some patients with proven infection diagnosed by traditional approaches had negative plasma mNGS. Importantly, 38.5% of patients in whom multiple genomic fragments of microorganisms were detected using mNGS did not have clinical evidence of infection either immediately or during follow-up. To some extent, our observation was in line with the finding that there is a circulating microbiome in individuals with cirrhosis.[25] Taken together, these data suggest that plasma mNGS results need to be interpreted in clinical context and may serve as an adjunct to currently used techniques rather than a replacement.

The limitations of this study should be considered when interpreting our results. First, untargeted plasma mNGS testing is not truly comprehensive as we focused on microbial cfDNA and RNA viruses, such as hepatitis C and E virus, were not included. The database approach used has limitations, as some members of the Anelloviridae family (which includes TTV) are excluded in the database. Second, the clinical relevance of the NHV signature detected here is still not fully clear as there are several confounding factors in this study. Third, microbial transcriptome assays for plasma cfRNA, which may be more specific to diagnose active infection, could only be performed in a small minority of individuals due to RNA degradation of the stored samples. Nevertheless, the data presented here provide compelling evidence for the potential importance of NHVs in the development and progression of AD.

In summary, this study used an untargeted plasma mNGS approach focusing on the genome fragments of circulating microorganisms and suggested that individuals with AD, particularly those with ACLF, had a marked NHV signature, which might complicate the course of AD. This observation was validated in a separate prospective study. Further refinement and validation are needed to define the clinical relevance of testing for NHVs in the routine management of individuals with AD.

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Abbreviations

AD, acute decompensation; ACLF, acute-on-chronic liver failure; CAID, cirrhosis-associated immune dysfunction; CMV, human cytomegalovirus; cfDNA, cell-free DNA; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HSV, herpes simplex virus; ICU, intensive care unit; mNGS, metagenomic next-generation sequencing; NHV, non-hepatotropic virus; qPCR, real-time PCR; RT-qPCR, quantitative reverse-transcription PCR; TTV, torque teno virus.

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Conflict of interest

Rajiv Jalan is the inventor of OPA, which has been patented by UCL and licensed to Mallinckrodt Pharma. He is also the founder of Yaqrit Discovery, a spin out company from University College London, Hepyx Limited and Cyberliver. He had research collaborations with Yaqrit Discovery. Y. W. and X.W. are employees of BGI-Shenzhen. S.L. and X.N. are employees of Guangzhou Goodwill Clinical Laboratories. B.L., H.C., Z.F. and S.C., Q.H., X.L., Q.L., Y.L., W.L., J. L., X.C., M.L., Y.G., G.L. and J.C. disclose no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

B.L.: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript. H.C.: acquisition of data, analysis and interpretation of data, drafting of the manuscript. Z.F. and S.C.: patients care and participated in data acquisition. Q.H., X.L., Q.L., Y.L., W.L., J. L., X.C., M.L., Y.G. and G.L.: patients care, participated in data acquisition and interpretation. Y. W., X.W., S.L., X.N.: performed the NGS testing, acquisition of sequencing data, analyzed and interpreted metagenome data. R.J.: study concept and design, interpretation of data, critical revision of the manuscript for important intellectual content and approval of the final version of the manuscript. J. C.: study concept and design, interpretation of data, drafting of the manuscript, critical revision of the manuscript, study supervision, obtained funding and approval of the final version of manuscript.

Data availability statement

The data that support the finding of this study are described in this manuscript and available upon reasonable request. Sequencing data of this study (with human reads removed) have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (BioProject accession number PRJNA865093).

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Supplementary data

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