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Original article

Genotype 4 HEV infection triggers the initiation and development of acute pancreatitis

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ABSTRACT

The role of HEV infection in AP remains unclear. 1000 patients with AP and 1000 HCs were enrolled, and pancreatitis was evaluated in HEV-infected rhesus macaques. The positive rates of anti-HEV IgG, IgM, and HEV RNA in the AP patients were significantly higher than HCs. With the increase in the severity of AP, the percentage of HEV infection increased. AP patients were divided into AP- and AP + AHE groups. The percentage of severe AP in the AP + AHE group was significantly higher than in the AP- group. HEV infection was one of the main independent risk factors and had high predictive power for AP outcomes. A high level of HEV titer would prolong the recovery time and increase the risk of recurrent AP. Moreover, AP + AHE patients receiving conservative treatment showed a better prognosis. Furthermore, HEV can replicate in the pancreas of rhesus macaques. The pancreatic islet structure was damaged, the tissue was loose after 272 dpi, and a large amount of hyperemia appeared after 770 dpi. HEV infection also caused a large number of inflammatory cells in the pancreas. The pancreas and liver had a comparable viral load. HEV infection affects AP's occurrence, development, and prognosis.

Abbreviations: AHE, acute hepatitis E; AP, acute pancreatitis; AMY, amylase; BMI, body mass index; CMV, cytomegalovirus; ERCP, endoscopic retrograde cholangiopancreatography; EBV, Epstein–Barr virus; HCs, healthy controls; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; LIP, lipase; OPLS-DA, orthogonal partial least-squares discrimination analysis; RAP, recurrent acute pancreatitis.

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Hepatitis E virus (HEV) belongs to the genus paslahepevirus in the Hepeviridae family. It is a nonenveloped, positive-sense, single-stranded RNA icosahedral virus with a diameter of 27–34 nm [1]. At least 20 million HEV infections are reported annually, including more than 3 million symptomatic cases and approximately 60,000 deaths [2]. There are 4 major human-pathogenic HEV genotypes. Genotypes 1 and 2 are mainly distributed in low-income countries, while genotypes 3 and 4 are common in high-income countries such as Europe, North America, and China [3]. HEV is considered to spread among humans through fecal-oral transmission, contaminated water, or animal hosts. HEV infection may cause symptoms of jaundice, dark urine, fever, nausea, vomiting, and fatigue. Most patients with acute hepatitis E (AHE) caused by genotype 3 and likely also 4 are asymptomatic [4,5].

Hepatitis E is more than solely a liver disease and should be considered a systemic disease. HEV infection can cause many extrahepatic manifestations, including nervous system diseases, renal system diseases, hematological diseases, male infertility, and autoimmune hepatitis [6–8]. In recent years, cases of acute pancreatitis (AP) caused by HEV infection have been reported. A 26-year-old Frenchman with HEV genotype 1 infection who developed AP 3 weeks after being diagnosed with HEV infection was described by Deniel et al. [9]. Similarly, a 70-year-old woman from Europe was also reported to have AP associated with HEV genotype 3 infection [10]. Jung et al. established a small pig model, demonstrating that infection of HEV genotype 3 could lead to pancreatic cell necrosis and AP [11]. So far, few studies have been conducted to assess the relationship between HEV infection and AP. In this study, we aim to investigate whether HEV infection is associated with the occurrence and development of AP and assess its impact on the disease characteristics.

1. Patients and methods

1.1. Patients

As shown in Figure S1, 1000 eligible patients with AP, 1000 healthy controls (HCs), and 300 patients with AHE were recruited from Suzhou Municipal Hospital, the People's Hospital of Jianhu City, the First People's Hospital of Yancheng City, the Second People's Hospital of Yancheng City, Zhejiang provincial Tongde hospital, the First Affiliated Hospital (College of Medicine, Zhejiang University), the Fifth People's Hospital of Wuxi and Linyi Traditional Hospital from January 1, 2016, to May 31, 2021.8 hospitals are located in different regions of China. HCs were all excluded from AP disease history in the recent two years. We judged the possible causes of AP according to the patient's consultation and diagnostic criteria at admission and followed up with all the enrolled patients for 4 months to see if these patients had recurrent AP or HEV infections. The protocol for the present study was endorsed by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (approval number: 2,020,454) and Suzhou Municipal Hospital (approval number: K-2022-080-H01). Informed consent was obtained from all participants or their families.

1.2. Construction of rhesus macaques with HEV infection model

The animal experiment was approved by the Animal Care and Use Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College. All procedures were performed under ketamine anesthesia by trained personnel under the supervision of veterinary staff. Rhesus macaques, negative for HEV RNA or anti-HEV IgG and IgM antibodies, were housed individually and fed with complete formula food. Rhesus macaques intravenously injected with chronic gt4 HEV described in our previous study (macKM01 strain, 5.1×10^5 copies/mL). The livers and pancreas were collected at 272 days post-infection (dpi) and 770 dpi.

Further details regarding the inclusion and exclusion criteria for AHE and AP patients and the methods used were shown in the Supplementary Information.

1.3. Statistical analysis

This study used GraphPad Prism 9, SPSS 22.0, and SIMCA for statistical analysis. The mean ± standard deviation was used for the normal distribution expression measurement data, and two groups were compared using the t-test. We represent the non-normal distribution measurement data by median (quartile), and two groups were compared using the Mann–Whitney U test. We compared the enumeration data between two groups using the $\chi 2$ test. The correlation between anti-HEV IgM (Y-axis) and the recovery time (X-axis) was analyzed to draw the linear regression standard curve. Univariable and multivariable logistic regression analyses were performed to identify AP patients' independent severity and prognosis indicators. Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to evaluate and rank the ability of the parameters with high predictive power for the outcome of AP using SIMCA software. Besides, the Kaplan–Meier analysis evaluated the improvement rate between patients who received hepatoprotective treatment and those who did not. $P < 0.05$ was considered statistically significant.

2. Results

2.1. Baseline characteristics

The baseline characteristics of 1000 AP patients and HCs were shown in Table 1, and there was no significant difference in age, gender, and BMI (all $P > 0.05$). No significant difference existed in anti-HAV IgG, anti-HAV IgM, HBsAg, and HCV-Ab (all $P > 0.05$). The positive rate of anti-HEV IgG in the AP group was significantly higher than in the HCs group (16.1% vs. 12.1%, $P = 0.01$). The positive rate of anti-HEV IgM in the AP group was 5.80%, significantly higher than that of 2.10% in the HCs group ($P < 0.001$). The positive rate of HEV RNA in the AP group was 1.30%, while the positive rate in the HCs group was 0 ($P < 0.001$).

Besides, significant differences were found among several laboratory parameters between these two groups, including WBC, GGT, ALB, TC, TG, AMY, LIP, CRP, LDH, GLU, and Ca^{2+} (all $P < 0.05$). Of note, the levels of AMY and LIP in the AP group were both significantly higher than those in the HCs group [AMY, AP: 519.00 (401.25–659.00) *vs*. HCs: 68.00 (46.25–89.00); LIP, AP: 608.00 (432.75–784.00) *vs*. HCs: 161.00 (90.25–232.00), both $P < 0.001$].

2.2. HEV infection was associated with the occurrence and development of AP

According to AHE diagnostic criteria, 23 of 1000 AP (2.3%) patients were also diagnosed with AHE, including 13 patients positive for HEV RNA and 10 with anti-HEV IgM positive accompanied by rising anti-HEV IgG titers. Then, we divided 1000 AP patients into those accompanied with AHE (AP + AHE group, n = 23) and those without AHE (AP- group, n = 977). The baseline characteristics of 977 AP patients and 23 patients with AHE + AP were compared in Table S1. No significant difference existed in age, gender, and BMI between the AP- and AP + AHE groups. The levels of ALT and GGT in the AP + AHE group were significantly higher than those in the AP- group (both $P < 0.05$). The level of TC in the AP + AHE group was

Table 1
Baseline characteristics of enrolled patients.

Variables	HCs group (n = 1000)	AP group (n = 1000)	*P*
Age (years)	52.00 (44.00–60.00)	53.00 (39.00–68.00)	0.654
Gender (M/F)	492/508	519/481	0.227
BMI	24.24 (21.88–26.27)	24.33 (22.32–26.21)	0.165
WBC ($\times 10^9$/L)	6.84 (5.28–8.55)	13.26 (9.28–16.90)	<0.001
ALT (U/L)	23.30 (11.70–35.40)	23.65 (17.62–31.08)	0.072
GGT (U/L)	22.00 (13.22–35.40)	135.80 (103.75–162.80)	<0.001
TBIL (μmol/L)	10.30 (6.60–14.80)	10.85 (6.40–15.30)	0.619
ALB (g/L)	45.55 (41.62–49.40)	25.95 (21.85–30.10)	<0.001
TC (mmol/L)	4.31 (3.58–4.94)	4.79 (4.05–5.47)	<0.001
TG (mmol/L)	1.36 (0.95–1.80)	3.22 (2.48–3.83)	<0.001
AMY (U/L)	68.00 (46.25–89.00)	519.00 (401.25–659.00)	<0.001
LIP (U/L)	161.00 (90.25–232.00)	608.00 (432.75–784.00)	<0.001
CRP (mg/L)	4.85 (2.39–7.50)	121.08 (74.63–166.60)	<0.001
BUN (mmol/L)	6.60 (5.24–7.82)	6.56 (4.89–7.97)	0.258
LDH (U/L)	165.00 (131.00–194.00)	187.00 (141.25–226.00)	<0.001
CREA (μmol/L)	75.05 (58.72–91.58)	72.55 (57.90–89.68)	0.435
Platelet ($\times 10^9$/L)	202.50 (162.00–243.00)	199.00 (153.00–244.00)	0.117
PT (s)	12.01 (11.53–12.50)	11.90 (9.60–14.20)	0.418
INR	1.03 (0.91–1.21)	1.06 (0.85–1.31)	0.335
GLU (mmol/L)	5.02 (4.46–5.59)	9.02 (6.48–11.44)	<0.001
Ca^{2+} (mmol/L)	2.43 (2.32–2.55)	2.13 (1.95–2.31)	<0.001
Anti-HAV IgG (P/N)	907/93	912/88	0.697
Anti-HAV IgM (P/N)	1/999	3/997	0.625
HBsAg (P/N)	65/935	62/938	0.783
HCV-Ab (P/N)	4/996	5/995	1.000
Anti-HEV IgG (P/N)	121/879	161/839	0.010
Anti-HEV IgM (P/N)	21/979	58/942	<0.001
HEV RNA (P/N)	0/1000	13/987	<0.001

Note: BMI: body mass index; WBC: white blood cell; ALT: alanine aminotransferase; GGT: γ-glutamyl transpeptidase; TBIL: total bilirubin; ALB: albumin; TC: total cholesterol; TG: triglyceride; AMY: amylase; LIP: lipase; CRP: C-reactive protein; BUN: blood urea nitrogen; LDH: lactate dehydrogenase; CREA: creatinine; PT: prothrombin time; INR: international normalized ratio; GLU: glucose.

significantly lower than that in the AP- group ($P < 0.05$). Besides, no significant difference was found between these two groups in WBC, TBIL, ALB, TG, AMY, LIP, CRP, BUN, LDH, CREA, platelet, PT, INR, GLU, and Ca^{2+} (all $P > 0.05$).

Moreover, no significant difference existed in anti-HAV IgG, anti-HAV IgM, HBsAg, and HCV-Ab between the AP- and AP + AHE groups (all $P > 0.05$). The percentage of patients with severe AP in the AP + AHE group was significantly higher than that in the AP- group (43.48% *vs*. 13.31%, $P < 0.001$), and the percentage of patients with mild AP in the AP + AHE group was significantly lower than that in the AP- group (17.39% *vs*. 56.81%, $P < 0.001$), while the percentage of moderate AP patients showed no significant difference between these two groups ($P > 0.05$). Of the 23 AP patients with AHE, most were asymptomatic. No liver failure, chronic hepatitis E, and neurological manifestations were observed, according to the recorded information.

To further assess the relationship between HEV infection and disease progression of AP, 1000 AP patients were divided into mild AP group (n = 559), moderate AP group (n = 301), and severe AP group (n = 140) based on the severity of AP. As shown in Table S2, the clinical characteristics were compared among the mild AP, moderate AP, and severe AP groups. The percentage of HEV infection in the severe AP group was significantly higher than in the moderate AP group (7.14% *vs*. 2.99%, $P < 0.05$). Similarly, the percentage of HEV infection in the moderate AP group was significantly higher than in the mild AP group (2.99% *vs*. 0.72%, $P < 0.05$).

12 patients were considered to have AP potentially associated with HEV infection after excluding common AP triggers such as intravenous drug use, blood transfusion, surgical history, gallstones, peptic ulcer, abdominal trauma, alcohol abuse, and drug use. The demographic, clinical, and diagnostic features of 12 AP patients potentially associated with HEV infection are shown in Table S3. Of these 12 patients, 9 were diagnosed with severe AP, 3 with moderate AP, and no with mild AP. Genome sequencing showed that 6 of 12 AP patients potentially associated with HEV infection had HEV genotypes 3 and 4 (Table S4). After receiving the conservative treatment, including protecting the liver, reducing enzymes, and eliminating jaundice, the treatment of 7 patients was markedly effective. The treatment of 3 patients was effective, while the treatment of 2 patients was ineffective.

In 12 patients with AP potentially associated with HEV infection, anti-HEV IgM titers at admission in the 9 severe AP patients were significantly higher than those in the 3 moderate AP patients (11.07 ± 5.07 *vs*. 2.90 ± 1.09, $P = 0.023$; Fig. 1A). The levels of ALT in the severe AP patients were also significantly higher than those in the moderate AP patients (871.68 ± 370.45 *vs*. 197.73 ± 61.53, $P < 0.001$), while the levels of TBIL showed no significant difference between the severe and moderate AP patients (15.78 ± 10.97 *vs*. 12.77 ± 4.82, $P > 0.05$).

2.3. HEV infection was related to the poorer outcome of AP

To explore the relationship between HEV infection and the outcome of AP patients, 1000 AP patients were divided into the markedly effective group (n = 746), an effective group (n = 209), and the ineffective group (n = 45). The multivariable logistic regression analysis showed that AMY, LIP, BUN, Ca^{2+}, BMI, TG, LDH, and HEV infection were the main independent risk factors for the outcome of AP patients (all $P < 0.05$; Table 2).

In addition, OPLS-DA was also used to rank and assess the risk factors for the outcome of AP patients. The results showed that the difference could be clearly distinguished among the markedly effective, effective, and ineffective groups (Fig. 1F and G).

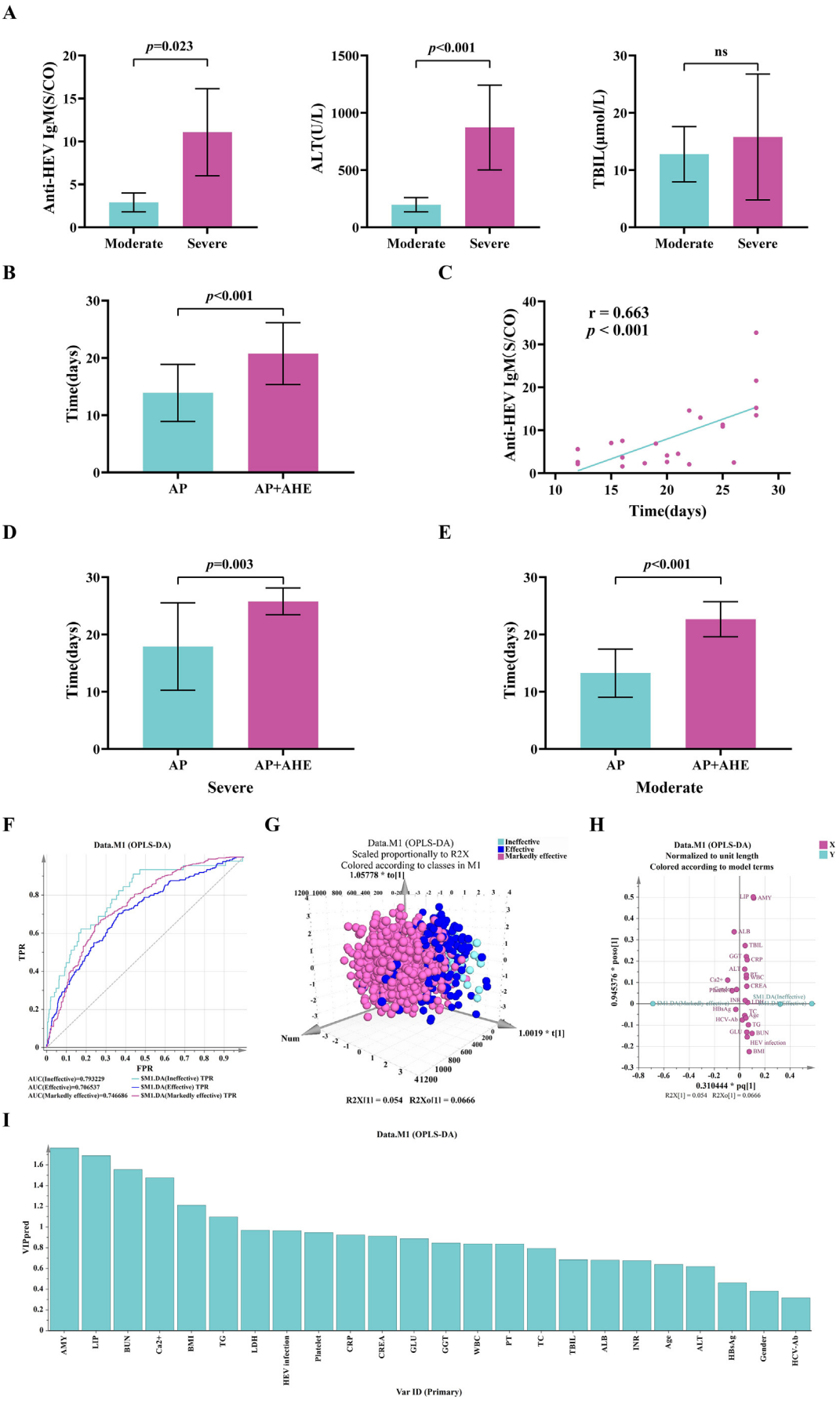
A
p=0.023
Anti-HEV IgM(S/CO)
Moderate
Severe
p<0.001
ALT(U/L)
ns
TBIL(μmol/L)
B
p<0.001
Time(days)
AP
AP+AHE
C
r = 0.663
p < 0.001
Anti-HEV IgM(S/CO)
Time(days)
D
p=0.003
Severe
E
p<0.001
Moderate
F
Data.M1 (OPLS-DA)
TPR
FPR
AUC(Ineffective)=0.793229
AUC(Effective)=0.706537
AUC(Markedly effective)=0.746686
SM1.DA(Ineffective) TPR
SM1.DA(Effective) TPR
SM1.DA(Markedly effective) TPR
G
Data.M1 (OPLS-DA)
Scaled proportionally to R2X
Colored according to classes in M1
Ineffective
Effective
Markedly effective
R2X[1] = 0.054 R2Xo[1] = 0.0666
H
Data.M1 (OPLS-DA)
Normalized to unit length
Colored according to model terms
X
Y
R2X[1] = 0.054 R2Xo[1] = 0.0666
I
Data.M1 (OPLS-DA)
VIPpred
AMY
LIP
BUN
Ca2+
BMI
TG
LDH
HEV infection
Platelet
CRP
CREA
GLU
GGT
WBC
PT
TC
TBIL
ALB
INR
Age
ALT
HBsAg
Gender
HCV-Ab
Var ID (Primary)

Table 2
Characteristics at admission according to outcomes for the 1000 AP patients.

Variables	Markedly effective (n = 746)	Effective (n = 209)	Ineffective (n = 45)	*P*	Univariate analysis		Multivariate analysis	
					OR (95% CI)	*P*	OR (95% CI)	*P*
Age (<53.00, %)	384 (51.47)	101 (48.33)	14 (31.11)	0.026	0.756 (0.569–1.005)	0.054		
Gender (Male, %)	394 (52.82)	103 (49.28)	22 (48.89)	0.610	0.867 (0.653–1.150)	0.322		
BMI (<24.33, %)	398 (53.35)	80 (38.28)	21 (46.67)	0.001	0.591 (0.443–0.787)	<0.001	0.521 (0.382–0.710)	<0.001
WBC (<13.26, %)	388 (52.01)	95 (45.45)	17 (37.78)	0.060	0.720 (0.541–0.957)	0.023		
ALT (<23.65, %)	382 (51.21)	102 (48.80)	16 (35.56)	0.116	0.791 (0.596–1.051)	0.106		
GGT (<135.80, %)	389 (52.14)	92 (44.02)	19 (42.22)	0.065	0.713 (0.536–0.948)	0.020		
TBIL (<10.85, %)	387 (51.88)	94 (44.98)	19 (42.22)	0.119	0.742 (0.558–0.986)	0.040		
ALB (<25.95, %)	358 (47.99)	115 (55.02)	27 (60.00)	0.077	1.382 (1.040–1.837)	0.026	1.886 (1.378–2.583)	<0.001
TC (<4.79, %)	384 (51.47)	99 (47.37)	13 (28.89)	0.010	0.720 (0.541–0.957)	0.024	0.679 (0.499–0.923)	0.013
TG (<3.22, %)	393 (52.68)	89 (42.58)	16 (35.56)	0.005	0.628 (0.471–0.836)	0.001	0.600 (0.441–0.816)	0.001
AMY (<519.00, %)	409 (54.83)	77 (36.84)	14 (31.11)	<0.001	0.459 (0.342–0.615)	<0.001	0.636 (0.414–0.976)	0.038
LIP (<608.00, %)	409 (54.83)	78 (37.32)	13 (28.89)	<0.001	0.456 (0.340–0.612)	<0.001	0.548 (0.359–0.837)	0.005
CRP (<121.08, %)	393 (52.68)	90 (43.06)	17 (37.78)	0.012	0.650 (0.489–0.866)	0.003	0.712 (0.520–0.976)	0.035
BUN (<6.56, %)	402 (53.89)	84 (40.19)	13 (28.89)	<0.001	0.521 (0.390–0.696)	<0.001	0.506 (0.371–0.690)	<0.001
LDH (<187.00, %)	391 (52.41)	89 (42.58)	19 (42.22)	0.024	0.674 (0.507–0.897)	0.007	0.696 (0.512–0.947)	0.021
CREA (<72.55, %)	390 (52.28)	93 (44.50)	17 (37.78)	0.034	0.691 (0.520–0.919)	0.011		
Platelet (<199.00, %)	355 (47.59)	118 (56.46)	26 (57.78)	0.043	1.439 (1.082–1.914)	0.012		
PT (<11.90, %)	378 (50.67)	90 (43.06)	18 (40.00)	0.075	0.719 (0.540–0.956)	0.023		
INR (<1.06, %)	381 (51.07)	97 (46.41)	18 (40.00)	0.206	0.785 (0.591–1.043)	0.095		
GLU (<9.02, %)	391 (52.41)	88 (42.11)	21 (46.67)	0.028	0.692 (0.520–0.920)	0.011	0.674 (0.496–0.915)	0.011
Ca^{2+} (<2.13, %)	343 (45.98)	125 (59.81)	30 (66.67)	<0.001	1.851 (1.387–2.471)	<0.001	1.921 (1.410–2.617)	<0.001
HBsAg (N, %)	695 (93.16)	199 (95.22)	44 (97.78)	0.382	1.648 (0.844–3.218)	0.143		
HCV-Ab (N, %)	743 (99.60)	208 (99.52)	44 (97.78)	0.264	0.415 (0.075–2.307)	0.315		
HEV infection (N, %)	734 (98.39)	202 (96.65)	41 (91.11)	0.007	0.321 (0.145–0.710)	0.005	0.260 (0.109–0.620)	0.002

Note: BMI: body mass index; WBC: white blood cell; ALT: alanine aminotransferase; GGT: γ-glutamyl transpeptidase; TBIL: total bilirubin; ALB: albumin; TC: total cholesterol; TG: triglyceride; AMY: amylase; LIP: lipase; CRP: C-reactive protein; BUN: blood urea nitrogen; LDH: lactate dehydrogenase; CREA: creatinine; PT: prothrombin time; INR: international normalized ratio GLU: glucose.

Encouragingly, HEV infection was also the main indicator with high predictive power (Fig. 1H and I).

2.4. HEV infection prolonged the hospital stay of AP patients

We further compared the recovery time between the AP + AHE group (n = 23) and the AP group (n = 977). It was found that the average recovery time in the AP group was significantly lower than that in the AP + AHE group (13.89 ± 4.98 days *vs*. 20.74 ± 5.40 days, $P < 0.001$; Fig. 1B). In the AP + AHE group, anti-HEV IgM titers at admission were proportional to the recovery time (r = 0.663, $P < 0.001$; Fig. 1C).

In 12 patients with AP potentially associated with HEV infection, the average recovery time of severe AP patients was significantly higher than that of severe AP patients in the AP- group (25.78 ± 2.33 days *vs*. 17.89 ± 7.63 days, $P = 0.003$; Fig. 1D), and the average recovery time of moderate AP patients was also significantly higher than that of moderate AP patients in the AP- group (22.67 ± 3.06 days *vs*. 13.25 ± 4.21 days, $P < 0.001$; Fig. 1E).

2.5. HEV infection was related to a higher risk of RAP

To assess the relationship between HEV infection and RAP, we compared the frequency of RAP in patients for anti-HEV IgG, anti-HEV IgM, and HEV RNA with those who were negative. 1000 AP patients were divided into the anti-HEV IgG-positive group (n = 161) and -negative group (n = 839), anti-HEV IgM-positive group (n = 58) and -negative group (n = 942), and HEV RNA-positive group (n = 13) and -negative group (n = 987). The results showed no significant difference between the anti-HEV IgG-positive and -negative groups, nor between the anti-HEV IgM-positive and -negative groups (Both $P > 0.05$; Fig. 2A). However, the percentage of RAP patients in the HEV RNA-positive group was significantly higher than that in the HEV RNA-negative group (53.85% *vs*. 20.97%, $P = 0.01$).

2.6. Timely hepatoprotective treatment contributed to the improvement in AP + AHE patients

Among the 23 AP + AHE patients, some presented mild hepatitis symptoms and did not receive conservative treatment, according to the medical records. To assess the effect of conservative treatment, 23 AP + AHE patients were divided into the treatment group (n = 15) and the non-treatment group (n = 8), depending on whether patients received treatment. The results showed that the improvement rate in the treatment group was significantly higher than that in the non-treatment group (HR = 6.275, $P < 0.0001$; Fig. 2B). Besides, the incidences of complications of diabetes and peripancreatic effusion in the treatment group were significantly lower than those in the non-treatment group (Diabetes: 13.33% *vs*.

Fig. 1. A high level of HEV titer aggravated AP and was related to poorer outcomes of AP. (A) Anti-HEV IgM titers, ALT and TBIL levels in the 9 severe and 3 moderate AP patients potentially associated with HEV infection (B) The average recovery time in the AP- and AP + AHE groups (C) The correlation between anti-HEV IgM titers and the recovery time in the AP + AHE group (D) The average recovery time of severe AP patients in 12 with AP potentially associated with HEV infection and the AP- group (E) The average recovery time of moderate AP patients in 12 with AP potentially associated with HEV infection and the AP- group (F) The receiver operating characteristics of OPLS-DA among the markedly effective, effective and ineffective groups (G) In the OPLS-DA model, the predictive component was employed to distinguish the markedly effective, effective and ineffective groups through the three-dimensional scatter plot (H) The relation of parameters to the predictive component and the first orthogonal component was revealed in the loading plot (I) Higher predicted VIP pred value on the left.

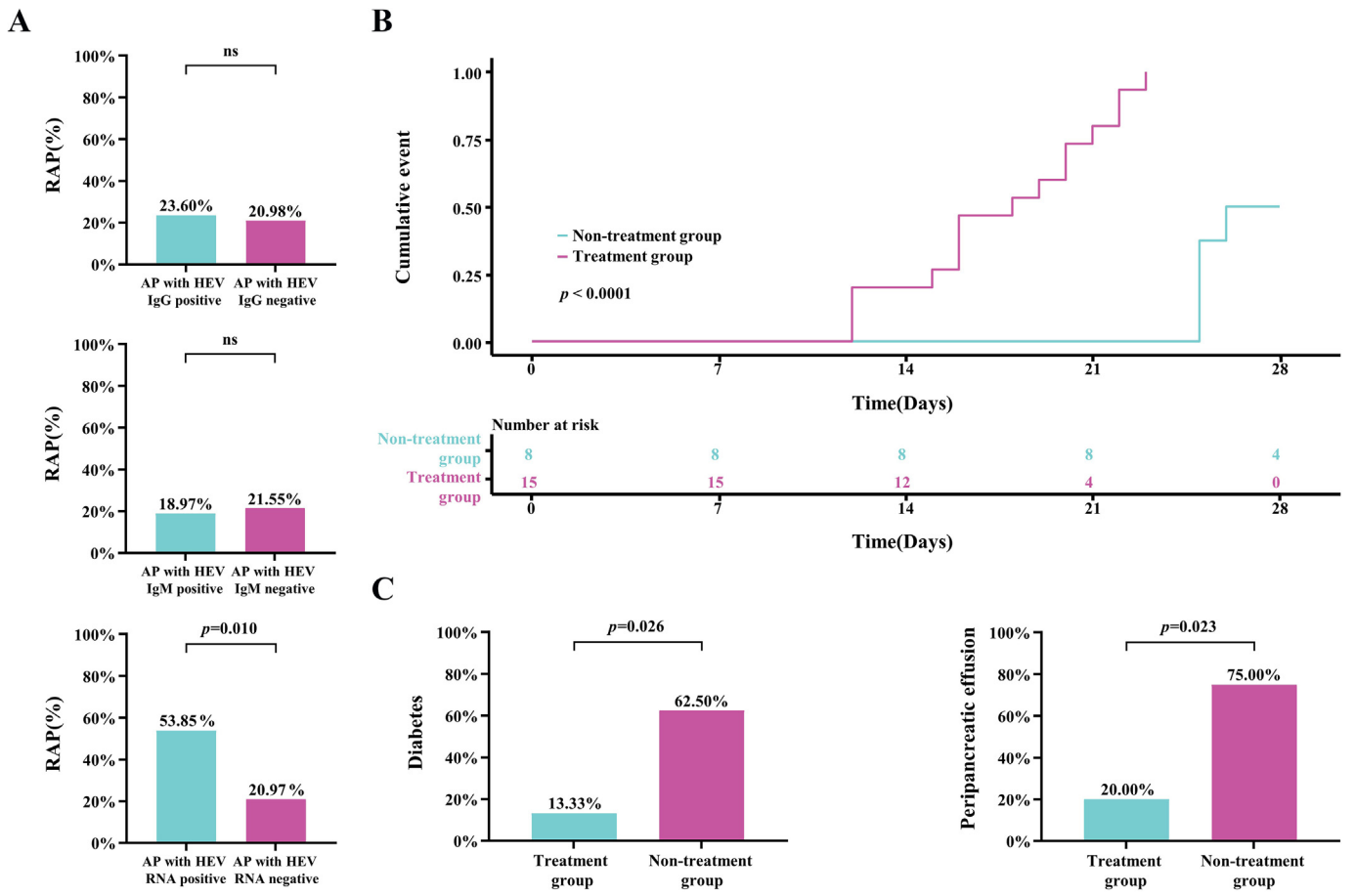

Fig. 2. A high level of HEV titer increased the risk of RAP, and conservative liver protection treatment contributed to the improvement. (A) The percentage of RAP patients in the AP + anti-HEV IgG-positive and negative groups, AP + anti-HEV IgM-positive and negative groups, and AP + HEV RNA-positive and negative groups (B) The improvement rate in the treatment and non-treatment groups (C) The incidences of diabetes and peripancreatic effusion in the treatment and non-treatment groups.

62.50%, $P = 0.026$; Peripancreatic effusion: 20.00% *vs.* 75.00%, $P = 0.023$; Fig. 2C).

2.7. Case presentations of AP in AHE patients

We retrospectively enrolled 300 AHE patients from the 8 hospitals. Among them, 5 patients were diagnosed with AP, including 1 case of mild AP, 3 of moderate AP, and 1 of severe AP. After excluding intravenous drug use, blood transfusion, surgical history, gallstones, peptic ulcer, abdominal trauma, alcohol abuse, and drug use, 2 patients with AP were considered potentially associated with HEV infection. Genome sequencing also showed that the two AHE patients had HEV genotype 4. The timeline of two cases with acute pancreatitis is revealed in Fig. 3. The details are as follows.

2.7.1. Case 1

A 54-year-old female farmer was admitted to the hospital with fever, fatigue, and nausea symptoms. The patient had no recent history of alcohol or drug abuse. Routine detection after admission found serum TBIL 286 μmol/L, DBIL 214 μmol/L, ALT 1493 U/L, AST 1215 U/L, GGT 48 U/L, and leukocytes ($4.34*10^9$/L). Tests were negative for HAV, HBV, HCV, cytomegalovirus (CMV), and Epstein–Barr virus (EBV). Serological tests also showed that anti-HEV IgM was positive (titer 1:28.9), anti-HEV IgG negative, and HEV RNA positive (20,918.83). She was diagnosed with AHE. The patient was given conservative treatment. Five days after admission, the patient presented with severe, persistent upper abdominal pain accompanied by vomiting and abdominal distention.

Further laboratory examination showed that serum amylase and lipase levels were 723.0 U/L and 1207.3 U/L, respectively. Imaging examinations showed liver enlargement, normal gallbladder and bile ducts, pancreatic edema, ascites, and pleural effusion. According to the AP classification, and excluding biliary disease, alcohol abuse, hypertriglyceridemia, hypercalcemia, drug use, trauma, family history, or recurrent pancreatitis, the case was finally considered moderate AP potentially associated with HEV infection.

The patients received conservative treatment and medical treatment observation, including fasting, anti-infection, inhibition of pancreatic secretion, and parenteral nutrition support. Fever, fatigue, nausea, and other symptoms associated with HEV infection were significantly improved, and jaundice subsided. The abdominal symptoms were also alleviated, including vomiting and upper abdominal pain. Eleven days after admission, serum amylase and lipase were near normal at 35.5 U/L and 103.6 U/L, respectively. Serum ALT and TBIL were 105 U/L and 36.8 μmol/L, respectively. On day 15 after admission, serum levels of TBIL, ALT, amylase, and lipase all normalized, and anti-HEV IgM and HEV RNA were both negative, while anti-HEV IgG was positive (titer 1: 9.1). Hence, the patient was approved for discharge. No recurrence of AHE or AP was observed after 4 months of follow-up.

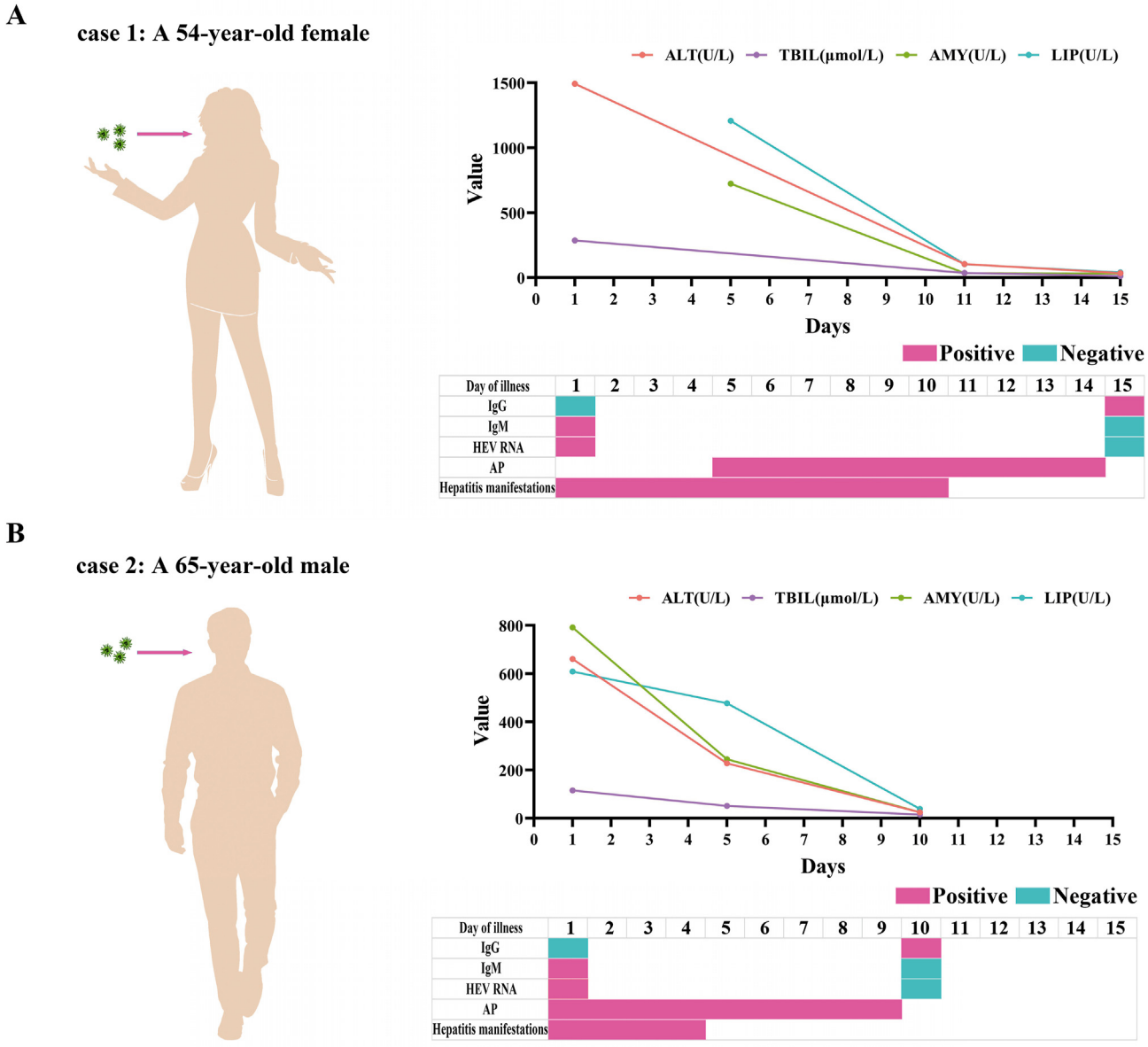

Fig. 3. Timeline of two cases of acute pancreatitis in AHE patients. Timeline of the laboratory parameters, HEV antibody levels, AP occurrence, and acute hepatitis symptoms of cases 1 (A) and 2 (B).

2.7.2. Case 2

A 65-year-old male patient was hospitalized with repeated vomiting, fever, anorexia, and upper abdominal pain. Routine tests after admission found TBIL 115.6 μmol/L and ALT 661 U/L. Serum amylase and lipase were 791.4 U/L and 608.3 U/L, respectively. Serological tests for HAV, HBV, HCV, CMV, and EBV were negative. Serological tests also showed that anti-HEV IgM was positive (titer 1: 26.6), anti-HEV IgG negative, and HEV RNA positive (804.26). Imaging examination confirmed liver enlargement, pancreatic edema, ascites, and pleural effusion. The patient had no history of intravenous drug use, blood transfusion, surgical treatment, gallstones, peptic ulcer, abdominal trauma, alcohol abuse, and recent drug use. The diagnosis was AHE complicated with moderate AP.

After 5 days of conservative treatment and medical treatment observation, liver function tests showed that TBIL and ALT had returned to 51 μmol/L and 228 U/L, respectively. The vomiting and abdominal pain symptoms were significantly relieved, and appetite gradually recovered. The serum amylase level decreased to 244.7 U/L and lipase to 477.3 U/L. On day 10 after admission, serum levels of TBIL, ALT, amylase, and lipase normalized. Serum anti-HEV IgM and HEV RNA were negative, while anti-HEV IgG was positive (titer 1: 6.3). The patient was discharged from the hospital. During the 4-month follow-up, the patient had no recurrence of AHE or AP.

2.8. Pancreatitis in rhesus macaques infected with HEV

To further study the manifestations of pancreatitis after HEV infection, we constructed rhesus macaques with the HEV infection model. First, we used ISH to detect the hybridization of HEV ORF3 RNA in the pancreas of rhesus macaques. The results showed that HEV RNA increased in the pancreas with the increase in infection time, indicating that HEV replicates in the pancreas (Fig. 4A). IHC was used to detect pancreatic antigen levels, which found that the distribution of HEV in the pancreas gradually expanded with the passage of infection time (Fig. 4A). HE staining showed that after 272 dpi of HEV infection, the structure of the pancreatic islets was

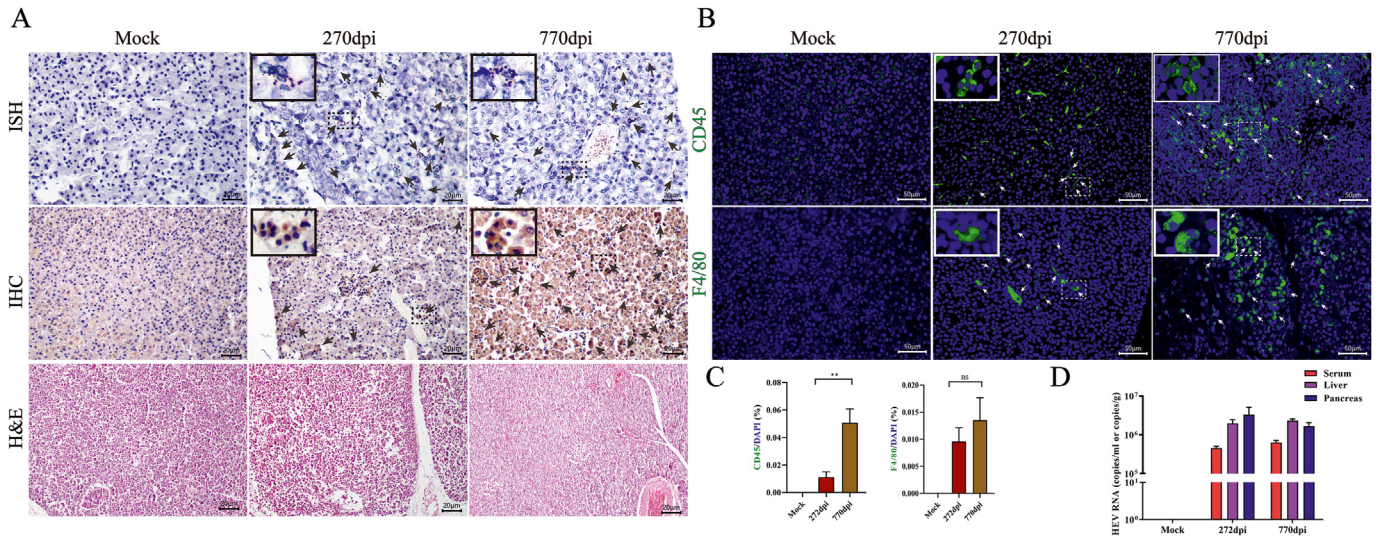

Fig. 4. Pancreatitis in rhesus macaques infected with HEV. (A) ISH showed that HEV RNA increased in the pancreas with the increase of infection time; IHC found that the distribution of HEV in the pancreas was gradually expanding with the passage of infection time; HE staining showed that after 272 dpi of HEV infection, the structure of the pancreatic islets was damaged and the tissue was loose. After 770 dpi, a large amount of hyperemia appeared in the pancreatic islets (B) IFA showed that CD45 and F4/80 positive particles in the pancreas increased significantly with the increase of infection time (C) Quantitative analysis also showed there were significant statistical differences of CD45 and F4/80 accompanied by increased infection time (D) Quantitative detection of HEV RNA in the serum, liver, and pancreas of rhesus macaques infected with HEV revealed that the pancreas and liver had a comparable viral load.

damaged, and the tissue was loose. After 770 dpi, a large amount of hyperemia appeared in the pancreatic islets, indicating that HEV infection caused pancreatitis (Fig. 4A).

IFA showed that CD45 and F4/80 positive particles in the pancreas increased significantly with the increase in infection time (Fig. 4B). Quantitative analysis also showed significant statistical differences between CD45 and F4/80 accompanied by increased infection time (Fig. 4C), indicating that rhesus macaques infected with HEV caused many inflammatory cells in the pancreas. Finally, quantitative detection of HEV RNA in the serum, liver, and pancreas of rhesus macaques infected with HEV revealed that the pancreas and liver had a comparable viral load, indicating that the pancreas was a new replication site for HEV (Fig. 4D).

3. Discussion

AP is an inflammatory pancreatic disease with substantial morbidity and mortality [12]. It is reported that the incidence of AP is 34 cases per 100,000 people worldwide and is on the rise [13]. Common causes of AP include gallstones, alcohol abuse, hypertriglyceridemia, hypercalcemia, drug use, and trauma [14,15]. In recent years, viral hepatitis has been considered a possible cause of AP. A 20-year-old woman was reported to have gallbladder sludge and AP caused by acute hepatitis A [16]. A 70-year-old woman from Brazil was also reported to have developed AP during HCV infection [17]. In 1999, Mishra et al. first reported a case of HEV infection complicated with AP, which indicated the association between the two diseases [18]. A single-center study from India also showed that 2.1% of AP patients had evidence of concomitant HEV infection [19]. In a prospective study, Jain et al. reported 4 cases (7.4%) diagnosed with HEV-related AP after a follow-up of 54 HEV-infected patients [20]. Previously, our team has identified many HEV-related extrahepatic manifestations in the early stage, focusing on neurological and autoimmune diseases [21,22]. Here, we explored the relationship between HEV infection and AP. This study is the first to investigate the relationship between HEV infection and AP through a multicentre clinical cohort.

Firstly, we found that the positive rates of anti-HEV IgG, anti-HEV IgM, and HEV RNA in the AP group were significantly higher than those in the HCs group. 23 AP patients were accordingly diagnosed with HEV infection. After excluding other common AP causes, AP was potentially associated with HEV infection in 12 patients, which implied that the existence of HEV infection could cause the occurrence of AP. Besides, the percentage of HEV infection in the severe AP group was significantly higher than that in the moderate AP group, consistent with the comparison between the moderate AP and mild AP groups. In 12 patients with AP potentially associated with HEV infection, the severity of AP was associated with a high level of HEV titer. It was speculated that the more severe the HEV infection, the more severe AP. Hence, HEV infection was also associated with the development of AP.

Secondly, we further explored the relationship between HEV infection and the outcome of AP patients. The multivariable logistic regression analysis showed that the main independent risk factors for the outcome of AP patients included AMY, LIP, BUN, Ca^{2+}, BMI, TG, LDH, and HEV infection. In several studies, AMY, LIP, BUN, Ca2+, BMI, TG, and LDH have been closely related to AP patients' prognosis [23–28]. HEV infection was considered a novel factor that can influence the outcome of AP patients. To rank the assess the risk factors for the outcome of AP patients, OPLS-DA was performed.

Interestingly, HEV infection was also considered the main indicator owning the high predictive power for the outcome of AP patients. It was concluded that HEV infection may influence the therapeutic effect of AP patients. In other words, HEV infection can result in poorer outcomes in AP patients.

Thirdly, the recovery time between the AP + AHE and AP groups was compared. The average recovery time in AP patients with HEV infection was significantly higher than in AP patients without HEV infection. In 23 AP + AHE patients, anti-HEV IgM titers at admission were proportional to the recovery time, indicating that the more severe the HEV infection, the more severe AP, leading to more recovery time. In 12 patients with AP potentially associated with HEV infection, the average recovery time of severe AP patients was significantly higher than that of severe AP patients in the AP- group, as the same in the moderate AP patients. The high HEV titer level will prolong AP patients' recovery time.

We next evaluated the percentages of RAP patients among patients positive for anti-HEV IgG, anti-HEV IgM, and HEV RNA

compared to negative patients. There was no significant difference between the anti-HEV IgG-positive and -negative groups, nor between anti-HEV IgM-positive and -negative groups. The percentage of RAP patients in the HEV RNA-negative group was 20.97%, consistent with the reported RAP rate of approximately 20% [29,30]. Nevertheless, the percentage of RAP patients in the HEV RNA-positive group reached 53.85%, significantly higher than that in the -negative group, indicating that AP patients with HEV infection may be more likely to develop RAP.

In 23 AP + AHE patients, 15 received conservative treatment, while 8 did not. Those receiving conservative treatment got better faster than those who did not. Moreover, 23% of patients were reported to be diagnosed with diabetes after AP. Therefore, the incidence of diabetes may also be considered a prognostic factor in AP patients [31]. However, the incidences of diabetes and peripancreatic effusion in the patients who did not receive treatment reached 62.5% and 75%, respectively, the incidences of complications of diabetes and peripancreatic effusion were significantly reduced in patients receiving conservative treatment. In AP + AHE patients, given conservative treatment may promote the improvement of the patient's condition.

We further retrospectively analyzed 300 AHE patients. There was 1 case of mild AP, 3 of moderate AP, and 1 of severe AP. After excluding the common causes that could have led to AP, two patients were considered to have AP potentially associated with HEV infection, both with moderate AP. Patients with AP occurred 5 days after admission in one patient and simultaneously after admission in the other. After conservative liver protection treatment and medical treatment observation, the symptoms of AHE were significantly relieved. The liver function of patients gradually recovered, while pancreatitis-related indexes and symptoms were significantly alleviated until recovery. In the 4-month follow-up after discharge, neither patient had a recurrence of AHE or AP.

Finally, we constructed rhesus macaques with HEV infection model to assess pancreatitis in rhesus macaques infected with HEV. Both ISH and IHC showed that HEV replicates in the pancreas. HE staining showed that the structure of the pancreatic islets was damaged, and the tissue was loose after 272 dpi of HEV infection. After 770 dpi, a large amount of hyperemia appeared in the pancreatic islets, indicating that HEV infection caused pancreatitis. Both IFA and Quantitative analysis of CD45 and F4/80 showed that rhesus macaques infected with HEV caused many inflammatory cells in the pancreas. Quantitative detection of HEV RNA in the serum, liver, and pancreas of rhesus macaques infected with HEV revealed that the pancreas and liver had a comparable viral load, indicating that the pancreas was a new replication site for HEV.

The mechanism of HEV infection mediating AP has always been a concern. Several studies demonstrated that the direct cytopathic effect and immune-related damage on the pancreatic acinar cells are plausible explanations supported by HBV infection [18,32]. HBsAg was found in the pancreatic juice and acinar cells [33,34]. Moreover, HBV was reported to infect and replicate in human pancreatic acinar cells [35]. The relationship between HBV infection and AP may provide deep insight into the mechanism of HEV infection mediating AP. Lysosomal enzymes from the infected liver are released into the circulation and activate trypsinogen to trypsin from virus-damaged acinar cell membranes, which may also cause pancreatic injury [36,37]. Besides, HEV genotype 3 infection can cause inflammation of pancreatic cells, leading to AP, which has been demonstrated in a small pig model [11]. Although our study has revealed that HEV infection plays an important role in AP's occurrence, development, and prognosis, the specific mechanism of HEV infection in the occurrence and development of AP needs to be clarified, and more studies are needed.

AHE patients usually do not require antiviral therapy. Most of them will receive hepatoprotective treatment without progressing to liver failure [38]. None of the AP + AHE patients received antiviral ribavirin treatment since no patients had liver failure. It has been reported that antiviral ribavirin treatment for HEV infection may cause AP [39]. Hence, our study has ruled out AP caused by antiviral ribavirin treatment. It also suggests whether patients with AP caused by HEV can be treated with antiviral ribavirin treatment when they progress to severe cases needs further investigation.

In conclusion, our results suggest that HEV infection plays an important role in AP's occurrence, development, and prognosis. These findings will have implications for managing AP patients complicated with AHE. Furthermore, timely screening and treatment of HEV infection in AP patients are recommended.

Author contribution statement

JW and FH is the guarantor of this work; JW, LH, ZX, FH, BJ, CC, and PD experimental design, acquisition of data, analysis, and interpretation of data, drafting of the manuscript, statistical analysis; CG, LT, BL, DW, GL, FJ, CJ, and XJ collected serum and completed the follow-up; MZ, PX, and YY: acquisition of data, statistical analysis, critical revision of the manuscript; FH, JW, and PD. obtained financial support for this study, study concept and design, study supervision, and critical revision of the manuscript for important intellectual content.

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Ethics approval

The protocol for the present study was endorsed by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (approval number: 2,020,454) and Suzhou Municipal Hospital (approval number: K-2022-080-H01). Informed consent was obtained from all participants or their families. The animal experiment was approved by the Animal Care and Use Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College.

Data availability statement

All data relevant to the study are included in the article.

Declaration of competing interest

The authors have declared no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105190.

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