Research Article
Viral Hepatitis

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Viral clade is associated with severity of symptomatic genotype 3 hepatitis E virus infections in Belgium, 2010–2018

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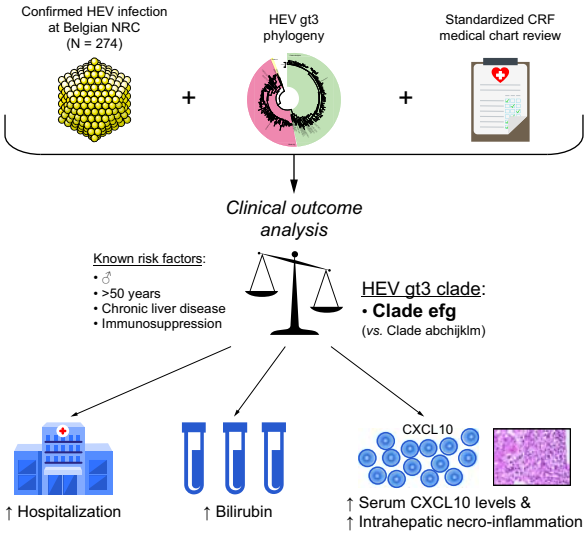
Graphical abstract



Highlights

- This is a study of clinical outcome, CXCL10 level, and viral phylogenetic analysis of 274 HEV infections in Belgium.
- HEV gt3 clade is the strongest predictor for outcomes in symptomatic infections.
- HEV gt3 clade efg is linked to higher hospitalisation rates, peak bilirubin levels, serum CXCL10 levels, and liver necro-inflammatory activity.

Impact and implications

HEV genotype (gt) 3 infections display a wide spectrum of clinical presentations currently ascribed to host factors. Here we examined the role of viral factors on liver disease outcomes by combining viral phylogeny with clinical, biochemical, cytokine, and histological data from 274 Belgian adults infected with HEV presenting between 2010 and 2018. HEV gt 3 clade efg infections were associated with a more severe disease presentation, higher serum CXCL10 levels and liver necro-inflammatory activity, irrespective of known host risk factors. HEV gt3 clade-dependent clinical outcomes call for broad HEV gt3 subtyping in clinical practice and research to help identify those at higher risk for worse outcomes and to further unravel underlying virus–host interactions.

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Viral clade is associated with severity of symptomatic genotype 3 hepatitis E virus infections in Belgium, 2010–2018

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Background & Aims: HEV genotype (gt) 3 infections are prevalent in high-income countries and display a wide spectrum of clinical presentations. Host – but not viral – factors are reported to be associated with worse clinical outcomes.
Methods: Demographic, clinical, and biochemical data of laboratory-confirmed HEV infections (by PCR and/or a combination of IgM and IgG serology) at the Belgian National Reference Centre between January 2010 and June 2018 were collected using standardised case report forms. Genotyping was based on HEV open reading frame 2 sequences. Serum CXCL10 levels were measured by a magnetic bead-based assay. H&E staining was performed on liver biopsies.
Results: A total of 274 HEV-infected individuals were included. Subtype assignment was possible for 179/218 viraemic cases, confirming gt3 as dominant with an almost equal representation of clades abchijklm and efg. An increased hospitalisation rate and higher peak serum levels of alanine aminotransferase, bilirubin, and alkaline phosphatase were found in clade efg-infected individuals in univariate analyses. In multivariable analyses, clade efg infections remained more strongly associated with severe disease presentation than any of the previously identified host risk factors, being associated with a 2.1-fold higher risk of hospitalisation (95% CI = 1.1–4.4, p = 0.034) and a 68.2% higher peak of bilirubin levels (95% CI = 13.3–149.9, p = 0.010), independently of other factors included in the model. In addition, acute clade efg infections were characterised by higher serum CXCL10 levels (p = 0.0005) and a more pronounced liver necro-inflammatory activity (p = 0.022).
Conclusions: In symptomatic HEV gt3 infections, clade efg is associated with a more severe disease presentation, higher serum CXCL10 levels, and liver necro-inflammatory activity, irrespective of known host risk factors.
Clinical Trial Registration: The protocol was submitted to clinicaltrials.gov (NCT04670419).

Introduction

HEV is the leading cause of acute viral hepatitis worldwide and is more common in high-income countries than initially thought.[1] Eight genotypes (gt) have been identified so far, of which gt3 is dominant in Europe and America.[2] HEV gt3 infections present as a zoonosis after the consumption of undercooked pig, wild boar, or deer meat, the main viral reservoirs.[1] Based on phylogenetic analyses, HEV gt3 subtypes can be assigned to 1 of 3 clades: abchijklm (HEV-3.1), efg (HEV-3.2), and ra (HEV-3.3).[2,3]

The clinical spectrum of HEV gt3 infections is highly variable: clinically silent in the vast majority, symptoms of acute hepatitis in <5% and acute liver failure in very rare cases.[4] The most frequent clinical picture in symptomatic cases is an acute self-limiting hepatitis.[1] Individuals with pre-existing cirrhosis can develop an acute-on-chronic liver failure, whereas up to two-thirds of immunocompromised individuals may fail to clear HEV, resulting in a chronic HEV infection.[4,5] Similar to other hepatitis viruses, extrahepatic manifestations have been reported, with a predominant neurological disease spectrum.[1,4]

Both host and viral factors may contribute to this wide spectrum of clinical disease presentations. Host factors identified until now include male sex, age above 50 years, pre-existing liver disease and an immunocompromised status.[4,6–8] Diabetes mellitus and alcohol consumption are other identified risk factors, most probably linked to an associated chronic

Keywords: Hepatitis E virus; Clade; Risk factor; Severity; Pathogenicity; CXCL10.
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liver disease.[4,6–8] It is currently unclear whether HEV gt3 virological factors, such as viral load or clades, can influence the course of the liver disease. A retrospective phylogenetic analysis of HEV gt3 infections in English blood donors found an association between virus variants and self-reported illness, whereas a larger European study could not corroborate this association in individuals with clinical hepatitis.[9,10] In contrast, we recently reported that Belgian adults infected with HEV gt3c are at lower risk of hospitalisation than individuals infected with gt3f in a retrospective survey of the hospitalisation status that has to be provided on Belgian National Reference Centre (NRC) HEV diagnostic test request forms.[11] A French study recently confirmed this observation in an analysis of patient-reported symptoms and medical history from individuals infected with HEV gt3f and gt3c.[12] However, these studies do not account for possible confounding factors, as some clinical and laboratory data were lacking, or not retrieved through medical chart review.

Here we aim to examine the role of viral and host factors on disease presentation by analysing clinical, biochemical, virological, and histological parameters of Belgian adults infected with HEV with disease signs over an 8-year timeframe. For the present study, we retrieved data from medical charts and re-analysed liver histology of available samples. Based on the most recent recommendations, viraemic cases were phylogenetically clustered in 1 of 3 clades instead of HEV subtypes.[2] In addition, we studied serum C-X-C motif chemokine ligand 10 (CXCL10) levels in an expanded cohort with acute HEV gt3 that was well balanced for age, sex, and viral clade. Our data show that HEV gt3 clade efg infections are associated with a more severe disease presentation, irrespective of all previously identified host factors, and lead to higher serum CXCL10 levels and liver necro-inflammatory activity.

Patients and methods

Study design and participants

This is a follow-up study of an identified and expanded cohort of individuals with confirmed HEV infection (*i.e.* a positive serum HEV-RNA and/or a combination of positive HEV-IgM and -IgG serology, as defined by EASL Clinical Practice Guidelines [CPG][4]) that were spontaneously reported to the Belgian NRC for viral hepatitis, Sciensano, between January 2010 and June 2018. Based on parameters reported by previous studies,[5–8,10–12] the following variables were systematically and retrospectively collected on a standardised case report form (CRF) via medical chart review by participating Belgian centres and physicians: (1) demographic variables (postal code, age, sex); (2) HEV disease characteristics (symptoms and duration, diagnosis date, HEV diagnosis in family members); (3) general and HEV disease specific risk factors (BMI, alcohol use, illicit drug abuse, pork meat consumption, history of travel, previous blood transfusion, pregnancy); (4) comorbidities (diabetes mellitus, pre-existing cirrhosis, profound immunosuppression, haematological or oncological disease, organ or stem cell transplantation, haemodialysis, other comorbidities); (5) biochemical parameters (alanine aminotransferase [ALT], alkaline phosphatase [ALP], bilirubin, International normalised ratio [INR], albumin, and estimated glomerular filtration rate); (6) HEV disease severity and outcome (full recovery, chronic infection, extrahepatic manifestations, hospitalisation and duration of stay in a general ward or intensive care unit [ICU], antiviral treatment, acute liver failure, decompensated liver cirrhosis, liver transplantation, death). Pre-existing cirrhosis was diagnosed through the combination of clinical and imaging studies and, where available, by elastography or histology.[13] Profound immunosuppression is defined according to the Green Book, a consensus document edited by the UK Health Security Agency that defines methods applicable for public health professionals working on immunisation against vaccine preventable infectious diseases.[14] Alcohol consumption was reported qualitatively at the discretion of the treating physician. A chronic HEV infection was defined by a persistent viraemia for at least 3 months, as recommended by EASL CPG.[4] Extrahepatic manifestations observed during the course of the infection were defined as described by EASL CPG.[4] Conversely, reported prolonged fatigue, asthenia, and viral syndrome signs were considered to be part of the normal clinical spectrum of HEV infection. Collaborating physicians and centres were approached to request available liver biopsy samples for centralised blinded reading. From the 26 specimens reported as available, 22 were received.

Laboratory investigations

RecomWell (Mikrogen Diagnostik GmbH, Neuried, Germany) and Wantai (Wantai BioPharm, Beijing, China) HEV-IgG and HEV-IgM immuno-assays were performed according to the manufacturer's instructions between January 2010 and October 2016 and from November 2016 onwards, respectively.[15] All positive and equivocal results obtained with Mikrogen kits were considered positive only after confirmation by Western blot (RecomBlot HEV-IgG/IgM [Mikrogen Diagnostik GmbH]). All equivocal HEV-IgM obtained with the Wantai assay were considered positive only if confirmed positive by a repeated Wantai HEV-IgM test.[15]

HEV-RNA detection was performed upon request by the referring physician and on all HEV-IgM-positive samples. RNA was extracted with the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany). Before September 2012, qualitative PCR followed by on-gel detection was performed. From October 2012, semi-quantitative (q)PCR was performed by using Altona HEV PCR 1.0 (Altona Diagnostics GmbH, Hamburg, Germany), following the manufacturer's guidance.[15] HEV viral loads are expressed by cycle threshold (Ct) values. Sanger sequencing was performed on all HEV-RNA positive samples, unless the residual sample volume was insufficient. Between 2010 and 2016, a protocol adapted from Huang *et al.*[16] was used to sequence 348 base pairs of Open Reading Frame (ORF) 2. From 2017, the protocol described by Boxman *et al.*,[17] amplifying 493 base pairs fragment of ORF2, was used. The obtained sequences were aligned against HEV reference genomes for assignment.

Phylogenetic tree analysis

Alignment of the sequences was performed using Clustal W.[18] The gap opening penalty and gap extension penalty were fixed to 15.00 and 6.66, respectively. The homologous regions of HEV gt1 (nucleotides 5985–6283 of LC225387), gt3 (nucleotides 5998–6314 of AB369687) and gt4 (nucleotides 6021–6342 of DQ279091) were applied to construct phylogenetic trees. Evolutionary analyses were conducted with MEGA X.[19] The

maximum likelihood method with the Tamura-Nei model was used.[3,15] The phylogenetic trees were constructed by applying Neighbor-Join and BioNJ algorithms to matrices of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value.

Serum CXCL10 measurement

A Bio-Plex Pro human CXCL10 (Bio-Rad, Temse, Belgium) assay was performed according to the manufacturer's instructions on leftover serum samples sent for HEV diagnosis to the Belgian NRC from: (1) an expanded cohort of individuals with an acute HEV gt3 infection (n = 200) presenting between 2010 and 2020 that was well balanced for age, sex, and clade; and (2) diseased controls (n = 50) for which HEV serology and PCR were negative. Seven-fold serial dilutions of the standards as well as 3 healthy control samples (with mean ± SD CXCL10 levels of 332 ± 71 pg/ml) were tested in duplicate on each plate. Interplate variability was below 20%. Plates were read by the Bio-Plex MAGPIX system (Bio-Rad) and raw data were analysed with the Luminex xPonent Software (Thermo Fisher Scientific, Waltham, MA, USA).

Histology

Received liver biopsies were evaluated in a blinded manner by an expert hepato-pathologist unaware of the individual's data. The following acute inflammatory histological parameters were scored in a semi-quantitative manner (score 0–3): portal and lobular inflammation, presence of interface hepatitis, and necrosis. The liver fibrosis degree was scored according to Metavir. All acute inflammatory changes (thereby excluding liver fibrosis, which reflects underlying concomitant chronic liver disease) were summed to obtain a global score, reaching a maximum of 12. Pictures were taken using a Leica DFC290HD camera (Leica Microsystems BV, Diegem, Belgium) on a Leica DM LB2 microscope (Leica Microsystems BV, Diegem, Belgium).

Statistical analyses

The characteristics of participants were compared by using the 2-sided X^2-test for categorical variables and 2-sided Student's *t* test (Welch test when variances were not equal and Wilcoxon Rank sum test when normality was not met) for the continuous variables. Numerical data are presented as the mean and standard deviation or 95% CI, and geometric mean and 95% geometric CI for semi-quantitative data (*i.e.* Ct values) and non-normal distributed continuous variables (*i.e.* CXCL10 levels), whereas qualitative data are expressed as counts and percentages. The *p* values of univariate analyses were adjusted by using the Benjamini and Hochberg correction method.[20] Multivariable linear regression analysis was performed for the laboratory defined liver disease outcomes (*i.e.* ALT, ALP, and bilirubin peak levels) and multivariable logistic regression for clinical outcome (*i.e.* hospitalisation). The independent variables were chosen based on reported HEV disease risk factors: viral load, diabetes, sex, age, profound immunosuppression, alcohol consumption, and pre-existing cirrhosis. As alcohol consumption linearly correlated with pre-existing cirrhosis ($p = 0.035$), only pre-existing cirrhosis was finally included as an independent variable. Because of minimal missing dependent variables in genotyped cases, no data imputation was deemed necessary. The ALT peak was square root transformed, whereas ALP and bilirubin levels were log transformed to meet the assumptions of linear regression. The log transformed response variables were expressed in percent increase/decrease for every 1-unit increase in the independent variable. The square root transformed response was back transformed by using an nlmixed model in SAS. CXCL10 levels and histological parameters were compared using 2-sided Wilcoxon Rank sum exact tests. Statistical analyses were performed with R (version 3.6.6, R Foundation for Statistical Computing, Vienna, Austria) and SAS (version 9.4, SAS Institute, Cary, NC, USA) for the ALT peak model.

Ethics

Permission of the ethical committee of the Antwerp University Hospital was obtained before the start of the study (number: 18/03/024, January 29, 2018).

Results

Demographic and clinical characteristics of the patient cohort

The flowchart of cases of HEV included in the study is presented in Fig. 1. Between January 2010 and June 2018, HEV infection was confirmed in 365 individuals by a positive serum HEV-RNA and/or both positive HEV-IgM and -IgG serology. No clinical data could be obtained from 91 cases because of the lack of response to the sent-out CRF (Table S1), leaving 274 cases (75.1%) with clinical data and 253 cases (69.3%) with laboratory values for further analyses (Table 1). Of these, 218 cases (79.6%) were HEV-RNA positive. No differences in

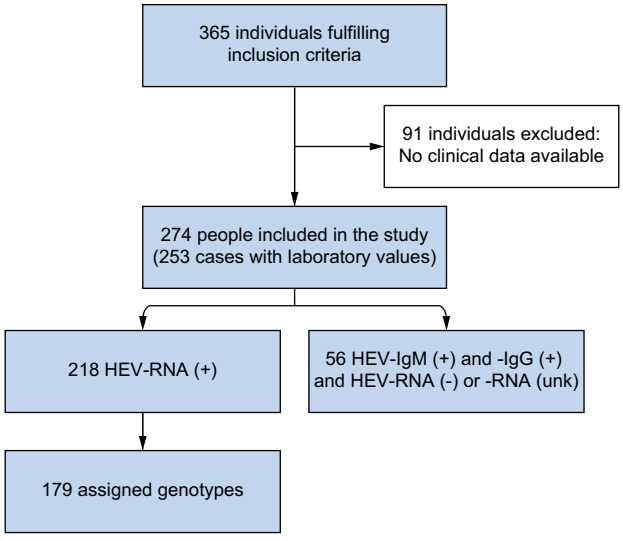

Fig. 1. Flowchart of cases. Of 365 individuals with a confirmed HEV infection diagnosed by a positive HEV-RNA and/or both HEV-IgM and -IgG positive serology at the NRC for viral hepatitis, Sciensano, between January 2010 and June 2018, 274 patients had available clinical data. Among them, PCR was positive for 218 patients and 56 patients had negative or unknown (unk) PCR results. From the 218 individuals with detectable HEV RNA, 179 were successfully sequenced.

Table 1. Demographic, laboratory, and outcome data of the study participants and their association with HEV gt3 clades.

	Total population	Viraemic gt3		
Demography	**(N = 274)**	**3abchijklm (n = 77)**	**3efg (n = 88)**	**Adjusted *p* values**
Region				0.54*
Flanders	158 (57.7%)	50 (64.9%)	48 (54.5%)	
Wallonia + Brussels	116 (42.3%)	27 (35.1%)	40 (45.5%)	
Age (years, mean, SD)	54.6 (13.5)	58.9 (11.9)	55.2 (13.1)	0.37†
Sex				1.00*
Male	191 (69.7%)	57 (74.0%)	64 (72.7%)	
Female	83 (30.3%)	20 (26.0%)	24 (27.3%)	
Pre-existing cirrhosis (yes)	21 (7.7%) (6 NA)	6 (7.8%) (1 NA)	5 (5.7%) (1 NA)	1.00‡
Alcohol consumption (yes)	92 (33.6%) (62 NA)	27 (35.1%) (20 NA)	35 (39.7%) (14 NA)	1.00*
Diabetes mellitus (yes)	50 (18.2%) (5 NA)	15 (19.5%) (1 NA)	20 (22.7%) (1 NA)	1.00*
Immunosuppression (yes)	44 (16.1%) (8 NA)	19 (24.7%) (1 NA)	12 (13.6%) (2 NA)	0.41*
Laboratory values (mean, CI)	**n = 253**	**3abchijklm n = 71**	**3efg n = 79**	**Adjusted *p* values**
Peak ALT (IU/L)	1,743.5 (1,540.8–1,946.1) (3 NA)	1,536.1 (1,249.8–1,823.1) (0 NA)	2,179.4 (1,834.6–2,524.4) (0 NA)	0.07†
Peak bilirubin (mg/dl)	6.1 (5.2–7.1) (7 NA)	4.4 (2.9–5.9) (0 NA)	8.3 (6.1–10.4) (1 NA)	0.006†
Peak ALP (IU/L)	267.0 (246.3–287.6) (13 NA)	276.2 (226.3–325.1) (2 NA)	305.9 (270.6–342.9) (3 NA)	0.37†
Peak INR	1.3 (1.2–1.4) (39 NA)	1.3 (1.1–1.6) (7 NA)	1.4 (1.2–1.5) (10 NA)	0.62†
Lowest albumin (g/L)	36.2 (35.3–37.1) (67 NA)	36.4 (34.5–38.3) (14 NA)	35.5 (33.9–37.0) (20 NA)	0.54†
HEV serum viral load (Ct)	**n = 218**	**3abchijklm n = 77**	**3efg n = 88**	**Adjusted *p* values**
HEV RNA (geometric mean, geometric CI)	27.5 (26.9–28.1) (21 NA)	26.9 (26.0–27.9) (3 NA)	26.3 (25.4–27.2) (13 NA)	0.54†
Outcome	**n = 274**	**3abchijklm n = 77**	**3efg n = 88**	**Adjusted *p* values**
Hospitalisation (yes)	127 (50.0%) (3 NA)	28 (36.4%) (0 NA)	53 (60.2%) (1 NA)	0.030*
Duration hospitalisation (days, mean, CI)	10.3 (9.1–11.5) (2 NA)	10.7 (8.7–12.7) (1 NA)	10.7 (8.4–12.9) (0 NA)	1.00†
ICU (yes)	16 (5.8%) (4 NA)	4 (5.2%) (1 NA)	5 (5.7%) (1 NA)	1.00‡
Duration ICU (days, mean, CI)	6.7 (6.1–7.3) (0 NA)	7.3 (6.0–8.5) (0 NA)	9.0 (7.6–10.5) (0 NA)	1.00§
Extrahepatic manifestations	23 (8.4%) (17 NA)	8 (10.3%) (2 NA)	11 (12.5%) (4 NA)	1.00‡
Neurological manifestations	17 (6.2%)	6 (7.8%)	8 (9.1%)	1.00‡
Other (renal, haematological, etc.)	6 (2.2%)	2 (2.6%)	3 (3.4%)	1.00‡
Chronic HEV infection¶	13 (4.7%)	7 (9.1%)	4 (4.5%)	0.54‡
Among immunosuppressed patients	12 (27.3%)	7 (36.8%)	4 (33.3%)	1.00‡
Death	6 (2.2%) (5 NA)	1 (1.3%) (0 NA)	4 (4.5%) (4 NA)	0.67‡

General demographic and outcome data are presented as means and percentages. Laboratory values, viral load, and durations are mentioned as means with 95% CI.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; Ct, cycle threshold; gt, genotype; ICU, intensive care unit; INR, international normalized ratio; NA, number of cases with missing information.
*X^2 test.
†Wilcoxon rank sum test.
‡Fisher exact test.
§*t* test.
¶HEV-RNA positive for >3 months.

demographic or viral parameters were observed between cases with completed CRFs and those without (Table S1).

Our cohort was representative for the whole country with a proportionate representation from the northern (57.7%) and southern (42.3%) parts of Belgium (Table 1). With a mean age of 54.6 years (range 23–90 years) and predominant male sex (69.7%), demographic characteristics matched those of other HEV European cohorts.[6–8] In addition, other established host risk factors for severe disease presentation were enriched in our cohort: diabetes mellitus (18.2%); pre-existing cirrhosis (7.7%) and alcohol consumption (33.6%) (Table 1).[6–8] Furthermore, 44 individuals (16.1%) were profoundly immunosuppressed, including 21 (7.7%) recipients of a solid-organ transplant (SOT) (Table 1).[5] Combined, 92.7% (254/274), 62.8% (172/274), 29.2% (80/274), 9.5% (26/274), and 1.8% (5/274) of individuals had at least 1, 2, 3, 4 or 5 known host risk factors (*i.e.* male sex, age above 50, diabetes mellitus, pre-existing cirrhosis, and alcohol consumption) for a worse acute HEV disease presentation, respectively. Corresponding to a severe symptomatic viral hepatitis infection, mean peaks of serum

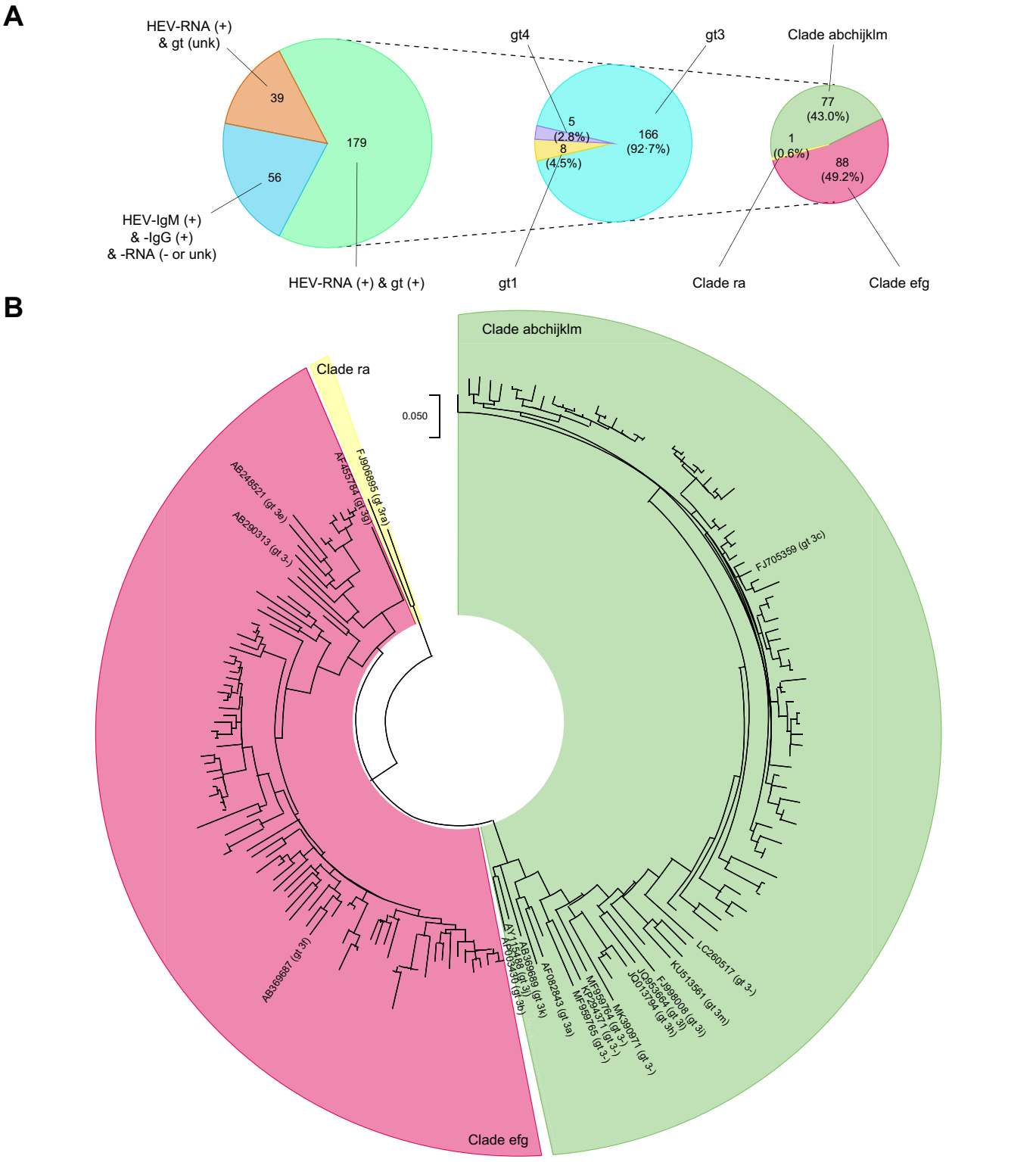

Fig. 2. HEV Gt3 and clade assignment among the study population. (A) HEV gt3 distribution among the study population. Genotype and subtype assignment was possible for 179 of the 274 patients included in our cohort. Among them, 166 patients were infected by HEV gt3. This genotype is further subdivided into 3 clades: abchijklm (HEV-3.1), efg (HEV-3.2), and ra (HEV-3.3). HEV-RNA (unk) refers to the absence of known PCR results. HEV gt (unk) is mentioned when HEV genotype assignment was not possible. (B) Phylogenetic analysis of a sequence of a 317 base-pair fragment from ORF2 of HEV gt3 (n = 166). The tree is at scale, with branch lengths measured as the number of substitutions per site. GenBank accession numbers are mentioned for all HEV gt3 reference strains used in the phylogenetic tree. Clade abchijklm, efg, and ra belongings are highlighted by green, red, and yellow circle arcs, respectively. gt3, genotype 3; ORF2, open reading frame 2.

ALT, bilirubin, and ALP levels reached a 34-fold upper limit of normal (ULN), 6-fold ULN and 2-fold ULN, respectively (Table 1). Peak INR and lowest serum albumin levels were not dramatically affected with means of 1.3 and 36.2 g/L, respectively. Twenty-three individuals presented mostly neurological extrahepatic manifestations and 13 developed HEV viraemia for more than 3 months (Table 1). Half of individuals were hospitalised for a mean of 10.3 days, 16 of them requiring an ICU admission (Table 1). Twenty-two individuals were treated with ribavirin (RBV) of whom 77.3% (17/22) were profoundly immunosuppressed. Six individuals died during the course of their HEV infection, representing a case fatality ratio of 2.2% (95% CI = 0.8 – 4.7; Table 1). These individuals had either extrahepatic manifestations or pre-existing cirrhosis and died because of liver failure, combined with multiple organ failure (Table S2).

Multivariable regression analysis identifies viral clade to be associated with clinical outcomes

In a first multivariable analysis, none of the previously identified host factors were associated with hospitalisation risk although pre-existing cirrhosis was associated with higher peak bilirubin levels and profound immunosuppression with lower peak ALT, bilirubin, and ALP levels (Table S3).

As it is currently unclear whether HEV gt3 clades can influence the course of the disease,[9,11,12] we analysed their association with disease severity. A viral genotype could be determined in 179 of 218 viraemic cases (82.1%), which predominantly clustered in gt3 (n = 166; 92.7%) (Fig. 2A). Only 8 and 5 viraemic cases belonged to gt1 and gt4, respectively (Fig. 2A and Fig. S1). Further phylogenetic analyses revealed an almost equal number of infections with gt3 clades abchijklm (HEV-3.1; n = 77; 43.0%) and efg (HEV-3.2; n = 88; 49.2%), and only 1 infection with the clade ra (HEV-3.3) (Fig. 2A and B). This allowed us to comprehensively analyse the contribution of HEV gt3 clades abchijklm and efg to disease severity.

Neither baseline demographic characteristics nor viral load differed significantly between the 2 clades (Table 1), nor was HEV viral load associated with baseline host characteristics (Table S4). In addition, RBV treatment was not associated with bilirubin levels or with risk for hospitalisation (data not shown). However, almost double peak bilirubin levels and higher peak ALT levels were found in individuals infected with HEV gt3 clade efg compared with abchijklm (Table 1). These differences in clinical presentation were associated with an almost double hospitalisation rate of individuals infected by clade efg (Table 1). In multivariable analyses, when accounting for gt3 clades, viral load and known host risk factors for HEV gt3 disease severity (Table 2), viral clade was the only variable associated with a higher hospitalisation rate, increasing risk by 2.1 (95% CI = 1.1–4.4, p = 0.034) fold. Moreover, clade efg infections were associated with a 68.2% higher peak bilirubin level (95% CI = 13.3–149.9, p = 0.010). Interestingly, HEV viral load did not influence any of the HEV outcome measures, whereas male sex remained associated with a 65.6% increase in peak bilirubin level (p = 0.024) as a previously established host risk factor. In contrast, profound immunosuppression diminished hepatitis parameters with lower peak bilirubin (-41.2%, 95% CI = -63.9 to -4.4, p = 0.032) and ALT (-1,287.8; 95% CI = -1,744.2 to -831.4, p = 0.001) levels (Table 2).

These data show that the HEV gt3 clade efg is associated with a worse HEV clinical phenotype and higher risk of hospitalisation, irrespective of known risk factors identified so far.

HEV genotype 3 clade efg infections are characterised by higher serum CXCL10 levels and intrahepatic necro-inflammatory activity

Intrahepatic transcripts of several interferon stimulated genes and chemokines, such as CXCL10, have been found to follow HEV and ALT kinetics in experimental HEV infections of rhesus macaques and chimpanzees.[21,22] To analyse differences in host immune responses between both clades, we now analysed serum CXCL10 levels in an expanded cohort of individuals with acute HEV gt3 that was well balanced for age, sex, and viral clades (Table S5). Serum CXCL10 levels were significantly higher in acute HEV gt3 infections compared with diseased controls (p <0.0001; Fig. 3A) and in clade HEV efg infections compared with clade abchijklm infections (p = 0.0005; Fig. 3B).

As serum CXCL10 levels have been linked to intrahepatic CXCL10 production and more severe liver histopathology in HCV infections, we next blindly re-analysed available liver biopsies (9 gt3c, 1 gt3h, 1 gt3e, and 11 gt3f specimens).[23] Liver test results from the subgroup of individuals with HEV

Table 2. Multivariable analyses of factors associated with disease presentation (peak ALT, bilirubin, and ALP levels) and outcome (hospitalisation rate).

	Multivariable linear regression			Multivariable logistic regression
	Peak ALT	Peak bilirubin	Peak ALP	Hospitalisation
	Difference of effect (95% CI; p value)	Difference % (95% CI; p value)	Difference % (95% CI; p value)	OR (95% CI; p value)
HEV gt3 clade (efg)	-384.6 (-831.1 to 62.0; 0.09)	68.2% (13.3 to 149.9; 0.010)	15.1% (-7.7 to 43.4; 0.45)	2.1 (1.1 to 4.4; 0.034)
HEV viral load (Ct)	-4.9 (-79.7 to 62.8; 0.91)	-2.0% (-6.9 to 3.0; 0.42)	-1.7% (-4.4 to 1.1; 0.16)	0.9 (0.8 to 1.0; 0.23)
Diabetes mellitus (yes)	-382.2 (-883.9 to 119.6; 0.13)	55.6% (-2.1 to 147.4; 0.06)	-11.4% (-31.3 to 14.2; 0.57)	1.0 (0.4 to 2.2; 0.99)
Sex (male)	276.4 (-274.6 to 826.5; 0.32)	65.6% (7.0 to 156.3; 0.024)	-15.5% (-34.0 to 8.2; 0.15)	0.9 (0.4 to 1.9; 0.75)
Age (>50 years)	251.8 (-334.9 to 838.1; 0.85)	5.0% (-35.5 to 70.9; 0.84)	13.1% (-14.7 to 49.8; 0.43)	1.4 (0.5 to 3.5; 0.47)
Pre-existing cirrhosis (yes)	-621.1 (-1,324.5 to -82.3; 0.08)	136.0% (13.7 to 390.0; 0.022)	11.6% (-25.6 to 67.2; 0.46)	2.3 (0.6 to 9.8; 0.22)
Immunosuppression (yes)	-1,287.8 (-1,744.2 to -831.4; 0.001)	-41.2% (-63.9 to -4.4; 0.032)	-22.7% (-41.2 to 1.8; 0.082)	0.4 (0.1 to 1.1; 0.09)

Viral clade, viral load, diabetes, sex, age, pre-existing cirrhosis and profound immunosuppression were considered as independent variables. As alcohol consumption linearly correlated with pre-existing cirrhosis, it was not included in the final multivariable analyses. Analyses are reported as difference of effect (in IU/L for peak ALT and in percentage for peak bilirubin and ALP levels), odds ratio (OR) for hospitalisation and 95% CI. p values were calculated using multivariable linear regression (peak ALT, bilirubin, and ALP levels) and multivariable logistic regression (hospitalisation).

ALP, alkaline phosphatase; ALT, alanine aminotransferase; Ct, cycle threshold; gt, genotype.

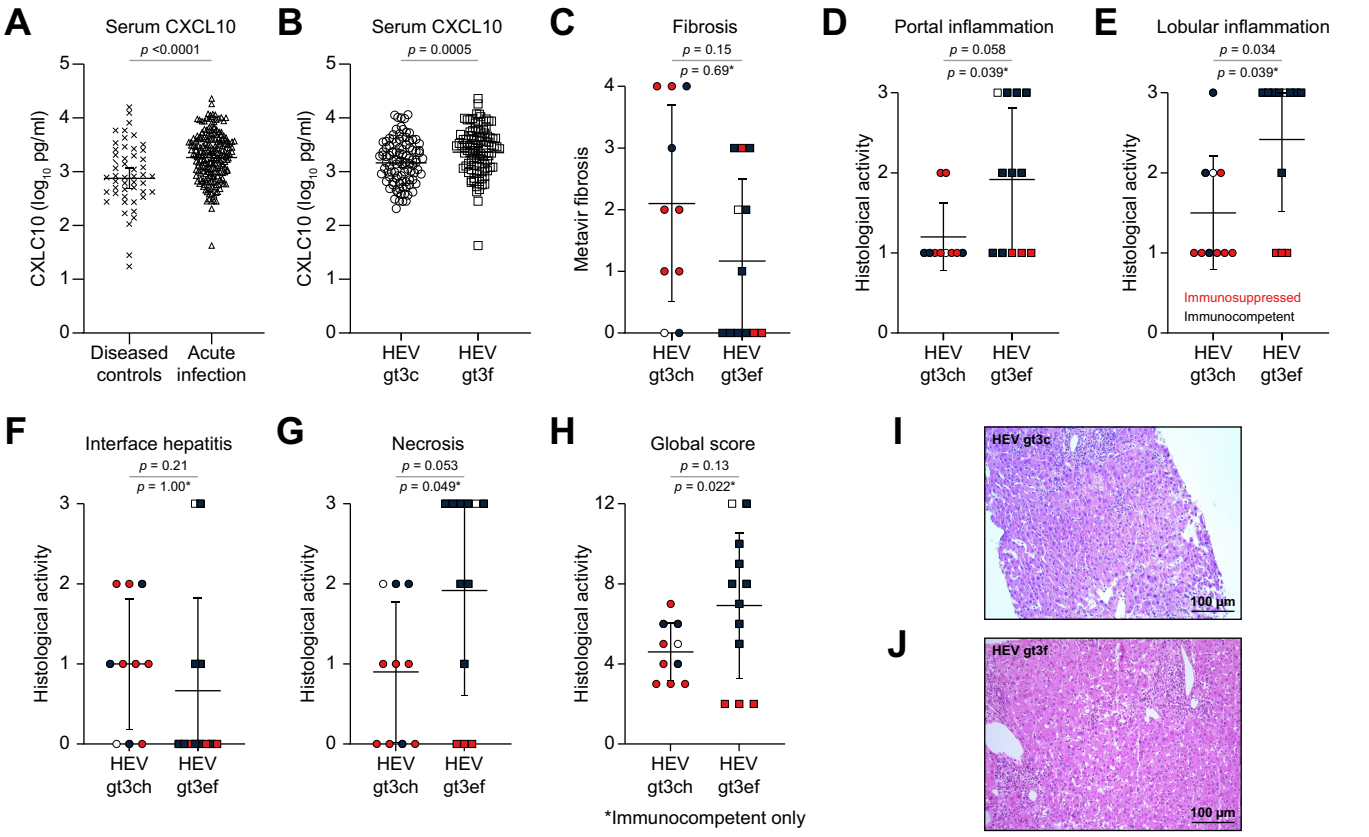

Fig. 3. Serum CXCL10 levels and histological scoring of patients infected with HEV gt3. (A,B) Serum CXCL10 levels (log_{10} pg/ml, geometric mean with 95% CI) were significantly higher in acute HEV gt3 infections compared with diseased controls (A) and in HEV gt3f infections compared with HEV gt3c (B). Acute infection: individuals with viraemic HEV gt3 with a successful ORF2 Sanger sequencing well balanced for age, sex, and clade. Diseased controls: patients for which samples were sent for HEV diagnosis, but with negative HEV serology and PCR. *p* values were calculated using the Wilcoxon Rank sum test. (C–J) HEV gt3 clade efg infections were associated with a higher intrahepatic necro-inflammatory activity. The following parameters were semi-quantitatively and blindly scored: degree of liver fibrosis according to Metavir (C), portal (D), and lobular (E) inflammation, presence of interface hepatitis (F) and necrosis (G). Reflecting the severity of the liver damage, a global score (H) based on the sum of individual portal inflammation, lobular inflammation, interface, and necrosis scores is presented for each patient. Patients infected with HEV gt3 subtypes ah and ef are presented by circles and squares, respectively. Red circles and red squares represent profoundly immunosuppressed patients. *p* values were calculated using the Wilcoxon Rank sum exact test for the 22 examined biopsies and for biopsies of patients who were immunocompetent, only (*). (I, J) Representative pictures of a liver biopsy from patients who were immunocompetent and infected with HEV gt3c and gt3f. They are presented by open circles and squares in panels C–H, respectively. The scale bar is shown. CXCL10, C-X-C motif chemokine ligand 10; gt3, genotype 3; ORF2, open reading frame 2.

infection who were biopsied did not differ from the overall cohort. However, 2 individuals who were biopsied died consecutively from an acute HEV gt3f infection, indicative of a severe infection (Table S6). Overall, individuals who were biopsied were older, more often immunocompromised (40.9%; 9/22 individuals) and chronically infected, had higher HEV viral loads, and were hospitalised for a longer duration (Table S7). Liver fibrosis did not significantly differ between both clades (Fig. 3C). As signs for acute inflammatory liver damage, portal and lobular inflammation, presence of interface hepatitis, and necrosis were scored semi-quantitatively from 0 to 3 (Fig. 3D–G). These individual scores were summed to obtain a global score with a maximum of 12 (Fig. 3H). Profoundly immunosuppressed individuals (light blue symbols) had generally lower acute inflammatory histological scores, irrespective of the infecting HEV clade, as recently also reported (Fig. 3D–H).[8] We therefore separately analysed histological scores for immunocompetent individuals (dark blue symbols): portal and lobular inflammation ($p = 0.039$), degree of necrosis ($p = 0.049$) and the global score ($p = 0.022$), representing the overall acute necro-inflammatory changes, were all found to be worse for clade efg infections compared with clade abchijklm (Fig 3 H and I–J). Finally, for the whole cohort of biopsied individuals, clade efg infections were associated with a more pronounced lobular inflammation ($p = 0.034$; Fig. 3D). Overall, these data show that efg infections are associated with a higher intrahepatic necro-inflammatory activity and suggest that innate immune responses are differentially induced by both viral clades.

Discussion

HEV gt3 infections are the leading cause of acute viral hepatitis in high-income countries and are increasingly being diagnosed in Europe and Belgium.[1,2,15,24] The pathophysiology of the remarkably diverse disease presentation remains poorly understood. In our retrospective study of HEV infections, we demonstrate that viral clade is strongly associated with HEV gt3 disease severity, irrespective of known host risk factors. Indeed, clade efg infections were associated with higher hospitalisation rates and higher peak serum bilirubin levels in individuals with disease signs, compared with

established risk factors. Accordingly, HEV clade efg infections were associated with higher serum CXCL10 levels and a more pronounced intrahepatic necro-inflammatory activity.

Unique to this cohort study is the identification of all cases of HEV infection over an 8-year timeframe by a single NRC, receiving referrals from about three-quarters of all hospitals and outpatient clinics throughout the country. In addition, here we grouped HEV gt3 viral subtypes into clades instead of analysing clinical differences among subtypes, as recently defined by Nicot *et al.*[2] and Smith *et al.*[3] This offers a more correct representation of inter-subtype distance. In our cohort, we also anticipated uniform countrywide hospitalisation policies, based on restrictions by the national health insurance system. Finally, we obtained demographic, clinical, and laboratory data from most participants, and histology data when available. This approach enabled an analysis of viral and host factors independently associated with disease presentations and outcomes.

In our study, HEV viral load was not significantly different between both gt3 clades. Furthermore, HEV viral load was not statistically associated with baseline host characteristics or disease outcomes. These findings are in contrast with a recent French study where HEV viraemia in individuals with gt3f infection was found to be higher compared with gt3c.[12] This discrepancy may be explained by: (1) the analysis of gt3c and gt3f only in the French report *vs* clades in the present study; (2) the inclusion of immunocompetent individuals only in the French paper; (3) the unequal and rather low representation of gt3c cases in the French study, compared with a well-balanced clade representation in our study; and (4) the variation in sampling during disease course and viraemia kinetics, inherent to retrospective analyses.

Alignment of reference genomes of HEV gt3 subtypes shows a trend of higher similarity within clades than between both clades. Subtypes within a same clade are thus more similar to each other.[2,3] As we observed similar viral loads for both clades, the differences at a genomic level most probably do not affect viral replication.

Proteins encoded by viruses from the 2 clades may have different post-translational modifications, which might result in different viral epitopes and subsequent host responses. For instance, ORF2 is known to be present under 3 different forms: ORF2i which is the component of infectious particles; ORF2g and ORF2c (cleavage product of ORF2g) which are glycosylated, secreted, and targets of humoral responses, inhibiting thereby antibody-mediated neutralisation of infectious particles.[25,26] Of note, no mutation in the described glycosylation sites and surrounding residues is observed in any subtype of the 2 clades. Bioinformatics analyses performed on ORF1, ORF2, and ORF3 of HEV gt3c and gt3f suggest some differences in post-translational modification profiles. ORF1 shows different phosphorylation and ubiquitination profiles especially in its hypervariable region. For instance, Lys_{763} of $ORF1_{3f}$ is potentially ubiquitinated whereas the corresponding site in $ORF1_{3c}$ is a Glu. The phosphorylation and O-glycosylation profiles of the 105 first amino acids of ORF2 differs between the 2 subtypes. Despite its small size, ORF3 might also show different phosphorylation and O/N-glycosylation profiles. Further analyses are required to confirm this.

HEV infections are considered to be non-cytopathic with a clinical picture that is dominated by host immune responses, amongst others a HEV-specific T cell response predominantly targeting ORF2.[27,28] In older persons, a symptomatic infection was very recently found to be associated with HEV-non-specific effector memory CD8 T cells and differences in cytokine production.[28] Furthermore, some case studies reported a pro-inflammatory HEV-associated cytokine storm syndrome, possibly related to pronounced innate immune responses.[29] In our cohort, serum CXCL10 levels were significantly higher in HEV gt3 clade efg compared with abchijklm infections. This interferon-induced, hepatocyte-secreted chemokine is known to recruit monocytes, natural killer cells, and T cells into the liver.[23,30] The HEVgt3 clade-dependent serum CXCL10 differences therefore support the more pronounced necro-inflammatory changes we observed in liver biopsy specimens. This suggests that intrahepatic innate immune responses are differentially induced by both viral clades. Further insight into the mechanisms governing these clade-dependent outcomes will require additional studies in optimised *in vitro* and *in vivo* models, as well as carefully designed prospective clinical cohort studies, with thoughtful collection of blood and liver samples.

Clinical outcome data and reported risk factors in our cohort were in line with those of previous studies.[5–8,10,31] Extrahepatic manifestations were reported for 8.4% individuals with a clear predominance of neurological symptoms, which is similar to other studies.[4,7] These included Guillain-Barre's, Parsonage-Turner's, and Miller Fischer syndromes as well as undetermined acute poly(radiculo)neuropathy for 23.5%, 11.8%, 5.9%, and 41.2% of the individuals with neurologic symptoms, respectively.[32–35] We observed a case fatality rate of 2.2%, which corroborates previously reported Scottish and English studies.[7,36] Fatal outcomes were exclusively seen in individuals with extrahepatic manifestations or pre-existing cirrhosis. About one-quarter of immunocompromised individuals suffered from a chronic HEV infection, a percentage similar to that found in the Scottish cohort.[7] Numeric higher fatal outcomes and lower chronicity rates were noted for clade efg infection, that did not reach statistical significance (Table 1). The only independent risk factor for chronicity identified until now is the use of tacrolimus over cyclosporine A in an international cohort of 85 SOT recipients.[5] A recent full-length HEV sequencing study of 35 individuals with chronic HEV gt3 found 29 to be infected with gt3c and 4 with clade efg, suggesting that viral clade may also impact the risk for chronicity.[37] The observed higher inflammatory activity in clade efg infections may explain this trend to more spontaneous clearance. Nevertheless, the number of deaths and chronic cases remain insufficient in our study to draw any firm conclusions. Pooling multinational cohorts will be required to further substantiate these observations.

We were able to collect data of 75.1% of individuals with a HEV infection confirmed at the NRC. More than 90% of genotyped cases were infected with gt3. The predominance of HEV gt3 infection in our study confirms what was observed in other European studies.[2,3,11,12,15,24] We may have missed some cases that were diagnosed in other laboratories, as HEV is not a notifiable disease in Belgium. We believe these limitations do not affect our results, as our cohort reflects the whole Belgian

population. Furthermore, demographic characteristics of reported cases matched those of other European cohorts and were similar to those for which the CRFs were not returned.[6–8]

Study limitations

Our study suffers from the inherent biases of a retrospective design, but is the first combining ORF2 sequence analysis and medical chart review applying a standardised CRF. Analysing laboratory values and hard clinical outcomes from patient charts, such as hospitalisation and death, will have circumvented most of these drawbacks. Responses for more subjective parameters, such as alcohol consumption, are nevertheless prone to recall bias. Furthermore, the timing of patient presentation and serum sampling during the disease course varied significantly, resulting in aviraemic samples in individuals who presented late and a broad range of observed viral loads. In our opinion, a multinational prospective study coordinated by several NRCs would be required to overcome these identified shortcomings.

Conclusions

In conclusion, we found for the first time that HEV gt3 clade efg infections are associated with worse clinical, biological, and histological outcomes, as well as higher serum CXCL10 levels, independently from known host risk factors. Systematically determining the clades of HEV gt3 infected individuals may help to identify those at higher risk for severe disease presentation as well as to unravel underlying mechanisms.

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Abbreviations

ALP, alkaline phosphatase; ALT, alanine aminotransferase; CPG, clinical practice guidelines; CRF, case report form; Ct, cycle threshold; CXCL10, C-X-C motif chemokine ligand 10; gt, genotype; HEV, hepatitis E virus; ICU, intensive care unit; INR, international normalised ratio; NRC, national reference centre; ORF, Open Reading Frame; PCR, polymerase chain reaction; RBV, ribavirin; SOT, solid-organ transplant; ULN, upper limit of normal.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

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

Authors' contributions

Conceptualisation: MP, TDS, SVG, TV. Data curation: MP, TDS Formal analysis: MP, JS, TR, SK, NH, TV. Funding acquisition: SVG, TV Investigation: MP, JS, TDS, TR, TL, SK, JD, PS, SDM, PW, IC, MVH, JVA, CVS, CM, FJ, MR, MS, WV, LL, CDG, AG, JM, MG, SVO, AM, HR, JD, EB, JS, JPM, SDG, MS, DD, SND, JB, JN, JB, NH, FN, SVG, TV. Methodology: MP, JS, TDS, TR, TL, SK, NH, SVG, TV. Project administration: MP, TDS, SVG, TV. Resources: JD, PS, SDM, PW, JVA, CVS, LL, AG, AM, JD, FN, TV. Supervision: NH, SVG, TV. Validation: SVG, TV. Visualisation: MP, JS, TDS, TR, TV. Writing – original draft: MP, JS, TDS, TR, TV. Writing – review & editing: MP, JS, TDS, TR, TL, SK, LS, VS, JD, PS, SDM, PW, IC, MVH, JVA, CVS, CM, FJ, MR, MS, WV, LL, CDG, AG, JM, MG, SVO, AM, HR, JD, EB, JS, JPM, SDG, MS, DD, SND, JB, JN, JB, NH, FN, SVG, TV MP, TDS, SVG and TV have directly accessed and verified the underlying data reported in the present manuscript.

Data availability statement

Data are available based on a motivated request sent to Steven.VanGucht@sciensano.be and after a MTA.

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Supplementary data

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Author names in bold designate shared co-first authorship

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