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Pathophysiology of hepatitis E virus infection: viral evolution during antiviral therapy and virus-specific T cell responses

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In Liebe für meine Familie.

Mama, Papa, Andreas und Alexander

„Die Kunst ist, einmal mehr aufzustehen, als man umgeworfen wird."

Winston Spencer Churchill

(1874-1965)

ZUSAMMENFASSUNG

Pathophysiologie der Hepatitis E Virusinfektion: Virale Evolution unter antiviraler Therapie und virus-spezifische T-Zell-Antworten

Anett Gisa

Hepatitis E ist eine Leberentzündung, die durch das Hepatitis E Virus (HEV) verursacht wird. In den letzten Jahren stieg die Anzahl der dem Robert Koch Institut gemeldeten Hepatitis-E-Fälle an; ebenso die Anzahl der in PubMed-gelisteten Publikationen. Lange Zeit galt Hepatitis E ausschließlich als Reisekrankheit in Industrienationen. Seit 2008 werden in Industrieländern vermehrt chronische HEV-Infektionen beobachtet, zumeist bei immunsupprimierten Patienten nach Organtransplantation. Chronische HEV-Infektionen sind bisher fast nur für den HEV-Genotyp 3 beschrieben. Unsere Arbeitsgruppe konnte eine Assoziation zwischen verminderten HEV-spezifischen T-Zell-Antworten und einer Chronifizierung der HEV-Infektion zeigen. Bislang liegen keine Daten zu HEV-spezifischen T-Zell-Antworten in Patienten mit akuter HEV-Genotyp 3 Infektion vor. Ribavirin ist die häufigste antivirale Therapie gegen HEV für immunsupprimierte Patienten. Jedoch wurden zunehmend Therapieversagen unter Ribavirin-Einnahme beschrieben.

HEV zeigt eine große genetische Diversität („Quasispezies Population“) aufgrund seines RNA Genoms. Antivirale Medikamente wie Ribavirin führen zu einem evolutionären Selektionsdruck auf das Virus.

Im ersten Teil meiner Arbeit konzentrierten wir uns auf den Einfluss von Ribavirin auf das HEV-Genom und fanden eine Mutation (G1634R) in der HEV-Polymerase im Zusammenhang mit einem Ribavirin Therapieversagen bei immunsupprimierten Organtransplantierten. Diese Mutation zeigte eine erhöhte Replikationsfähigkeit, jedoch keine Resistenz gegen Ribavirin *in vitro*. Ergänzend wurde die HEV-Quasispezies-Evolution der HEV-Polymerase durch Tiefensequenzierung untersucht. Für die chronische Hepatitis-E Infektion konnte gezeigt werden, dass die HEV-Quasispezies-Population relativ beständig war über längeren Zeitraum. Jedoch erschienen temporäre Aminosäure Veränderungen während einer Behandlung mit Ribavirin.

Im zweiten Teil untersuchten wir in akut infizierten HEV-Genotyp 3 Patienten T-Zell-Antworten und fanden eine breite und starke HEV-spezifische T-Zell-Antwort während und nach einer akuten Infektion. Diese T-Zell-Antworten waren Cross-Genotyp spezifisch gegen

HEV-Genotyp 1. Dies lässt auf eine mögliche Rolle der T-Zellen in heterologen HEV Infektionen vermuten.

Abschließend wurde ein möglicher Zusammenhang zwischen HEV und Autoimmunhepatitis (AIH) festgestellt. Während unserer Untersuchungen zu HEV-spezifischen T-Zell-Antworten während einer akuten Hepatitis E, konnten HEV-spezifische T-Zell-Antworten in anti-HEV-IgG positiven AIH-Patienten nachgewiesen werden und mit einer erhöhten HEV-Seroprävalenz in Verbindung gebracht werden.

Insgesamt ergab diese Arbeit neuartige Erkenntnisse hinsichtlich der HEV-Evolution während der chronischen Hepatitis E durch *in vitro* und *in vivo* Studien, die eine Ribavirin-induzierte Mutagenese in der HEV-Polymerase zeigten. Diese Erkenntnisse könnten wichtige Auswirkungen auf eine personalisierte antivirale Therapie haben.

Des Weiteren könnte der Nachweis von starken antigen-spezifischen T-Zell-Antworten in einer akuten Hepatitis E ein Hinweis auf die Wichtigkeit von T-Zell-Antworten beim Bekämpfen von HEV sein. Cross-Genotyp-spezifische T-Zell-Antworten in der akuten Hepatitis E könnten einen erheblichen Einfluss auf die Impfstoffentwicklung haben.

ABSTRACT

Pathophysiology of hepatitis E virus infection: Viral evolution during antiviral therapy and virus-specific T cell responses

Anett Gisa

Hepatitis E is an inflammation of the liver, which is caused by the hepatitis E virus (HEV). In recent years, the number of cases reported to the Robert Koch Institute has risen gradually, as has the number of PubMed publications on HEV. Hepatitis E was mainly considered to be travel associated in industrialized countries. However, since 2008 chronic HEV infections have been repeatedly observed in developed countries, e.g. in immunocompromised patients after organ transplantation. Chronic HEV infection has been described mainly for HEV genotype 3 infections. Our group was able to show an association between reduced HEV-specific T cell responses and chronicity of HEV infection. Nothing is known about HEV-specific T cell responses in acute HEV genotype 3 infection. The preferred antiviral therapy against HEV in immunocompromised patients is Ribavirin. Recently, the first cases of ribavirin treatment failure have been described.

HEV displays a high genetic diversity of its RNA genome ("quasispecies population"). Antiviral therapy (e.g. ribavirin) can lead to an evolutionary pressure of the virus.

In the first part of my thesis we focused on the impact of ribavirin on the HEV genomes and found a mutation (G1634R) in the HEV polymerase associated with ribavirin treatment failure in immunosuppressed organ transplant recipients. This mutation showed an increased replicative capacity, however, no ribavirin resistance *in vitro*. In addition, the HEV quasispecies evolution of the polymerase region was studied using deep sequencing. We found that the HEV quasispecies composition was relatively stable in chronic infection without ribavirin therapy; however temporary amino acid changes occurred during ribavirin therapy.

In the second part we investigated T cell responses in acute HEV genotype 3 infected patients and found broad and functional HEV-specific T cell responses during and after acute HEV infection. These T cell responses are cross-genotype-specific against HEV genotype 1 suggesting a potential protective role of T cells in heterologous HEV infections.

Finally, a possible link between HEV and autoimmune hepatitis (AIH) should be considered During our T cell studies in acute hepatitis E we were able to measure HEV-specific T cell

responses in anti-HEV IgG positive AIH patients and could linked it to increased HEV seroprevalence

Overall, the work of my thesis revealed novel insights in the HEV evolution during chronic hepatitis E by *in vitro* and *in vivo* evidence for ribavirin-induced mutagenesis in the HEV polymerase. These findings could have important implications for personalized antiviral therapy. Moreover, the detection of antigen-specific T cell responses in acute hepatitis E might indicate the importance for resolving HEV infection. Cross-genotype-specific T cell responses in acute hepatitis E might have an influence regarding implications for vaccine development.

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ABBREVIATIONS

α , β, γ	alpha, beta, gamma
ALT	alanine transaminase
APC	antigen presenting cell
AST	aspartate Transaminase
CTL	cytotoxic T lymphocyte
DC	dentritic cell
ER	endoplasmic reticulum
FDA	Food and Drug Administration
GT	glutamyltranspeptidase
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C Virus
HDV	hepatitis D Virus
Hel	helicase
HEV	hepatitis E Virus
HIV	human immunodeficiency virus
HVR	hypervariable region
IFN	interferon
IMPDH	ionosine-5´-monophosphate dehydrogenase
INR	International Normalized Ratio
IL	interleukin
ISG	interferon-stimulated genes
MeT	NH_2-methyltransferase
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	natural killer
ORF	open reading frame
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PRR	pattern recognition receptor
RdRp	RNA dependent RNA polymerase
RBV	Ribavirin
RNA	ribonucleic acid
SOT	solid-organ transplanted
TCR	T cell receptor
T_H	T helper cell
TLR	toll like receptor
WHO	World Health Organisation

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I. INTRODUCTION

1.1 Hepatitis E

1.1.1 Epidemiology

In 1978, a novel non-A, non-B water-borne hepatitis was recognized during an epidemic of hepatitis in India (Khuroo., 1980). The existence of a novel virus was confirmed by Balayan using immune electron microscopy in 1983 and the virus afterwards named hepatitis E virus (HEV) (Balayan et al., 1983, Tam et al., 1991). Interestingly, it has been shown in the recent years that the clinical presentation of the hepatitis E infection as well as transmission routes and geographical distributions seem to be much broader than expected. Hepatitis E appears to be an under-reported infectious disease worldwide with an increasing awareness and relevance in recent years (Figure 1) (Pischke et al., 2014a).

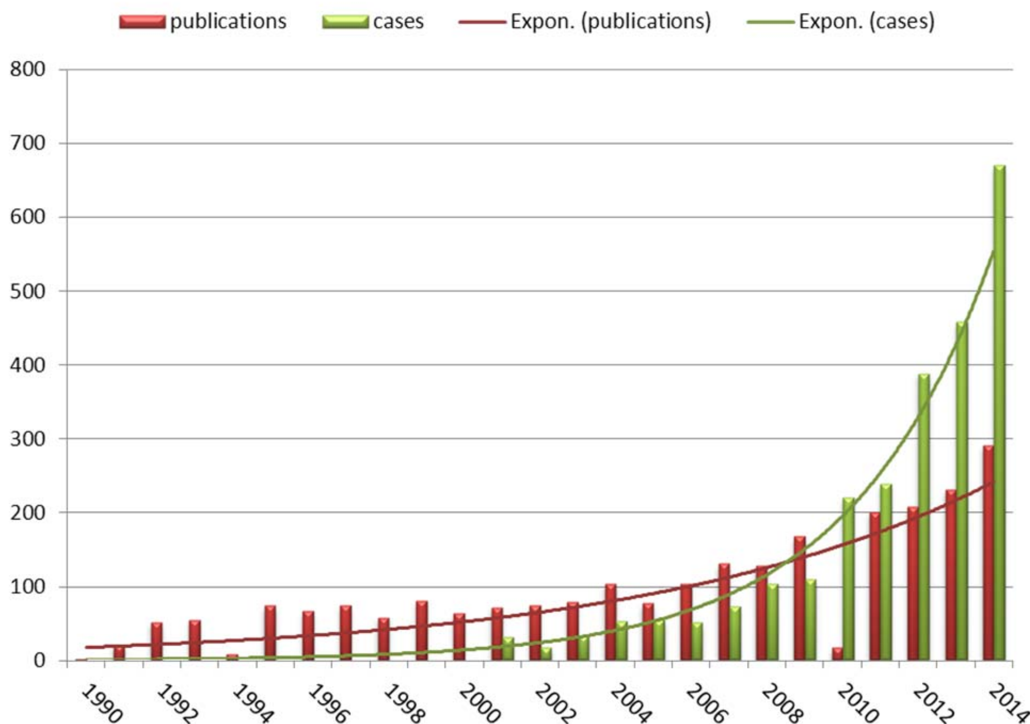


Figure 1: Number of cases of hepatitis E reported in Germany.

Publications on hepatitis E worldwide in the period of 1990 to 2014 (red; PubMed). In Germany the number of cases reported to the Robert Koch Institute has increased in recent years (green; Robert Koch Institute); *adapted from* (Pischke et al., 2014a).

World-wide, more than 3 million symptomatic HEV cases occur per year with an estimated 70,000 deaths annually (Pischke and Wedemeyer., 2010). Four human HEV genotypes have been reported. Genotypes 1 and 2 are transmitted predominantly via the fecal-oral route and are responsible for the majority of endemic hepatitis E cases in subtropical and tropical regions like India, South-East Asia and Sub-Saharan Africa (Velazquez et al., 1990, Maila et al., 2004, Sugitani et al., 2009, Teshale et al., 2010). On the other hand, genotypes 3 and 4 are zoonotic with their main animal reservoir in domestic pigs, wild boar and shellfish (Takahashi et al., 2004, Li et al., 2009, Meng., 2010a, Colson et al., 2010) and infections often result from consumption of undercooked meat or fish. Additionally, HEV can also be transmitted by infected blood products and vertical (materno-fetal) transmission (Gallian et al., 2014, Hewitt et al., 2014, Krain et al., 2014a)(Aggarwal., 2011). Autochthonous HEV genotype 3 and 4 infections have been described in Western Europe, North America and partly in Asia (Wedemeyer et al., 2012, Kamar et al., 2012). Although exact epidemiological data are missing, these infections seem to be a lot more common than initially thought (Dalton et al., 2014). Furthermore, an increasing number of new non-human HEV viruses are discovered showing an expanding family of HEV in vertebrates. Occurrence of HEV genotype 5 formed by one wild boar isolate and HEV genotype 6 formed by rat and ferret isolates was reported (Johne et al., 2014a, Li et al., 2015). Still, there is evidence that all HEV genotypes have the same serotype (Li et al., 2015).

HEV genotype 3 infections are in most cases asymptomatic. However in patients infected with HEV genotype 3, but not with genotype 1, 2 or 4, prolonged viremia as well as persistence of HEV has been reported (Kamar et al., 2013). This has first been observed in solid organ transplanted individuals taking immunosuppressive drugs (Kamar et al., 2008, Kamar et al., 2012, Behrendt et al., 2014, Pischke et al., 2014d). In addition, cases of chronic hepatitis E were reported also in other conditions of immunosuppression including advanced HIV infection (Dalton et al., 2009) and hereditary immunodeficiency syndromes (Honer zu Siederdissen et al., 2014). Chronic hepatitis E is frequently associated with a particular severe course of liver disease with development of liver cirrhosis within 1-2 years of infection (Behrendt et al., 2014, Pischke et al., 2014d, Pischke et al., 2012).

1.1.2 Clinical Syndromes/Course of Disease

HEV is one of the most common causes of acute viral hepatitis worldwide and clinical presentations in infected carriers varies from being completely asymptomatic, to patients with mild symptoms (malaise, anorexia, nausea, abdominal pain) up to fulminant hepatitis in rare cases (Khuroo., 1980, Pischke et al., 2014a). Symptomatic patients show increased liver function parameters as liver transaminases, bilirubin and gamma-glutamyl-transferase. The acute phase of hepatitis E infection lasts 4-6 weeks; liver enzymes normalized after 6 weeks after hepatitis on-set (Figure 2A) (Pischke et al., 2014d, Hoofnagle et al., 2012).

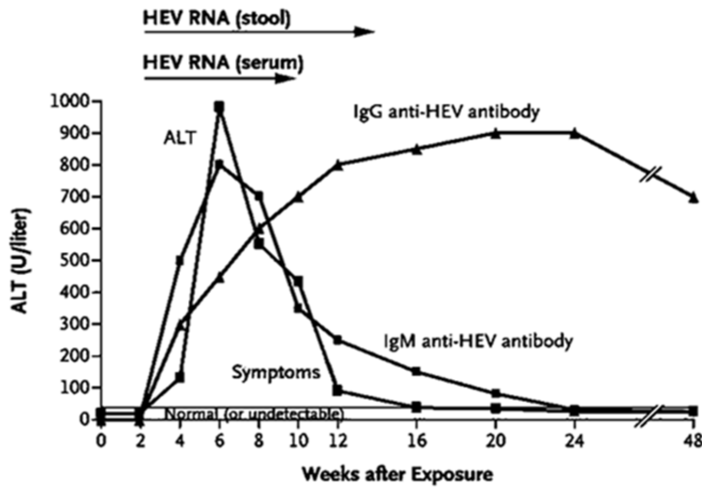
HEV infections usually resolve spontaneously in immunocompetent individuals but severe cases of acute HEV infection can be found especially in pregnant women infected with HEV genotype 1 and 2 (Tsega et al., 1993, Ramdasi et al., 2014). The underlying mechanisms why HEV is particular severe during pregnancy are unclear to date but hormonal, immunological and genetic factors might be involved (Jayanthi and Udayakumar., 2008, Navaneethan et al., 2008).

Even though HAV and HEV have similar symptoms and HEV initially thought to appear similar to HAV with acute, resolved infection only, chronic HEV infections have been reported since 2008 in immunocompromised patients, including organ transplant recipients (Kamar et al., 2008, Pischke et al., 2010, Kamar et al., 2012), HIV patients (Dalton et al., 2009, Robbins et al., 2014) and cancer patients receiving chemotherapy (Ollier et al., 2009). HEV persistence (HEV RNA$^+$) longer than 6 months were considered as chronic HEV infection (Figure 2B) (Wedemeyer et al., 2012, Hoofnagle et al., 2012, Kamar et al., 2014).

Furthermore, extrahepatic manifestations such as neuralgic amyotrophy (van Eijk et al., 2014, Pischke et al., 2014b) and Guillain-Barré syndrome (van den Berg et al., 2014) are described during or after resolution of HEV infections (Pischke et al., 2014a) as well as nephrological manifestations such as glomerulonephritis (Kamar et al., 2012).

In addition, we described in patients suffering from autoimmune hepatitis, which is a chronic immune-mediated liver disorder (Liberal et al., 2014), an increased HEV seroprevalence (Pischke et al., 2014c, Manns et al., 2015)(Liberal et al., 2014, Pischke et al., 2014c). It has been shown for other virus infections that associations between viral infections and autoimmune diseases exist (Vogel et al., 2002, Strassburg et al., 2003). Even though the knowledge on the epidemiology and clinical courses of HEV infection considerably improved in recent years (Figure 3), HEV remains frequently underdiagnosed and various questions are regarding incidence, transmission and natural history are remain unanswered.

(A)



(B)

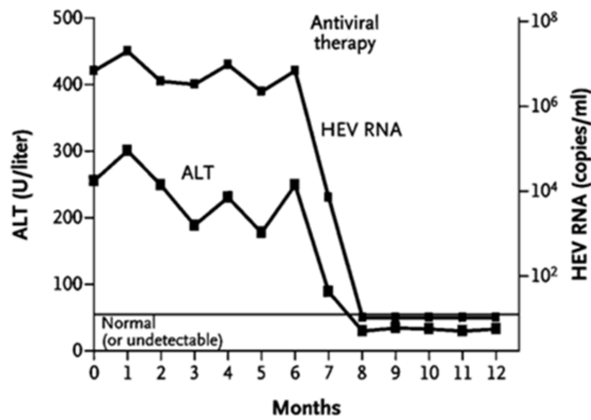


Figure 2 Clinical course of hepatitis E virus infection.

Typical evolution of the serologic titer of HEV-specific antibodies and levels of alanine aminotransferase (ALT) levels during **(A)** acute HEV Infection **(B)** chronic HEV infection. *Reproduced with permission from* (Hoofnagle et al., 2012)*, Copyright Massachusetts Medical Society.*

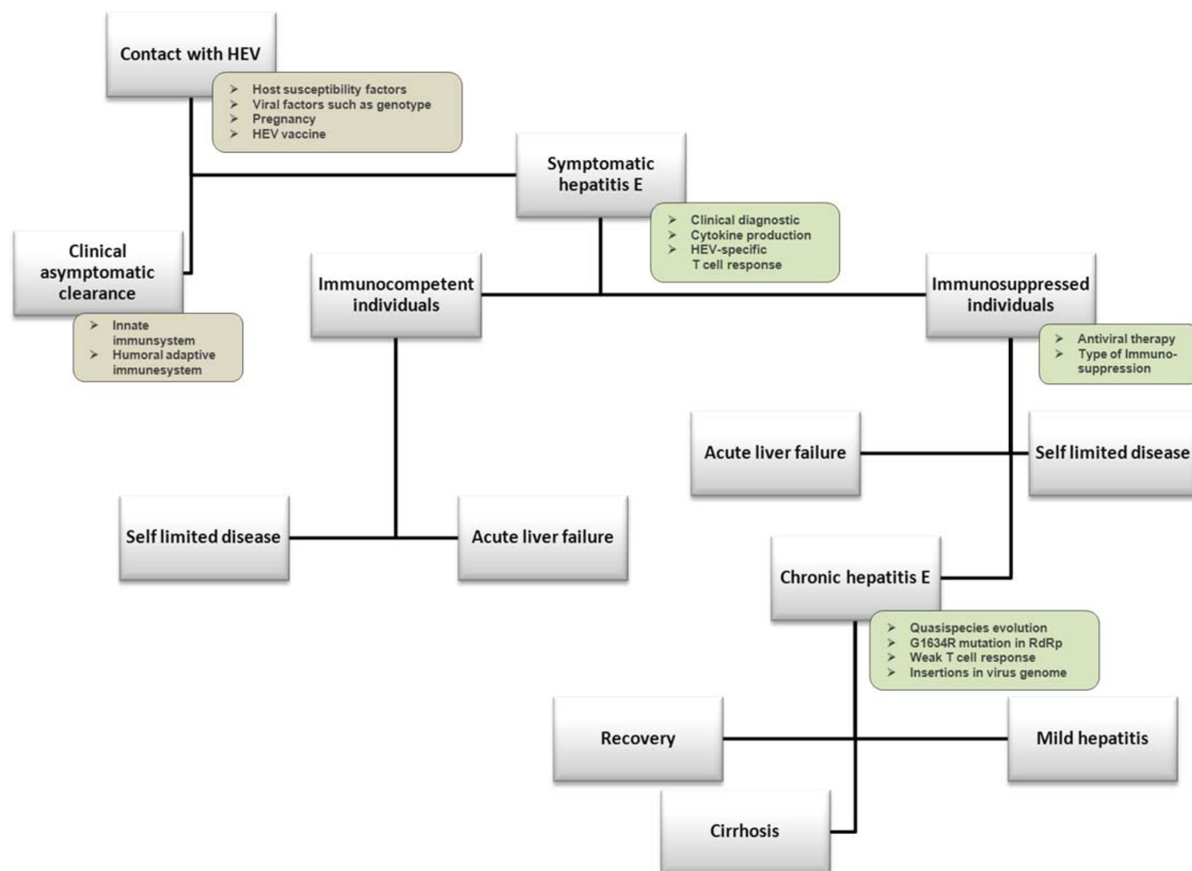


Figure 3 Course of hepatitis E and role of immune responses.

Adapted from (Wedemeyer et al., 2013)**.**

1.1.3 Prevention

HEV genotype 1 infections occur mainly in developing countries with a fecal-oral transmission route. Therefore, HEV infections can be prevented improving the inadequate sanitary infrastructure as well as individual hygiene (Kamar et al., 2014). During epidemics the most common vehicle of transmission is fecally contaminated water. On the other hand, HEV genotype 3 is transmitted predominantly by undercooked meat and selfish and thus can be prevented by heating meat, particularly pork products, above 70°C to inactivate the virus (Emerson et al., 2005). Furthermore, additional transmission routes are via blood products by transfusions and organ transplantations (Figure 4).

Recently, a recombinant HEV genotype 1 vaccine (Hecolin®) has been approved by the Chinese Food and Drug Administration in China (Zhang et al., 2014). This vaccine induced HEV-specific antibodies in 100% of vaccinated healthy individuals and clinical data suggest

cross-protection against genotype 4 (Zhu et al., 2010, Zhang et al., 2015). This vaccine is so far only licensed for the People's Republic of China. Thereby, it is essential to recognize the value of vaccination in high-risk populations like pregnant women, patients with chronic liver diseases and patients undergoing immunosuppression therapy (Zhang et al., 2012). Additionally, Liu et al could show that Hecolin® is even protective in rabbits challenged with genotype 4 rabbit HEV and swine HEV with strong anti-HEV antibody response (Liu et al., 2014). Similar findings were reported in mice and rhesus monkeys (Li et al., 2005).

Figure 4 Confirmed, suspected and potential transmission routes of HEV in developed regions.

Red lines, confirmed route; continuous black lines, strong evidence for route; broken black lines, suspect or potential route**.** *Reproduced with permission from* (Dalton et al., 2008), *Copyright Elsevier provided by Copyright Clearance Center.*

1.1.4 Current Standard of Care

Immunocompetent patients with HEV infection usually do not need any specific treatment since the course of disease is mostly asymptomatic and self-resolving (Pischke et al., 2014a). However, HEV can induce chronic hepatitis and cirrhosis in immunosuppressed patients like in recipients of solid organ transplants, haematological malignancies, HIV patients and those on haemodialysis. (Kamar et al., 2013, Abbas and Afzal., 2014). HEV clearance in solid organ transplanted patients can be achieved by reduction of immunosuppressants in about one third of cases (Abbas and Afzal., 2014, Kamar et al., 2010b, Kamar et al., 2011). In this context, the type of immunosuppression may play a crucial role in clinical use. *In vitro,* the combination of mycophenolic acid (IL-2 dependent T cell proliferation inhibitor) and ribavirin has been found to inhibit the HEV replication more efficient than ribavirin alone (Wang et al., 2014). In contrast, the HEV replication is enhanced by calcineurin inhibitors (e.g. tacrolimus) and mTOR inhibitors (e.g. sirolimus, everolimus) *in vitro* (Kamar et al., 2015).

Nevertheless, when the reduction is unsuccessful there are two therapeutic options which have been found to be efficacious: the use of pegylated interferon alpha (PEG-IFN-α) and/or ribavirin (RBV) (Pischke et al., 2014d, Pischke et al., 2012, Kamar et al., 2010, Kamar et al., 2010a). However, IFN-α is contraindicated in heart, lung and kidney transplant recipients due to the risk of inducing rejections (Pischke et al., 2012). Recently, in a large retrospective study in solid organ transplanted patients infected chronically with HEV ribavirin alone has been found to be efficient to clear the virus (Kamar et al., 2015). In general, organ transplanted patients receiving 8.1 mg of ribavirin per day and kilogram of bodyweight which is a median dose of 600 mg per day for an average duration time for 3 month (Kamar et al., 2014). The optimal individual dose and duration of RBV therapy needs to be adjusted over time to avoid: (I) decreasing hemoglobin levels and the possibility of anemia, (II) treatment failure after lowering the ribavirin dose (Pischke et al., 2014d, Pischke et al., 2012, Pischke et al., 2013, Guo et al., 2015).

Ribavirin (RBV) is a ribonucleoside analog (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) which has antiviral activity against a variety of RNA viruses (Sidwell et al., 1972, De Clercq., 1993). The antiviral mechanism of RBV against HEV is not fully elucidated. In recent years *in vitro* cultures for HEV have been established which have provided a better understanding of antiviral activities of RBV *in vitro* (Shukla et al., 2012, Okamoto., 2013, Debing and Neyts., 2014). Recently, *in vitro* results have shown that intracellular guanosine triphosphate

(GTP) depletion can be one of the major mechanisms contributing to RBV antiviral activity (Debing et al., 2014a). Other known GTP-depleting agents are mycophenolic acid (MPA; an immunosuppressive drug) and EICAR, a 5-ethynylimidazole analogue of RBV (Sintchak and Nimmesgern., 2000). MPA was associated with HEV clearance in a single study in heart transplant patients (Pischke et al., 2012).
Furthermore, it has been shown for both poliovirus genomic RNA and hepatitis C replicon that RBV increases the error rate of viral genome replication (Pfeiffer and Kirkegaard., 2003). Interestingly, a poliovirus with a point mutation in the RdRp with an increased replicative fidelity and RBV resistance has been described (Pfeiffer and Kirkegaard., 2003, Vignuzzi et al., 2005). RBV has also immunomodulatory properties by enhancing the host T cell-mediated immunity and supporting the T_H2 to T_H1 switch (Ning et al., 1998). Consequently, antiviral T_H1 cytokines and antiviral gene expression via the interferon-stimulated response element are increased (Vignuzzi et al., 2005, Zhang et al., 2003).

1.2 HEV Virology

Previously, HEV was classified in the family Calciviridae, 2003 shortly unclassified (Emerson and Purcell., 2003) but declassified and placed in a sole genus Hepevirus within a new family Hepeviridae (SU et al., 2004, Meng., 2010b, Smith et al., 2013). HEV is a spherical, non-enveloped virus of about 27–34 nm (Krawczynski et al., 2000). The viral genome is a positive, single-stranded RNA genome of ~7.2 kb in length, which is capped with 7-methylguanosine and polyadenylated at the 5´- and 3´-termini respectively (Okamoto., 2007). Besides short 5′ and 3′ untranslated regions (UTRs), it is subdivided in three partially overlapping open reading frames (ORF), called ORF1, ORF2 and ORF3 (Tam et al., 1991, Mori and Matsuura., 2011).
ORF1 starts 27 nucleotides downstream of the 5′ end and encodes a non-structural polyprotein (Panda and Varma., 2013) of 1693 amino acids which contains several putative functional motifs and domains: the NH_2-methyltransferase (MeT), a cysteine protease, a polyproline hypervariable region (HVR), a ADP-ribose phosphorylase (macro domain), a RNA helicase (Hel) and a RNA dependent RNA polymerase (RdRp)-COOH (Figure 5) (Panda and Varma., 2013, Chandra et al., 2008, Kenney and Meng., 2015). Recently, insertions and rearrangements within the HVR have been described and may play a role in the development of chronic HEV in solid organ transplanted which would be in line with persistent HIV and HCV infections where it is assumed that the HVR is involved in the escape from host

immunological responses (Shukla et al., 2012, Smith et al., 2012, Lhomme et al., 2014, Johne et al., 2014b).

The viral capsid protein ORF2 is 660 amino acids long and is positively charged at its N-terminal (Ahmad et al., 2011) and consists of three domains: the S (shell), M (middle) and P (protruding) domain (Figure 5) (Lhomme et al., 2014). Among HEV genotypes the S domain is highly conserved (Xing et al., 2010). The P domain functions as a putative binding site for both cellular receptors and neutralizing antibodies (Xing et al., 2010, He et al., 2008). The ORF2 protein was proposed to encapsidate the negatively charged viral RNA genome (Tam et al., 1991, Ahmad et al., 2011). A precursor of ORF2 co-translationally is translocated into the endoplasmic reticulum (ER) using an N-terminal signal sequence (Panda and Varma., 2013). It carries three potential N-glycosylation sites which are used in the ER (Ahmad et al., 2011, Zafrullah et al., 1999) for the formation of virions (Graff et al., 2006). The ORF2 capsid protein is immunogenic and provides a basis for used for all vaccine studies so far (Ahmad et al., 2011, Kamili., 2011). Epitopes located in the ORF2 protein between the amino acids 458-607 have been shown the majority of anti-HEV antibody responses (Kamili., 2011). One bacterially expressed recombinant peptide called HEV239 is encompassing the amino acids 368–606 aa of ORF2 designed from an HEV genotype 1 Chinese strain (Kamili., 2011). This recombinant peptide underwent later pre-clinical studies followed by the clinical trial Phases and has been in use in China since 2012 under the vaccine name Hecolin® (Zhang et al., 2015). Recently, the first long-term efficacy study of Hecolin® was published (Zhang et al., 2015).

ORF3, the smallest open reading frame, partially overlaps with ORF1 and ORF2 is 114 amino acids long (Figure 5) (Tam et al., 1991, Holla et al., 2013). It appears to fulfil several functions such as host cell environment regulation through its interaction with various intracellular pathways, modulation of acute phase responses and induction of relative immunosuppression (Chandra et al., 2008, Tyagi et al., 2004, Tyagi et al., 2005). Furthermore, ORF3 is associated with the cytoskeleton (Zafrullah et al., 1997) and phosphorylated ORF3 interacts with the non-glycosylated ORF2 possibly leading to internal regulation of viral replication (Tyagi et al., 2002).

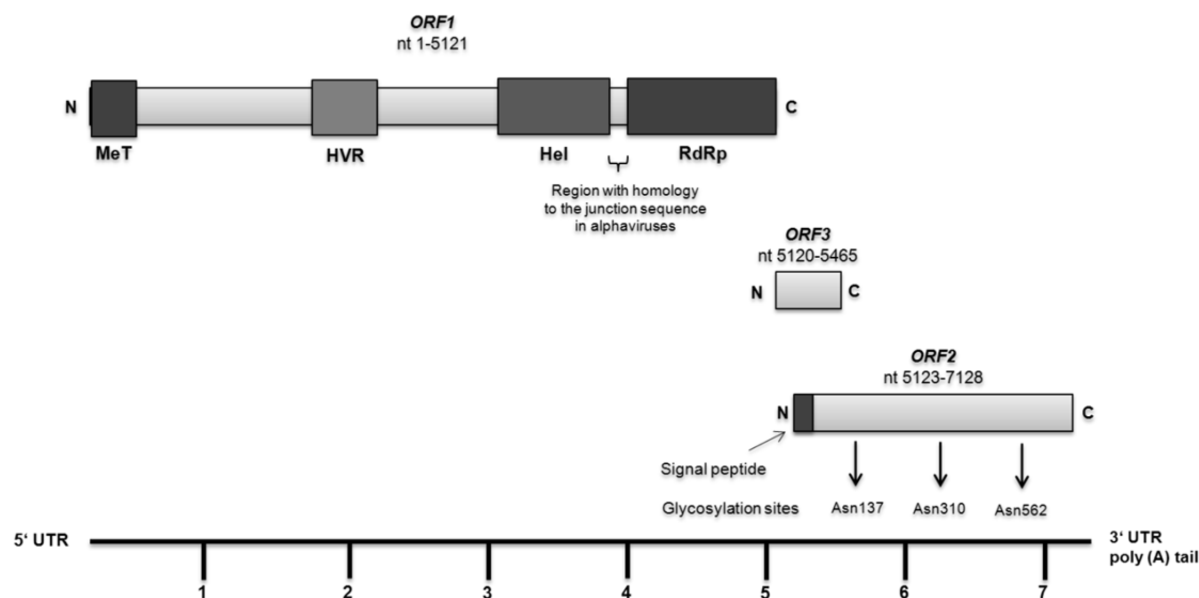


Figure 5 Genomic organization of HEV including the 3 ORFs.

The scale shows nucleotides in thousands *adapted from* (Wedemeyer et al., 2012, Panda and Varma., 2013).

The HEV replication cycle is still poorly understood (Figure 6). The original view of the HEV replication cycle was based on its genome analysis and analogy to other positive-strand RNA viruses (Ahmad et al., 2011, Holla et al., 2013, Reyes et al., 1993). Little is known about the binding and entry of HEV (Chandra et al., 2008, Holla et al., 2013). In Huh-7 cells it has been shown that for the capsid binding the cell surface heparan sulfate proteoglycans plays an important role (Kalia et al., 2009). Furthermore, it has been proposed that HEV enters liver cells through a Dynamin-2, clathrin-mediated endocytosis by a membrane cholesterol dependent pathway (Kapur et al., 2012, Holla et al., 2015). Still, there is nothing known about the mechanism of uncoating of HEV to release viral RNA in the cytosol (Ahmad et al., 2011). As it is the case for all positive-sense RNA viruses, the HEV RNA is translated directly into the ORF1-encoded nonstructural polyprotein (Chandra et al., 2008, Ahmad et al., 2011, Holla et al., 2013). Cleavage of the nonstructural polyprotein is accomplished by a papain-like cysteine protease (Ropp et al., 2000) and necessary for subsequent replication from genomic RNA into negative-sense RNA intermediates (Chandra et al., 2008, Holla et al., 2013). The negative-strand RNA serves as template for the synthesis of genomic as well as subgenomic positive-sense RNAs (Ahmad et al., 2011, Holla et al., 2013). These subgenomic RNAs are translated into the ORF2 and ORF3 proteins (Ahmad et al., 2011, Graff et al., 2006). The ORF2 capsid protein assembles the genomic positive-sense RNA into progeny virions

(Balayan et al., 1983, Ahmad et al., 2011) and virions are released from cell through an undefined non-lytic pathway (Chandra et al., 2008). Current evidence proposes that the ORF3 protein together with lipids coats this particle (Holla et al., 2013) that are removed later by bile salts and enteric proteases respectively (Yamada et al., 2009, Takahashi et al., 2010).

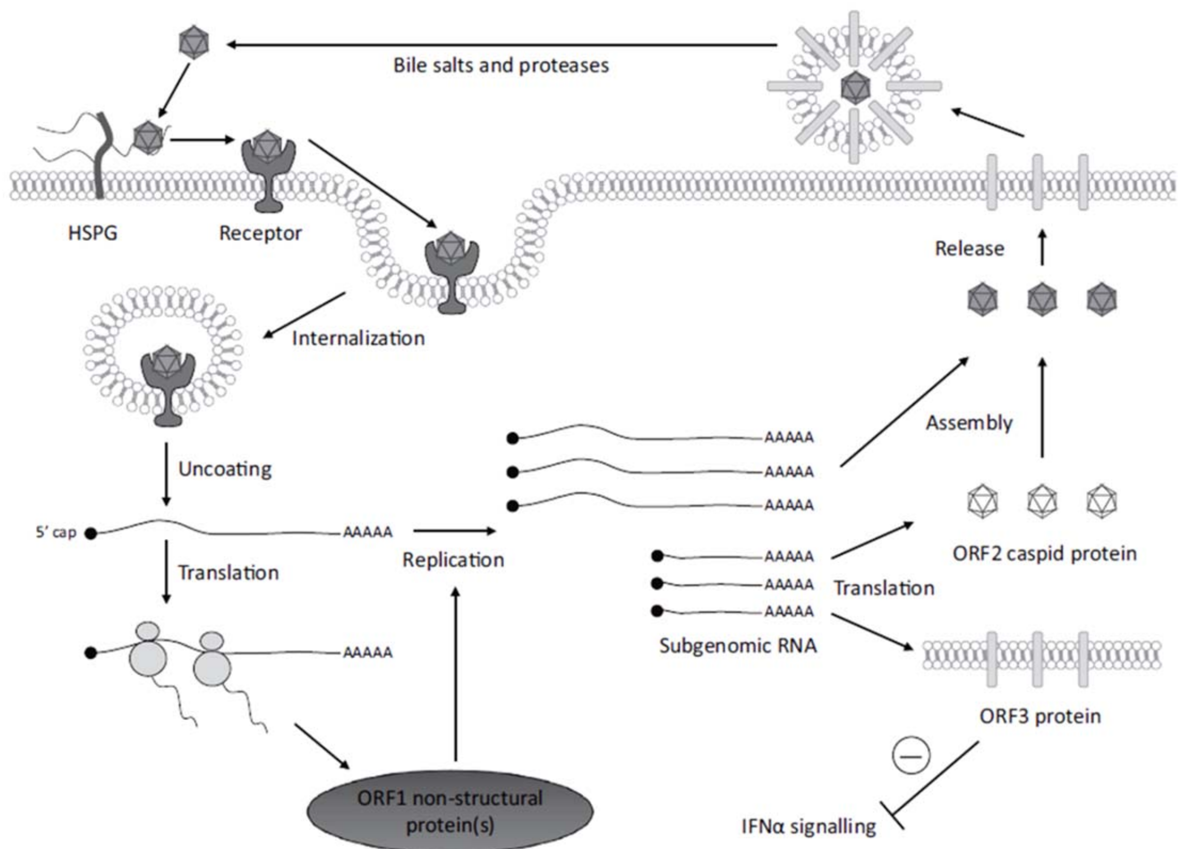


Figure 6 HEV replication cycle.

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1.3 Antiviral Immunity

The immune system is a system of biological structures and processes within an organism that protects against disease, not only from foreign pathogens like bacteria and viruses but also internal dangers like cancer. To function appropriately, an immune system must be able to distinguish between harmful substances from the organisms own healthy tissue to avoid failure in the regulation of these processes (Janeway., 2001).

The immune system can be classified into subsystems, the innate immune system which is the evolutionary older part (Litman et al., 2005, Mesquita Junior et al., 2010) and the adaptive immune system which first evolutionary developed in early vertebrates (Litman et al., 2005). The non-specific innate immune system is immediately active and includes physical barriers as well as cellular and humoral parts. The cellular fraction of the innate immune system includes macrophages, neutrophils, dendritic cells (DC), mast cells, eosinophils, basophils and natural killer (NK) cells (Janeway and Medzhitov., 2002). Importantly, these cells are able to produce chemical signaling proteins called cytokines and chemokines that have important effects on both the innate and adaptive systems (Janeway and Medzhitov., 2002). Whereas the humoral part of the innate immune system consists of the complement system, which is a group of serum proteins that can kill pathogens directly (lysis) or mark them (opsonization) for later destruction (phagocytosis) by certain immune cells (Janeway and Medzhitov., 2002). However, the adaptive immune system is mobilized slower but is a more sophisticated defense mechanisms which is able to adapt over time to recognize specific pathogens more efficiently (Janeway., 2001, Mesquita Junior et al., 2010, Kasahara et al., 2004). The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation and is able to create afterwards an immunological memory ("memory cells") which leads to an enhanced response to subsequent encounters (Janeway., 2001, Mesquita Junior et al., 2010). The main effector cells of the adaptive response are T and B lymphocytes which in contrast to the cells of the innate immune system undergo somatic rearrangement in the antigen recognizing receptors (T or B cell receptor) during cell maturation (Janeway., 2001, Kasahara et al., 2004). Most T cells are either cytotoxic T lymphocytes (CTLs) or T helper cells (T_H cells). CTLs kill cells which are infected with intracellular pathogens (e.g. virus). T_H cells help to determine which immune response is necessary against the pathogen by producing cytokines and link the cellular adaptive immune system with the humoral adaptive immune system by providing extra stimulatory signals to activate antibody producing B cells (Mesquita Junior et al., 2010).

Humoral and cell mediated as well as innate (non-specific) and adaptive (specific) responses are essential for antiviral defense to obtain immunity and each interacts with the other in critical and complex ways (Klimpel., 1996, Guidotti and Chisari., 2001). The contribution of each varies, depending on the virus and the host.

1.3.1 Immune responses against viral infections: viral hepatitis

Before a virus can reach the site of virus replication it has to overcome physical barriers to enter its host target cell to establish an infection (Bhella., 2015). After overcoming the first barrier the immune system has to sense the virus. Therefore, multiple immune responses are critical for eliminating virus. The most effective mechanisms of the early innate response against viral infections are mediated by non-specific inhibition, interferon alpha and by the NK cell activity (Klimpel., 1996, Snell and Brooks., 2015). Furthermore, the innate immune system has evolved pattern recognition receptors (PRRs) like toll-like receptors (TLRs) which are expressed by a variety of host cells to recognize pathogen-associated molecular patterns (PAMPs) (Balenga and Rafati., 2007, Gayed., 2011). After sensing PRR the intracellular signaling cascades are initiated and lead to a significantly up-regulation of pro-inflammatory cytokines/chemokines, type I interferons and antimicrobial proteins (Snell and Brooks., 2015). Especially type I interferons are important to link later on the innate and adaptive immune responses (McNab et al., 2015). This is achieved by the maturation and differentiation of DCs, NK cells, B and T cells. T cells are not able to recognize whole viral particles. Instead, T cells recognize short peptide fragments presented at the cell surface from professional (DCs, macrophages. certain B cells) and non-professional APC (e.g. fibroblasts) in association with MHC molecules (Guidotti and Chisari., 2001). After the adaptive immune system is activated it has to distinguish between viral particles and infected cells to respond in the most efficient way. In the first case, the antigenic viral capsid/particles are able to induce antibody production to neutralize (IgG, IgM and IgA) or agglutinate (IgM) the virus. The latter the classical complement activation, phagocytosis and/or CTL are used to attack virus infected cells. However, viruses have adapted to the defensive strategies employed by the immune system for example by evasion from the immune system via mutations in the viral genome (Plauzolles et al., 2013, Echeverria et al., 2015).

One of the major global health problems is the inflammation of the liver caused by the hepatitis A, B, C, D, and E virus called viral hepatitis (Acorn et al., 1995). The outcome and severity of a viral hepatitis infection can be tremendously variable from the subclinical to the

seriously acute to the chronic among these virus types (Acorn et al., 1995). Especially, HBV and HCV are unique because of their ability to cause persistent infection, cirrhosis, and liver cancer (Rosenberg., 1999, Guidotti and Chisari., 2006). Recently, also HEV has been reported to cause chronic infections (Kamar et al., 2008, Dalton et al., 2009). Virtually, the adaptive immune system mediates most of the liver diseases associated viral hepatitis.

1.3.1.1 Immune responses against HBV and HCV

Acute HBV infection during adulthood results often in self-resolving hepatitis by a strong adaptive immune response with a clearance in 90% of the cases and protective immunity (Guidotti and Chisari., 2006, Holz and Rehermann., 2015). The outcome of HBV infection is determined by early HBV-specific $CD4^+$ T cell responses to develop strong $CD8^+$ T cell responses (Maini et al., 1999, Asabe et al., 2009, Isogawa and Tanaka., 2015). Furthermore, clearance of acute HBV infection is associated with vigorous $CD8^+$ T cells responses and persistence with impaired and complex dysfunctional $CD8^+$ T cells responses (Asabe et al., 2009, Rehermann et al., 1996, Ferrari et al., 2003, Schuch et al., 2014). Additionally, it has been shown in chimpanzees that a $CD4^+$ T cell depletion also may result in chronic infection (Asabe et al., 2009, Rehermann et al., 1996). Overall, similar to HCV and HEV infection T cell responses in chronic HBV infections are weak (Holz and Rehermann., 2015, Isogawa and Tanaka., 2015, Suneetha et al., 2012). Further, HBV-specific T cell responses in chronic infected patients are negatively regulated by NK cells which is partly mediated through TNF-related apoptosis-inducing ligand (TRAIL) (Peppa et al., 2013). Simultaneously, an up-regulation of the TRAIL-R2 death-inducing receptor was found on HBV-specific $CD8^+$ T cells resulting in the deletion of these (Peppa et al., 2013).
Focusing on HBV and T cells it is worth to mention the peripheral regulatory T cell (T_{reg}) population inhibiting the effector functions of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells (Stoop et al., 2005). Compared to healthy controls it has been shown for chronic HBV infected patients that the T_{reg} population in peripheral blood is greater (Stoop et al., 2005).

Hepatitis C virus (HCV) is well known to develop persistent infection in up to 70% of immunocompetent patients (Holz and Rehermann., 2015). Studies have been shown that the persistence of HCV is based on the ability to counteract, suppress or evade immune mechanisms (Holz and Rehermann., 2015). Therefore, NK cells and T cells play a major role to eliminate virus-infected cells (Rehermann., 2009). Typically, patients who have been

shown strong and functional $CD4^+$ and $CD8^+$ T cell responses targeting multiple epitopes in the acute phase of infection resolve the hepatitis C virus (Diepolder et al., 1995). Failure in developing strong and functional T cell responses will lead to viral persistence and further establishment of chronic infection. Similar to HBV, the depletion of $CD4^+$ T cells resulted in chronic HCV infection (Grakoui et al., 2003). In contrast, HCV-specific $CD8^+$ T cells can be detected in the early acute phase of infection regardless of the outcome (Panther et al., 2004). However, viral clearance is associated with the secretion of $CD8^+$-specific antiviral cytokines in the later phase of HCV infection (Thimme et al., 2002). The importance of $CD8^+$ T cells to clear HCV infection is shown and that depletion of CD8+ T cells in experimentally infected chimpanzees led to the persistence of HCV viremia (Rehermann., 2009, Shoukry et al., 2003). Overall, Takaki et al. have been shown that HCV-specific $CD4^+$ and $CD8^+$ T cell responses persist after resolving of HCV infection (Takaki et al., 2000). Similar to T cell responses, chronic HCV infection is characterized by altered dysfunctional NK cell responses (Holder et al., 2014).

Besides T cell regulation, the immune system exert pressure on the viral variants and thus high mutation rate occur throughout the course of the infection and often results in immune escape which may predict the outcome of infection (Holz and Rehermann., 2015, Panther et al., 2004, Farci et al., 2000, von Hahn et al., 2007). So far, no protective HCV vaccine could be developed due to the heterogeneity in the HCV genome known as quasispecies population (Houghton., 2011).

1.3.1.2 Immune responses against HEV

Humoral adaptive immune responses against HEV

In the clinical outcome of acute HEV infection, humoral immune responses seem to play a serious role (Shata et al., 2012) which could be demonstrated by strong anti-HEV antibody responses during the early course of infection (Shata et al., 2012, Ke et al., 1996, Koshy et al., 1996). After the onset of infection anti-HEV IgM peaks within four weeks and in most patients anti-HEV IgM becomes undetectable after a period of three month (Figure 2) (Shata et al., 2012). Furthermore, IgM anti-HEV precedes the IgG anti-HEV by a few days. HEV-IgG seems to persist for decades after recovery in the majority of patients, however, there are largely diverse data from different countries due to the differences in sensitivity of available HEV ELISA assays. However, a wide variation within the antibody levels in affected

individuals was suggesting that host factors beyond antibody levels might be also important (Zhang et al., 2015). Nevertheless, a 3-shoot vaccination with Helicon® provided protection for up to 4.5 years with a vaccine efficiency of 86.8% (Zhang et al., 2015). Additionally, a 2-shot vaccination with Helicon® within one month could be an alternative for travelers in endemic areas to prevent illness and HEV infection (Zhang et al., 2015). However, future studies are necessary to explore alternative vaccine strategies.

Few studies are available investigating HEV-specific T cell responses in relation to antibody responses. Wu et al. studied HEV-specific T cell responses together with anti-HEV IgM antibodies in patients with acute hepatitis E and found that HEV-specific cellular immune responses decreased along with anti-HEV antibody titers and normalization of liver function (Wu et al., 2008). In contrast, there is no correlation between HEV-specific CTL responses and IgM levels between acute and recovered HEV infected individuals (Tripathy et al., 2012a). IgG levels were negatively correlated with HEV-specific CTL responses (Tripathy et al., 2012a). Further studies are required to investigate in more detail kinetics and relationships between HEV-specific humoral and cellular immune responses in acute infection or during the recovery phase. Differences in assays and post onset days of illness of study subjects must be taken into account which would be also important to improve HEV vaccines (Wedemeyer et al., 2013, Krain et al., 2014b).

Cellular adaptive immune responses against HEV

T cell–mediated adaptive immune responses are important for elimination of viral infections. $CD4^+$ and $CD8^+$ T cell responses have been shown to be of importance in the control of both HBV (Rehermann et al., 1995, Das and Maini., 2010) and HCV infection (Rehermann., 2009, Chang., 2003, Lauer et al., 2005).

In general, most available reports on the role of T cell immunity in acute and persistent HEV infection are based on HEV genotype 1 due to the periodically occurrence throughout the developing world. However, previous studies are partially contradictory with either absent or even very strong T cell responses during acute HEV infection (Wu et al., 2008, Tripathy et al., 2012a, Aggarwal et al., 2007).

Aggarwal et al. could show lymphoproliferative responses in acute infected patients after stimulation with overlapping peptide pools for ORF2 but not for ORF3 and significant stimulation after stimulating PBMCs with recombinant HEV ORF2 protein (Aggarwal et al., 2007). Another study in acute HEV infected patients described HEV-specific T cells after

in vitro stimulation with a particulate HEV capsid protein (HEV 239) which is consistent with the observation of Aggarwal et al. (Wu et al., 2008). *In vitro*, no immune activation was found in $CD4^+$ and $CD8^+$ T cells in the acute phase of HEV infection after stimulation with a recombinant HEV ORF2 protein (Zafrullah et al., 2004, Srivastava et al., 2007). Nevertheless, it could be shown that IFNγ levels after stimulating PBMCs with ORF2 protein in culture supernatants were higher in patients with acute HEV infection.
Taken all these data together, evidence is given for the activation of effector T cells during acute hepatitis E infection which might play a role in viral clearance from patients infected with HEV (Husain et al., 2011). Different methodological assays were used which makes it difficult to compare the current data on HEV T cell responses.
In Western countries HEV genotype 3 is responsible for the majority of autochthonous HEV infections as outlined above and patients infected with HEV genotype 3, but not with genotype 1, may develop chronicity of HEV infection. Thus, HEV genotype 3 may also show a distinct pattern in terms of T cell responses. So far, we are not aware of any study investigating T cell immunity in acute HEV genotype 3 infection (Wu et al., 2008, Tripathy et al., 2012a, Aggarwal et al., 2007).
Similar to persistently HBV or HCV infected individuals; very weak HEV-specific T cell responses were detectable in patients with persistent HEV infection. However, HEV clearance in solid organ transplanted patients with chronic hepatitis E was associated with HEV-specific T cell responses (Suneetha et al., 2012). Interestingly, restauration of HEV-specific T cell responses could be shown by blocking PD-1 or CTLA-4 co-inhibitory molecule pathways which is comparable to HCV (Suneetha et al., 2012, Owusu Sekyere et al., 2015).

Thus, these data indicate that regulation of HEV-specific T cell immunity may be similar to other hepatitis virus infections, even though more studies are needed in different patient cohorts.

Regarding cross-genotype-specific T cell responses, a study reported HEV-specific T cell responses in chronically and revolved patients carrying HEV genotype 3 using genotype 1-derived peptides (Suneetha et al., 2012). But nothing is yet known if cross-genotype-specific T cell immunity may contribute to a relative protection of HEV genotype 3 exposed individuals from Western countries travelling to HEV genotype 1 endemic area.
Furthermore, heterogeneity between genotypes and additionally mutations are often a hurdle especially to find epitopes for vaccine development for cross-genotype protection.

For HCV, partial cross-genotype-reactivity of one immune dominant HCV $CD8^{+}$ T cell epitope could be shown (Fytili et al., 2008).
For HEV it is known that all major genotypes in humans occur to only one serotype. Due to this observation the development of recombinant HEV vaccine (Hecolin®) has been facilitated for genotype 1 (Zhang et al., 2015). Hecolin® is able to induce HEV-specific antibodies and clinical data suggest cross-protection against genotype 4 (Zhu et al., 2010), but still there is no formal proof that HEV 239 also protects from HEV genotype 3 infection, which can induce persistent infection in organ transplanted patients (Pischke et al., 2014d, Pischke et al., 2012, Kamar et al., 2011, Wedemeyer and Pischke., 2011). However, for HEV no study has been performed mapping peptide epitopes among the different HEV genotypes.

1.4 Quasispecies, heterogeneity and immune pressure

The non-proofreading polymerase of RNA viruses lead to high mutation rates during replication which causes genomic diversity called quasispecies (Grandadam et al., 2004). Such viral diversity is a challenge for the host immune system to develop virus-specific T cells. Immune pressure on the pathogen can in turn lead to quasispecies selection or specific immune escape. Furthermore, quasispecies populations may allow adaptation to antiviral drugs and inducing e.g. resistance or enhanced viral fitness. These mechanisms mediate adaptability on viruses for persistence and increases a rapid evolution during passage from host to host which are partly responsible for current difficulties of viral disease prevention and control (Schneider and Roossinck., 2001, Domingo et al., 2012). Correlations between quasispecies diversity and slow or fast progression of the disease may indicate that viral diversity is a significant pathogenicity factor (Grandadam et al., 2004).
Although the hepatitis B virus (HBV) is a DNA virus the absence of viral-encoded RdDp proofreading capacity combined with a complex replication cycle and high replication rate generates great mutation rates, leading to a quasispecies population (Domingo et al., 2012, Nishijima et al., 2012). Especially the therapy with lamivudine compared to other HBV drugs results in amino acid substitutions at polymerase and subsequently resistant population (Kim et al., 2015). Furthermore, it is also known for the hepatitis C virus (HCV) which has a single stranded, positive sense RNA genome that it is existing as a quasispecies. Viral populations as well as host genetic determinations control the response of HCV to different available drugs

(Domingo et al., 2012). Particularly for RBV monotherapy in HCV genotype 1 infected patients the mutagenic effect of RBV was described (Dietz et al., 2013).

Little is known how the quasispecies population evolves during HEV infection and how drugs like RBV are influencing the heterogeneity of the population. First studies from Lhomme and colleagues have demonstrated that a greater HEV quasispecies heterogeneity was associated with chronic evolution of HEV infection in organ transplant recipients (Wedemeyer et al., 2013, Lhomme et al., 2012). Furthermore it is examined that during the acute phase of HEV infection the diversity of quasispecies in the M and P domains of ORF2 is associated with the progress of HEV persistence (Lhomme et al., 2012). Therefore, it would be of further interest to study the effect of RBV on the HEV quasispecies population in chronic infected patients especially in patients who failed RBV treatment (Debing et al., 2014b).

Collectively, the hepatitis E virus can be a particular problem in immune compromised individuals. Therefore, it is necessary to understand the two major directions: the molecular virology and the host immune system. For this reason we ask ourselves following questions and set ourselves the following aims in this thesis.

II. AIMS OF THE THESIS

RNA viruses face dynamic environments and are masters in adaptation within short generation times due to relatively high mutation rates. Coevolution of virus and host is a process that emerges in persistent virus infections. Until now, there is nothing known about the survival of the hepatitis E virus population in patients over time in the presence of the selective pressure of the antiviral ribonucleoside analog ribavirin, which is the therapy of choice for patients with chronic hepatitis E.

T cell responses are important for viral clearance in hepatitis B (HBV) and hepatitis C virus (HCV) infection as well as in patients with chronic hepatitis E. So far, information on cellular responses during acute hepatitis E is limited. Furthermore, nothing is known about potential HEV-specific cross-genotype T cell responses between genotype 1 and 3.

Therefore, I wanted to address two major parts with the following questions:

Part I: The ribavirin influence of the HEV genome.

a. Identification of mutations in the HEV genome in patients with chronic HEV infection resistant to RBV therapy using Sanger sequencing.

b. To ascertain the temporal evolution of replication fitness HEV polymerase mutant G1634R during course of RBV therapy we used Illumina next generation sequencing.

Part II: Antiviral immunity of HEV infection.

a. Are HEV-specific T cells detectable in acutely HEV genotype 3 infected patients?

b. Are HEV-specific T cells detectable after an acute resolved HEV infection?

c. Can cross-genotype specific HEV-specific T cell responses between HEV genotype 1 and 3 be found?

III. RESULTS

3.1 A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients.

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3.2 *In vivo* evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome.

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3.3 Cross-genotype-specific T cell responses in acute hepatitis E virus (HEV) infection.

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Published in Journal of Viral Hepatitis 2016

3.4 Increased HEV seroprevalence in patients with autoimmune hepatitis.

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Published in PLoS One 2014

IV. DISCUSSION

HEV can develop a broad variety of possible infection outcomes from asymptomatic cases to fulminant hepatic failure with fatality rates up to 15-20% in certain risk groups (Wedemeyer et al., 2012). Little is known about the specific role of individual host factors explaining disease severity.

Therefore, we wanted to address two major parts. The first part focused on the molecular virology in particular on the impact of ribavirin on the HEV RNA genome and the HEV viral quasispecies evolution in the HEV polymerase. The second part focused on the antiviral immunity investigating HEV specific T cell responses mainly in acute HEV infection.

<u>The ribavirin influence of the HEV genome.</u>

HEV genome mutations or rearrangements have already been described in different genome regions and disease outcomes (Liang et al., 2010, Cordoba et al., 2011, Devhare et al., 2014). It has been shown for various other viruses like HCV that mutations in the virus affect viral replication and drug resistance which is a major problem for antiviral treatment (Holysz et al., 2015). We here hypothesized that ribavirin-induced viral mutagenesis could be of importance also for HEV (Pfeiffer and Kirkegaard., 2003, Dietz et al., 2013, Beaucourt and Vignuzzi., 2014). Ribavirin (RBV) is the first treatment of choice for patients with chronic hepatitis E, but the influence of RBV on the HEV genome is unknown (Dalton et al., 2014, Kamar et al., 2015). Consequently, we studied the RBV pressure on the HEV genome in 15 solid organ transplanted patients at different time points: before on-set of treatment, during and after RBV treatment and compared afterwards the viral sequences. As a result, we described for the first time a mutation (G1634R) in the C-terminal region of the HEV polymerase in solid organ transplanted patients who failed RBV treatment [see Addendum I (page 68); Figures 1 A and B] (Debing et al., 2014b). In general, published HEV genotype 3 sequences from the National Center for Biotechnology Information (NCBI) showed a natural predominance for the amino acid (aa) G1634 (77%) than the aa R1634 (22%) in the HEV polymerase [see Addendum I (page 68); Figure 1 C]. No differences in RBV sensitivity could be observed after introducing the 1634R mutation into a HEV genotype 3 replicon compared to the wild type replicon. However, we could show that 1634R mutant revealed higher luminescent signals suggesting an increased viral RNA replication capacity *in vitro* [see Addendum I (page 68); Figure 1 D and E] (Debing et al., 2014b). Additionally, we used a competition assay to compare the

fitness of G1634 and 1634R and were able to show a relative fitness advantage for 1634R for genotype 3 [see Addendum I (page 68); Figure 2 C, D and Suppl. Figure 3]. Similar to genotype 3, we determined the influence of a vice versa 1634 mutation in a genotype 1 replicon which predominantly carries the aa K/R1634 in nature. As predicted, a decreased replication signal for G1634 could indeed be observed [see Addendum I (page 68); Figure 2 E]. Overall, this data suggests that HEV genotype 3 replicates to lesser extent than genotype 1 *in vitro* and likely also *in vivo* (Debing et al., 2014b). This might be a correlate for the usually milder infection course in HEV genotype 3 infected patients compared to HEV genotype 1 infected patients where severe cases occur of acute hepatitis occure more frequently which can lead a fulminant hepatitis and even death.

As RBV resistance could not be determined in the *in vitro* assays other patient- and virus-related factors may contribute to the RBV treatment failure (Debing et al., 2014b). Considering the clinical context, in this patient cohort, the type of immunosuppression may play an important role. *In vitro* data showed that the combination of mycophenolic acid as an immunosuppressant drug in combination with ribavirin inhibited the HEV replication more efficient than ribavirin alone (Wang et al., 2014). On the other hand, immunosuppressant drugs like tacrolimus, sirolimus and everolimus have been used in solid organ transplanted patients to reduce organ rejection. *In vitro* studies revealed that HEV replication can be enhanced by these types of immunosuppressive drugs (Wang et al., 2014, Zhou et al., 2014). Furthermore, little is known about the combination of these different drugs and the optimal individual dose and duration especially for RBV therapy which needs to be adjusted over time to avoid the possibility of anemia and treatment failure after lowering the ribavirin dose (Pischke et al., 2014d, Pischke et al., 2012, Kamar et al., 2014, Pischke et al., 2013, Guo et al., 2015). The possibility of testing for the 1634 mutant as an inexpensive prognostic marker to predict the outcome of patients with chronic infections in the clinic might be interesting. E.g, determination of the 1634 mutant could be used to adjust dose and duration of RBV therapy based on the presence of the G1634R variant. Transplanted patients carrying the G1634R mutation before or during RBV therapy might benefit from longer treatment duration and higher doses of RBV to avoid treatment failure.

HEV genome diversity represents a potential mechanism to escape successful immune response while on the other hand immune pressure may drive viral evolution (Duggal and Emerman., 2012). For HEV, chronicity has been linked with greater quasispecies heterogeneity (Lhomme et al., 2012). We monitored the evolution of the G1634R variant to

determine if the G1634R variant may have been present already before therapy as a minor population in persistently infected patients by novel deep sequencing methods. Deep sequencing confirmed that the G1634R variant was already present as a minor population before therapy in two already published non-responder [see Addendum II (page 69); Figure 4 A and B] (Debing et al., 2014b). In addition, two more patients not clearing HEV by ribavirin therapy could be analyzed showing the 1634R mutation already before therapy or no changes in the 1634 region. Moreover, further mutations could be detected in all four non-responders within the HEV polymerase during ribavirin treatment [see Addendum II (page 69); Figure 4]. Ribavirin seemed to have a broader impact on the HEV genome but after stopping treatment the quasispecies population returned back to the initial population [see Addendum II (page 69); Figure 4]. To check whether the additional mutations in the HEV genome of the first non-responder (Debing et al., 2014b) can cause a drug resistance, we tested the ribavirin sensitivity and viral replication efficiency by inducing the triple mutation 1383N / 1587F / 1634R into a HEVp6 genotype 3 replicon *in vitro*. Surprisingly, the 1383N / 1587F / 1634R combination showed an increased sensitivity to RBV but similar viral replication compared to the HEVp6 genotype 3 wild type or 1634R construct [see Addendum II (page 69); Figure 5 A and B]. This data indicates the complex dynamics of viral evolution and underlies that multiple amino acid variations may have different outcomes. Future studies will be needed to investigate in more detail how multiple amino acid variations influence the RBV sensitivity and HEV replication efficiency, also in the context of various immunosuppressant drugs.

In a previous study from our lab, we showed weak or hardly detectable HEV specific T cell responses in chronic hepatitis E (Suneetha et al., 2012). It is believed that immune pressure may drive viral evolution and thus increase viral diversity which is a potential viral mechanism to escape successful immune responses (Duggal and Emerman., 2012). It is already shown for HEV that greater quasispecies heterogeneity is linked with chronicity (Lhomme et al., 2012). Overall, in our deep sequencing study in immunocompromised patients with chronic hepatitis E we found no major intra-individual short-term variations in untreated patients indicating that there is no major immune selection pressure on HEV - which is consistent with our previous finding of weak HCV-specific T cell responses in chronically infected individuals.

Regarding the overall HEV polymerase heterogeneity in patients receiving ribavirin therapy, RBV treatment was associated with a marked increase in synonymous (silent) and non-synonymous (amino acid replacement) nucleotide substitutions [see Addendum II (page 69); Figures 2 A and B]. However, as mentioned above, the HEV quasispecies composition in the HEV polymerase was rather stable over time in most patients with chronic hepatitis E without ribavirin therapy [see Addendum II (page 69); Suppl. Figure 1]. The finding of ribavirin-therapy-associated mutagenesis of HEV is partially in line with recent data generated in patients with hepatitis C receiving ribavirin monotherapy (Dietz et al., 2013). Also in that study mutations occurred apparently randomly throughout the viral genome. Thus, these two studies suggested that ribavirin-induced mutagenesis might indeed be a relevant antiviral mechanism of ribavirin leading to error catastrophe as suggested previously in modeling work (Graci and Cameron., 2002). We also suggest that deep sequencing could be useful to identify patients at risk for not achieving a sustained virological response.

However, our results need to be confirmed by a larger cohort in a long-term study. Beyond patients with chronic hepatitis E it would be of importance to compare these data with patients acutely infected with the hepatitis E virus. Additionally, chronicity is described only for HEV genotype 3 infections. The reason behind is still unknown. Deep sequencing might be helpful to explain these differences.

Antiviral immunity of HEV infection.

HEV-specific T cell responses in patients with chronic HEV genotype 3 were previously described from our lab (Suneetha et al., 2012). In line with that study we further wanted to ascertain HEV-specific T cell responses during and after acute hepatitis E infection [see Addendum III (page 70); Figures 1 and 3]. T cells from the blood of acutely HEV infected patients showed proliferation upon stimulation with HEV-specific overlapping peptides derived from genotype 3 [see Addendum III (page 70); Figure 1 B, right panel]. Along with the T cell proliferation, high levels of antigen-specific IFNγ$^+$, TNF$^+$ and IFNγ$^+$/TNF$^+$ T cells could be detected in all acute hepatitis E virus infected patients and exert the functional capacity of both CD4$^+$ and CD8$^+$ T cells [see Addendum III (page 69); Figure 1 D; data not shown].

Little is known about the production of circulating cytokines as well as their correlation with elevated liver transaminases in acute hepatitis E. To address this, we determined 50 cytokine/ chemokine in plasma of acute infected hepatitis E patients and correlated those with the increased liver transaminases (ALT and AST). Up-regulated ALT and AST levels are an indicator of liver damage. During the acute phase of HEV infection we could show strong correlations between liver transaminases and several cytokines/ chemokines [see Addendum III (page 70); Suppl. Table 2 and 3].

Correlations between circulating cytokines and liver enzymes in plasma indicate an activated T cell response during acute hepatitis E infection. Similar to acute HAV, HBV and HCV infection (Duffy et al., 2014), elevated plasma levels of IFNγ$^+$ and TNFα$^+$ were found. This finding is in line with a report in solid organ transplanted patients. In that study higher TNFα$^+$ concentrations were found in resolving patients than in individuals who became chronic (Lhomme et al., 2012). These data support the concept that IFNγ$^+$ and TNFα$^+$ are main mediators of acute inflammatory responses as suggested for other hepatotropic viruses (Duffy et al., 2014).

Furthermore, strong correlations between IL-2 and liver transaminases were detected. IL-2 is mainly produced by T_H cells and is a major growth factor for T cells acting in an autocrine fashion. Activation of T cells results in expression of IL-2Rα. Lhomme et al. described positive correlations between liver transaminases and the T cell activation marker IL-2Rα in acute infected solid organ transplanted patients (Lhomme et al., 2012). Also in pregnant women ALT levels correlated with IL-2Rα (Ramdasi et al., 2014). This correlation was not observed in our study cohort. Nevertheless, it would be of interest whether the correlation

between IL-2Rα and ALT would disappear after resolving HEV in solid organ transplanted and pregnant women as the stimulating antigen would no longer be present.

IL-4 and IL-10 showed strong correlations to both liver transaminases in the acute phase of infection. Both are important for antiviral responses. Correlations between IL-10 and ALT were also found in during HEV infection in pregnant women (Ramdasi et al., 2014). IL-4 induces the differentiation from naïve helper T cells into T_H2 cells. Additionally, IL-4 is activating B and T cell proliferation which is in line with the strong T cell proliferation observed in our study during the acute hepatitis E infection. However, limitations of this part of the study need to be considered. We had no control group to state if elevated or reduced cytokine levels were present in plasma of acute infected hepatitis E patients. Overall, the simultaneous strong correlation between liver transaminases and several T_H1 and T_H2 related cytokines may indicate that both T_H1 and T_H2 cytokines are involved in the pathogenesis of HEV infections. Similar findings are assumed for HCV (Rehermann., 2009). Our study did not demonstrate a clear T_H1 and T_H2 shift which could be an additional explanation for our observed decline in $CD4^+$ T cell proliferation after viral HEV infection. Further investigations are therefore necessary.

Additional, Lhomme et al. showed for patients who cleared HEV infection higher IL-1Rα concentrations in serum than in patients who became chronic (Lhomme et al., 2012). In our study we found strong correlation between liver transaminases and IL-1Rα indicating that IL-1Rα could play a role in resolving HEV infection. Furthermore, we found significant correlations between liver transaminases in the acute phase of HEV infection for the cytokines IL-4, IL-10, IL-13. On top of that, IL-7, IL-15 and GM-CSF correlated with liver transaminases which is in line with data from solid organ transplanted patients in the acute phase of HEV infection and partially with data from pregnant women (Ramdasi et al., 2014, Lhomme et al., 2012). Those cytokines are implicating cell differentiation and survival.

Declined IP-10, IL-13 and IL-9 levels were observed after viral clearance [see Addendum III (page 70); Figure 2]. IP-10 is a pro-inflammatory interferon-inducible chemokine secreted by several cell types in response to IFNγ and plays an important role infectious diseases (Fabiani., 2015). Moreover, IP-10 appears to be involved on the pathogenesis of liver damage and on extrahepatic manifestations with chronic HBV infection (Fabiani., 2015). Interestingly, also for acute HAV, HBV and HCV infected patients an IP-10 up-regulation compared to healthy controls was found (Duffy et al., 2014). During acute hepatitis C, IP-10 levels were predictive for spontaneous HCV clearance during acute HCV infection as well as

for treatment induced clearance in patients with chronic HVC (Grebely et al., 2013). IP-10 is secreted by hepatocytes in inflammatory areas to recruit T cells in different hepatitis virus infections (Fabiani., 2015). If IP-10 levels in HEV infected immunocompromised patients might predict HEV clearance needs further investigation. Of note, ALT and IP-10 also correlated in HEV-infected pregnant women (Ramdasi et al., 2014).

Chemokines are supposed to recruit activated lymphocytes to the liver. In the acute phase of infection we found correlations between the chemokines IL-8 and MCP-1 and liver transaminases while after viral clearance no such correlations were evident. Chemokines like RANTES, MIP-1α and MIP-1β can recruit T cells to an inflamed liver and were also higher in the previous study in solid organ transplanted patients with HEV infection (Lhomme et al., 2012). We found no correlation between those chemokines and liver transaminases during and after acute hepatitis E. Whereas, Duffy et al. described an up-regulation for RANTES, MIP-1α and MIP-1β in acute HAV, HBV and HCV compared to healthy controls as a common hepatic signature of viral infection (Duffy et al., 2014). In fact, studying both acute and chronic infected HEV patients would be of further interest to clarify the question whether high concentrations of those chemokines during the acute phase may be the first step toward HEV persistence (Lhomme et al., 2012).

Our group has generated preliminary data that cytokine patterns in acute HEV infection may differ from acute HAV, HBV and HCV infection by altered plasma IL-13 and GM-CSF (Hartdke et al., unpublished data 2015). IL-13 declined significantly upon HEV RNA clearance from blood suggesting also a possible involvement of IL-13 in resolving HEV infection. Comparing only acute HAV with acute HEV infection significant higher levels of VCAM 1, ICAM 1 and MIG could be found for HAV infection (Hartdke et al., unpublished data 2015).

Of note, correlations between cytokines/chemokines and liver transaminases observed during in the acute phase of HEV infection could no longer be observed after viral clearance. However and interestingly, other correlations were detected including GROα, MIF, PDGFbb, IL-1α, MCP-3, SCGFβ, IL-18 and IL-13 early after recovery. MIF is a pro-inflammatory cytokine and often elevated in various inflammatory diseases. For HBV, MIF together with TNF-alpha and IL-6 are strongly correlated with ALT in chronic HBV infection (Zhang et al., 2002). The role of MIF after HEV infection is unknown. GROα, also known as CXCL1, is a chemokine and a ligand for the CXC chemokine-receptor 2 expressed on hepatic stellate cells. IL-1 α is also a pro-inflammatory cytokine and is produced mainly by activated macrophages.

MCP-3 attracts monocytes, and regulates macrophage function. IL-18 is also mainly produced by macrophages. Taken together, most of these cytokines/chemokines are connected with macrophages. Liver macrophages are critical components of the innate immune and their role in the pathogenesis of inflammatory liver diseases gained significant interest in recent years (Marra and Tacke., 2014). The specific role of liver macrophages in hepatitis E requires further investigation.

We further had the chance to study T cell responses longitudinally in four patients during and after acute HEV infection [see Addendum III (page 70); Figure 3]. In all patients HEV specific memory T cells were found. Interestingly, for the first time we found that the proliferative capacity of the HEV specific $CD4^+$ T cell responses decreased to almost undetectable levels early after HEV clearance (1-2 month). Proliferative capacity of $CD4^+$ T cells was detectable again at later time points (> 1 year after first visit). The mechanism why $CD4^+$ T cells are unable to proliferate early after recovery is not clear but one possibility is that cytokines/ chemokines are involved in the lack of $CD4^+$ T cell proliferation at this time point. Similar to our data, a $CD4^+$ T cell slope has been described for HAV infected chimpanzees. Once, HAV RNA became undetectable in the serum the $CD4^+$ T cell contraction stopped in that study (Zhou et al., 2012). Still, HAV RNA could be measured for several weeks in the liver after post infection. In contrast to our results, the $CD4^+$ T cell slope in the chimpanzees was described for cytokine producing $CD4^+$ T cell with a strong decline of cytokine production in PBMCs (Zhou et al., 2012). In our study, we showed a lack of $CD4^+$ T cell proliferation but with a still high $IFN\gamma^+$ production. Nevertheless, antigen-specific $IFN\gamma^+/TNF^+$ T cells could be detected in both HAV infected chimpanzees (Zhou et al., 2012) and in HEV infected patients (Gisa et al., 2015) considering a highly effective $CD4^+$ T cell immunity and an overall important role for $CD4^+$ T cells in HAV and HEV clearance. Therefore, further investigations are needed.

Moreover, it would be of further interest whether blocking co-inhibitory receptors (e.g. PD-1 or CTLA-4) can restore the lack of $CD4^+$ T cell proliferation early after HEV clearance (1-2 month) as it was described previously in patients with chronic hepatitis E (Suneetha et al., 2012) and various other viruses such as HIV (Day et al., 2006).

Previously, we reported HEV-specific T cell responses in chronically and revolved patients carrying HEV genotype 3 using genotype 1-derived peptides (Suneetha et al., 2012). To address this topic in more detail we evaluated HEV-specific T cell responses derived from genotype 1 and genotype 3 overlapping peptide pools. Afterwards, we could describe for the first time functional HEV cross-genotype-specific T cell responses between HEV genotype 3 and 1 during acute hepatitis E genotype 3 virus infections. These results might have implications for vaccine development. Studies in rhesus macaques and preliminary data from the first commercially available HEV vaccine Hecolin® showed potential cross-genotype- and cross-species-specific protection against re-infection with a homologous and heterologous HEV (Zhang et al., 2015, Liu et al., 2014, Purcell et al., 2003, Huang et al., 2008, Sanford et al., 2011, Sanford et al., 2012). The degree of cross-genotype protection was strongly associated with the level of the HEV IgG titer prior challenge (Huang et al., 2008). To our knowledge, no studies have been performed investigating cross-genotype and cross-species-specific T cells in patient acutely infected with HEV. These T cell responses, especially $CD4^+$ T cells, might play an important role to develop durable functional antibody responses after vaccination. Many $CD4^+$ T cell functions in HEV infection are only partially understood. $CD4^+$ T cell responses induced by vaccines may also play an important role in HEV infections as it was reported in different vaccine approaches (Rodriguez et al., 2001, Streeck et al., 2013). It might be of interest whether Hecolin® can additionally provide protection against HEV genotype 3 infection in organ transplanted patients. In summary, our findings might also be important for vaccine development and application in developed countries.

Limitations of the T cell studies need to be considered. Due to low numbers of diagnosed acute hepatitis E genotype 3 cases in Germany, we had only a small study cohort. Blood sampling were often collected in a routing clinical setting and samples are often retrospectively examined based on availability. Moreover, different methods were applied in different studies investigating HEV specific immune responses must be considered (Wedemeyer et al., 2013). In different publications PBMCs were either stimulated with recombinant ORF2 or 3 proteins or with overlapping peptide pools. In the latter case, the length of peptides, the number of overlapping amino acids and the antigenic regions were used are different (Wedemeyer et al., 2013). Besides, usually only one functional read out was applied and there was seldom a distinction between $CD4^+$ and $CD8^+$ T cell responses (Suneetha et al., 2012). As a descriptive additional study, screening lymphocyte responses against ORF1 using overlapping peptide pools might be of interest. Previous studies only investigated antigen specific T cell responses using recombinant ORF2 or 3 proteins or

peptide pools (Suneetha et al., 2012, Aggarwal et al., 2007) studying antigen-specific T cell responses in acute hepatitis E, we found out that especially our overlapping peptide pool 2 derived from ORF2 showed stronger $CD8^+$ T cell responses compared to other peptide pools [see Addendum III (page 70); Figure 1 B, right panel]. Therefore, it would be of interest to screen down this peptide pool to find HEV derived epitopes. Another method to identify HLA class-I restricted HEV epitopes for ORF1, 2 and 3 would be to predict them *in silico*, and test these epitopes *ex vivo* and *in vitro* to monitor antigen-specific T cell responses. Until date there are no HLA class-I restricted HEV epitopes published. Detailed knowledge about HLA-restricted epitopes in HEV might be crucial to improve the knowledge about the immunopathogenesis of hepatitis E. Only one publication describes $CD4^+$ T cell responses connected with epitope mapping in HEV genotype 1 infected patients (Aggarwal et al., 2007). An association between one HLA-DR allele and reactivity to a particular peptide pool was found. HEV genotype 3 and HEV genotype 1 infections result in different clinical courses [see Addendum III (page 70); Suppl. Figure 3]. Therefore, a more detailed comparison of both HEV genotypes would be interesting. Moreover, HEV specific T cell responses had only been studied in the peripheral blood so far. Investigating lymphocytes from liver biopsies would therefore be of interest. One method could fine needle aspirations as previously been reported from our group (Hengst et al., 2015).

Finally, a possible link between the hepatitis E virus and induction of autoimmune hepatitis (AIH) is currently a matter of debate. During our T cell studies in acute hepatitis E we were able to measure HEV-specific T cell responses also in anti-HEV positive AIH patients and could thereby confirm that the increases HEV seroprevalence in AIH patients can indeed by linked to previous contact to HEV [see Addendum IV (page 71); Figure 3] (Pischke et al., 2014c). Conversely, the actual prevalence of AIH in HEV patients is unknown and remains to be addressed in future studies. Molecular mimicry as a mechanism for the cause of autoimmune liver diseases has been suggested. Viruses may trigger autoimmune hepatitis through sequence or structural similarities with host self-antigens (Manns et al., 2015, Liberal et al., 2013). In case of HCV infection, the virus shares high amino acid sequence homology with the auto-antigenic target of anti-LKM-1 autoantibodies in AIH-2 and cytochrome P4502D6 (CYP2D6) (Liberal et al., 2013). Additionally, HBV, cytomegalovirus and herpes simplex virus are further potential viral triggers for AIH (Liberal et al., 2013). For these reasons, it would be interesting to study cross-reactive T cells to self-epitopes also during and after HEV infection to elucidate the topic whether previous acute HEV infection might also trigger AIH.

Further projects to better understand the immunopathophysiology of HEV infection could be studying regulatory immune cells (T_{reg}). Up to now, only one study investigated T_{reg} cells in acute hepatitis E showing higher T_{reg} frequencies and increased IL-10 levels similar to HBV and HCV infection (Tripathy et al., 2012b). Additional, limited information is available about the role of innate immunity in HEV infection, e.g. the specific phenotype and function of NK cells (Srivastava et al., 2008).

In summary, we have gained new insights into the pathophysiology of hepatitis E virus infection in the context of both molecular virology and antiviral immunity. For the first time a higher replicating mutation (G1634R) in the HEV polymerase was described *in vitro*; selection of this variant may explain ribavirin treatment failure in some patients. We also described that ribavirin therapy is increasing the amount of nucleotide substitutions *in vivo* and that ribavirin also caused transient amino acid changes. We suggest that a better understanding of hepatitis E viral evolution during antiviral therapy may help to guide development of personalized antiviral strategies against the virus.

Moreovoer, we characterized antigen specific T cell responses in the acute phase of hepatitis E indicating that T cell responses might be crucial in resolving HEV. Of note, 50 pro- and anti-inflammatory cytokines/ chemokines were measured in patients with acute hepatitis E which showed distinct associations with biochemical disease activity. Cross-genotype-specific T cell responses were measureable in acute hepatitis E which is of importance for vaccine development as well as for the basic understanding of immune responses in the different HEV genotypes.

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VI. APPENDIX

a. Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel „Pathophysiology of Hepatitis E Virus Infection: viral evolution during antiviral therapy and virus-specific T cell responses“ selbständig verfasst habe.

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Ich habe die Dissertation an folgendem Institut angefertigt:
Klinik für Gastroenterologie, Hepatologie und Endokrinologie,
Arbeitsgruppe Prof. Wedemeyer, Medizinische Hochschule Hannover,
Carl-Neuberg-Str.1 30625 Hannover.

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

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Ort, Datum Unterschrift (Anett Gisa)

b. Acknowledgments

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d. Publications

A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients.

Debing Y[§], **Gisa A[§]**, Dallmeier K, Pischke S, Bremer B, Manns M, Wedemeyer H, Suneetha PV, Neyts J., [§] contributed equally

Published in Gastroenterology 2014

Contribution:

I performed half of the experiments, data analyses and figures as well as writing half of the manuscript.

In vivo **evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome**

D. Todt[§], A. Gisa[§], A. Radonic, P. Behrendt, PV Suneetha, S. Pischke, J. Hinzmann, B.Bremer, R.Brown, MP. Manns, M. Cornberg, T. Bock[#], E. Steinmann[#],
H. Wedemeyer[#1,6]; [§] /# contributed equally

Accepted in Journal of Hepatology 2016

Contribution:

I performed the HEV polymerase amplification, analysed and prepared the SNV figures, table 1 and wrote half of the material/method and introduction as well as the complete result part.

Cross-genotype-specific T cell responses in acute hepatitis E virus (HEV) infection.

A. Gisa, PV Suneetha, P. Behrend, S. Pischke, B. Bremer, MP Manns, M. Cornberg, H. Wedemeyer and A.R.M. Kraft

Published in Journal of Viral Hepatitis 2016

Contribution:

I performed the experiments, analyzed the data, prepared the figures and wrote the manuscript.

Increased HEV seroprevalence in patients with autoimmune hepatitis.

Pischke S, **Gisa A**, Suneetha PV, Wiegand SB, Taubert R, Schlue J, Wursthorn K, Bantel H, Raupach R, Bremer B, Zacher BJ, Schmidt RE, Manns MP, Rifai K, Witte T, Wedemeyer H.

Published in PLoS One 2014

Contribution:

Before reviewing and editing the manuscript, I performed for figure 3 the experiments, the data analyses and figure.

Course and treatment of chronic hepatitis E virus infection in lung transplant recipients.

Pischke S, Greer M, Hardtke S, Bremer B, **Gisa A**, Lehmann P, Haverich A, Welte T, Manns MP, Wedemeyer H, Gottlieb J; Hepatitis E study group.

Transpl Infect Dis. 2014

Contribution:

I reviewed the manuscript.

Relevance of chronic hepatitis E in liver transplant recipients: a real-life setting.

Galante A, Pischke S, Polywka S, Luetgehethmann M, Suneetha PV, **Gisa A**, Hiller J, Dienes HP, Nashan B, Lohse AW, Sterneck M.

Transpl Infect Dis. 2015

Contribution:

I reviewed the manuscript.

Addendum I

A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients.

Debing Y.[§], Gisa A.[§], Dallmeier K., Pischke S., Bremer B., Manns M., Wedemeyer H., Suneetha P.V., Neyts J.

§ contributed equally

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BRIEF REPORT

A Mutation in the Hepatitis E Virus RNA Polymerase Promotes Its Replication and Associates With Ribavirin Treatment Failure in Organ Transplant Recipients

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This article has an accompanying continuing medical education activity on page e15. Learning Objective: Upon completion of this CME exercise, successful learners will be able to describe the current treatment options of chronic hepatitis E infections and explain the potential implications of the presence of the G1634R mutation in the hepatitis E virus (HEV) polymerase.

We analyzed blood samples collected from 15 patients with chronic hepatitis E who were recipients of solid-organ transplants. All patients cleared the hepatitis E virus (HEV) except for 2 (nonresponders); 1 patient died. A G1634R mutation in viral polymerase was detected in the HEV RNA of the nonresponders; this mutation did not provide the virus with resistance to ribavirin in vitro. However, the mutant form of a subgenomic replicon of genotype 3 HEV replicated more efficiently in vitro than HEV without this mutation, and the same was true for infectious virus, including in competition assays. Similar results were obtained for genotype 1 HEV. The G1634R mutation therefore appears to increase the replicative capacity of HEV in the human liver and hence reduce the efficacy of ribavirin.

Keywords: RNA-Dependent RNA Polymerase; Virulence; Drug Resistant; Mechanism.

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Hepatitis E virus (HEV) is a feco-orally transmitted RNA virus and a common cause of acute hepatitis worldwide.[1,2] Genotypes 1 and 2 cause water-borne outbreaks in developing countries and exclusively infect humans, and genotypes 3 and 4 are zoonotic and consumption of undercooked pig meat is the most documented route of transmission.[1,3] Although HEV infections are often asymptomatic, they can manifest as acute hepatitis, but usually resolve spontaneously. However, genotype 3 infections may evolve to chronicity in immunocompromised patients, for example, transplant or human immunodeficiency virus patients,[4,5] and can lead to cirrhosis, graft loss, and death.[6] Ribavirin (RBV) monotherapy is the treatment of choice for most patients.[7,8] However, treatment failure has been observed, either as a partial response to RBV or viral recurrence after therapy cessation, and is possibly linked to dose reductions because of severe anemia in some cases.[7,8]

We report on 15 solid-organ transplant patients with chronic hepatitis E (11 with genotype 3c, 3 genotype 3f, and 1 genotype 3e). RBV treatment was successful in all but 2 patients (both genotype 3c) who failed to clear the virus (13%, which is comparable with other studies reporting failure rates of 15%–18%).[7,8] The first patient was a male heart/kidney recipient who was treated for 9 months and showed an initial decrease in viral RNA load upon initiation of RBV treatment but never became HEV RNA negative (ie, <1000 copies/mL, Figure 1*A*). After 4 months of RBV therapy, HEV RNA loads increased again to baseline levels (even before a transient dose reduction because of anemia) and persisted over time. The patient died of hepatic decompensation as described previously.[9] The second patient is a female lung/kidney/bone marrow recipient who underwent 2 consecutive RBV treatments of 4 and 7 months, respectively.[10] Although at the end of each treatment period, HEV RNA status was negative, viral RNA was detected again shortly after stopping therapy. Alanine aminotransferase levels normalized rapidly, but γ-glutamyltransferase remained above normal and strongly increased during the second RBV course (Figure 1*B*). To explore the underlying causes of nonresponsiveness to RBV in both patients, complete HEV genome sequences before, during, and after treatment were compared. In both patients, a unique G-to-A nucleotide substitution was identified, resulting in a G1634R mutation in the C-terminal

*Authors share co-first authorship; §Authors share co-senior authorship.

Abbreviations used in this paper: **HEV, hepatitis E virus; RBV, ribavirin; wt, wild-type.**

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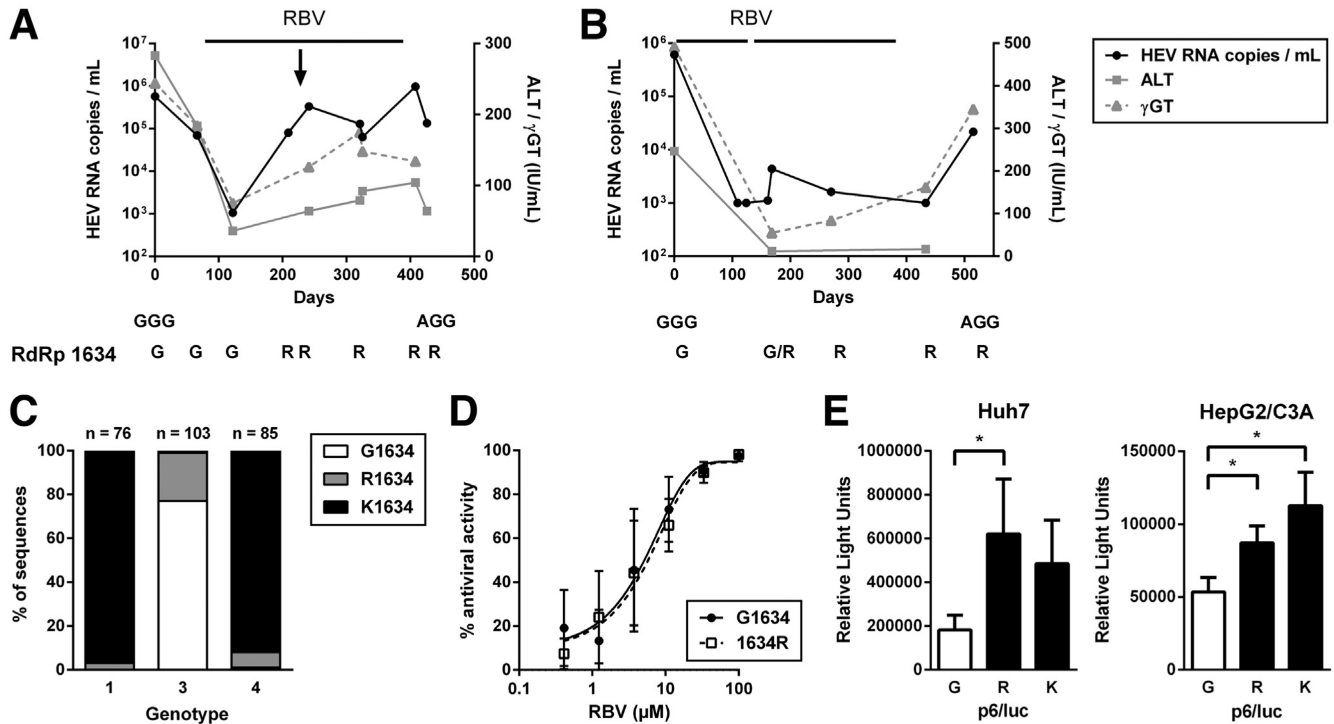

Figure 1. RBV treatment failure is associated with a G1634R mutation. (*A, B*) Clinical course and sequencing results for patients experiencing RBV failure; *arrow* indicates RBV dose reduction. (*C*) Prevalence of G, R, and K at position 1634 per genotype. (*D*) G1634R does not alter RBV sensitivity. (*E*) Increased luminescence read-out in 1634R/K compared with G1634 in Huh7 and HepG2/C3A cells. *$P < .05$; **$P < .01$.

region of the HEV polymerase. Comparison of HEV sequences in GenBank revealed that K1634 was the predominant amino acid in genotype 1 and 4, and in genotype 3, G1634 was more common (77%) than R1634 (22%, mostly subgenotypes 3e-f) (Figure 1*C*, Supplementary Table 1).

Because the clinical course and evolution of viral loads was reminiscent of antiviral resistance development, the 1634R mutation was introduced into a genotype 3 replicon and its sensitivity to RBV was compared with that of the wild-type (wt) replicon.[11] No difference in RBV sensitivity was observed, with calculated 50% effective concentration values of 5.1 ± 3.7 μM and 5.1 ± 4.1 μM for G1634 and 1634R, respectively (Figure 1*D*). The 1634R construct, however, consistently yielded higher luminescence signals than its wt counterpart, suggesting increased viral RNA replication. To further explore this, 2 hepatoma cell lines were transfected with capped replicon RNA for wt, 1634R, and 1634K (predominant in genotype 1). In Huh7 cells, the 1634R construct resulted in a 3.4-fold increase in luminescence signal compared to the G1634 construct ($P = .04$) and the 1634K construct yielded a 2.7-fold higher signal than wt ($P = .07$, Figure 1*E*). In HepG2/C3A cells, both mutants resulted in a significantly increased signal: 1.6-fold for 1634R ($P = .02$) and 2.1-fold for 1634K ($P = .02$) (Figure 1*E*). Similar results were obtained when 10:1, 1:1, or 1:10 mixtures of wt and/or mutant RNA were tested (Supplementary Figure 1).

Next, the impact of the 1634R/K mutations on replication and production of viral progeny of full-length genotype 3 was studied by transfection of Huh7 cells and quantification of released viral RNA. The 1634R and 1634K variants replicated to higher titers than the wt at every time point studied (Figure 2*A*). When treated with RBV at 10 or 25 μM, replication was partially reduced, but the same pattern in relative replication efficiency was observed for the 3 variants. Intracellularly, viral RNA copies per μg RNA were at least 2-fold higher for 1634R/K compared with G1634 (Figure 2*B*). In untreated HepG2/C3A cells, a comparable pattern was observed, although somewhat less pronounced than in Huh7 cells (Supplementary Figure 2). Treatment of transfected HepG2/C3A cells with RBV at 10 or 25 μM resulted in a strong inhibition of viral replication so that no differences could be observed.

To compare the fitness of G1634 and 1634R variants, replication was analyzed in direct competition assays. To this end, cells were transfected with mixtures of G1634:1634R full-length RNA in 10:1, 1:1, or 1:10 ratios (90%, 50%, and 10% G, respectively) and were cultured for 20 days. The evolution of the fraction of both variants released into the culture medium was monitored by allele-specific reverse-transcription quantitative polymerase chain reaction. The proportion of G1634 was experimentally confirmed to be 12% ± 1%, 45% ± 1%, and 88% ± 1% in the input RNA and decreased to respectively 2.2% ± 0.4%, 17% ± 1%, and 61% ± 2% in Huh7 and 2% ± 2%, 18% ± 4%, and 64% ± 2% in HepG2/C3A cells (Figure 2*C*). This corresponds to a relative fitness gain for 1634R of 7%−9% over 20 days and up to 15% for the first 10 days. A similar pattern was noted in cell lysates ($P < .001$; Figure 2*D*, Supplementary Figure 3). These results provide a strong

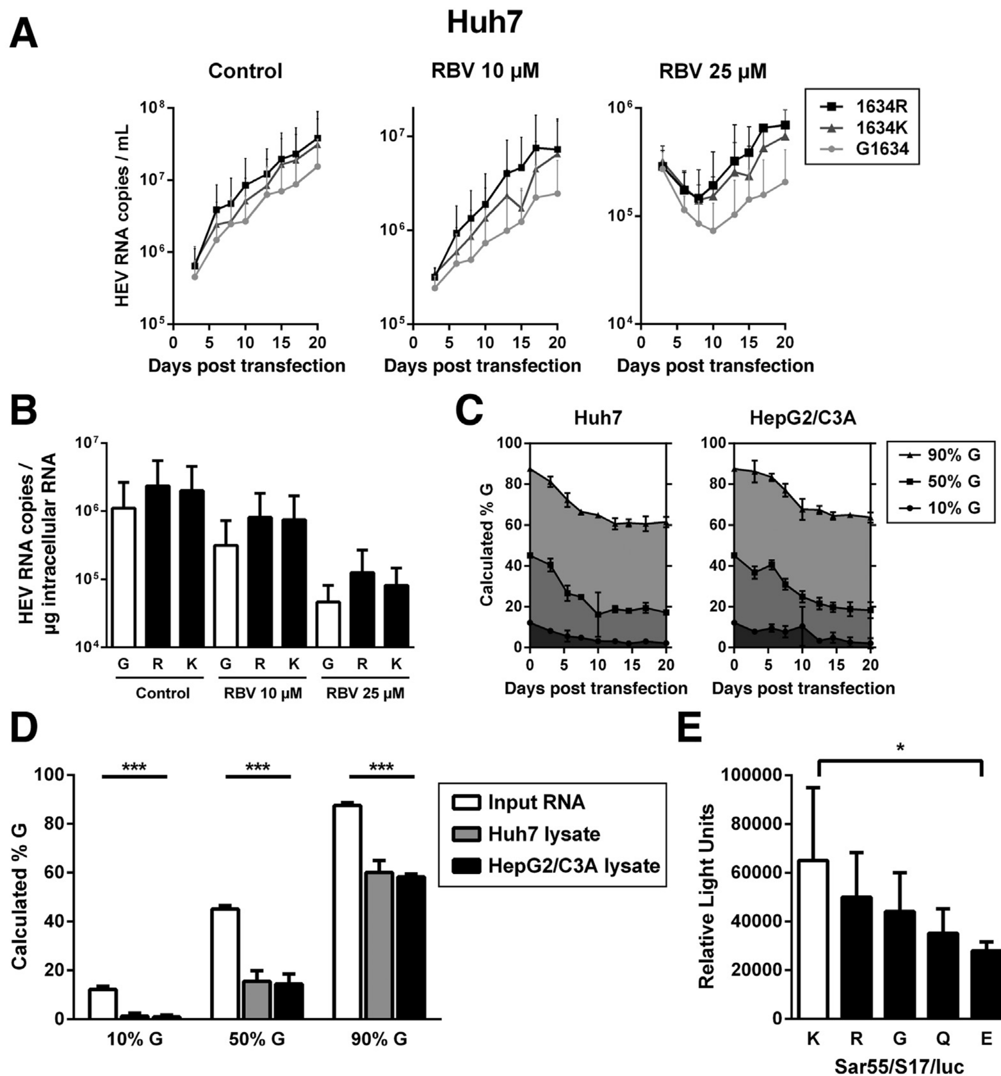

Figure 2. 1634R/K has an increased fitness compared with G1634. (*A*) Kinetics of released genotype 3 RNA indicate increased replication of 1634R/K strains compared with G1634, both in untreated or RBV-treated cells. (*B*) Intracellular viral RNA reveals a similar pattern. (*C*) Mixtures of G1634 and 1634R full-length RNA were transfected into Huh7 or HepG2/C3A cells and the fraction of wt G1634 in released viral RNA was monitored, indicating outcompeting of G1634 by 1634R. (*D*) Comparing input RNA with intracellular viral RNA 20 days post transfection shows a similar decrease in the G1634 fraction. (*E*) In a genotype 1 replicon, replication is decreased for 1634G/R/Q/E compared with the wt K1634 in Huh7 cells. *$P < .05$; ***$P < .001$.

indication that for genotype 3, 1634R has an increased fitness compared with G1634.

To determine the influence of the 1634 mutations on viral fitness in other genotypes, a genotype 1 replicon in Huh7 cells was employed in which the original K1634 was mutated to 1634R, 1634G, 1634Q, or 1634E. As expected, 1634G yielded a decreased signal compared with K1634 (0.7-fold, Figure 2*E*). Surprisingly, the 1634R mutant also displayed decreased replication (0.8-fold), albeit not statistically significant. A single genotype 1 strain was found with Q1634 (Accession Number JF443723),[12] but in our replicon, 1634Q decreased replication (0.5-fold; $P = .11$). Introduction of 2 other mutations unique to this strain yielded replication-impaired or lethal phenotypes (Supplementary Figure 4). Finally, the 1634E mutant displayed a significantly reduced replication (0.4-fold; $P = .049$) as expected, given that this is a negatively charged amino acid, contrary to the positively charged R and K.

In conclusion, this is the first reported virulence mutation in the HEV genome that was confirmed in vitro. The increased replication capacity of the mutant may have contributed to the persistent disease courses despite RBV treatment, although other patient- and virus-related factors could have contributed as well.[6,13] It may be interesting to assess the possible use of position 1634 as a prognostic marker and, accordingly, to adjust dose and duration of RBV therapy based on the presence of the G1634R variant.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2014.08.040.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

Study Subjects

All patients were recruited at Hannover Medical School between 2008 and 2014. Serum samples were collected from 15 solid organ transplant recipients who developed chronic hepatitis E and received ribavirin (RBV) treatment. All patients were infected with genotype 3 HEV. Serum samples were collected before (I), during (II), and after RBV treatment (III). At least one serum sample from each time point (I–III) was used for further studies. Written informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the Institutional Review Committee.

RBV (Rebetol or Copegus) was administered orally twice daily with an initial daily dose of 600–1000 mg, depending on the patients' hemoglobin level and comorbidities.[1] Dose reductions were performed if hemoglobin levels declined and/or patients developed symptoms associated with anemia. At each visit, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltranspeptidase and HEV serology and HEV RNA viremia were determined. Anti-HEV status was determined using Wantai HEV IgG (Beijing, China). HEV RNA from serum was quantified by one-step reverse transcription quantitative polymerase chain reaction (PCR) as described.[2] All patients cleared HEV RNA by the end of treatment, except for 2 chronically infected transplant recipients (both infected with genotype 3c).

Extraction and Sequencing of Hepatitis E Virus RNA From Serum Samples

Total RNA was extracted from 200 μL serum or EDTA-plasma using Cobas AmpliPrep total nucleic acid isolation kit (Roche, Basel, Switzerland). Total RNA concentration was measured using the NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, UK). Complementary DNA was synthesized from 4–8 μL purified total RNA using the SuperScript III first-strand synthesis system (Life Technologies, Carlsbad, CA) with the external reverse primer from each set (set1-4 A2 [ex]) at a final concentration of 2 μM (see section Primers and Probes). A touchdown nested polymerase chain reaction with 4 sets of specific external and internal primer pairs listed in the section Primers and Probes was used to amplify the coding regions of the HEV genome. The first PCR round was carried out with TaKaRa Ex Taq Hot Start Version (Dalian, China) using 8 μL of synthesized complementary DNA and an external primer pair at a final concentration of 1 μM each in a 50 μL reaction with 18 cycles of 30 seconds at 94°C, 45 seconds at 62°C with a reduction of 0.5°C/cycle and 1 min/kb at 72°C, followed by 14 cycles of 30 seconds at 94°C, 45 seconds at 53°C, and 1 min/kb at 72°C. A final extension of 10 minutes at 72°C followed the final cycle. The second PCR round was carried out using internal primer pairs and 5 μL of the first-round PCR product with identical amplification parameters to the first round. The resulting amplicons were separated by agarose gel electrophoresis and purified using Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and concentrations were measured using NanoVue Plus spectrophotometer (for Sanger sequencing).

Sanger Sequencing and Analysis of Hepatitis E Virus Coding Regions

The purified PCR products were sequenced commercially (GATC Biotech, Konstanz, Germany) in both forward and reverse directions using the internal primer pairs from set 1–4 (see section Primers and Probes) on an automatic DNA sequencer (Sanger ABI 3730xl). To identify nucleotide and amino acid variations between different time points (before, during, and after treatment) the nucleotide sequences from all sets were assembled using Sequencher 4.9 software by Gene Codes (Ann Arbor, MI).

Cells, Viruses, and Replicons

Huh7 cells (a kind gift from Ralf Bartenschlager, University of Heidelberg, Germany) and HepG2/C3A cells (a kind gift from Luc Verschaeve, Scientific Institute of Public Health, Brussels, Belgium) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified 5% CO_2 incubator at 37°C.

Genotype 3 full-length and reporter replicon viral RNA was derived from plasmids encoding Kernow-C1 p6 (GenBank accession number JQ679013) and Kernow-C1 p6/luc HEV strains, respectively.[3] Genotype 1 replicon RNA was derived from Sar55/S17/luc-encoding plasmid.[4] Both were kind gifts from Suzanne U. Emerson (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Amino acid numbering is according to NCBI reference sequence NP_056779.

Site-Directed Mutagenesis and Plasmid Preparation

Mutations were introduced by PCR amplification of overlapping fragments with specifically mutated primers. To introduce the 1634R mutation into the Kernow-C1 p6/luc replicon, PCR amplifications were performed with primer pairs KC1p6-*Afl*II-5′f + KC1p6-1634Rr and KC1p6-1634Rf + KC1p6/luc-*Nru*I-3′r (see section Primers and Probes for primer sequences). Consequently, both fragments were combined in a fusion PCR with primers KC1p6-*Afl*II-5′f and KC1p6/luc-*Nru*I-3′r. PCRs were performed with KAPA HiFi HotStart ReadyMix PCR kit (Kapa Biosystems, Wilmington, MA). The resulting fragment was digested with *Afl*II, phosphorylated and ligated into *Afl*II- and *Nru*I-digested Kernow-C1 p6/luc plasmid. Similarly, primer pairs KC1p6-*Afl*II-5′f + KC1p6-1634Kr and KC1p6-1634Kf + KC1p6/luc-*Nru*I-3′r were used to introduce the 1634K mutation. To mutate the full-length Kernow-C1 p6 plasmid, PCRs were performed with the same primer sets except KC1p6-*Pml*I-3′r was used as the ultimate 3′-end primer. The resulting fusion fragment was digested with *Afl*II, phosphorylated and ligated into *Afl*II- and *Pml*I-digested Kernow-C1 p6 vector.

To mutate the genotype 1 Sar55/S17/luc construct (wild-type [wt]: K1634), PCRs were performed with primer pairs

SarLuc-*Sfi*I-5′f + SarLuc-1634Gr and SarLuc-1634Gf + SarLuc-*Nhe*I-3′r (1634G), SarLuc-*Sfi*I-5′f + SarLuc-1634Rr and SarLuc-1634Rf + SarLuc-*Nhe*I-3′r (1634R), SarLuc-*Sfi*I-5′f + SarLuc-1634Er and SarLuc-1634Ef + SarLuc-*Nhe*I-3′r (1634E), SarLuc-*Sfi*I-5′f + SarLuc-1634Qr and SarLuc-1634Qf + SarLuc-*Nhe*I-3′r (1634Q), SarLuc-*Sfi*I-5′f + SarLuc-1346Gr and SarLuc-1346Gf + SarLuc-*Nhe*I-3′r (1346G), SarLuc-*Sfi*I-5′f + SarLuc-1498Pr and SarLuc-1498Pf + SarLuc-*Nhe*I-3′r (1498P), SarLuc-*Sfi*I-5′f + SarLuc-1346Gr and SarLuc-1346Gf + SarLuc-1498Pr and SarLuc-1498Pf + SarLuc-*Nhe*I-3′r (1346G + 1498P). Fragments were combined in fusion PCRs with primers SarLuc-*Sfi*I-5′f and SarLuc-*Nhe*I-3′r and digested with *Nhe*I and *Sfi*I. The resulting fragments were ligated into *Nhe*I- and *Sfi*I-digested Sar55/S17/luc vector.

Escherichia coli Top10 cells (Life Technologies) were transformed with ligated plasmids. Kernow-C1−related constructs were cultured in 500 mL Super Broth with ampicillin and maxiprepped (Plasmid maxi kit; Qiagen). Sar55-related plasmids were cultured in 100 mL LB medium with ampicillin and midiprepped (NucleoBond Xtra midi kit, Macherey-Nagel, Düren, Germany). The cloned regions in each of the constructs were sequenced to ensure that no additional mutations had been introduced.

In Vitro Transcription and Capping

Viral RNA was in vitro transcribed from *Mlu*I- (Kernow-related) or *Bgl*II- (Sar55-related) linearized plasmid DNA with the RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI) and capped with the ScriptCap m7G capping system (Cellscript, Madison, WI). A firefly luciferase-based transfection control was generated as described.[5] Nucleic acid concentrations were determined by spectroscopy (Nanodrop ND-1000; Thermo Fischer Scientific, Waltham, MA).

Antiviral Assay

Luminescence-based antiviral assays were performed essentially as described.[5] RBV (1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide [Virazole]) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). A stock solution was prepared in dimethyl sulfoxide and stored at 4°C.

Replicon Assays

Huh7 and HepG2/C3A cells were seeded into 6-well plates at 2×10^5 cells per well and transfected with capped RNA transcripts (1 μg per well) 24 hours later using Lipofectin (Life Technologies) according to the manufacturer's instructions. After 72 hours of incubation at 35°C, *Gaussia* luciferase activity was measured in 20 μL culture medium with the *Renilla* luciferase assay system (Promega).

For replicon competition assays, Huh7 and HepG2/C3A cells were seeded into 12-well plates at 8×10^4 cells per well. RNA was produced for each wt and mutant Kernow-C1 p6/luc replicon and 2 types of uncapped RNA were mixed in 10:1, 1:1, and 1:10 ratios. Mixtures were capped and 0.4 μg per well was transfected into cells 24 hours after seeding with Lipofectin. After 72 hours of incubation at 35°C, *Gaussia* luciferase activity was determined.

Full-Length Hepatitis E Virus Replication Kinetics and Competition Assays

To determine the growth kinetics of wt and mutant full-length HEV (G1634, 1634R, and 1634K), Huh7 and HepG2/C3A cells were seeded into 6-well plates at 2×10^5 cells per well and transfected with capped RNA transcripts for Kernow-C1 p6 wt or mutants (1 μg per well). Dimethyl sulfoxide (0.125%) or RBV at 10 or 25 μM were included in the culture medium. One milliliter of the medium was removed every 2–3 days, stored at −80°C until RNA extraction and 1 mL of fresh medium (with dimethyl sulfoxide or RBV) was added to each well. After 20 days, cell layers were lysed and intracellular RNA was extracted with the Qiagen RNeasy kit. To extract viral RNA from culture medium, RNase A (Promega) was added to 100 μL of thawed medium to a final concentration of 200 ng/mL and incubated at room temperature for 5′ to reduce the amount of residual in vitro transcripts from RNA transfection. Viral RNA was extracted with the NucleoSpin RNA virus kit (Macherey-Nagel) and quantified by reverse transcription quantitative PCR (RT-qPCR).

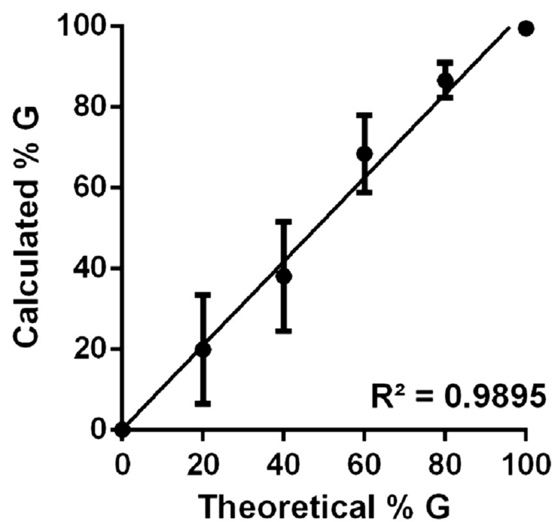
For infectious virus competition assays, Huh7 and HepG2/C3A cells were seeded into 6-well plates at 2×10^5 cells per well. RNA was produced for Kernow-C1 p6 wt and 1634R mutant and was mixed in 10:1, 1:1, and 1:10 ratios. Mixtures were capped and transfected with Lipofectin into cells 24 hours after seeding. One milliliter of the medium was removed every 2–3 days, stored at −80°C until RNA extraction and 1 mL of fresh medium was added to each well. RNase treatment and RNA extractions were performed as described. RNA extracts were analyzed by allele-specific multiplex RT-qPCR. Input RNA mixtures and RNA extracts from cell lysates were subjected to RT-PCR with primers KC1p6-*Afl*II-5′f and KC1p6-*Pml*I-3′r (Onestep RT-PCR kit; Qiagen) and subsequent Sanger sequencing with primer KC1p6-*Afl*II-5′f (BigDye Terminator v3.1 cycle sequencing kit; Life Technologies) for semiquantitative assessment of the G1634:1634R ratio. For calculation of relative fitness gains, the fitness of G1634 and 1634R strains is considered 1 and $1+s$ respectively with s being the relative fitness gain; s is calculated as described.[6]

Reverse Transcription Quantitative Polymerase Chain Reaction

Quantification of total HEV RNA was performed essentially as described with primers HEVqf and HEVqr and probe HEVqp.[5] For absolute quantification of intracellular viral RNA, copy numbers were normalized to total RNA per sample as determined by spectroscopy.

Allele-specific multiplex RT-qPCR was performed with primers KC1p6-asqf and KC1p6-asqr. As probes, KC1p6-asqpG and KC1p6-asqpR were used (see section Primers and Probes). Reactions were performed with One-Step qRT-PCR mix (Eurogentec, Seraing, Belgium) in a final volume of 25 μL containing 375 nM of each primer, 125 nM of each probe and 5 μL of RNA sample using the ABI 7500 Fast

Real-Time PCR System (Applied Biosystems, Foster City, CA) under following conditions: 30 minutes at 48°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Data were analyzed with ABI PRISM 7500 SDS software (version 1.3.1, Applied Biosystems). For absolute quantification, standard curves were generated using 10-fold dilutions of the cloned target complementary DNA. Concentrations of DNA standards were calculated by spectroscopy (for regular RT-qPCR) or RT-qPCR (for allele-specific multiplex RT-qPCR). In order to validate the allele-specific multiplex RT-qPCR, mixtures of G1634 and 1634R RNA were made in known ratios (0%, 20%, 40%, 60%, 80%, and 100% wt) and analyzed. Results show a strong correlation between expected and experimentally obtained fractions of wt ($R^2 = 0.9895$, n = 3).



Primers and Probes

Primers for analysis of clinical samples

Set	Primer	Sequence
1	Set1-S1 (ex)	5′-AGGCTCCTGGCATTACTACTG-3′
	Set1-S2 (in)	5′ -GCCTTGGCGAATGCTGTG-3′
	Set1-A1 (in)	5′ -GGCCGGGGATGTARTCACG-3′
	Set1-A2 (ex)	5′ -CGGCACTGRGCATARAACTG-3′
2	Set2-S1 (ex)	5′ -ATGACATACCTYCGTGGYATTAG-3′
	Set2-S2 (in)	5′ -GTYGCYAATGAGGGKTGGAA-3′
	Set2-A1 (in)	5′ -TTGTGGTCTCTGTRAARGTRGCRCCCT-3′
	Set2-A2 (ex)	5′ -CRGCCGTRGCTATAATTGTRGTCT-3′
3	Set3-S1 (ex)	5′ -GYTTTGCGGCCTTYACACCYCAYAC-3′
	Set3-S2 (in)	5′ -CGYCGTGTTGTKATTGAYGAGGC-3′
	Set3-A1 (in)	5′ -GRCCRGCAAAHCGCACYACAT-3′
	Set3-A2 (ex)	5′ -AACACARACCTGCGCRACATTCGT-3′
4	Set4-S1 (ex)	5′ -CCTGGYACCCTYCTYTGGAAYAC-3′
	Set4-S2 (in)	5′ -TGGGYTGTATGCYGGTGTGGTRGT-3′
	Set4-A1 (in)	5′ -CAGCCGACGAAATCAATTCTG-3′
	Set4-A2 (ex)	5′ -CCCTTATCCTGCTGYGCATT-3′

Primers for mutagenesis and in vitro studies

Primer name	Sequence
KC1p6-*Afl*II-5′ f	5′ -TGATCACTTAAGGGTTTCTGGAA GAAGC-3′
KC1p6-1634Rr	5′ -TCAA**CCT**TCGAAGGAAATCACAAACAG-3′
KC1p6-1634Kr	5′ -TCAA**CTT**TCGAAGGAAATCACAAACAG-3′
KC1p6-1634Rf	5′ -CTGTTTGTGATTTCCTTCGA**AGG**TTGA CGAACG-3′
KC1p6-1634Kf	5′ -CTGTTTGTGATTTCCTTCGA**AAG**TTGAC GAACG-3′
KC1p6/luc-*Nru*I-3′ r	5′ -CGAAGTTGCTGGCCACGGCCAC-3′
KC1p6-*Pml*I-3′ r	5′ -GTGAATCAACATCAGGTACAGGGGCTG-3′
SarLuc-*Sfi*I-5′ f	5′ -AGATCTGGCCGTTATGGCCGCCGCAC AAAG-3′
SarLuc-1634Gr	5′ -TGAG**CCC**GCGGAGAAAATCACTCACAG-3′
SarLuc-1634Rr	5′ -TGAG**CCT**GCGGAGAAAATCACTCACAG-3′
SarLuc-1634Er	5′ -TGAG**CTC**GCGGAGAAAATCACTCACAG-3′
SarLuc-1634Qr	5′ -TGAG**CTG**GCGGAGAAAATCACTCACAG-3′
SarLuc-1634Gf	5′ -CTGTGAGTGATTTTCTCCGC**GGG**CTCA CGAATG-3′
SarLuc-1634Rf	5′ -CTGTGAGTGATTTTCTCCGC**AGG**CTCA CGAATG-3′
SarLuc-1634Ef	5′ -CTGTGAGTGATTTTCTCCGC**GAG**CTCA CGAATG-3′
SarLuc-1634Qf	5′ -CTGTGAGTGATTTTCTCCGC**CAG**CTCAC GAATG-3′
SarLuc-1346Gr	5′ -GCTCGTACAATTC**CCC**GGTTGTAACC-3′
SarLuc-1346Gf	5′ -GGTTACAACC**GGG**GAATTGTACGAGC-3′
SarLuc-1498Pr	5′ -GCAGAATCCACGC**AGG**CCTTATAAG GTGG-3′
SarLuc-1498Pf	5′ -CCACCTTATAAGG**CCT**GCGTGGATT CTGC-3′
SarLuc-*Nhe*I-3′ r	5′ -CAAGCAATGCTAGCACAGAGTGG-3′
HEVqf	5′ -GGTGGTTTCTGGGGTGAC-3′
HEVqr	5′ -AGGGGTTGGTTGGATGAA-3′
HEVqp	5′ -6FAM-TGATTCTCAGCCCTTCGC-MGBNFQ-3′ (from Life Technologies)
KC1p6-asqf	5′ -TGCTGAGCAGCTACGTC-3′
KC1p6-asqr	5′ -GGGCTAACTCCATAGACACG-3′
KC1p6-asqpG	5′ -6FAM-TCCTTCGAG-ZEN-GGTTGACGA-IBFQ-3′
KC1p6-asqpR	5′ -HEX-TCCTTCGAA-ZEN-GGTTGACGAA-IBFQ-3′

NOTE. Restriction sites (or partial restriction sites) are underlined and mutated codons are in bold type. All primers for quantification and analysis of clinical samples were purchased at Eurofins MWG Operon (Germany), and primers and probes for in vitro studies were purchased at Integrated DNA Technologies (Coralville, IA), unless otherwise indicated. K=G/T/U, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T/U, N=A/T/G/C, H=A/T/C, V=A/G/C, D=A/T/G.
6FAM, 6-fluorescein amidite; HEX, hexachlorofluorescein; IBFQ, Iowa black fluorescent quencher; MGBNFQ, minor groove-binding nonfluorescent quencher; ZEN, internal quencher.

Statistical Analysis

Results for all experiments are derived from at least 3 independent experiments and were analyzed by 2-tailed Student *t* test.

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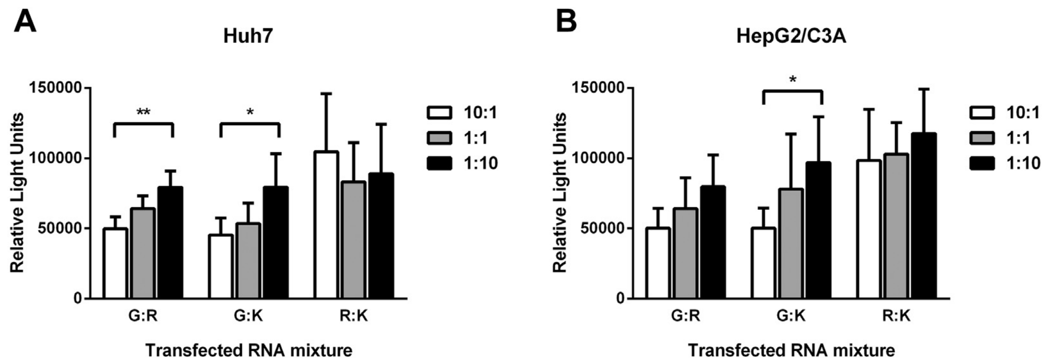
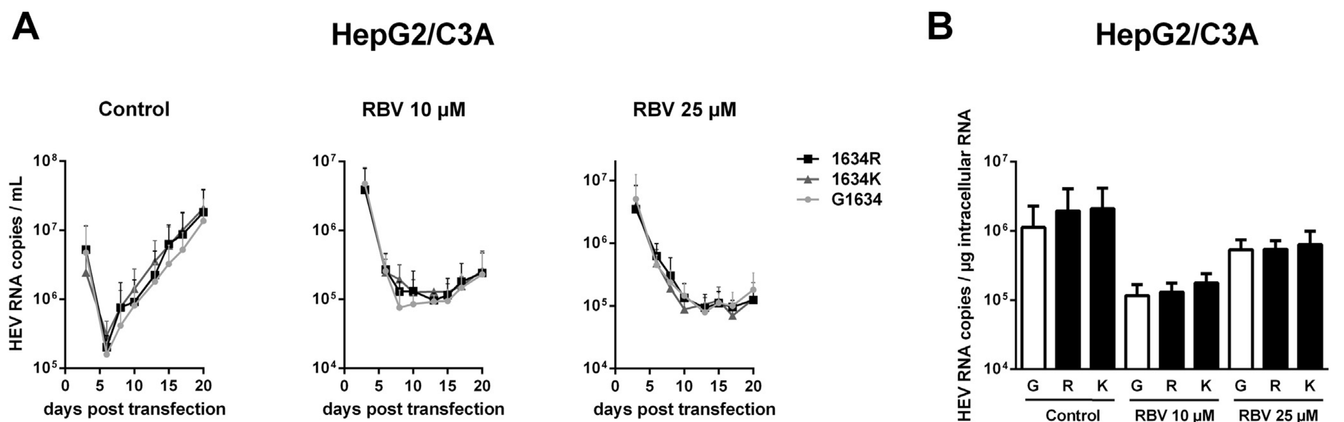
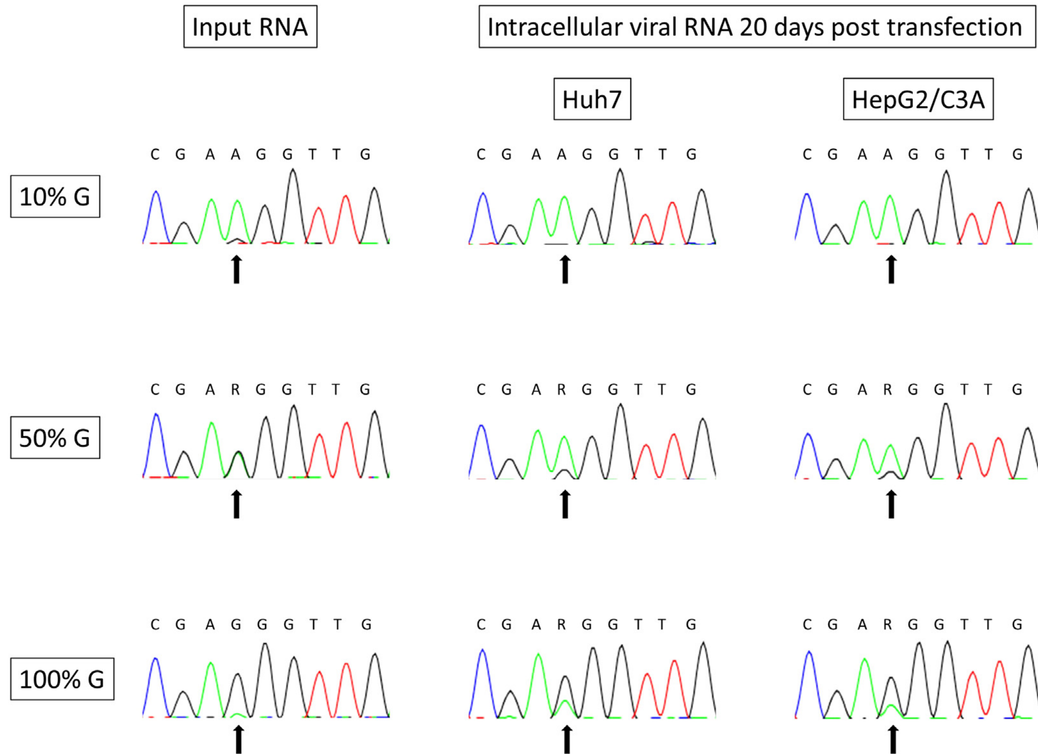
Author names in bold designate shared co-first authorship.



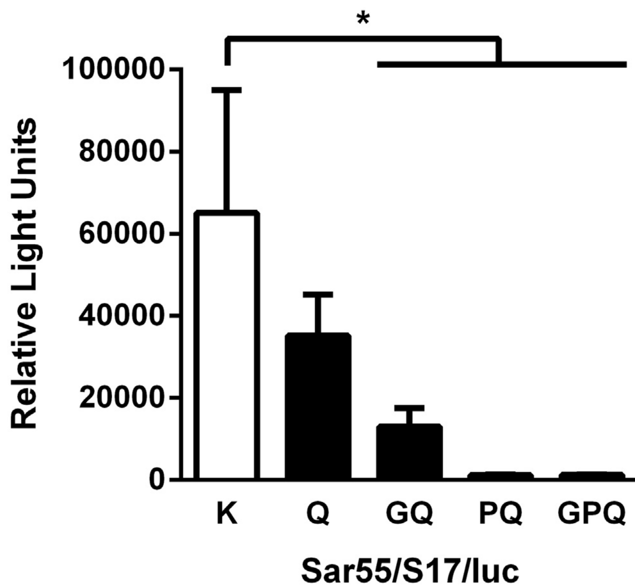
Supplementary Figure 1. 1634R/K display increased replication compared to G1634 in a genotype 3 replicon assay when mixtures of G1634:1634R (G:R), G1634:1634K (G:K) and 1634R:1634K (R:K) in 10:1, 1:1, or 1:10 ratios are transfected into Huh7 (*A*) or HepG2/C3A (*B*) cells. For Huh7, significant differences were found between 10:1 and 1:10 mixtures of G:R ($P = .006$) and G:K ($P = .04$). Results in HepG2/C3A cells were fully in line with this. *$P < .05$; **$P < .01$.



Supplementary Figure 2. Effect of G1634R/K on full-length genotype 3 HEV replication in HepG2/C3A cells. (*A*) Kinetics of released viral RNA after transfection in untreated HepG2/C3A cells indicate an increased replication for 1634R/K strains compared with wt G1634. Treatment with RBV at 10 or 25 μM results in a strong inhibition of viral replication. As a result, no differences in replication can be observed. (*B*) Intracellular viral RNA from transfected HepG2/C3A cells reveals a similar pattern as in released RNA. The higher RNA levels observed in cells treated with 25 μM RBV compared with 10 μM are due to cytostatic effects on HepG2/C3A cells at this concentration and a strong decrease in total cellular RNA.



Supplementary Figure 3. Sequencing of input and intracellular viral RNA from competition assay. Mixtures of full-length G1634 and 1634R HEV RNA were transfected into Huh7 and HepG2/C3A cells. RT-PCR and subsequent Sanger sequencing were performed on input and intracellular viral RNA 20 days post transfection to compare relative amounts of G and R at position 1634 (marked with *arrow*). A gradual increase in the fraction 1634R can be observed in all mixtures in both cell lines.



Supplementary Figure 4. 1634Q and other unique mutations in a genotype 1 HEV replicon. Viral replication is impaired for 1634Q compared to wt K1634 and even further for a combination of 1346G+1634Q (GQ). The combinations 1498P+1634Q (PQ) and 1346G+1498P+1634Q (GPQ) result in a lethal phenotype. *$P < .05$.

Supplementary Table 1. Prevalence of G, R, and K at Position 1634 per Genotype

Genotype	No. of sequences	Sequences with G1634, n (%)	Sequences with R1634, n (%)	Sequences with K1634, n (%)
1	76	0 (0)	2 (3)	73 (96)
2	1	0 (0)	1 (100)	0 (0)
3	103	79 (77)	23 (22)	1 (1)
4	85	1 (1)	6 (7)	78 (92)
Total	265	80 (30)	32 (12)	152 (57)

HEV sequences from genotypes 1 to 4 for which a sequence of the C-terminal RdRp was available were used to calculate the prevalence of each amino acid at position 1634. Sequences obtained through BLASTP alignment, genotyping based on phylogenetic grouping with known genotype sequences (ClustalW2).

Addendum II

In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome.

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Keywords:	HEPATITIS E, CHRONIC HEPATITIS, ANTIVIRAL THERAPY

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In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome

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ABSTRACT

Objective: Hepatitis E virus (HEV) infection can take chronic courses in immunocompromised patients potentially leading to liver cirrhosis and liver failure. Ribavirin (RBV) is currently the only treatment option for many patients, but treatment failure can occur which has been associated with the appearance of a distinct HEV polymerase mutant (G1634R). Here, we performed a detailed analysis of HEV viral intra-host evolution during chronic hepatitis E infections.

Design: Illumina deep sequencing was performed for the detection of intra-host variation in the HEV genome of chronically infected patients. Novel polymerase mutants were investigated *in vitro* using state-of-the-art HEV cell culture models.

Results: Together, these data revealed that (i) viral diversity differed markedly between patients but did not show major intra-individual short-term variations in untreated patients with chronic hepatitis E, (ii) RBV therapy was associated with an increase in viral heterogeneity which was reversible when treatment was stopped, (iii) the G1634R mutant was detectable as a minor population prior to therapy in patients who subsequently failed to achieve a sustained virological response to RBV therapy and (iv) in addition to G1634R further surfacing variants in the polymerase region were detected impacting HEV replication efficiency *in vitro*.

Conclusion: In summary, this first investigation of intra-host HEV population evolution indicates that RBV causes HEV mutagenesis in treated patients and that an emergence of distinct mutants within the viral population occurs during RBV therapy. We also suggest that next generation sequencing could be useful to guide personalized antiviral strategies.

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69 ## SIGNIFICANCE OF THIS STUDY

70 **What is already known on this subject?**

71 • RNA viruses like HEV establish populations with high intrahost variability,
72 which enables them to rapidly adapt to changing immune responses.
73 • HEV is the major cause of acute hepatitis, but can also establish chronic
74 infections in immunocompromised patients. Ribavirin (RBV) is currently the
75 only treatment option available.
76 • RBV inhibits HEV replication *in vitro* by, among other mechanisms, increasing
77 the error rate of viral RNA-dependent RNA polymerases. A mutation (G1634R)
78 in the polymerase region of HEV can lead to treatment failure during RBV
79 therapy.

80 **What are the new findings?**

81 • Viral diversity differed markedly between patients but did not show major intra-
82 individual short-term variations in untreated patients with chronic hepatitis E.
83 • RBV therapy was associated with an increase in viral heterogeneity in all open
84 reading frames which was reversible when treatment was stopped.
85 • The G1634R mutant was detectable as a minor population prior to therapy in
86 patients who subsequently failed to achieve a sustained virological response
87 to RBV therapy
88 • Additional dominant variants in the polymerase emerged during RBV therapy
89 impacting HEV replication efficiency *in vitro*

90 **How might it impact on clinical practice in the foreseeable future?**

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91 • Investigation of HEV intrahost population evolution indicates that RBV causes
92 HEV mutagenesis in treated patients and that an evolution of viral dominances
93 may occur during RBV therapy.
94 • Next generation sequencing methods could be diagnostically used to rapidly
95 identify patients at risk for treatment failure and predict therapy outcomes of
96 chronically infected patients in clinics and could be a useful tool for
97 personalized antiviral strategies.

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99 ## INTRODUCTION

100 Hepatitis E virus (HEV) is a non-enveloped single-stranded RNA virus and a common 101 cause of acute hepatitis worldwide.[1 2] More than 3 million symptomatic hepatitis E 102 cases occur each year accounting for an estimated 70,000 deaths.[1] Four different 103 HEV genotypes infecting humans have been described. HEV genotypes 1 and 2 104 have been linked with water-borne outbreaks in developing countries and exclusively 105 infect humans. In contrast, HEV genotypes 3 and 4 can be found in various animal 106 species, with the major route of HEV transmission to humans via consumption of 107 undercooked meat.[1 3 4] It is now well established that prolonged HEV viremia and 108 even courses of chronic hepatitis E may occur in immunocompromised patients 109 potentially leading to liver cirrhosis and liver failure.[5 6]

110 Pathogenesis, epidemiology and evolution of RNA viruses are influenced by the 111 composition of the viral population.[7] Genetic diversity is achieved by high mutation 112 rates and as a consequence, quasispecies populations are generated which may 113 allow adaptation to antiviral drugs, potentially inducing resistance or enhanced viral 114 fitness.[8] In addition, viral diversity represents a potential mechanism to escape a 115 successful immune response while in turn immune pressure may drive viral 116 evolution.[9] For HEV, greater intra-host heterogeneity has been linked with evolution 117 to chronicity.[10] The immune pressure on HEV maybe weak in chronic hepatitis E 118 where HEV-specific T cell responses are barely detectable, but various cytokines and 119 chemokines are elevated in acute and chronic hepatitis E correlating with disease 120 activity and progression of liver disease.[10 11] However, the mode and tempo of HEV 121 evolution in persistently infected patients undergoing therapy is currently unknown.

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RBV monotherapy is currently considered as the treatment of choice for patients with chronic hepatitis E.[12 13] While the majority of patients clear HEV after 3-5 months of RBV therapy cases of on-treatment failures or post-treatment relapses have been reported.[14-16] Recently, the selection of a distinct HEV mutant in the C-terminal region of the HEV polymerase (G1634R) during RBV therapy was reported. The G1634R mutant was still sensitive to RBV but conferred an enhanced replication-fitness to the virus *in vitro*.[17] Consequently, the selection of the G1634R mutant likely contributed to treatment failure. It is currently unknown if a minor part of the viral population harbored this particular mutation prior to therapy commencement.

RBV inhibits HEV replication *in vitro* by a depletion of cellular GTP pools.[18] Additional modes of actions of RBV might be important in patients as inosine-5'-monophosphate dehydrogenase (IMPDH) inhibition by mycophenolic acid does not always prevent chronicity in HEV infected solid organ transplant recipients.[6] Intra-host populations of RNA viruses are believed to exist at the edge of a genomic error threshold and exceeding the threshold by RBV-induced additional mutations could lead into a sequence of error catastrophes resulting in viral extinction.[19 20] Here, we hypothesized that RBV-induced increased mutagenesis may be implicated in HEV treatment outcomes as has been shown for various other viruses.[21]

The aims of the current study were to investigate HEV genome evolution in all open reading frames (ORFs) prior to and during RBV therapy in patients with chronic hepatitis by Illumina deep sequencing. Furthermore, we wanted to determine if the G1634R mutant was present already before therapy as a minor population in patients not achieving a sustained virological response to RBV therapy. Finally, we aimed to explore if additional amino acid changes were selected during therapy and if these mutations had an impact on HEV replication *in vitro*.

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SUBJECTS, MATERIALS, AND METHODS

Study subjects

All patients were recruited at Hannover Medical School between 2008 and 2015. Overall, twelve patients with chronic hepatitis E were included, for whom at least two stored plasma samples with detectable HEV ribonucleic acid (RNA) were available. In addition, for RBV-treated patients, we aimed to study at least one viremic sample during treatment and in patients not achieving a sustained virological response, a post-treatment HEV RNA-positive sample was required to be available. RBV was administered orally twice daily with an initial daily dose of 600–1000 mg, depending on the patients' hemoglobin level and comorbidities, as previously described.[15] Dose reductions were performed if hemoglobin levels declined and/or patients developed symptoms associated with anemia. At each visit, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltranspeptidase and HEV RNA viremia were determined. Anti-HEV status was determined using Wantai HEV IgG ELISA (Beijing, China). The study protocol was in line with the ethical guidelines of the Institutional Review Committee. The study was approved by the ethics committee of Hannover Medical School in Hannover, Germany (record 930-2011), and it conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients gave written informed consent to participate in this study. Healthy volunteers were also recruited from Hannover Medical School.

Cell culture

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Human Huh7.5 hepatoma cells (kindly provided by Prof. Charles Rice) were kept in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10 % fetal bovine serum (FBS; Invitrogen), 1% nonessential amino acids (Invitrogen), 100 µg/mL of streptomycin (Invitrogen), and 100 IU/mL of penicillin. Cells were kept at 37 °C in a 5% (v/v) CO_2-incubator.

HEV constructs, cloning and *in vitro* transcription

A plasmid construct containing a subgenomic HEV Kernow-C1 p6 clone (GenBank Accession Number JQ679013) sequence coupled with a Gaussia luciferase reporter gene (p6-Gluc) were used to generate HEV *in vitro* transcripts as previously described with an additional capping step (m7G Cap Analog, Promega, Madison, WI, USA).[22] [23] Single nucleotide variations were introduced using site-directed mutagenesis PCR or gBlocks Gene Fragments (IDT, Coralville, IA, USA) (detailed information and primer sequences available on request).

HEV replication assay

HEV subgenomic RNAs were transfected into cells via electroporation as previously described.[24] In brief, 5×10^6 cells/mL Huh7.5 cells in 400 µL Cytomix containing 2 mM ATP and 5 mM glutathione were mixed with 3 µg of p6-Luc subgenomic HEV RNA. Electroporation was carried out with a Gene Pulser system (Bio-Rad, Munich, Germany). Cells were immediately transferred to 12.1 mL of DMEM complete, and 50 µL containing 2×10^4 cells/well were seeded in 96-well plates. After four hours, 50 µL DMEM complete containing RBV at indicated concentrations, or DMEM

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complete only, were added. Viral replication was determined by measuring luciferase activity in the supernatant of 96-well plates 72 h after transfection.

Luciferase reporter assay

HEV subgenomic replicon replication was assessed as described before.[25] In brief, 20 µL of supernatant was added per well of a 96-well white, flat-bottom microplate followed by the detection of luminescence for HEV encoding Gaussia luciferase using a microplate reader (CentroXS3 LB960, Berthold technologies, Bad Wildbad, Germany) using Coelenterazine as a substrate.

Statistics

Data were analyzed using GraphPad Prism v6.0b (GraphPad software, La Jolla, CA, USA). Comparisons of single nucleotide variants (SNV; a single nucleotide variation among an individual) between RBV treated patients and patients that did not receive RBV were performed using Mann-Whitney test. P values of $p<0.05$ were considered to be significant (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$ or **** = $p<0.0001$). GraphPad Prism 5 software was used for dose-response curve calculation.

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211 # RESULTS

212 ## Patient Characteristics

213 We studied twelve selected patients with chronic HEV genotype 3 infections (eight
214 patients with genotype 3c, three patients with genotype 3f, and one patient with
215 genotype 3e). The median age of the patient cohort was 46 years (range 14–70
216 years) and eight patients were male. Eleven of the twelve patients were organ
217 transplant recipients (Table 1). Three patients cleared HEV after reduction of
218 immunosuppressive medication while antiviral therapy with RBV was initiated in nine
219 subjects. RBV treatment was successful in five patients and failed in four organ
220 transplant recipients (patients#1-4). Initial immunosuppressive medications are listed
221 in Table 1 and included corticosteroids, proliferation inhibitors, calcineurin inhibitors
222 and mTOR inhibitors. Immunosuppressive regimens were adjusted over time
223 depending on the clinical parameters of the patients.

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5 224 **Table 1: Characteristics of chronical infected HEV patients.** Clinical parameter represent situation of first deep sequencing samples drawn. *adjusted individually over time
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Patient	Age	Sex	SOT	Steroid	Proliferation Inhibitors	Calcineurin Inhibitors	mTOR Inhibitors	Treatment for HEV infection	ALT [U/L]	AST [U/L]	Bilirubin [µmol/L]	HEV genotype
patient#1	61	m	heart, liver	Prednisolone		Tacrolimus	Everolimus	Ribavirin	283	296	13	3c
patient#2	39	f	liver, kidney stem cells, lung	Prednisolone	Mycophenolate Mofetil	Tacrolimus	Everolimus, Sirolimus	Ribavirin	247	121	8	3c
patient#3	52	f	heart, lung	Prednisolone	Mycophenolic Acid	Tacrolimus, Cyclosporine		Ribavirin	42	42	17	3e
patient#4	18	f	kidney			Tacrolimus	Everolimus	Ribavirin	294	138	6	3c
patient#5	45	f	kidney	Prednisone	Mycophenolic Acid		Everolimus	Ribavirin	374	139	9	3c
patient#6	60	m	heart	Prednisone	Azathioprine	Cyclosporine		Ribavirin	108	68	15	3c
patient#7	70	m	heart	Prednisone	Azathioprine	Cyclosporine	Everolimus	Ribavirin	165	91	10	3c
patient#8	14	m	liver	Prednisolone	Mycophenolate Mofetil	Tacrolimus		Ribavirin	354	168	41	3f
patient#9	35	m	multilocular fibrosis	Prednisone			Sirolimus	Ribavirin	473	296	n.d.	3c
patient#10	47	m	liver	Prednisolone		Cyclosporine		Reduction of immunosuppression	78	78	45	3c
patient#11	65	m	liver	Prednisolone	Mycophenolate Mofetil	Tacrolimus		Reduction of immunosuppression	43	30	14	3f
patient#12	47	m	kidney	Prednisolone	Mycophenolate Mofetil	Tacrolimus		Reduction of immunosuppression	359	171	16	3f

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225 **Evolution of HEV intra-host populations in patients with chronic hepatitis E**

226 RNA viruses exist as a diverse population of genetically related but distinct variants. 227 For HEV, viral heterogeneity determined at a single time point has been associated 228 with the outcome of infection.[10] We here first performed an analysis of intra-host HEV 229 viral population evolution by Illumina deep sequencing of the HEV ORF1 region 230 encoding for the RNA dependent RNA polymerase (RdRp) of at least two time points 231 in patients not receiving antiviral treatment with RBV (Figure 1). Significant inter- 232 individual variability of both synonymous (silent) as well as non-synonymous (amino 233 acid replacement) single nucleotide variants (SNVs) was evident (Figure 1). 234 However, within a given patient (#1, #4, #8, #9, #10, #11), the HEV population 235 composition was rather stable over several weeks or even months for the majority of 236 patients before on-set of RBV treatment as well as in patients who never received 237 antiviral therapy.

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239 **Effect of RBV on the heterogeneity in the HEV ORF1 encoding for the RNA** 240 **dependent RNA polymerase**

241 To ascertain whether RBV therapy has as an influence on viral heterogeneity, we 242 compared differences in the number of sites exhibiting non-synonymous and 243 synonymous SNVs between RBV treated and non-treated patients with chronic 244 hepatitis E via next generation sequencing methods. Indeed, patients receiving RBV 245 showed higher frequencies of both synonymous (Figure 2A) and non-synonymous 246 (Figure 2B) SNVs compared to samples drawn when patients were untreated. This 247 finding indicates that RBV therapy leads to an increased nucleotide substitution rate 248 in patients with HEV. We next analyzed the long-term evolution of the total number of

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synonymous and non-synonymous SNV in three patients not achieving a sustained virological response (patients#1, #2, #4). For these patients, samples were available before, during and after RBV medication (Figure 3). HEV RNA copy numbers during the course of infection ranged for patient#1 from 5.6×10^5 to 1.09×10^7, for patient#2 from 1.66×10^4 to 1.2×10^7 and for patient#4 from 1.54×10^4 to 6.75×10^8 RNA copies/mL, respectively. Overall, there was a progressive increase in both synonymous and non-synonymous SNVs during RBV medication with higher changes in the number of sites exhibiting substitutions for synonymous SNV (Figure 3). In patient#1 a marked SNV decline appeared after RBV was stopped and in patient#2 a gradually decline became evident three to six months after treatment for both, synonymous as well as non- synonymous SNV (Figure 3A, B). Patient#4 showed a marked increase in SNVs in particular during a second course of RBV therapy, however, no post-treatment samples were available in this case (Figure 3C). In all three patients the occurrence of nucleotide substitutions was mainly caused by transitions, i.e. a purine-purine or pyrimidine-pyrimidine exchange, and only to a lesser extent due to nucleotide transversions (purine-pyrimidine or pyrimidine-purine substitution, Figure 3A-C). In summary, the analysis of viral evolution during and after therapy in individual patients shows that increasing SNVs over time correlate with continued administration of RBV. Furthermore, RBV-induced mutagenesis seemed to be reversible when treatment was stopped.

Time course of distinct non-synonymous SNVs in patients not achieving a sustained virological response to RBV therapy

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We next analyzed, if viral heterogeneity and detection of the G1634R mutant via deep sequencing would be predictive for subsequent responses to RBV treatment. There was no significant difference between the numbers of synonymous and non-synonymous SNVs detected in responding patients and patients not achieving a sustained virological response to RBV therapy (suppl. Figure 1). In most patients that cleared the HEV infection, the G1634R mutation could not be identified or was present at very low levels in the viral population. In two patients the arginine at position 1634 was already the dominant strain from the beginning onwards. However, the G1634R mutant that has been selected during therapy in patients with treatment failure was detectable as minor population before therapy in patient#1 and patient#2 (Figure 4). Deep sequencing revealed a gradual increase of the G1634R variant with continued RBV therapy until month eleven, where it comprises more than two thirds of the HEV population (Figure 4A). In patient#2 the G1634R variant also gradually increased with progression of RBV therapy until the ninth month (Figure 4B). Of note, the relative dominance of G1634R declined around three months after RBV medication was stopped and decreased to one third of the population (Figure 4B). G1634R was the dominant strain in patient#3 already before therapy which did not change during treatment (data not shown) while in patient#4 the G1634R variant was only detectable in the population at month eleven and only at as very low frequency of about 3.4 % (data not shown). Thus, the overall HEV heterogeneity does not seem to be associated with response to RBV therapy. However, variants possibly being associated with treatment failure may already be detectable before antiviral therapy as minor populations.

In addition to G1634R, two further mutations (K1383N and Y1587F) appeared in patient#1 in the C-terminal region of the HEV ORF1 at month five of therapy (Figure

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297 4A). The variant Y1587F did not exist and variant K1383N was present at extremely
298 low frequencies prior to initiation of RBV medication (Figure 4A). For the second non-
299 responder (patient#2), we also found three additional amino acid changes in the HEV
300 polymerase over time (K1383N, D1384G and V1479I; Figure 4B). Similar to G1634R,
301 the selection of these variants was reversible when RBV was stopped. Figure 4C
302 shows the time course of SNV of the only non-responder not harbouring the G1634R
303 (patient#4). In patient#4 we observed three amino acid changes previously detected
304 in patient#1 (K1383N and Y1587F) and patient#2: (K1383N and D1384G).
305 Additionally, one variant (K1398R) was identified which was not detectable in the
306 other patients (Figure 4). A linkage analysis using clonal sequencing methods
307 employing amplicons obtained from time points with highest viral heterogeneity
308 revealed different combinations of the identified variations on individual viral
309 genomes (suppl. Figure 2), that were present at different frequencies. In viral
310 populations of all three patients, the wild type sequences were no longer detectable
311 anymore. For patient#1, viral genomes harboring all three mutations could be
312 detected (suppl. Figure 2A), whereas for patient#2 and patient#4 several
313 combinations of mutations were detected, but no viral genomes containing all four
314 amino acid changes (suppl. Figure 2B and C). In summary, the G1634R mutation
315 was already detectable as a minor variant prior to therapy and became dominant
316 during ribavirin administration. Furthermore, additional amino acid changes in ORF1
317 could be identified that emerged in various combinations in the viral population.

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319 RBV sensitivity and viral replication of HEV subgenomic replicon harboring
320 identified SNVs

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3 321 In our previous study, it was demonstrated that the G1634R mutant was still sensitive
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5 322 to RBV but conferred an enhanced replication-fitness to the virus *in vitro*.[17] Here, we
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7 323 first confirmed that the previously described G1634R mutant (EC_{50} = 12. 9 µM, 95 %
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9 324 CI 11.91 – 13.98) introduced into a genotype 3 replicon has comparable RBV
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11 325 sensitivity to the HEVp6 wild type (wt) replicon (EC_{50} = 13.61 µM, 95 % CI 12.35 –
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13 326 15.00) (Figure 5A). However, the G1634R mutation resulted in an increase
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15 327 luminescence signal compared to the wild type construct suggesting increased viral
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17 328 RNA replication (Figure 5B). RBV was further used as control for active viral RNA
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19 329 replication in this assay setup. Consequently, after revealing two additional dominant
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21 330 amino acid changes close to the G1634R site in patient#1 using deep sequencing,
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23 331 we also introduced all three mutations (K1383N/Y1587F/G1634R) into the HEVp6
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25 332 replicon to investigate the RBV sensitivity and viral replication compared to the
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27 333 HEVp6 wt replicon and HEVp6 G1634R replicon. Interestingly, the
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29 334 K1383N/Y1587F/G1634R combination showed an increased sensitivity to RBV when
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31 335 compared to the wt or G1634R mutant (EC_{50} = 4.95 µM, 95 % CI 4.47 – 5.49)
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33 336 (Figure 5A). Similar viral replication could be detected for the
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35 337 K1383N/Y1587F/G1634R construct compared to the wt-construct after transfecting
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37 338 Huh7.5 cells (Figure 5B). A comparable phenotype could be observed for the
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39 339 mutations found in patient#2 (K1383N/D1384G/V1479I/G1634R, EC_{50} = 3.03 µM, 95
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41 340 % CI 2.80 – 3.28), whereas a HEV construct harboring the mutations identified in
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43 341 patient#4 (K1383N/D1384G/K1398R/Y1587F, EC_{50} = 11.98 µM, 95 % CI 10.39 –
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45 342 13.82) showed replication levels as well as RBV sensitivity like the wt replicon (Figure
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47 343 5A, B). In summary, next to the G1634R additional ORF1 mutations could be
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49 344 identified that abrogated the improved replication fitness and showed an increased
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51 345 RBV sensitivity.
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Effect of RBV on the heterogeneity of HEV in ORF2 and ORF3 genome regions

To extend the ribavirin-induced mutagenesis analysis to HEV genome regions other than the RdRp in ORF1, we next performed deep-sequencing approach of ORF2 and ORF3 regions with RBV treated and non-treated patients with chronic hepatitis E (Figure 6). In line with the findings made for ORF1, patients receiving RBV showed higher numbers of sites exhibiting both non-synonymous (Figure 6A and C) and synonymous (Figure 6B and D) in ORF2 and ORF3. The long-term evolution of the total number of SNVs in ORF2 and ORF3 for the three patients not receiving a sustained virological response demonstrated an increase of SNVs over time which also correlated with the administration of RBV and findings made for ORF1 (Figure 7; compare Figure 3). As the amplicon size for each ORF reference was different, we performed a normalized comparison of the number of sites exhibiting SNVs as well as average frequencies of SNVs in percent between ORF1, 2 and 3 (suppl. Figure 3A-D). These results show that in all genome regions comparable mutagenic effects of RBV can be observed. Only the average frequencies of synonymous substitutions in ORF1 under RBV treatment were not significantly increased compared to the non-treated patient samples (suppl. Figure 3C). Monitoring the changes in nucleotide frequencies in ORF2 and ORF3 resulting in alterations of the predominant amino acid over time in the three chronically infected patients identified four mutations in the ORF2 for patient#2 (P25S, G71R, P95S and V245I) and patient#4 (G38S, A64T, P79S, T324S) and additionally one mutation in the ORF3 of patient#4 (S82N) (suppl. Figure 4). In summary, in line with the results described for ORF1, the increase of SNVs in individual patients receiving RBV could also be observed in the HEV ORF2 and ORF3.

DISCUSSION

Chronic hepatitis E is an increasing problem in immunocompromised patients, with RBV being the only treatment option in many cases. Recently, the emergence of a distinct HEV polymerase mutation (G1634R) during RBV therapy of chronic HEV was reported demonstrating enhanced replication dynamics which likely contributed to treatment failure and a poor clinical long-term outcome.[17] In that study, selection of the HEV-G1634R mutant was identified by standard population sequencing. Here we performed a detailed analysis of intra-host HEV viral population evolution via deep-sequencing of the HEV genome in a larger cohort of patients at multiple time points. We show that (i) the HEV population composition was stable over several weeks or even months in most patients with chronic HEV although it was variable between patients, (ii) that RBV therapy was associated with a marked increase in viral population heterogeneity and (iii) that in addition to G1634R further mutants comprising the majority of the viral population emerged during RBV therapy which impacted HEV replication efficiency.

HEV generally causes acute infections with rather short durations of viremia in infected hosts. Prolonged courses of HEV infections or even chronicity only occur with the introduction of immunosuppressive medical therapies. Yet, the evolution of the HEV population during prolonged viremia has never been previously studied. The potential importance of HEV heterogeneity for both, development of chronic infections, as well as fibrosis progression has been suggested previously in an elegant study from Toulouse.[10] In line with that study, we here also observed extensive variability in the complexity of the HEV population between different patients. However, HEV diversity remained rather stable within a given patient followed for several months. This observation indicates a low level of immune

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pressure in these patients with different types of immunosuppressive medications. This is in agreement with our earlier finding that HEV-specific T cell responses are rather weak in chronically infected solid organ transplant recipients while much stronger T cell responses can be observed in immunocompetent patients with acute hepatitis E virus genotype 3 infections.[11] More and larger studies are required to better define the importance of viral diversity for disease progression once chronic hepatitis E infection has been established.

RBV displays antiviral activities against a broad range of RNA and DNA viruses.[26] Inhibition of HEV replication *in vitro* by RBV has been shown to be mediated by a depletion of cellular GTP pools.[18] However, additional modes of actions of RBV might be important as patients can develop chronic hepatitis E even in the presence of mycophenolic acid which also inhibits IMPDH and thereby alters cellular GTP pools.[6] We here suggest that RBV-induced viral mutagenesis is important for HEV therapy outcomes. Various *in vitro* studies showed that RBV exhibits mutagenic properties against different viruses such as GB virus B, poliovirus, hantaan virus and foot-and-mouth disease virus.[27-30] Yet, few studies presented *in vivo* evidence that RBV-induced mutagenesis is of relevance. The best studied example is hepatitis C where partially contradicting results were observed by direct sequencing of PCR products of about 30 clones per time point.[31-34] However, deep sequencing of patients being treated with RBV monotherapy revealed that, even though no overall increase of nucleotide substitutions of hepatitis C occurred during therapy, a mutagenic effect was demonstrated with more frequent G-to-A and C-to-U nucleotide transitions.[35] We here demonstrate that RBV therapy is associated with an increased HEV heterogeneity indicating that nucleotide substitutions accumulate over time during treatment. Of note, this diversity partially declined again in two patients upon RBV

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421 therapy cessation, further supporting that RBV is inducing HEV mutagenesis and that
422 this effect is reversible. Similar to the findings for HCV, we also observed an excess
423 of transitions compared to transversions in the HEV polymerase and, in addition, we
424 observed a surplus of synonymous vs. non-synonymous substitutions in the majority
425 of the cases (Figure 1, Figure 3 and Figure 7).[35] Nucleotide changes occurred
426 throughout the studied HEV regions in ORF1, ORF2 and ORF3 with no preferential
427 location of SNVs – which is also in line with findings in hepatitis C where respective
428 SNVs were not limited to specific non-structural proteins as suggested previously but
429 occurred ubiquitously in the HCV genome.[32 35] Overall, our findings could indicate
430 that the RBV-induced mutagenesis of HEV may lead to an exceeding of a genomic
431 error threshold followed by lethal mutagenesis as it has been shown for other
432 viruses.[19] Still, further studies in more patients are needed which should try to
433 determine correlations between viral declines and RBV-induced viral diversity.

434 The HEV G1634R mutation was selected during RBV therapy in two patients not
435 achieving a sustained HEV clearance and this mutant had a better replication
436 capacity *in vitro*.[17] We here provide a more detailed analysis how this variant became
437 dominant in the viral population over time. Importantly, deep sequencing revealed
438 that the G1634R mutant was already present as a minor population before therapy in
439 patient#1 and the proportion gradually increased until treatment month eleven, when
440 G1634R was the dominant strain representing more than two thirds of the HEV
441 population (Figure 4A). The mutant was also detectable before treatment in
442 patient#2, who failed to achieve a sustained virologic response (SVR), and also in
443 the third patient G1634R was the dominant strain throughout follow-up. Overall, these
444 data extend on our previous observation and suggest that selection of G1634R may
445 have contributed to treatment failure. Next generation sequencing maybe used

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446 diagnostically to early identify patients at risk for treatment failure who may require
447 alternative treatment approaches.

448 Importantly, next generation sequencing revealed that the situation might be even
449 more complex, as various additional amino acid changes were detected in some
450 patients (Figure 4). These variants may have additional implications for the
451 replication efficiency as demonstrated in Figure 5. Viral constructs harbouring
452 additional mutations in the ORF1 region next to the G1634R mutation abrogated the
453 improved replication fitness of G1634R viruses and showed an increased RBV
454 sensitivity. One has to keep in mind that the p6 HEV clone is a cell culture adapted
455 isolate, which might not reflect the individual dominant patient strain found *in vivo* in a
456 given patient. Furthermore, due to the limitations of Illumina sequencing, whereby no
457 linkage between polymorphisms can be assigned, we also performed a linkage
458 analysis via clonal sequencing methods, where single viral amplicons are cloned into
459 a vector and subsequently sequenced by standard Sanger sequencing. Here, we
460 were able to identify viral genomes harbouring all three SNVs in patient#1. Although
461 no genomes with all four SNVs for patient#2 and patient#4 were detected, different
462 combinations with up to three SNVs were also present in these populations.

463 The study has several strengths as this was the very first study evaluating viral
464 evolution over time in patients with chronic hepatitis E by deep sequencing. We could
465 study a cohort of patients with and without sustained response to RBV treatment and
466 as single patients could be studied for up to nine different time points. However,
467 limitations need to be considered. Blood sampling was performed in a routine clinical
468 setting and samples were retrospectively analysed based on availability. The number
469 of patients is still limited and a larger cohort needs to be investigated. Moreover,
470 different immunosuppressive regimens were used in different patients and specific

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drugs may interfere with viral RNA replication and thereby also affect the viral population composition.[36 37] Finally, the dose of RBV varied between patients and was even adapted during treatment based on tolerability, haemoglobin levels and kidney function. Thus, drug exposure may have differed between individuals.

In summary, this first investigation of HEV intra-host evolution revealed that viral diversity differs between patients but does not show major intra-individual short-term variations in untreated patients indicating that there is no major immune selection pressure on HEV in immunocompromised patients with chronic hepatitis E. Furthermore, we provide strong evidence that RBV causes HEV mutagenesis in treated patients and that an emergence of distinct viral populations may occur during RBV therapy. Next generation sequencing could be useful to rapidly identify patients at risk for not achieving a sustained virological response. Overall, this study gives novel insights to better understand the pathophysiology of chronic hepatitis E and may guide development of personalized antiviral strategies.

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FIGURE AND TABLE LEGENDS

Figure 1: Changes in total number of sites exhibiting variations in the HEV ORF1 region without the influence of RBV in chronically infected HEV patients over time. The time course of the appearance of non-synonymous (black lines) and synonymous (gray lines) single nucleotide variations (SNVs) in six chronically infected patients included in the study is depicted.

Figure 2: Effect of RBV on the heterogeneity of HEV ORF1 region in the viral intra-host population in chronically infected patients. The total numbers of nucleotide sites exhibiting synonymous (**A**) as well as non-synonymous (**B**) single nucleotide variations (SNVs) identified in the hepatitis E viral intra-host population in solid organ transplant patients are compared. Open circles represent analyses of available samples of patients before receiving RBV or before and during other antiviral treatment. Black dots show the number of SNVs in HEV ORF1 identified in patients during RBV treatment. Horizontal bars indicate the median, significance was tested with the Mann-Whitney test (*** = $p<0.001$; **** = $p<0.0001$).

Figure 3: Course of number of sites exhibiting nucleotide substitutions over time in three chronically HEV infected solid organ transplant recipients experiencing RBV treatment failure. Shown are the total numbers of synonymous (solid black lines) and non-synonymous (dashed black lines) single nucleotide variations (SNVs), as well as the numbers of nucleotide transitions (solid gray lines) and transversions (dashed gray lines) in the HEV ORF1 identified in three chronically

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3 509 infected patients (**A**, patient#1; **B**, patient#2; **C**, patient#4). Black bars indicate
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5 510 duration of RBV treatment starting with the first RBV dose at month 0.
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12 512 **Figure 4: Time course of non-synonymous single nucleotide variations at**
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14 513 **certain positions of the HEV ORF1 in patient samples.** Changes in nucleotide
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16 514 frequencies (x-axes) resulting in alterations of the predominant amino acids over
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18 515 monitoring time (y-axes) of chronically infected patients are depicted at amino acid
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20 516 positions indicated above the plots. The altered nucleotides in the coding triplets are
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22 517 underlined. White bars indicate proportions of adenine, light gray bars of guanine,
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24 518 dark grey bars represent cytosine and black bars show amount of thymine. **A)** Three
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26 519 positions with a change in the dominant amino acid were identified for patient#1, four
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28 520 non-synonymous SNV were found in patient#2 **(B)** and four in patient#4 **(C)**. Black
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30 521 vertical lines indicate course of administration of RBV.
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37 523 **Figure 5: RBV sensitivity and replication fitness of HEV SGR wt and mutants**
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39 524 **harboring the single nucleotide variations identified in patients experiencing**
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41 525 **RBV treatment failure. A)** Antiviral activity of RBV against HEVp6 wt replicon (solid
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43 526 line, EC_{50} = 13.61 µM, 95 % CI 12.35 – 15.00), the G1634R mutant (dashed line,
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45 527 EC_{50} = 12.9 µM, 95 % CI 11.91 – 13.98) the K1383NY1587FG1634R mutant (dotted
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47 528 line, EC_{50} = 4.95 µM, 95 % CI 4.47 – 5.49), the K1383ND1384GK1398RY1587F
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49 529 mutant (dotdashed line, EC_{50} = 11.98 µM, 95 % CI 10.39 – 13.82) and the
50
51 530 K1383ND1384GV1479IG1634R mutant (dotdotdashed line, EC_{50} = 3.03 µM, 95 % CI
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53 531 2.80 – 3.28) replicons in Huh7.5. **B)** Luminescence read-out of Huh7.5 cells
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transfected with the five constructs 72 h post transfection. As a control for replication specific read-out, the wt replicon was treated with 62.5 μM RBV.

Figure 6: Effect of RBV on the heterogeneity of HEV ORF2 and ORF3 in the viral intra-host population in chronically infected patients experiencing RBV treatment failure. The total numbers of sites exhibiting synonymous (**A, C**) as well as non-synonymous (**B, D**) single nucleotide variations (SNVs) identified in ORF2 (**A, B**) and ORF3 (**C, D**) of the hepatitis E viral intra-host population found in patient#1, patient#2 and patient#4 at time points of available serum samples before (open circles) and during RBV treatment (black dots) are compared. Horizontal bars indicate the median, significance was tested with the Mann-Whitney test (* = $p<0.05$; ** = $p<0.01$).

Figure 7: Course of number of sites exhibiting nucleotide substitutions over time in three chronically HEV infected solid organ transplant recipients experiencing RBV treatment failure. Shown are the total numbers of synonymous (solid black lines) and non-synonymous (dashed black lines) single nucleotide variations (SNVs), as well as the numbers of nucleotide transitions (solid gray lines) and transversions (dashed gray lines) in the HEV ORF2 (left panels) and ORF3 (right panels) identified in three chronically infected patients not achieving a sustained virological response (**A**, patient#1; **B**, patient#2; **C**, patient#4). Black bars indicate duration of RBV treatment starting with the first RBV dose at month 0.

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Supplement Figure 1: Comparison of the initial numbers of sites exhibiting nucleotide substitutions in the HEV ORF1 of chronically infected patients responding to RBV treatment vs. non-responder before the application of the first dose. Synonymous (**A**) and non-synonymous (**B**) nucleotide variants are depicted for four patients responding to RBV treatment (patient#5, #7, #8, and #9) vs. four non-responding patients (patient#1-4). Horizontal lines indicate the median, significance was tested using the Mann-Whitney test (n.s. = not significant).

Supplement Figure 2: Linkage analysis of the identified mutations and their appearance in the viral populations. The single nucleotide variations (SNVs) identified in the three patients not achieving sustained virological responses after RBV treatment were analyzed in regards to their linkage and combinations in single viral genomes via clonal sequencing. The left panels show the distribution of the SNVs in HEV ORF1 and their amino acid position for patient#1 (**A**), patient#2 (**B**) and patient#4 (**C**), as well as the respective wild type and mutated amino acids (black boxes). Light gray boxes represent amino acid changes that were not taken into account for deep sequencing analyses, because they did not reach threshold levels (**B**), or were not picked up during deep sequencing at all (**C**). The right panels represent the frequencies of the different identified residue combinations in the viral population at indicated time points.

Supplement Figure 3: Comparison of normalized numbers and frequencies of identified variations with and without RBV administered. The relative numbers of sites exhibiting synonymous (**A**) and non-synonymous (**B**) single nucleotide

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variations (SNVs), as well as the average frequencies of synonymous (**C**) and non-synonymous (**D**) SNVs identified in all three open reading frames of the hepatitis E viral intra-host population found in patient#1, patient#2 and patient#4 at time points of available serum samples before (open circles) and during RBV treatment (black dots) are depicted. Horizontal bars indicate the median, significance was tested with the Mann-Whitney test (* = p<0.05; ** = p<0.01, n.s. = not significant).

Supplement Figure 4: Time course of non-synonymous single nucleotide variations at certain positions of the HEV ORF2 and ORF3 in patients experiencing RBV treatment failure. Changes in nucleotide frequencies (x-axes) resulting in alterations of the predominant amino acids over monitoring time (y-axes) of chronically infected patients are depicted at amino acid positions indicated above the plots. The altered nucleotides in the coding triplets are underlined. White bars indicate proportions of adenine, light gray bars of guanine, dark grey bars represent cytosine and black bars show amount of thymine. **A)** Four positions with a change in the dominant amino acid were identified for patient#2 in ORF2. **B**) Four non-synonymous SNV were found in patient#4 in ORF2 and one in ORF3. Black vertical lines indicate course of administration of RBV.

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604 provide material for this study.

605

606 CONFLICT OF INTEREST

607 The authors do not have a conflict of interest.

608

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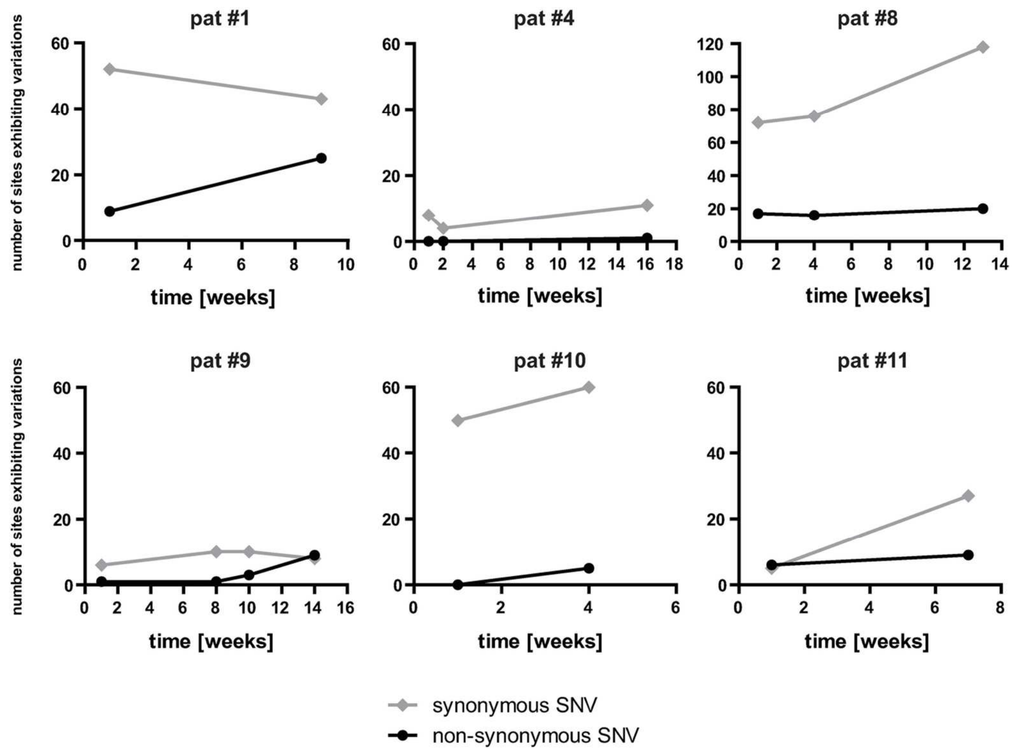

Figure 1: Changes in total number of sites exhibiting variations in the HEV ORF1 region without the influence of RBV in chronically infected HEV patients over time. The time course of the appearance of non-synonymous (black lines) and synonymous (gray lines) single nucleotide variations (SNVs) in six chronically infected patients included in the study is depicted.
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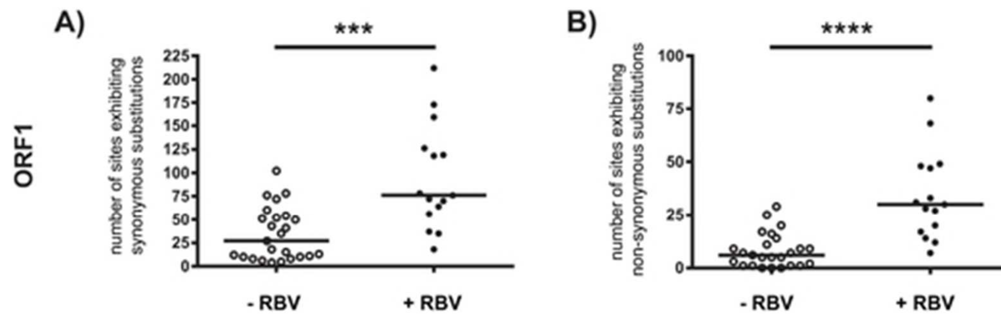

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Figure 2: Effect of RBV on the heterogeneity of HEV ORF1 region in the viral intra-host population in chronically infected patients. The total numbers of nucleotide sites exhibiting synonymous (A) as well as non-synonymous (B) single nucleotide variations (SNVs) identified in the hepatitis E viral intra-host population in solid organ transplant patients are compared. Open circles represent analyses of available samples of patients before receiving RBV or before and during other antiviral treatment. Black dots show the number of SNVs in HEV ORF1 identified in patients during RBV treatment. Horizontal bars indicate the median, significance was tested with the Mann-Whitney test (*** = $p<0.001$; **** = $p<0.0001$).

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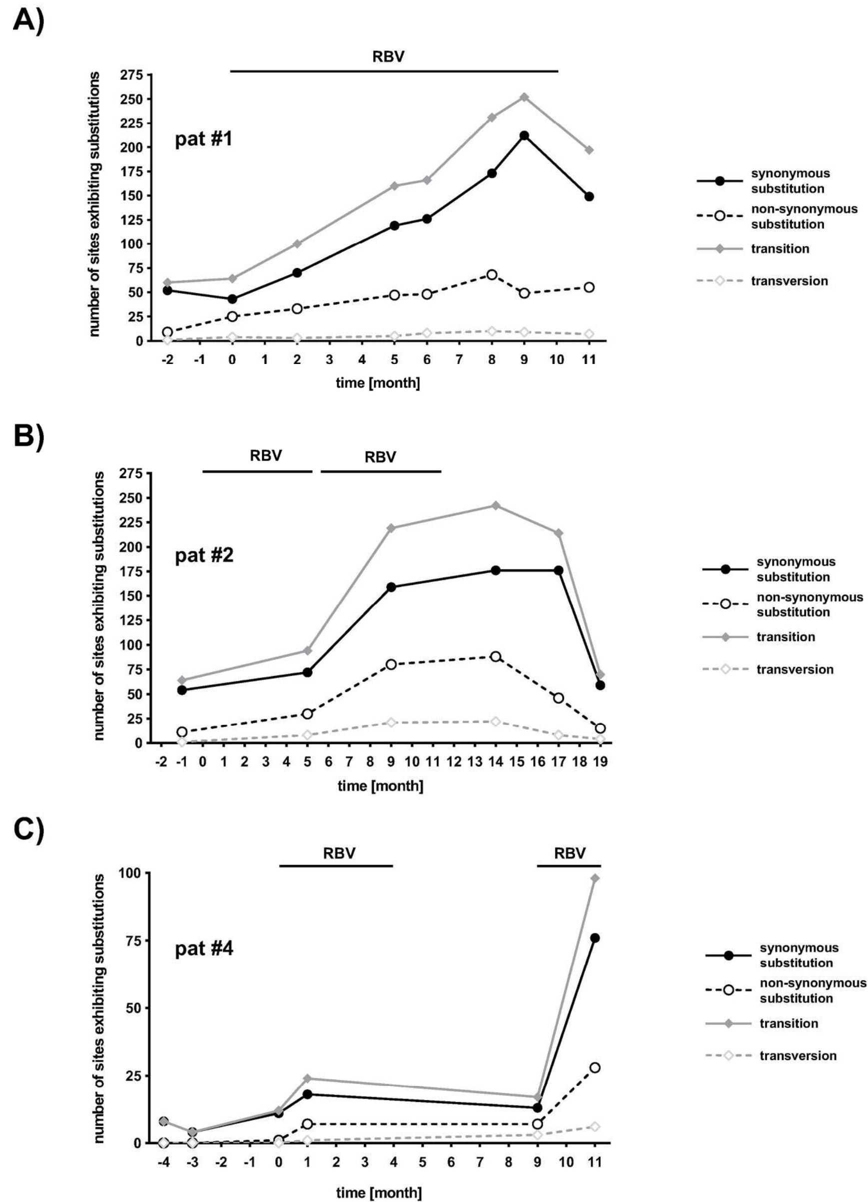

Figure 3: Course of number of sites exhibiting nucleotide substitutions over time in three chronically HEV infected solid organ transplant recipients experiencing RBV treatment failure. Shown are the total numbers of synonymous (solid black lines) and non-synonymous (dashed black lines) single nucleotide variations (SNVs), as well as the numbers of nucleotide transitions (solid gray lines) and transversions (dashed gray lines) in the HEV ORF1 identified in three chronically infected patients (A, patient#1; B, patient#2; C, patient#4). Black bars indicate duration of RBV treatment starting with the first RBV dose at month 0.
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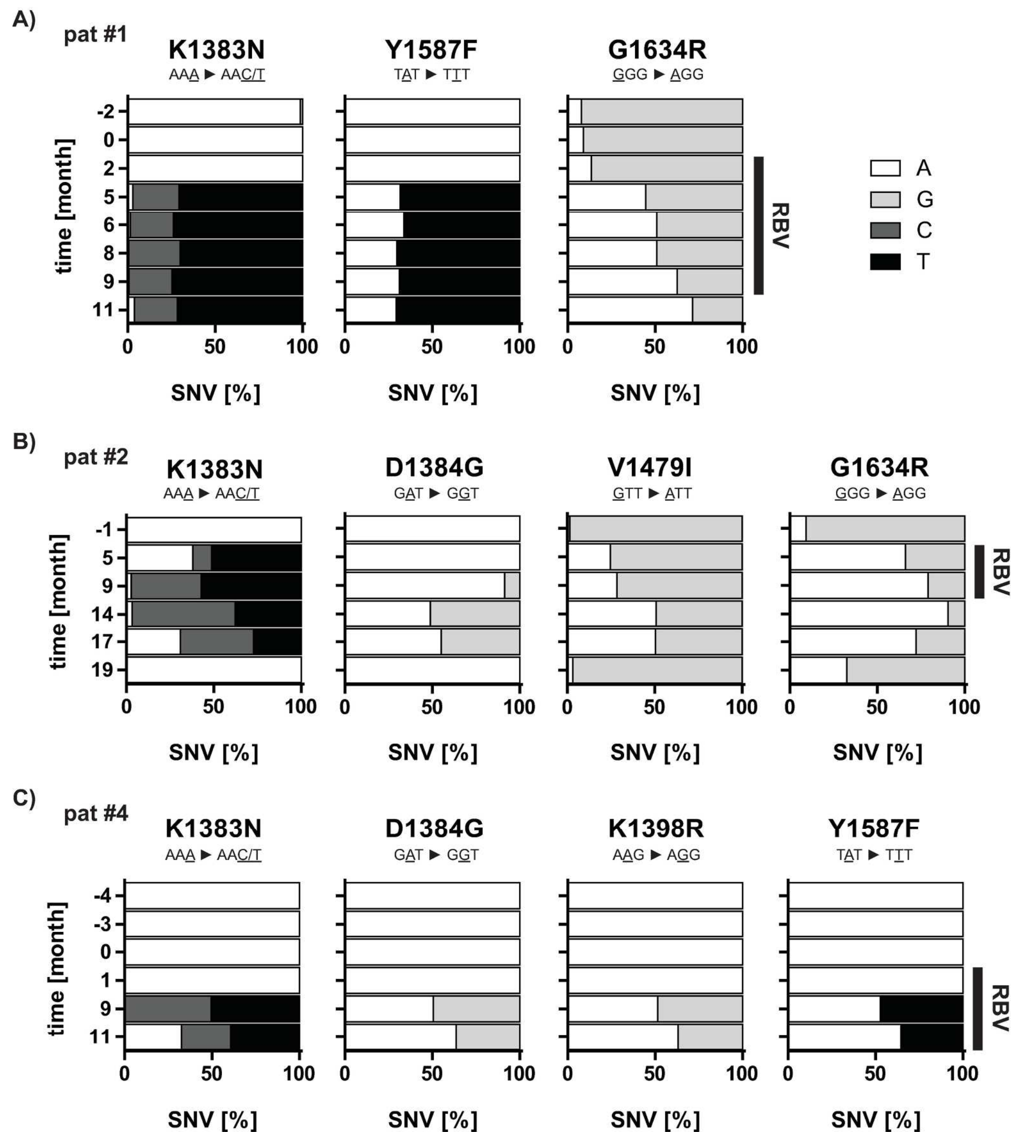

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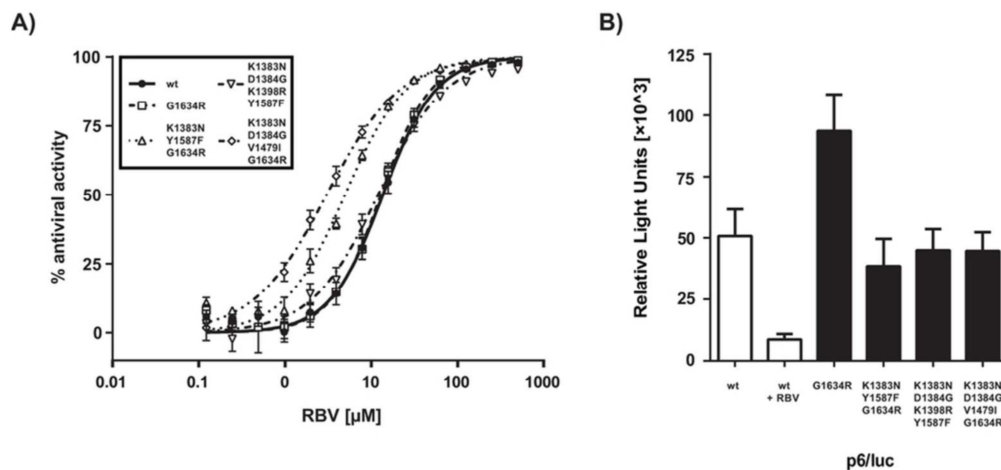

23 Figure 5: RBV sensitivity and replication fitness of HEV SGR wt and mutants harboring the single nucleotide
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28 CI 10.39 – 13.82) and the K1383ND1384GV1479IG1634R mutant (dotdotdashed line, EC50 = 3.03 µM, 95
29 % CI 2.80 – 3.28) replicons in Huh7.5. B) Luminescence read-out of Huh7.5 cells transfected with the five
30 constructs 72 h post transfection. As a control for replication specific read-out, the wt replicon was treated
with 62.5 µM RBV.
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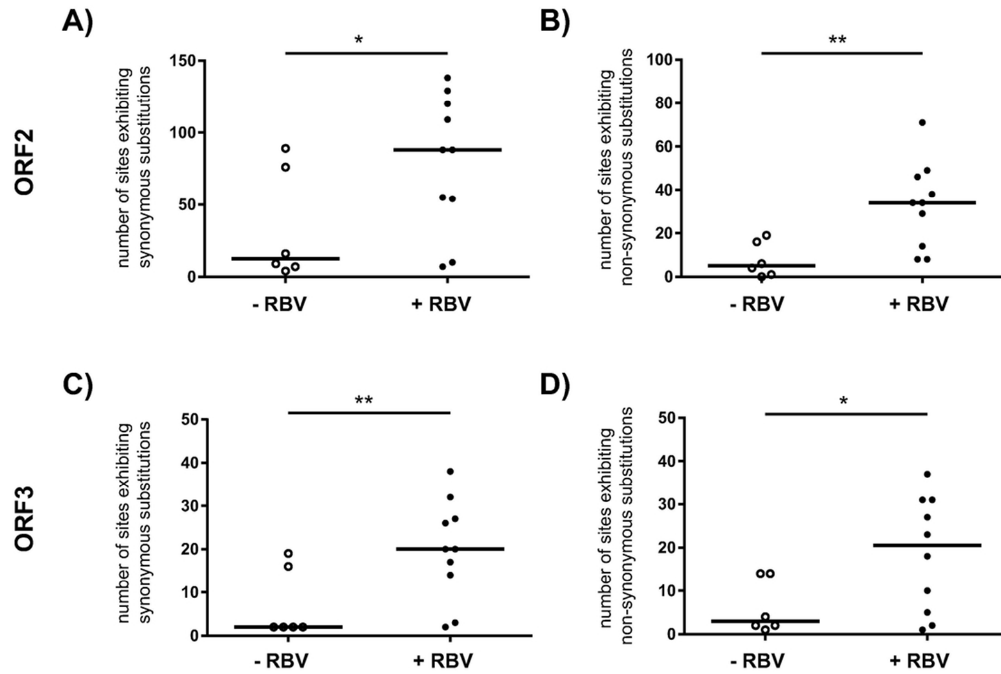

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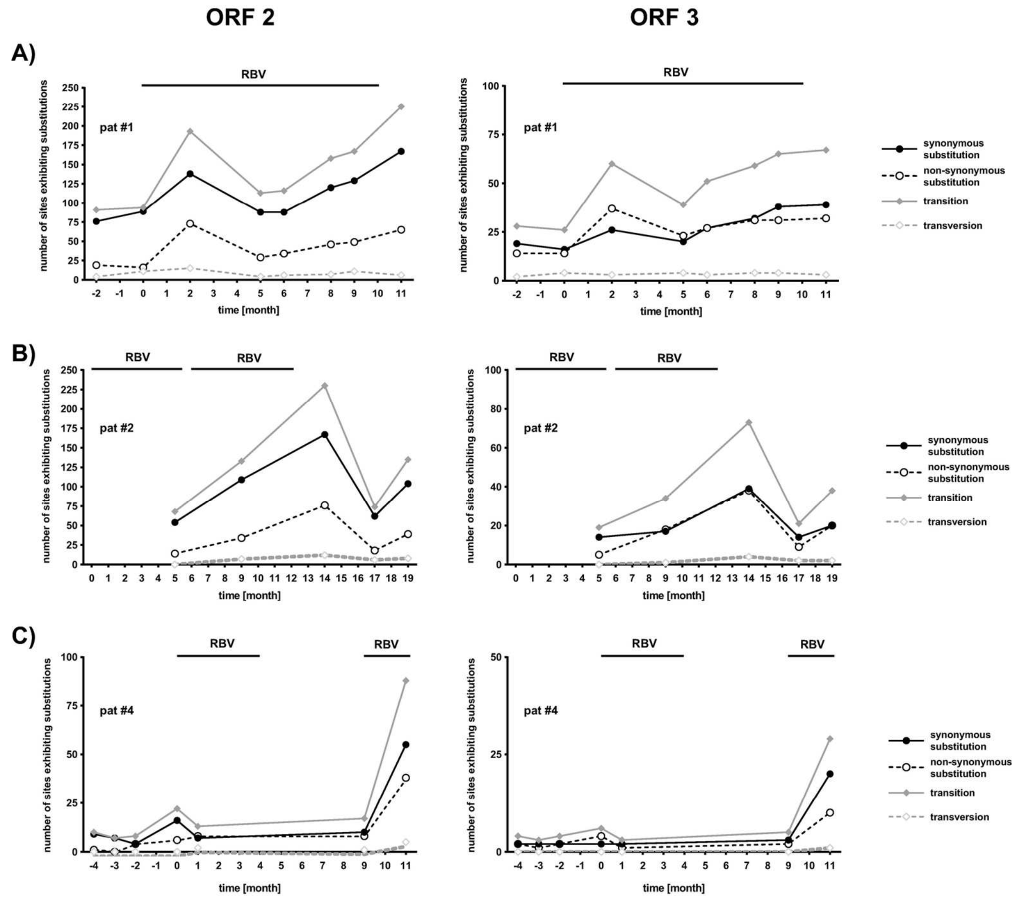

Figure 7: Course of number of sites exhibiting nucleotide substitutions over time in three chronically HEV infected solid organ transplant recipients experiencing RBV treatment failure. Shown are the total numbers of synonymous (solid black lines) and non-synonymous (dashed black lines) single nucleotide variations (SNVs), as well as the numbers of nucleotide transitions (solid gray lines) and transversions (dashed gray lines) in the HEV ORF2 (left panels) and ORF3 (right panels) identified in three chronically infected patients not achieving a sustained virological response (A, patient#1; B, patient#2; C, patient#4). Black bars indicate duration of RBV treatment starting with the first RBV dose at month 0.
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SUPPLEMENTARY MATERIAL AND METHODS

Amplification of HEV ORF1, ORF2 and ORF3 genome regions

Total RNA was extracted from 200 µL EDTA plasma using Cobas AmpliPrep total nucleic acid isolation kit (Roche, Basel, Switzerland). Complementary DNA (cDNA) was synthesized from 2–8 µL purified total RNA using the SuperScript III first-strand synthesis system (Life Technologies, Carlsbad, CA) with primer 5'AGGGGTTGGTTGGATGAATA3' for ORF1 and 5'CCCTTATCCTGCTGYGCATT3' for ORF2/3 at a final concentration of 2 µM. A touchdown nested polymerase chain reaction (PCR) with external and internal primer pairs was used to amplify the desired regions of the HEV genome. The first PCR round was carried out with TaKaRa Ex Taq Hot Start Version (Dalian, China) using 1-4 µL of synthesized cDNA and an external primer pair (ORF1: forward 5'ACGCTYGTGGGYAGGTACGG3' and reverse 5'AGCAYGARGARCAGCAACAC3'; ORF2/3: forward 5'CCTGGYACCCTYC TYTGGAAYAC3' and reverse 5'CCCTTATCCTGCTGYGCATT3') at a final concentration of 100 µM each in a 50 µL reaction with 18 cycles of 30 sec at 94°C, 45 sec at 62°C with a reduction of 0.5°C/cycle and 2 min at 72°C, followed by 14 cycles of 30 sec at 94°C, 45 sec at 53°C, and 2 min at 72°C. A final extension of 10 minutes at 72°C followed the final cycle. The second round PCR was carried out using an internal primer pair (ORF1: forward 5'YTCTGAYGTCCGTGAGTCCC3' and reverse 5'TATGYACCARBCCRGGRCTA3'; ORF2/3: forward 5'TGGGYTGTATGCY GGTGTGGTRGT3' and reverse 5'CAGCCGACGAAATCAATTCTG3') and 5 µL of the first-round PCR product with following parameters: 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 1.25 min/kb at 72°C. A final extension of 10 minutes at 72°C

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followed the final cycle. The resulting amplicons were analyzed for correct size on 1.5% agarose gels stained with ethidium bromide. For purification, a Qiaquick gel extraction kit was used (Qiagen, Hilden, Germany) and concentrations were measured using Qubit dsDNA HS Assay Kits (Life Technologies). For deep sequencing DNA-concentrations were adjusted to 0.2 ng/µL.

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Library preparation and Illumina sequencing

Briefly, for the preparation of sequencing-ready libraries for Illumina deep sequencing, 5 µL equimolarly pooled amplicons were fragmented and tagged using Nextera XT DNA Library Preparation Kit following the manufacturer's guidelines (Illumina, San Diego, CA, USA). For patient#1 no normalization was done for the samples and every sample was quantified single using qPCR and KAPA Library Quantification Kit for Illumina (Kapa Biosystems, Wilmington, MA, USA). For quality control of sequencing libraries the Agilent High Sensitivity DNA Kit (Agilent Technologies, Waldbronn, Germany) and a 2100 Bioanalyzer Instrument (Agilent Technologies) were used. Any additional samples were normalized and quantified using the KAPA Library Quantification Kit for Illumina (Kapa Biosystems, Wilmington, MA, USA). For cluster-generation and subsequent sequencing resulting DNA libraries were prepared with the MiSeq Reagent Kit v3 (Illumina). Deep sequencing was carried out on the Illumina MiSeq platform using the paired-end sequencing protocol for 2x 300-bp runs (Illumina).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Evaluation of deep sequencing data

Deep sequencing raw data was analyzed using CLC Genomics Workbench 8.0.1 (http://www.clcbio.com). Quality trimmed paired-end Illumina reads were mapped to the respective extracted consensus sequences of the first available time point for each patient. Amplicon sequences obtained from the ORF1 PCR were mapped to respective optimized ORF1 references (999 nt), amplicons from ORF2/3 PCR were mapped to ORF2 (1110 nt) and ORF3 (342 nt) references. Single nucleotide variations with minimum frequencies of 1% and $p < 0.01$ were called after base quality filtering.

Clonal Sequencing

ORF1 amplicons derived from all three non-responding patients obtained from ORF1 nested PCR were cloned into the pGEM-T vector (Promega) according to the manufacturer's recommendations. JM109 bacteria were transformed and colonies carrying a single copy of the viral ORF1 amplicon were selected on X-Gal, IPTG and ampicillin Lauria Broth (LB) agar plates. For each patient, 25 individual clones were extracted using NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany) and inserts were Sanger sequenced (GATC Biotech, Konstanz, Germany) with vector specific primers (detailed information and primer sequences available on request).

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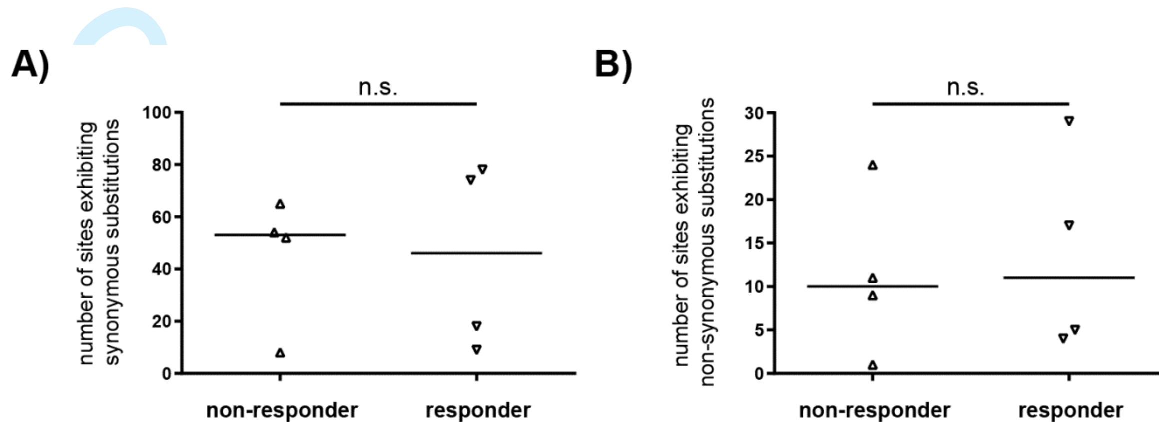
SUPPLEMENTARY FIGURES



Supplement Figure 1: Comparison of the initial numbers of sites exhibiting nucleotide substitutions in the HEV ORF1 of chronically infected patients responding to RBV treatment vs. non-responder before the application of the first dose. Synonymous (**A**) and non-synonymous (**B**) nucleotide variants are depicted for four patients responding to RBV treatment (patient#5, #7, #8, and #9) vs. four non-responding patients (patient#1-4). Horizontal lines indicate the median, significance was tested using the Mann-Whitney test (n.s. = not significant).

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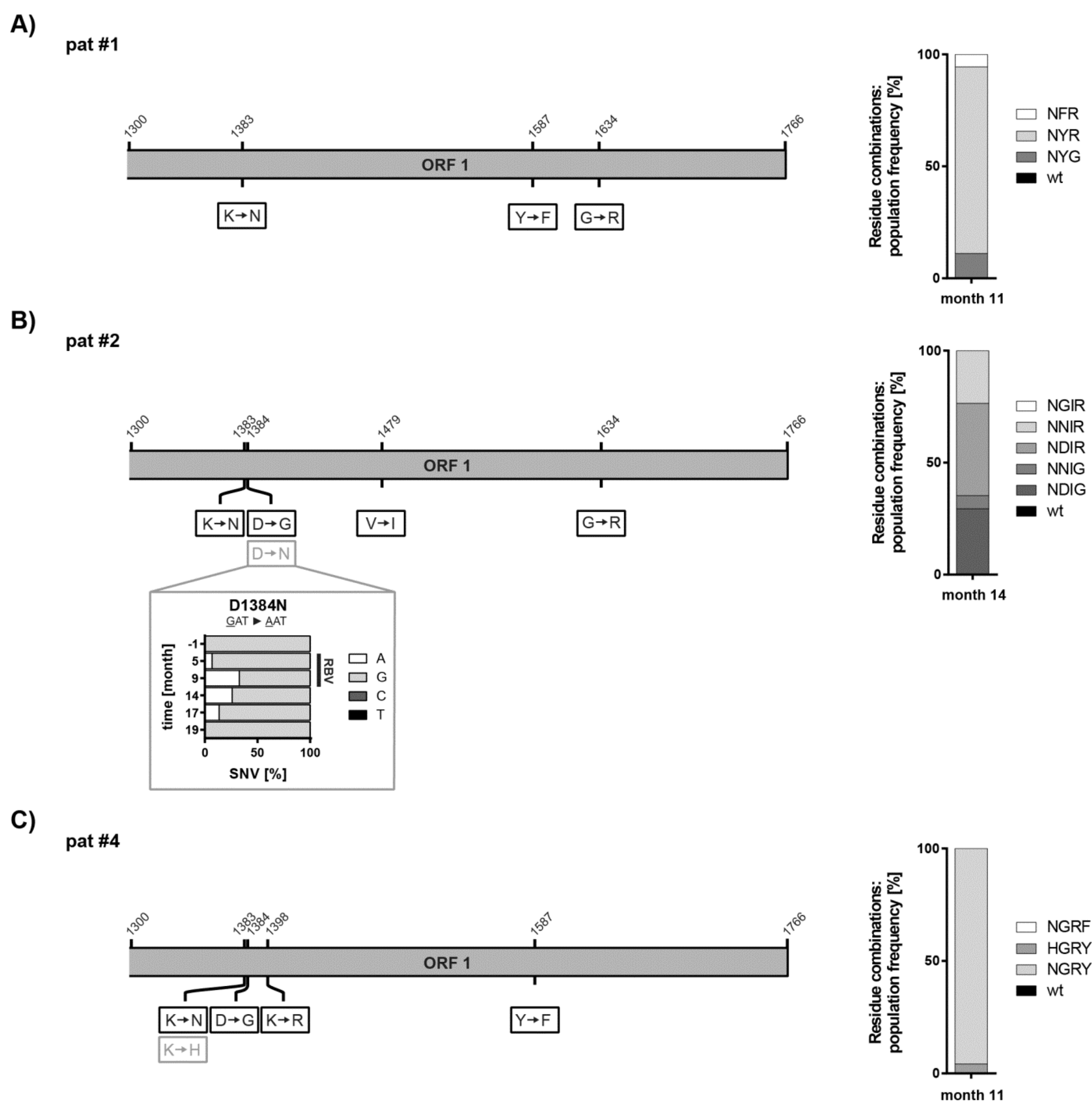

Supplement Figure 2: Linkage analysis of the identified mutations and their appearance in the viral populations. The single nucleotide variations (SNVs) identified in the three patients not achieving sustained virological responses after RBV treatment were analyzed in regards to their linkage and combinations in single viral genomes via clonal sequencing. The left panels show the distribution of the SNVs in HEV ORF1 and their amino acid position for patient#1 (**A**), patient#2 (**B**) and patient#4 (**C**), as well as the respective wild type and mutated amino acids (black boxes). Light gray boxes represent amino acid changes that were not taken into

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account for deep sequencing analyses, because they did not reach threshold levels (**B**), or were not picked up during deep sequencing at all (**C**). The right panels represent the frequencies of the different identified residue combinations in the viral population at indicated time points.

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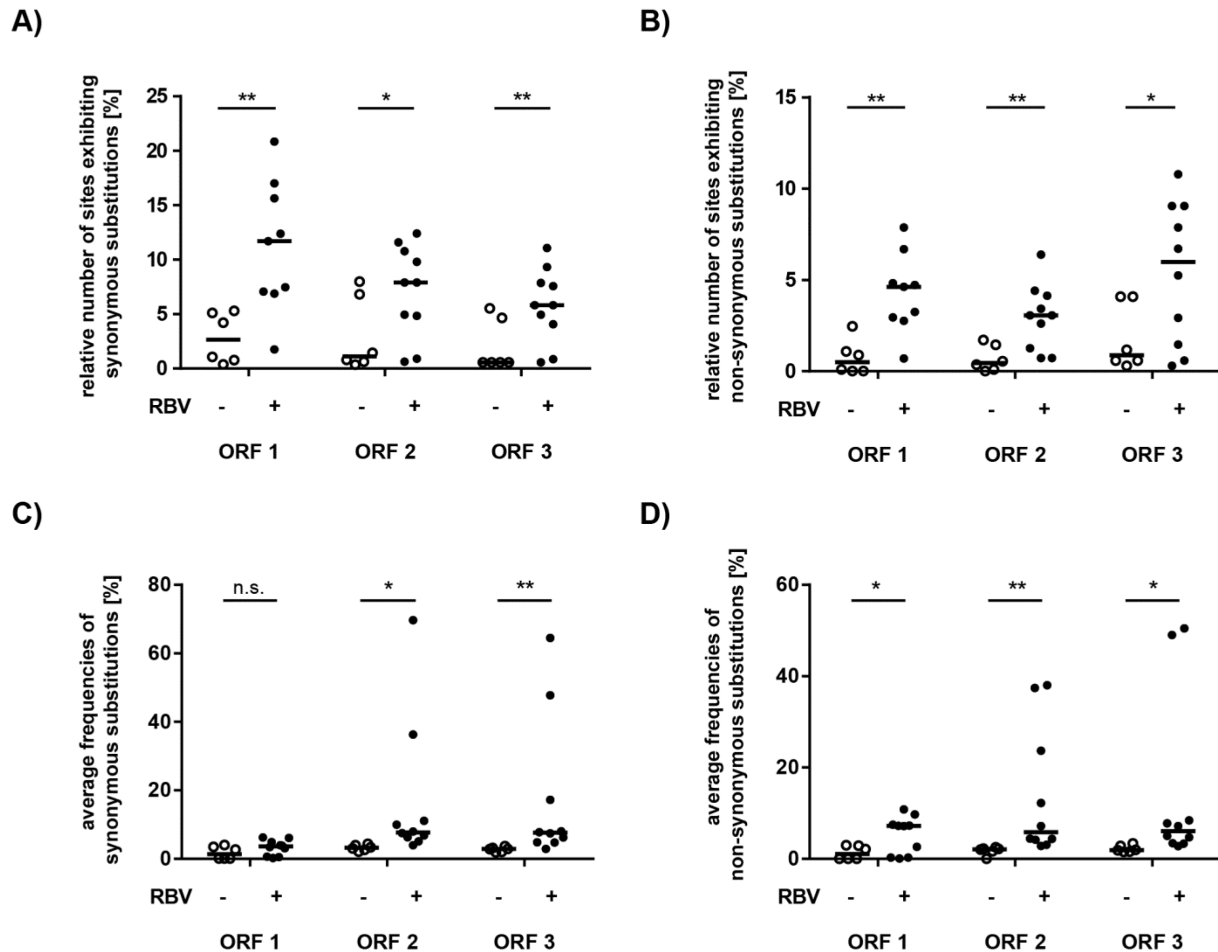

Supplement Figure 3: Comparison of normalized numbers and frequencies of identified variations with and without RBV administered. The relative numbers of sites exhibiting synonymous (**A**) and non-synonymous (**B**) single nucleotide variations (SNVs),as well as the average frequencies of synonymous (**C**) and non-synonymous (**D**) SNVs identified in all three open reading frames of the hepatitis E viral intra-host population found in patient#1, patient#2 and patient#4 at time points of available serum samples before (open circles) and during RBV treatment (black dots) are depicted. Horizontal bars indicate the median, significance was tested with the Mann-Whitney test (* = p<0.05; ** = p<0.01, n.s. = not significant).

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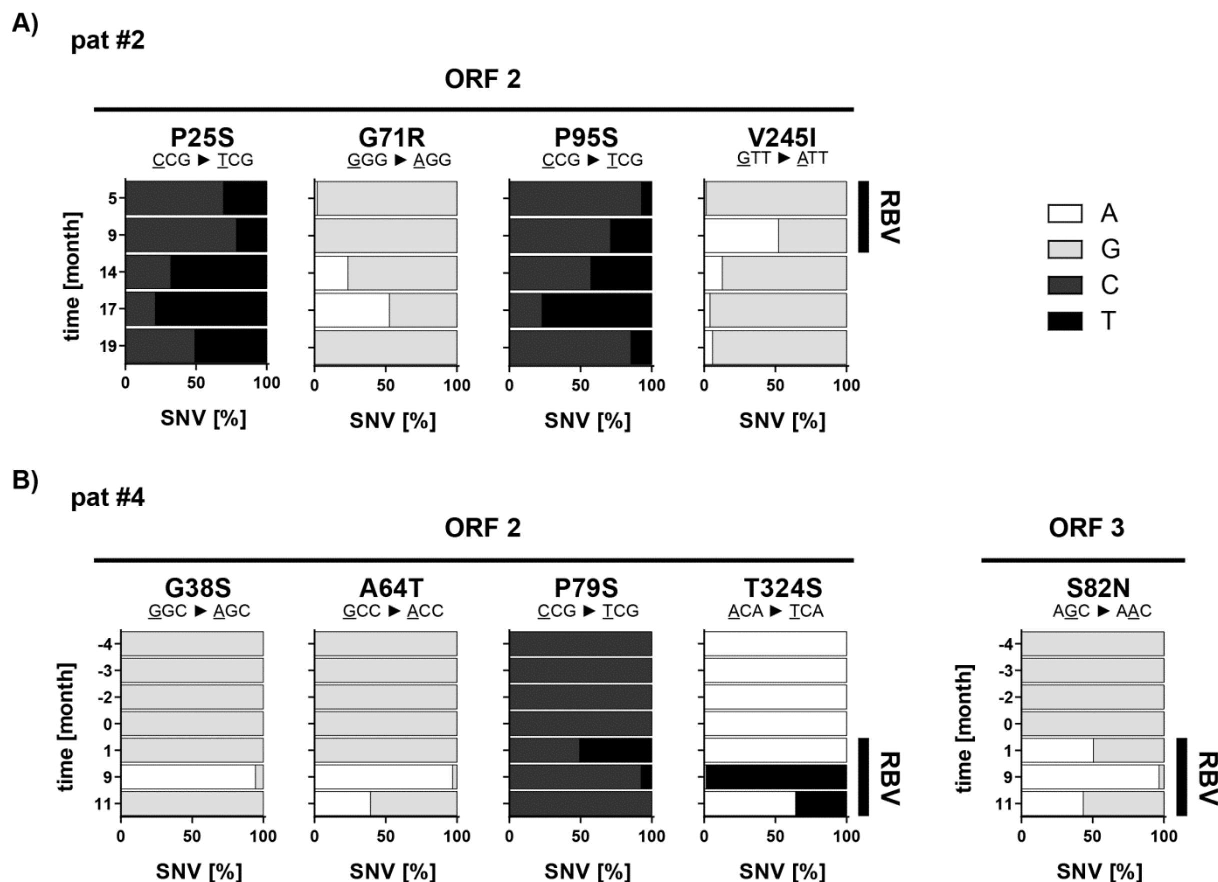

Supplement Figure 4: Time course of non-synonymous single nucleotide variations at certain positions of the HEV ORF2 and ORF3 in patients experiencing RBV treatment failure. Changes in nucleotide frequencies (x-axes) resulting in alterations of the predominant amino acids over monitoring time (y-axes) of chronically infected patients are depicted at amino acid positions indicated above the plots. The altered nucleotides in the coding triplets are underlined. White bars indicate proportions of adenine, light gray bars of guanine, dark grey bars represent cytosine and black bars show amount of thymine. **A)** Four positions with a change in the dominant amino acid were identified for patient#2 in ORF2. **B**) Four non-synonymous SNV were found in patient#4 in ORF2 and one in ORF3. Black vertical lines indicate course of administration of RBV.

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Addendum III

Cross-genotype-specific T cell responses in acute hepatitis E virus (HEV) infection

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Cross-genotype-specific T-cell responses in acute hepatitis E virus (HEV) infection

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SUMMARY. Hepatitis E is an inflammatory liver disease caused by infection with the hepatitis E virus (HEV). In tropical regions, HEV is highly endemic and predominantly mediated by HEV genotypes 1 and 2 with >3 million symptomatic cases per year and around 70 000 deaths. In Europe and America, the zoonotic HEV genotypes 3 and 4 have been reported with continues increasing new infections per year. So far, little is known about T-cell responses during acute HEV genotype 3 infection. Therefore, we did a comprehensive study investigating HEV-specific T-cell responses using genotypes 3- and 1-specific overlapping peptides. Additional cytokines and chemokines were measured in the plasma. In four patients, longitudinal studies were performed. Broad functional HEV-specific $CD4^+$ and $CD8^+$ T-cell responses were detectable in patients acutely infected with HEV genotype 3. Elevated of pro- and anti-inflammatory cytokine levels during acute HEV infection correlated with ALT levels. Memory HEV-specific T-cell responses were detectable up to >1.5 years upon infection. Importantly, cross-genotype HEV-specific T-cell responses (between genotypes 1 and 3) were measurable in all investigated patients. In conclusion, we could show for the first time HEV-specific T-cell responses during and after acute HEV genotype 3 infection. Our data of cross-genotype HEV-specific T-cell responses might suggest a potential role in cross-genotype-specific protection between HEV genotypes 1 and 3.

Keywords: cellular immune response, cross-genotype, hepatitis, hepatitis E virus (HEV), T cell.

INTRODUCTION

Hepatitis E virus (HEV) is a nonenveloped single-stranded RNA virus and a common cause of acute hepatitis worldwide [1–3]. More than 3 million symptomatic HEV cases occur per year with around 70 000 deaths [2]. Four human pathogenic genotypes of HEV have been described. Genotypes 1 and 2 are predominantly transmitted via the faecal-oral route and are responsible for the majority of endemic hepatitis E cases in subtropical and tropical regions such as India, South-East Asia and sub-Saharan Africa. Although HEV infections are in the majority of cases asymptomatic and are usually self-limiting, severe cases of acute HEV infection can be found especially in pregnant women infected with HEV genotype 1 or 2 and in individuals with pre-existing chronic liver diseases [4]. Recently, a recombinant HEV genotype 1 vaccine (Hecolin®) has been approved by the Chinese Food and Drug Administration in China [5]. This vaccine induces HEV-specific antibodies in 100% of vaccinated healthy individuals resulting in protection against homologous (genotype 1) and in part against heterologous (genotypes 2–4) HEV infections in humans and animal studies [6–9]. The efficiency of protection is dependent on the antibody level prior HEV infection [9].

HEV genotypes 3 and 4 are zoonotic pathogens with major animal reservoirs in pigs, wild boar and shellfish [10]. These autochthonous HEV genotypes 3 and 4

Abbreviations: aa, amino acid; ALT, alanine transaminase; AST, aspartate aminotransferase; BL, baseline; CD, cluster of differentiation; CFSE, carboxyfluorescein diacetate succinimidyl ester; FU, follow-up; GM-CSF, granulocyte-macrophage colony-stimulating factor; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; ICS, intracellular cytokine staining; IFN-γ, interferon-gamma; IgG, immunoglobulin G; IL, interleukin; IP, interferon-gamma-induced protein; MCP, monocyte chemoattractant protein; n.d., not determined; ns, not significant; ORF, open reading frame; PBMCs, peripheral blood mononuclear cells; RNA, ribonucleic acid; SEM, standard error of the mean; SI, stimulation index; TNF, tumour necrosis factor.

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infections have been stated in western Europe and North America and partly in Asia [2,11]. Hepatitis E is still a frequently under-reported infectious disease in Western countries [12]. HEV genotype 3 is predominately transmitted by consumption of undercooked pork [13,14] and transfusion of contaminated blood products [15]. In general, HEV genotype 3 infections are asymptomatic; however, in immunosuppressed patients infected with HEV genotype 3, a prolonged viremia as well as a chronicity of HEV has been detected. In particular, this has been observed in solid organ transplanted individuals who take immunosuppression [4,16,17]. Chronic hepatitis E is frequently associated with a particular severe course of liver disease with development of liver cirrhosis within 1–2 years of infection [18,19]. No vaccine against HEV genotype 3 is available, and the HEV genotype 1 vaccine (Hecolin®) is so far not solid outside of China. Ribavirin and Peg-IFN-alpha treatments have been successfully used to treat HEV [20–22,23]. Treatment contraindications have been reported in some patients, for example organ rejection and pregnancy as well as ribavirin resistance [3,24]. Therefore, other antiviral strategies or vaccination are needed. Furthermore, extrahepatic manifestations such as arthralgia and Guillain–Barré syndrome are described during or after resolution of HEV infections [12,25–27].

Virus-specific T-cell responses are important for viral clearance and are involved in immunopathology [28–30]. For other hepatotropic virus infection such as hepatitis B (HBV) and hepatitis C (HCV), cross-genotype-specific T-cell responses are reported [31,32]. HEV-specific T-cell responses were studied and detected in patients from endemic regions with presumably genotype 1 infection [33–36]. Previously, our laboratory showed that chronic hepatitis E genotype 3 is associated with impaired HEV-specific T-cell response and that interfering with checkpoint inhibitor PD-1 and CTLA-4 can enhanced HEV-specific T-cell responses [37]. However, to our knowledge, no T-cell responses have yet been studied during and after acute HEV genotype 3 infections.

The aim of our study was to investigate HEV-specific T-cell responses during and after acute HEV genotype 3 infection. We could show detectable HEV genotype 3-specific T-cell responses which were also cross-genotype-specific against HEV genotype 1. Elevated chemokines/cytokines in plasma during acute HEV infection correlated with liver inflammation. Memory HEV-specific T cells could be found after HEV clearance.

PATIENTS, MATERIALS AND METHODS

Patients

The study protocol conformed to the ethical guidelines of the Institutional Review Committee and written informed consent was obtained from each patient included in this study. All subjects were recruited at Hannover Medical School between 2008 and 2014. Plasma samples and PBMCs were collected from twelve acutely HEV-infected immunocompetent patients which cleared HEV. All patients were tested HEV-RNA positive and/or anti-HEV IgM positive in blood at day of PBMC isolation. Patient characteristics are shown in Table 1. As control, plasma and PBMCs from 27 anti-HEV-IgG-negative healthy subjects were collected. Anti-HEV-IgG status was determined using Wantai HEV-IgG (Beijing, China), and HEV-RNA from plasma was quantified by one-step RT-qPCR [38].

Isolation and storage of PBMC and blood plasma samples

From fresh whole blood samples, peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll density gradient centrifugation. Cells were cryopreserved in

Table 1 Patient baseline characteristics: n.d. = not determined

Patients	Age	Gender	Peak ALT [U/L]	Peak AST [U/L]	Total Bilirubin [μmol/L]	INR	Geno type	Travel associated
1	64	M	3876	1923	211	1.18	3	No
2	57	M	396	70	11	1.15	3	No
3	79	M	263	140	218	1.12	3	No
4	38	F	1486	476	41	1.02	3	No
5	58	F	4196	3604	116	1.80	3	No
6	41	M	3039	761	140	1.48	3	No
7	62	M	340	82	57	1.03	3	No
8	59	F	n.d	n.d	n.d	n.d	3	No
9	52	M	n.d	n.d	n.d	n.d	3	No
10	52	M	2518	1545	90	1.14	3	No
11	32	M	n.d	n.d	n.d	n.d	3	No
12	44	F	3315	1826	137	1.26	1	Yes

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freezing medium consisting of 30% RPMI-1640 medium (Invitrogen, Karlsruhe, Germany), 60% foetal bovine serum (PAA, Pasching, Austria) and 10% DMSO (Sigma-Aldrich, Munich, Germany) and stored in nitrogen liquid. Plasma were collected from EDTA-treated blood samples and stored at −20 °C.

Hepatitis E virus overlapping peptide pool

HEV-specific overlapping peptides (15 mers, overlapping by 5aa) spanning ORF2 and ORF3 of the genome corresponding to the amino acid sequences of the genotype 1 (GenBank accession number: AF459438) and genotype 3 (GenBank accession number: AF455784) were synthesized from PEPscreen custom peptide library (Proimmune, Oxford, UK). Synthetic peptides were dissolved in DMSO. Six overlapping HEV-specific peptide pools were created; pool 1–4 containing 13 peptides, pool 5 contains 14 peptides, and pool 6 contains 12 peptides. Peptides in pool 1–5 are corresponding to ORF2 and peptides in pool 6 to ORF3. Final concentration for each peptide was 5 μg/mL. DMSO concentration did not exceed 0.2% in cultures [39].

Antibodies

Cell-surface staining was performed with the following antibodies: anti-CD4 (clone: L200, RPA-T4) and anti-CD8 (clone: SK1), and intracellular staining was performed with anti-IFN-γ (clone: B27) and anti-TNF (clone: MAb11). To exclude monocytes/macrophages, NK cells and B cells, anti-CD14 (clone: M5E2), anti-CD19 (clone: SJ25C1) and anti-CD56 (clone: B159) were used. All antibodies were obtained from BD Biosciences, CA, USA.

HEV-specific T-cell proliferation in vitro

Briefly, PBMCs were stained with 4 μM carboxyfluorescein diacetate succinimidyl ester (CFSE), washed and suspended in medium as described previously [39]. Afterwards, 3×10^5 CFSE-labelled PBMCs were stimulated with HEV-specific overlapping peptide pools or as control with medium alone at 37 °C and 5% CO_2 for 7 days. IL-2 (5 IU/mL) was added on day 3. On day 7, cells were stained with anti-CD8 and anti-CD4. Samples were acquired using a flow cytometer (FACSCalibur, BD), and data were analysed with FlowJo (TreeStar, Inc., Ashland, OR, USA). Proliferation data are shown as Stimulation Index (SI): SI = percentage $CSFE^{low}$ peptide-stimulated cells/percentage $CSFE^{low}$ medium alone-stimulated cells.

Intracellular cytokine staining

IFN-γ- and TNF-producing HEV-specific $CD4^+$ and $CD8^+$ T cells were determined by intracellular cytokine staining (ICS) as described previously [39]. 3×10^5 PBMCs were stimulated with HEV overlapping peptide pools at 37 °C and 5% CO_2 for 10 days or cultured in medium alone as control. On day 7, cells were fed with IL-2 (5 IU/mL). On day 10, PBMC were stimulated with the respective peptide pool or medium for 1 h followed by additional 5 h in the presence of brefeldin A (2 μg/mL). After surface staining with anti-CD4, anti-CD8 and the exclusion markers (anti-CD19, anti-CD14 and anti-CD56), cells were permeabilized using BD Cytofine/Cytoperm. Afterwards, IFN-γ and TNF-α were stained intracellularly in BD permwash buffer, washed and suspended in FACS buffer. Samples were measured and analysed as described above.

Measurement of cytokine and chemokine in plasma

Multi-analyte profiling of 50 cytokine, chemokines and angiogenic factors in the plasma were performed using multiplex technology (Bio-Plex Pro Human Cytokine Panel, Bio-Rad, Hercules, CA, USA) according to the manufacture guidelines.

Statistical analysis

Data were analysed using GraphPad Prism v6.0b (GraphPad software, La Jolla, CA, USA). Correlations between cytokines/chemokines and clinical parameters were calculated using linear regression (R^2 and *P*-value). Comparisons of cytokines/chemokines between baseline and follow-up time point were performed using Wilcoxon's matched-pairs signed-rank test. In general, *P* values of $P < 0.05$ were considered to be significant ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$ or $****P < 0.0001$).

RESULTS

Detection of HEV-specific T-cell responses in patients during acute HEV genotype 3 infection

First, HEV-specific T-cell responses were investigated during acute HEV genotype 3 infection. The cohort contained 11 patients infected with HEV genotype 3 (#1-11) and one patient infected with genotype 1 (#12) (Table 1). PBMCs of these eleven acutely HEV-infected patients were stimulated with HEV genotype 3 overlapping peptide pools *in vitro*. PBMCs from the genotype 1-infected patient were stimulated with HEV genotype 1 overlapping peptide pools. As control, 27 healthy anti-HEV-IgG-negative subjects were stimulated with HEV genotype 3 overlapping peptide pools.

Proliferation of HEV-specific T-cell responses was determined by FACS analysis, and gating strategy is shown in representative FACS plots (Fig. 1a). A broad proliferative HEV-specific T-cell response was found in all investigated acutely HEV-infected patients (Fig. 1b). Interestingly, in 10 of 11 patients' proliferation of $CD8^+$ T cells was predominate

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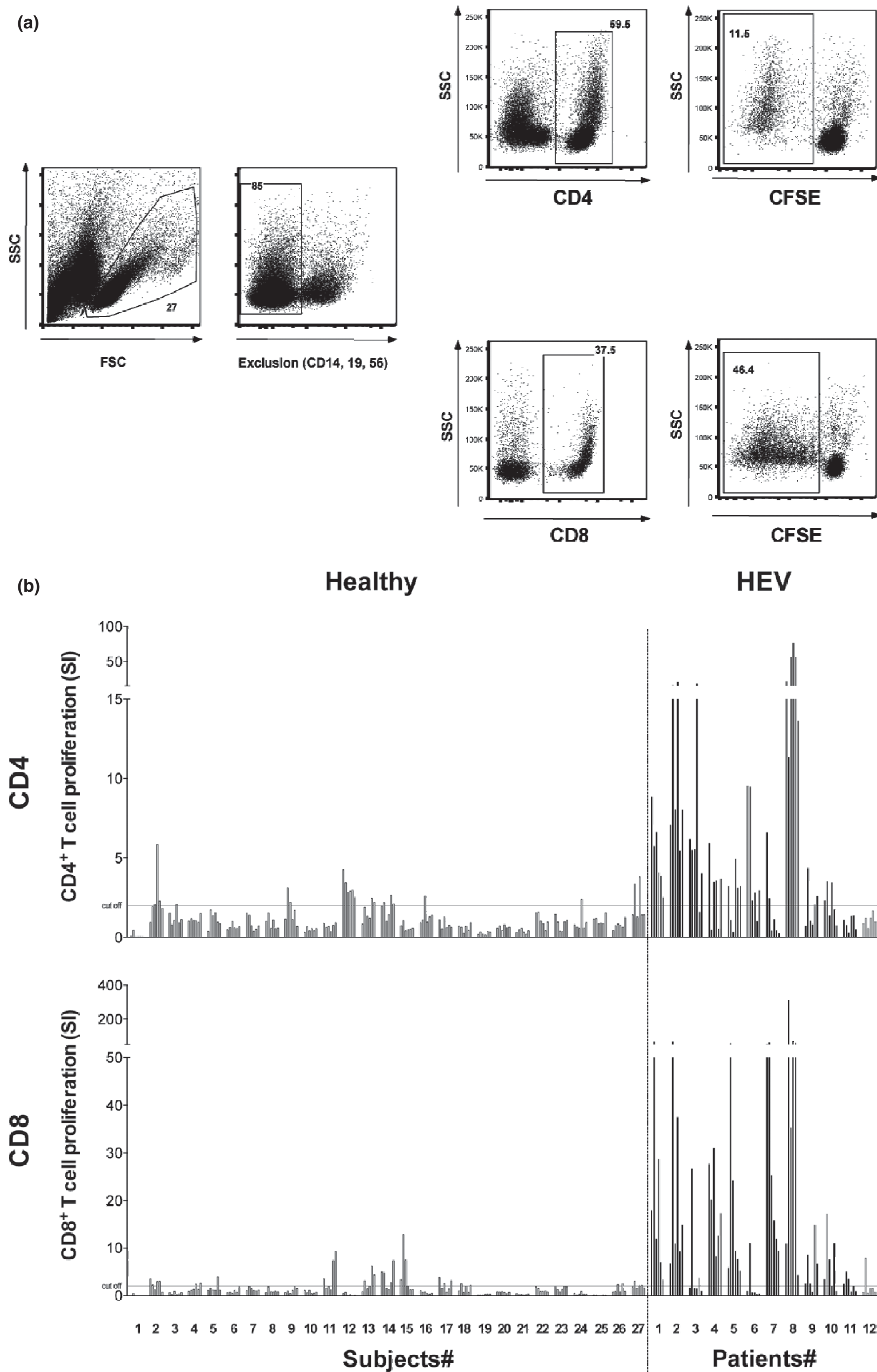

Fig. 1

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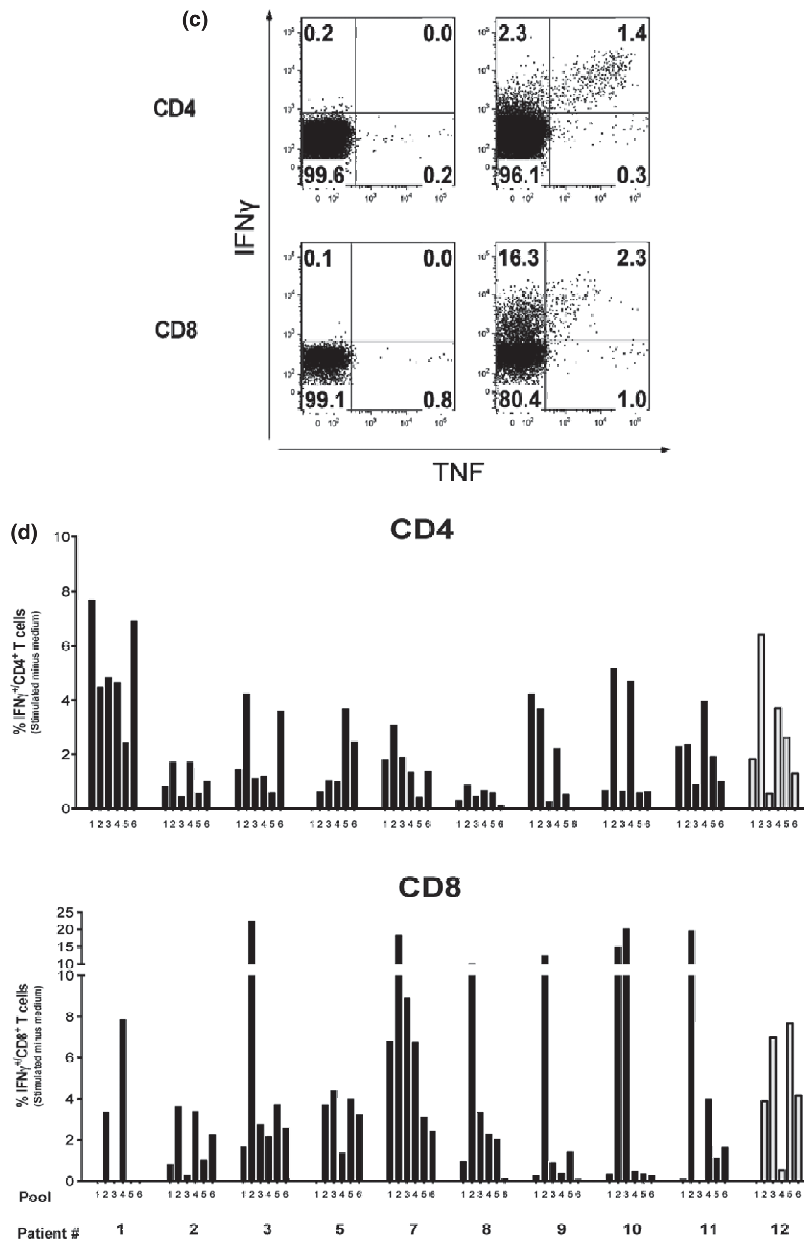

Fig. 1 HEV-specific T-cell responses in patients with acute hepatitis E and healthy subjects: (A+B) PBMCs of patients were stimulated with 6 HEV-specific overlapping peptide pools for 7 days, respectively, to their genotype. PBMCs of healthy controls were stimulated for 7 days with genotype 3 overlapping peptide pools as control. **(a)** A representative staining from PBMCs stimulated with one overlapping peptide pool indicates the gating strategy. **(b)** HEV-specific $CD4^+$ and $CD8^+$ T-cell proliferation were studied in healthy subjects (subject #1-27, left side) and patients with acute HEV genotype 3 (subject #1-11, black bar, right side) and genotype 1 (subject #12, grey bar, right side) infection. T-cell response is considered positive if the stimulation index (SI) is more than 2 (cut-off). Each bar per subject represents one pool (pool 1–6). (C+D) Functionality of HEV-specific T-cell responses were studied in patients with acute HEV genotype 3 (#1-11, black bar) and 1 (#12, grey bar) infection. Therefore, PBMCs were stimulated with overlapping peptide pools for 10 days followed by a 6-h restimulation with the respective peptide pool. **(c)** A representative staining from PBMCs stimulated with one overlapping peptide pool is depicted. IFN-γ and TNF shown is gated on $CD4^+$ (upper row) and $CD8^+$ (low row) T cells. **(d)** Percentage of IFN-γ^+ HEV-specific $CD4^+$ and $CD8^+$ T cells is shown for each patient and peptide pool. Each bar per subject represents one pool (pool 1–6).

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in PBMCs stimulated with overlapping peptide pool 2 (Fig. 1b), whereas it was more diverse for the $CD4^+$ T-cell response. Overall, lower responses were seen against ORF3 (pool 6) compared to ORF 2 (pool 1–5). In healthy subjects, T-cell proliferation upon HEV-specific peptide stimulation is hardly detectable (average sum of SI ± SEM CD4: 7 ± 0.8 *vs* 56 ± 23.6, CD8: 9 ± 1.4 *vs* 155 ± 52, respectively, healthy subjects *vs* acute HEV patients). To investigate whether the HEV-specific T cells in patients during acute HEV infection were also functional, cytokine (IFN-γ and TNF)-producing HEV-specific T cells were determined by intracellular cytokine staining (Fig. 1c+d, data not shown). Both, HEV-specific $CD4^+$ and $CD8^+$ T cells were capable of producing cytokines upon restimulation with HEV-specific overlapping peptides and revealed a broad functional T-cell response similar as seen in the proliferation assay. Interestingly, HEV-specific $CD8^+$ T cells predominantly produced IFN-γ alone (24.2 ± 4.2% IFN-γ^+TNF$^-$, 2.7±0.6% IFN-γ^+TNF$^+$, ratio 9:1, n = 10), whereas a higher proportion of HEV-specific $CD4^+$ T cells are multifunctional (IFN-γ/TNF double positive) (6.4 ± 2.1% IFN-γ^+TNF$^-$, 11.9 ± 2.6% IFN-γ^+TNF$^+$, ratio 1:2) (representative example Fig. 1c).

Biochemical disease activity is associated with elevated cytokine/chemokine levels in acute hepatitis E

As broad T-cell responses were detected, we next questioned whether this adaptive immune response is associated with an altered systemic cytokine profile in acute hepatitis E. Fifty different cytokines and chemokines were measured in plasma using multiplex technology. Indeed, pro- and anti-inflammatory cytokines and chemokines were elevated during viremia (first visit) compared to the follow-up visits. IL-2, -4, -6, -7, -8, -9, -10, -13, -15, -17, IL-1R-α, MCP-1, TNF-α and IFN-γ strongly correlated with both ALT and AST levels (Fig. 2, Tables S2 and S3). In follow-up visits, a significant decrease in plasma of IP-10, IL-13 and IL-9 was detected whereas no changes were found for IL-2, IL-10 and IFN-γ early after viral clearance (Fig. 2, Table S4).

HEV-specific T-cell responses after clearance of HEV infection

Finding elevated cytokine and chemokine pattern during the acute phase of HEV, we questioned whether HEV-specific memory T cells might be generated. To test this, we were able to study HEV-specific T-cell responses prospectively in four patients 1–2 month and >1 year after the baseline time point (Fig. 3). All patients were HEV-RNA negative in blood at all follow-up time points. HEV-specific T-cell responses were detectable after clearance of HEV-RNA from blood indicating HEV-specific memory T cells. Whereas the proliferative capacity of HEV-specific $CD8^+$ T cells were comparable in the follow-ups after *in vitro* stimulation with overlapping peptides, the proliferative capacity of HEV-specific $CD4^+$ T cells decreased to almost undetectable level early after HEV clearance (1–2 month), but had comparable responses >1 year after HEV clearance compared to baseline (Fig. 3a). This phenomenon could not be detected in their functionality (cytokine production upon restimulation) (Fig. 3b). Functional HEV-specific T cell could be measured at all follow-up time points (Fig. 3b). As cytokines and chemokine might alter the proliferative capacity of the $CD4^+$ T cells early after HEV clearance, we tried to link the measured cytokines and chemokines in the plasma of the patients to their $CD4^+$ T-cell proliferation capacity. So far, no direct association between altered cytokine/chemokine profile and decreased proliferative capacity of $CD4^+$ T cells could be detected (data not shown).

HEV-specific T-cell responses are cross-genotype specific

Finding detectable HEV-specific T-cell responses during acute HEV genotype 3 infection and knowing that the HEV genotype 1 vaccine might be cross-protective against other HEV genotypes, we question whether potential cross-genotype-specific T cells could be involved.

We had the unique opportunity to compare HEV-specific T-cell responses of the same 12 HEV-infected patients (Table 1) against overlapping peptide pools derived from HEV genotypes 1 and 3 in parallel experiments (Table 2, Fig. 4). Of note, HEV genotypes 1 and 3 have about 91% amino acid sequence homology for ORF2 and 3 (Fig. 2). Most amino acid (aa) sequence differences were found in ORF2 between 1–120 aa (20%, pool 1) and in ORF3 between 6–104 aa (18%, pool 6).

In all acutely genotype 3-infected patients, HEV-specific proliferative and functional T-cell responses were also detected against overlapping peptides derived from HEV genotype 1 (Table 2, Fig. 4). Additionally, similar broad HEV-specific T-cell responses against genotype 1 compared to 3 were found in these patients. Comparing the average sum of SI and percentage of IFN-γ^+ T-cell responses, an overall slight increase in the strength was found in genotype 3 *vs* 1 derived overlapping peptides with a significant difference in only HEV-specific $CD8^+$ T-cell proliferation (SI ± SEM: genotype 3 155 ± 52 *vs* genotype 1 62 ± 17; n = 8, P = 0.016) (Table 2). Similar effects were seen for the HEV genotype 1-infected patient towards genotype 3-derived overlapping peptides.

DISCUSSION

We had the unique opportunity to investigate the eleven patients infected with HEV genotype 3 plus one additional genotype 1-infected patient. $CD4^+$ and $CD8^+$ HEV-specific T-cell responses were detectable in all patients using overlapping peptides from ORF2 to ORF3 derived from their respective genotype. Our data are in line with findings

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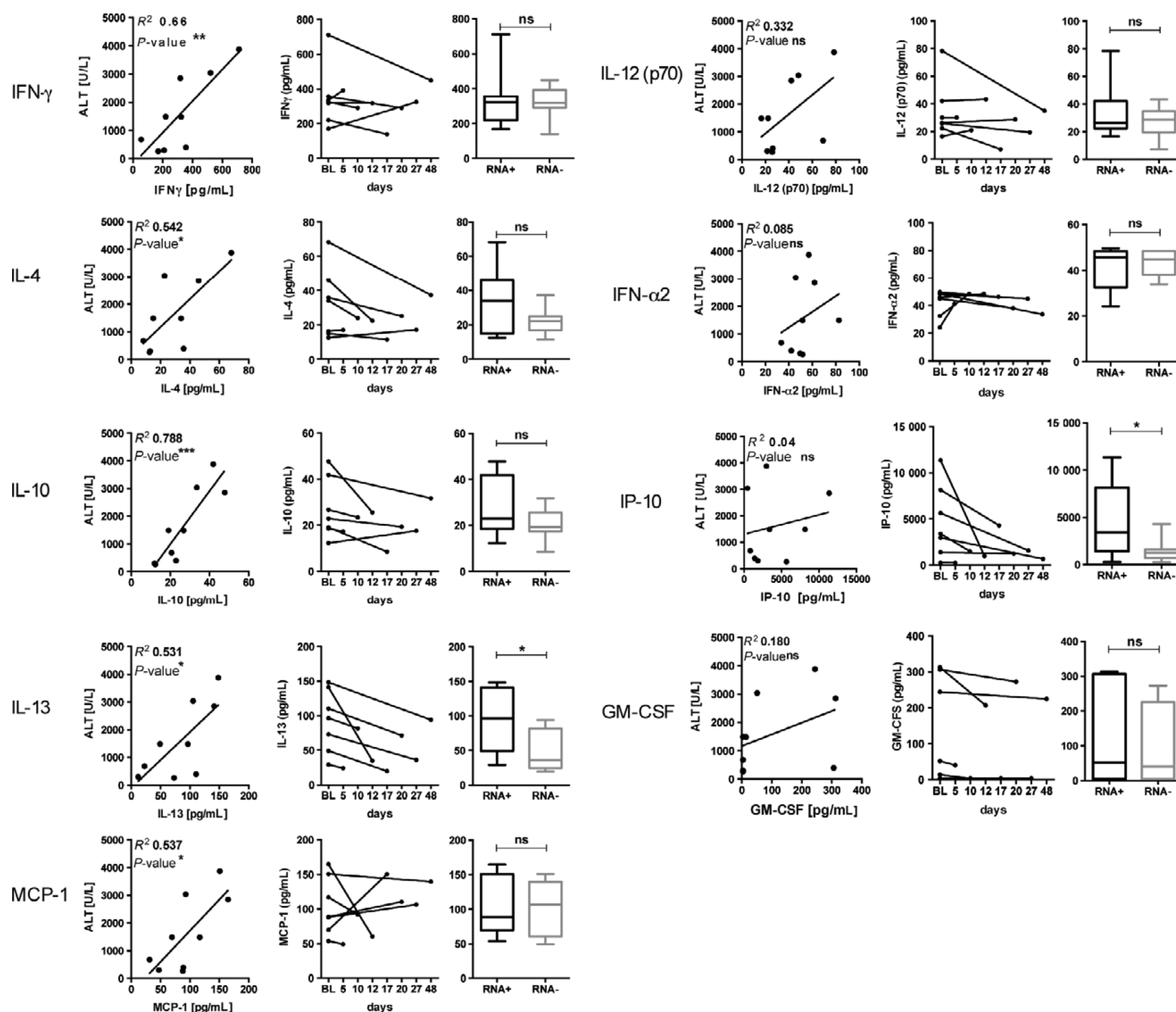

Fig. 2 Cytokines/chemokines profile in plasma of patients with acute hepatitis E: ALT [U/L] values were correlated to plasma levels (pg/mL) of nine cytokines/chemokines in nine patients at baseline (BL, first visit). Plasma levels (pg/mL) of nine cytokines/chemokines in seven patients at baseline (HEV-RNA positive, first visit) were compared to the next follow-up (HEV-RNA negative) time point. Box and whisker plots BL *vs.* FU (n = 7 subjects); BL = baseline; FU = follow-up. Statistical analysis: linear regression and Wilcoxon's test; ns= not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

from HEV infection of endemic region which were presumably HEV genotype 1 infection [33–36].

Similar to findings in acute hepatitis A virus infection (HAV), we also found that HEV-specific CD8 T cells predominantly produced only IFN-γ during acute HEV infection, whereas $CD4^+$ T cells were mainly IFN-γ/TNF double positive [40]. These might be of further interest as additional similarities between HAV and HEV have been reported, for example both viruses are nonenveloped in the faeces but are enveloped in host-derived membranes in the blood [41,42]. For hepatitis A, the importance of host-derived membranes to induce adaptive immunity has recently been established [42]. Whether similar mechanisms are also important in hepatitis E remains to be investigated.

Furthermore, we had the opportunity to study a panel of fifty cytokines and chemokines in plasma at different time points during and after acute HEV infection. Cytokines are important immunological molecules which have multiple direct/regulatory functions during infections. Most of the common pro- and anti-inflammatory cytokines correlated with ALT and AST suggesting an association of these cytokines with ongoing liver disease during acute HEV infection. Our data are in line with findings by Ramdasi *et al.* [43] where they showed elevated cytokine and chemokine levels between acute HEV-infected and healthy women. Importantly, all our acutely HEV-infected patients were admitted with symptoms to our clinic but had an uncomplicated course of disease. No correlation of HEV-

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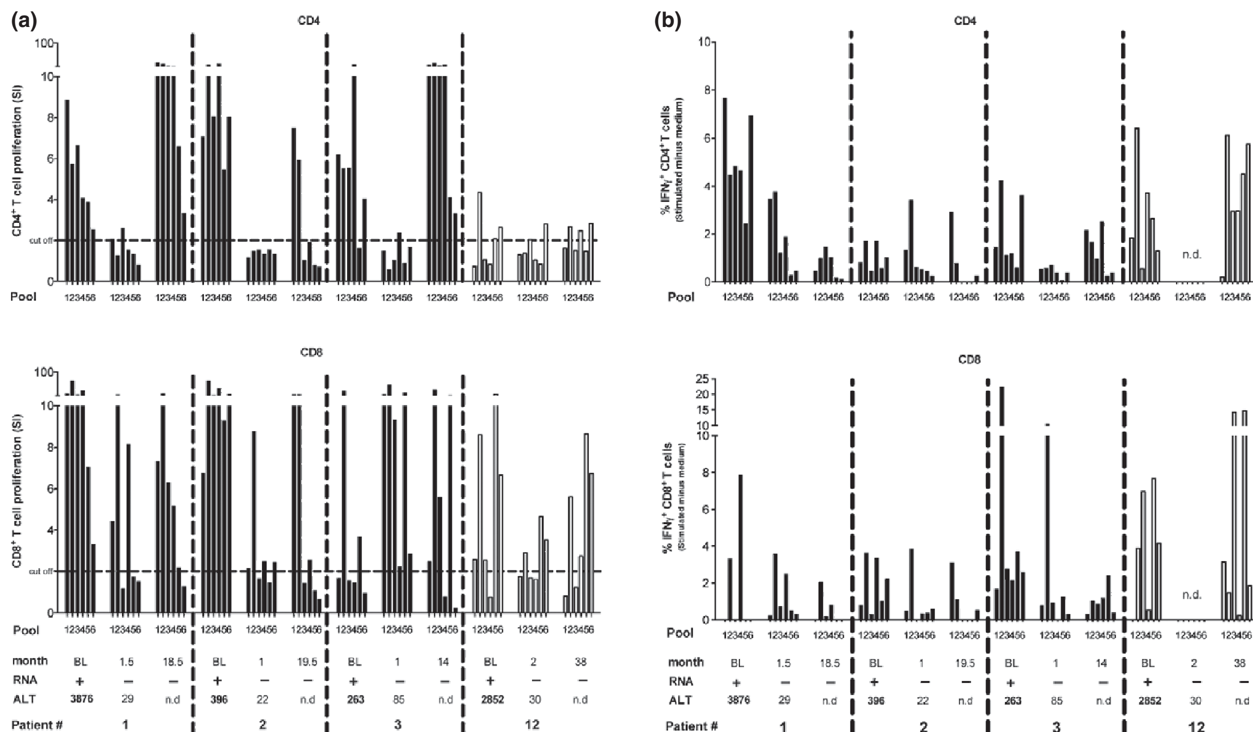

Fig. 3 Longitudinal T-cell responses in patients with acute resolved hepatitis E: HEV-specific T-cell responses were studied longitudinal in three patients infected with HEV genotype 3 (#1-3) and 1 patient with genotype 1 (#12). **(a)** T-cell proliferation and **(b)** IFN-γ^+ HEV-specific T cells were studied at baseline (HEV-RNA positive, BL), follow-up 1 (HEV-RNA negative, 1–2 month) and 2 (HEV-RNA negative, >2 years). PBMCs were stimulated with overlapping peptides as described in Fig. 1. The data from baseline time point for each patient is shown from the same patient in Fig. 1. T-cell response is considered positive if the stimulation index (SI) is more than 2 (cut-off). n.d. = not determined.

Table 2 Cross-genotype-specific T-cell response: T-cell responses of acutely HEV genotype 3-infected patients stimulated with overlapping peptide pools from genotype 3 were compared to genotype 1 peptide pools. All patients were infected with HEV genotype 3 (proliferation n = 8, ICS n = 4)

		Proliferation			IFN-γ response		
	Geno-type	Patients responding to at least one pool	Mean positive pools/patient ± SEM	Average sum of SI per patient ± SEM	Patients responding to at least one pool	Mean positive pools/patient ± SEM	Average sum of % IFN-γ per patient ± SEM
CD4	3	8/8	4.8 ± 0.6	56 ± 26.3	4/4	5.8 ± 0.5	15 ± 5.6
	1	8/8	3.9 ± 0.9	35 ± 11.6	4/4	5.3 ± 0.3	12 ± 4.1
CD8	3	8/8	4.9 ± 0.7	155 ± 52.0*	4/4	4.8 ± 0.9	19 ± 5.7
	1	8/8	4.4 ± 0.7	62 ± 17.3	4/4	3.8 ± 0.9	10 ± 3.9

Statistically, differences in T-cell responses between the different genotypes are indicated; Wilcoxon's test: *$P < 0.05$.

specific T-cell responses to ALT or AST was found suggesting that HEV-specific T cells play no major role in liver damage during uncomplicated HEV genotype 3 infection. Interestingly, pro- and anti-inflammatory cytokines correlated with ALT only when patients were acutely HEV infected but no correlation could be found in the follow-up visits. Whereas most of the pro- and anti-inflammatory cytokines stayed elevated during follow-up (till day 48), a significant reduction of IP-10, IL-13 and IL-9 as well as a reduction in GM-CSF and IL-6 was found. The level of IP-10 during acute HCV has predictive capacity in spontaneous HCV clearance during acute HCV infection as well as in treatment induced clearance in patients with chronic hepatitis C [44]. Whether IP-10 levels in HEV-infected immunocompromised patients, who have a reduced HEV-specific T-cell response and become chronically HEV

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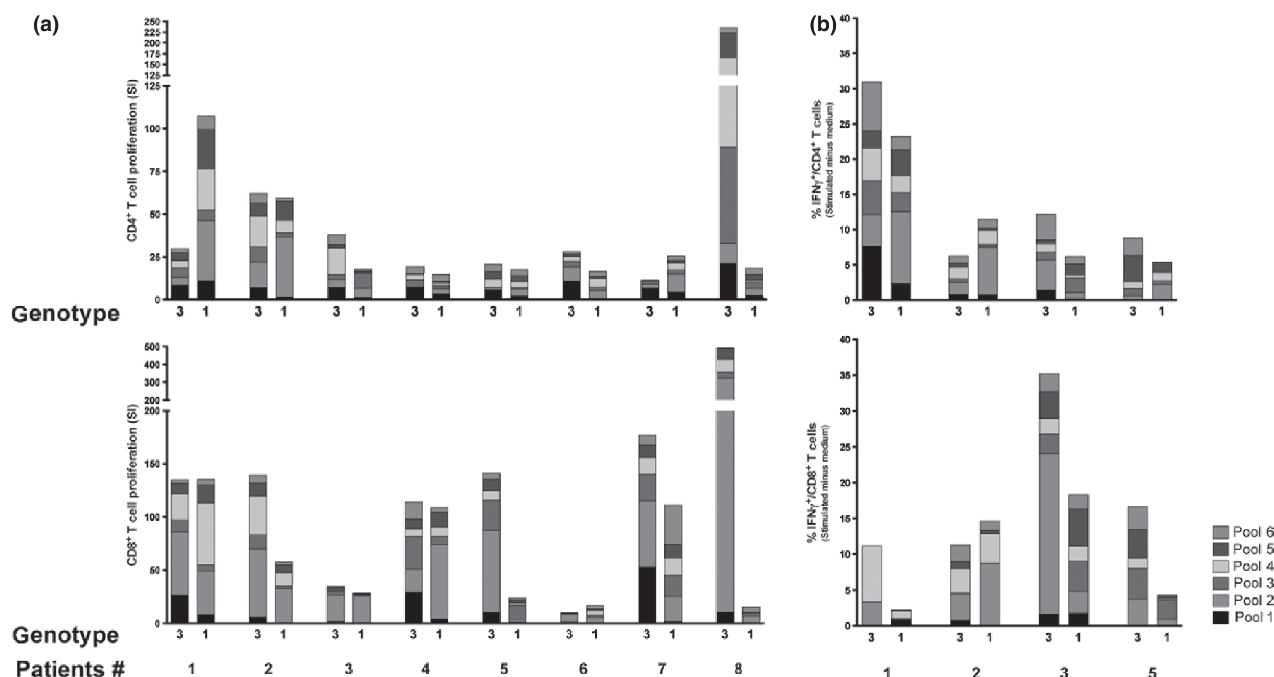

Fig. 4 Cross-genotype-specific T-cell response: T-cell responses of acutely HEV genotype 3-infected patients stimulated with overlapping peptide pools from genotype 3 were compared to genotype 1 peptide pools. **(a)** HEV-specific T-cell responses are shown indicating the response of each peptide pool for total proliferation (SI) and **(b)** percentage of total IFN-γ^+ HEV-specific T-cell responses per patient.

infected, might be able to predict HEV clearance in these patients needs further investigation.

To investigate the HEV-specific memory T cells, we measured HEV-specific T-cell responses upon HEV clearance during and after acute HEV infection. Overall, HEV-specific T-cell responses were detectable in terms of proliferation and functionality (cytokine producing T cells) indicating HEV-specific memory T cells. Interestingly, in all four follow-ups, we found that proliferative capacity of the HEV-specific $CD4^+$ T-cell responses decreased to almost no detectable T-cell proliferation *in vitro* early after HEV clearance (1-2 month). At >1 year after the first visit, $CD4^+$ T cells were able to proliferate similar to baseline level *in vitro*. The mechanism behind this lack of $CD4^+$ T-cell proliferation needs further investigation.

Studies in rhesus macaques and preliminary data from the Hecolin® vaccine trail showed potential cross-genotype- and cross-species-specific protection against re-infection with a homologous and heterologous HEV [5,7,9,45–48]. The degree of cross-genotype protection was strongly associated with the level of the HEV-IgG titre prior challenge [9]. In all indicated studies, HEV-IgG titre's prechallenge, ALT level, HEV-RNA and liver pathology were used to assess the cross-protective capability. To our knowledge, no study in terms of cross-genotype and cross-species-specific T cells was determined in patient acutely infected with HEV. These T-cell responses, especially $CD4^+$ T cells, induced by vaccines play also an important role as it has been reported in different vaccine approaches. For example, in HCV, an effective HCV genotype 1b vaccine might be protective against HCV genotype 4a infection by HCV cross-genotype-specific T-cell responses [31]. Previously, we reported HEV-specific T-cell responses in chronically and revolved patients carrying HEV genotype 3 using genotype 1-derived peptides [37]. However, no direct comparison was carried out in that study. To address this topic in more detail, we evaluated HEV-specific T-cell responses derived from genotype 1 and genotype 3 overlapping peptide pools for ORF2 and ORF3 in 8 of 12 acute HEV-infected patients. HEV-specific T-cell responses were detected in all patients regardless from which genotype the peptides were derived, with enhanced responses towards the homologous peptide stimulation. For the region with the most amino acid (aa) sequence differences between genotypes 1 and 3 (ORF2 between 1 and 120 aa (20%, pool 1); ORF3 between 6 and 104 aa (18%, pool 6)), an overall lower HEV-specific T-cell proliferation and IFN-γ^+ T-cell responses towards genotype 1 were found indicating that the cross-specific T-cell response might be presumably directed against conserved regions of HEV. However, further investigations are needed. In general, our data provide an indication that HEV-specific T-cell responses could contribute to the cross-genotype-specific protection seen in animal studies and in long-term follow-up of Hecolin®-vaccinated individuals [5,9,45–48]. Of

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note, our overlapping peptide pools 4 and 5 cover the HEV region which is included in the Hecolin® vaccine. How T cells, especially $CD4^+$ T cells, might be involved in the observed cross-genotype protection and how a booster in HEV-specific $CD4^+$ T-cell responses during vaccination might enhance vaccine efficiency need further investigation. We suggest that cross-genotype-specific T-cell immunity can be involved in protecting HEV genotype 3 exposed individuals from Western countries travelling to HEV genotype 1 endemic area. As 20–50% of people living in Europe are anti-HEV positive, the majority of these may at least partially be protected to genotype 1 according to our data which would need to be further investigated.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1: Correlation of ALT against 50 cytokines/chemokines in plasma of patients with acute HEV: ALT [U/L] values were correlated to plasma levels (pg/mL) of 50 cytokines/chemokines in nine patients at baseline (first visit).

Figure S2: Alignment of the amino acid (aa) sequences of HEV protein ORF2 and ORF3: AA sequences of HEV protein ORF2 and ORF3 for genotype 3 and 1 were aligned using MultAlin. ORF2 of HEV genotype 3 shares about 92% sequence identity with HEV genotype 1 at aa level, ORF3 shares 82% aa identity. (Differences in aa sequence are underlined; ORF2: peptide pool 1 – aa 1-130; peptide pool 2 – aa 131-265; peptide pool 3 – aa 266-395; peptide pool 4 – aa 396-522; peptide pool 5 – aa 523-660; ORF3: peptide pool 6 – aa 1-123).

Table S1: Details of amino acid sequences of peptides spanning entire region of ORF2 (peptides 1–66) and ORF3 (peptides 67–78) regions of the genotype 3 HEV genome.

Table S2: Cytokine and chemokine profile in plasma of patients with HEV: Correlation of 50 cytokines and chemokines to (A) ALT at baseline (first visit, $n = 9$) and to (B) ALT at follow-up ($n = 6$). Statistic: linear regression, ns = not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table S3: Correlation of AST against 50 cytokines/chemokines in plasma of patients with acute hepatitis E: Correlation of cytokine/chemokine to (A) AST at baseline (first visit, $n = 9$) and (B) to AST at follow-up ($n = 6$).

Table S4: Course of cytokines/chemokines levels in plasma of patients with throughout acute HEV infection: Plasma levels (pg/mL) of cytokines/chemokines in seven patients at baseline (HEV-RNA positive, first visit) were compared to next follow-up time-point (HEV-RNA negative).

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Addendum IV

Increased HEV seroprevalence in patients with autoimmune hepatitis.

Pischke S, Gisa A, Suneetha PV, Wiegand SB, Taubert R, Schlue J, Wursthorn K, Bantel H, Raupach R, Bremer B, Zacher BJ, Schmidt RE, Manns MP, Rifai K, Witte T, Wedemeyer H.

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Increased HEV Seroprevalence in Patients with Autoimmune Hepatitis

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Abstract

Background: Hepatitis E virus (HEV) infection takes a clinically silent, self-limited course in the far majority of cases. Chronic hepatitis E has been reported in some cohorts of immunocompromised individuals. The role of HEV infections in patients with autoimmune hepatitis (AIH) is unknown.

Methods: 969 individuals were tested for anti-HEV antibodies (MP-diagnostics) including 208 patients with AIH, 537 healthy controls, 114 patients with another autoimmune disease, rheumatoid arthritis (RA), and 109 patients with chronic HCV- or HBV-infection (HBV/HCV). Patients with AIH, RA and HBV/HCV were tested for HEV RNA. HEV-specific proliferative T cell responses were investigated using CFSE staining and in vitro stimulation of PBMC with overlapping HEV peptides.

Results: HEV-antibodies tested more frequently positive in patients with AIH (n = 16; 7.7%) than in healthy controls (n = 11; 2.0%; p = 0.0002), patients with RA (n = 4; 3.5%; p = 0.13) or patients with HBV/HCV infection (n = 2; 2.8%; p = 0.03). HEV-specific T cell responses could be detected in all anti-HEV-positive AIH patients. One AIH patient receiving immunosuppression with cyclosporin and prednisolone and elevated ALT levels had acute hepatitis E but HEV viremia resolved after reducing immunosuppressive medication. None of the RA or HBV/HCV patients tested HEV RNA positive.

Conclusions: Patients with autoimmune hepatitis but not RA or HBV/HCV patients are more likely to test anti-HEV positive. HEV infection should been ruled out before the diagnosis of AIH is made. Testing for HEV RNA is also recommended in AIH patients not responding to immunosuppressive therapy.

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Introduction

Autoimmune hepatitis (AIH) is an immune mediated liver disease more often affecting women than men. AIH is characterised by elevated serum IgG levels, the presence of certain autoantibodies and distinct histological features in the absence of other causes of liver disease [1]. The underlying pathomechanisms leading to autoimmune hepatitis are not well defined. One possibility is that viral infections trigger break of immunotolerance. Already 20 years ago an association between herpes simplex virus 1 (HSV 1) infection and autoimmune hepatitis has been described [2,3]. Other infectious agents including hepatitis C virus (HCV), cytomegalovirus, human T lymphotropic viruses 1 and 2 or salmonella typhimurum have been suggested to induce autoimmune liver disease [4]. If infections with the hepatitis E virus (HEV) are associated with AIH is unknown.

HEV infection takes a clinically silent course in the far majority of patients [5]. Few subjects may develop acute liver disease which can take a more severe course in particular in pregnant women or patients with underlying chronic liver diseases [6]. In recent years it became evident that HEV infection is not necessarily self limiting in all cases but may progress to chronic infection in immuno-compromised individuals [5,7]. Chronic hepatitis E has been described in liver and kidney transplant recipients [8] and also in some HIV-infected patients [9]. In Northern Germany, chronic hepatitis E was identified as the cause of graft hepatitis in 3% of liver transplant recipients with elevated liver enzymes [10]. Importantly persistent HEV infections have been associated with progressive liver disease [7,8,10,11]. To what extend HEV infections may lead to chronic hepatitis E in other patient groups receiving immunosuppressive medications including patients with autoimmune liver disease and rheumatoid arthritis is currently unknown.

The aims of this study were therefore, (i) to investigate the prevalence of antibodies to HEV in patients with autoimmune hepatitis and (ii) to determine if AIH patients receiving standard immunosuppressive medications are at risk for chronic hepatitis E in a low endemic Central European country and (iii) to rule out that rare cases of immunocompetent patients with chronic HEV infection had been misdiagnosed as autoimmune hepatitis.

Methods

From October 2009 until March 2010 all consecutive patients with AIH (n = 127) presenting our outpatient clinic were tested prospectively for presence of HEV RNA and anti HEV IgG. In addition, a control group of patients with viral hepatitis B or C (n = 109) was recruited. The diagnosis of autoimmune hepatitis was based on internationally accepted criteria [12]. Patients after liver transplantation were excluded. To compare the results with a cohort of patients with another autoimmune disease 114 consecutive patients receiving immunosuppressive medications followed by our Rheumatology outpatient clinic were studied between January 2012 and March 2012.

To enlarge the overall study cohort of patients with AIH, 81 additional patients were studied retrospectively. All retrospectively investigated patients were recruited at Hannover Medical School between 1998 and 2008. Furthermore 537 healthy subjects (employees of Hannover Medical School (n = 167) and blood donors (n = 370)) were studied for anti-HEV as already described as part of another project [10].

All AIH, HBV/HCV and RA patients were tested for the presence of HEV IgG antibodies by the MP assay (MP Biomedicals, formerly Genelabs Diagnostics, Singapore) according to the manufacture's instruction. Details of the primers used in the nested HEV RNA PCR were reported previously (11). Healthy subjects were only tested for anti-HEV-IgG.

Most AIH-patients received standard immunosuppressive regiments according to current guidelines [13] including mainly corticosteroids and partially azathioprine. Overall 70% of the AIH patients were female (n = 145). Age, gender and ALT levels of the overall study cohort are shown in table 1.

A subset of AIH patients (n = 123) was tested in addition with the Wantai anti-HEV assay (Wantai, Bejing). The frequency of anti-HEV in this subset was compared to 90 additional immunocompetent patients with chronic hepatitis B or C. Borderline test results were considered as positive for further statistical analysis.

Analysis of HEV-specific T cell responses

Cellular immune responses against HEV were studied in 1 AIH-patient with acute hepatitis E who tested HEV RNA and anti-HEV positive. Additional samples could be investigated from this subject 2 years after recovery. Furthermore, 4 anti-HEV-positive patients were tested for HEV-specific T cell responses. PBMC were stimulated with HEV overlapping peptide pools (spanning ORF2 and ORF3) as described previously [14,15]. T-cell proliferation was measured after 7 days by CFSE (5, 6-carboxyfluorescein diacetate succinimidyl ester) assay [14,15].

Statistics

Data for the different patient groups are presented as means and standard deviations. A comparison of continuous and categorical data between groups was performed using the chi-square test. Comparison of quantitative data between groups was performed using the Mann-Whitney test. A p-value<0.05 was considered significant.

Ethics

This study (including the analysis of HEV specific T-cell responses) was approved by the local research Ethics Committee (Ethics Committee of Hannover Medical School, Hannover, Germany). No research was conducted outside of our country. Patients agreed to hepatitis E testing as part of routine clinical work-up and thus no written informed consent was required. Retrospective testing of 81 samples from AIH patients was performed as part of the approved protocol and data were analysed anonymously according to the institutional and national ethics rules.

The need for written informed consent of participants of this study has been waived by the institutional review board according to our guidelines.

Results

Prevalence of HEV antibodies in patients with autoimmune hepatitis or persistent HBV- or HCV-infections

Anti HEV antibodies were detected in 16 out of 208 patients with autoimmune hepatitis (7.7%; 95% confidence interval 4.8–12). In contrast, anti HEV antibodies were detected in only 2/109 of control subjects with HCV or HBV infection (1.8%; CI 0.6–6.4). RA patients and healthy controls tested anti-HEV positive in 3.5% (4/115; CI 1.4–8.6) and 2.0% (11/537; CI 1.2–3.6), respectively (figure 1). Thus, anti HEV antibodies were more

Table 1. Prevalence of HEV antibodies and HEV-RNA in different patient groups.

	Patients with AIH (n = 208)	Patients with HBV or HCV infections (n = 109)	Patients with RA (n = 114)
Male	63 (30%)	59 (54%)**	29 (25%)
Age in years, mean (range, SD)	51 (18–84, 15)	49 (18–79, 13)	59 (19–85, 14)**
AST in U/ml	71 (15–684, 103)	69 (15–369, 59%)*	26 (13–63, 9)**
ALT in U/ml	94 (8–1422, 181)	82 (12–419, 78), p = 0.003**	26 (8–340, 32)**
HEV RNA positive	1 (0.5%)	0 (0%)	0 (0%)
Anti HEV IgG positive	16 (7.7%)	2 (1.8%)*	4 (3.5%)

p-values for comparison with AIH patients:
*p$<$0.05;
**p-values$<$0.01.
doi:10.1371/journal.pone.0085330.t001

frequent in AIH patients than subjects with chronic viral hepatitis B or C (p = 0.03) or healthy individuals (p<0.001). The difference between the anti-HEV seroprevalence rate in AIH and RA patients did not reach the level of significance (p = 0.13), however, AIH patients were younger than individuals with RA (mean age 51 years vs. 59 years, Mann-Whitney test: p<0.001).

Anti HEV IgG-positive AIH patients were older than in anti-HEV-negative patients (mean 58 years, SD 13 years vs mean 50 years, SD 15 years, p = 0.032 Mann-Whitney test). There was no difference regarding gender distribution between anti-HEV positive versus negative AIH patients (4/16 male vs 59/192 male, p = 0.44 chi-square test), ALT levels (mean 154 U/ml, SD 347 U/ml vs mean 89 U/ml, SD 160 U/ml, p = 0.26 Mann-Whitney test) or AST levels (mean 95 U/ml, SD 157 U/ml vs mean 69 U/ml, SD 98 U/ml, p = 0.07 Mann-Whitney test).

As this study was based on the anti-HEV MP assay, we next aimed to confirm the findings by a second, independent sero-assay, the Wantai assay, which has been suggested to be more sensitive than the MP assay [16,17]. Sera of 123 AIH patients and 90 patients with chronic hepatitis B or C were investigated. Indeed, the testing with the Wantai assay confirmed that AIH patients were numerically more likely to test anti HEV positive (33%, CI 25–42) than in HBV/HCV patients (21%, CI 14–31) even though only borderline statistical significance was reached (p = 0.05).

Prevalence of HEV RNA in patients with autoimmune hepatitis

All patients with AIH, RA or HBV/HCV were also tested for HEV RNA irrespective of the presence of anti HEV antibodies. None of the RA patients or HCV/HBV-infected subjects tested HEV RNA positive. HEV RNA was detected in only one AIH patient. This was a 70 years old female patient in whom AIH had been diagnosed 5 years earlier. Immunosuppressive medication consisted of cyclosporine (125 mg/d) and prednisolone (50 mg/d). The high dose of 50 mg prednisolone had been prescribed as liver enzymes had increased despite treatment with 20 mg prednisolone per day. A liver biopsy was performed showing a lymphoplasmacytic infiltration and signs of acute inflammation and portal fibrosis (Ishak-score A3,B0,C2,D3,F3 and steatosis S1, figure 2). Immunosuppression was reduced after the HEV RNA tested positive. HEV RNA became negative after reducing the dose of prednisolone from 50 mg/d to 15 mg/d. Anti HEV IgG antibodies remained positive. Retrospective analysis of stored serum samples revealed that this patient had no detectable HEV RNA or anti HEV IgG antibodies 4 months before this episode of ALT elevation indicating that the patient had indeed acute but not chronic hepatitis E at the time of investigation.

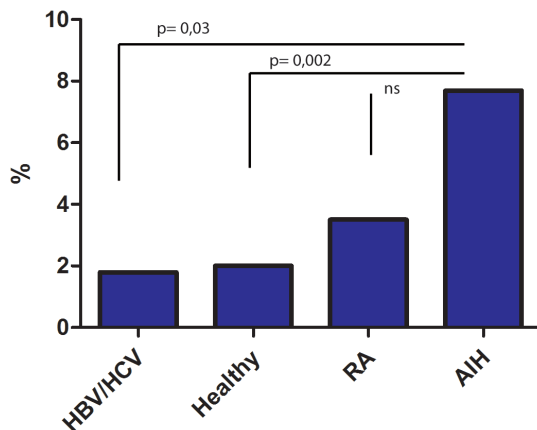

Figure 1. Seroprevalence rate of HEV specific antibodies in healthy controls, patients with chronic HBV- or HCV-infection and patients with autoimmune hepatitis.
doi:10.1371/journal.pone.0085330.g001

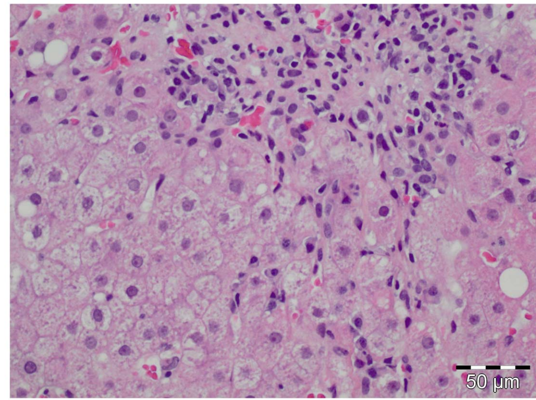

Figure 2. Histology of an autoimmune hepatitis patient with acute HEV infection. (H.E. stain, original magnification 200×).
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HEV-specific T-cell responses

HEV-specific T cell responses could be studied in the anti-HEV-positive AIH patient, who tested positive for RNA and in 4 further anti-HEV positive AIH patients (figure 3). HEV-specific proliferation of CD4+ T cells was detectable in all 4 anti-HEV-positive/HEV RNA-negative AIH patients with previous HEV infection. In addition, HEV-specific CD8+ T cell responses were detectable in two of the four patients. The AIH-patient with acute hepatitis E showed a low HEV-specific T cell response in the peripheral blood when she was still viremic. However, two years after recovery, a multispecific CD4+ and CD8+ T cell response became detectable (figure 3).

Discussion

We here show that HEV antibodies may be more frequent in patients with autoimmune hepatitis than in individuals with other chronic viral liver diseases or patients with other autoimmune diseases and healthy controls. We also show that chronic hepatitis E seems not to be a major clinical problem in individuals with autoimmune hepatitis receiving standard immunosuppressive medications. However in single cases HEV infection can occur and may contribute to the severity of hepatitis.

A higher prevalence of anti HEV antibodies in patients with AIH patients has already been described previously in two smaller studies from California [18] and Sweden [19] performed in the 1990ies. Thus, our findings obtained in a larger case series seem to be in line with these earlier observations. AIH patients with anti-HEV-IgG antibodies did not show any significant differences in specific characteristic as compared to anti-HEV-negative AIH patients. In contrast to the previous studies, two cross-sectional control groups of patients another hepatic disease or with another autoimmune respectively have been studied. Of note, even though RA patients were older than patients with AIH, the anti-HEV prevalence was more than two-fold higher in AIH as compared to RA patients. Moreover, the seroprevalence rate of HBV- or HCV-infected patients was the similar to healthy individuals and more than three times lower than in AIH patients. Of note, the higher anti-HEV seroprevalence rate of AIH patients compared to

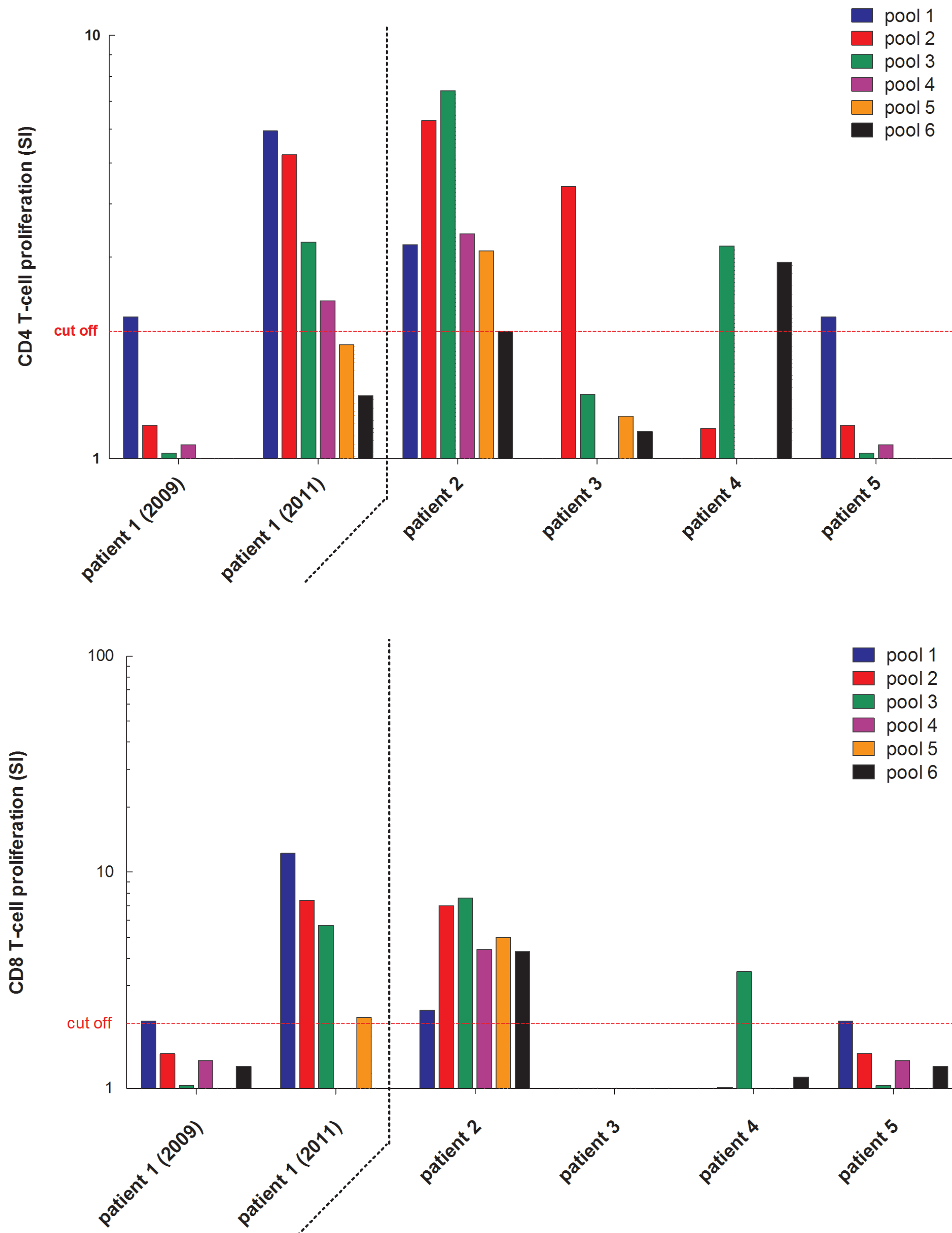

Figure 3. HEV specific T-cell responses in AIH patients (anti-HEV IgG positive).
doi:10.1371/journal.pone.0085330.g003

patients with chronic viral hepatitis was confirmed by a second independent sero-assay, the Wantai assay, which has been suggested to be more sensitive and to detect much higher frequencies of anti-HEV [16,17]. Even though the "background" seroprevalence rate in chronic viral hepatitis patients was indeed almost 10-fold higher using the Wantai-assay, AIH patients were again about 1.5 fold more likely to test anti-HEV positive. Still, 95% confidence intervals overlapped in this analysis and larger cohorts would therefore need to be studied to confirm or disprove our findings.

What could be possible explanations for an increased prevalence of anti-HEV antibodies in patients with autoimmune hepatitis? One possibility might be that previous subacute HEV infections could have triggered immune events leading to the manifestation of AIH. This hypothesis would be in line with several previous studies describing an association between viral

infections and subsequent development of AIH (2, 4). Specific cross-reactive humoral or T cell epitopes cross-reacting with possible auto-antigens have been described for HCV infection [20]. If these are also present during HEV infections remains to be shown. Larger studies are necessary to confirm the association between AIH and HEV-seropositivity and to get more insights into the underlying pathophyisiology.

Second, patients with autoimmune hepatitis may have distinct risk factors for acquiring HEV infections. This could include more frequent blood transfusions and medical procedures as well more frequent contacts to animals representing a potential zoonotic reservoir for HEV infection. Of note, not only pigs [21] but also several other animal species such as cats [22], shellfish [23], deer [24] or rats [25] can be carriers of HEV. Third, cross-reactive non-HEV-specific antibodies could be present in patients with AIH. The specificity of most HEV ELISA tests is not 100% but conditions determining "false-positive" results are currently unknown. Fourth, acute hepatitis E may present with histological and biochemical features of AIH and thus acute disease may be misclassified as de novo onset of AIH if HEV infection has not been excluded [26].

Persistent HEV infections have been described in organ transplant recipients receiving higher doses of immunosuppression and HIV-infected individuals with particular low CD4 counts. Studying 208 AIH patients receiving low doses of steroids alone or in combination with azathioprine, we could not identify a single case of chronic hepatitis E. This is an important clinical information suggesting that the risk to develop chronic hepatitis E should not been overestimated in patients with lower levels of immunosuppression in a non-endemic area. This finding is also well in line with our experience in patients with HIV-infection [27]. However, HEV infections can occur and thus hepatitis E should be considered in the differential diagnosis when AIH patients seem to be non-responder to immunosuppressive treatment.

Control of viral infections is believed to be mediated by both humoral and cellular immune responses. Interestingly, we detected a strong and multispecific HEV-specific T cell responses in an AIH patient who had cleared acute HEV infection previously while responses were much weaker when she was still HEV viremic. In addition, HEV-specific T cell responses were detected in three anti-HEV-positive AIH patients suggesting that these individuals had indeed contact with HEV and that unspecific cross-reactivity of the antibody assay seems to be unlikely. Future studies should investigate mechanisms potentially leading to the failure to mount an efficient adaptive cellular immune response against HEV. Overall, the findings presented in this paper are well in line with our experience in studying HEV-specific T cell responses in organ transplant recipients where both CD4+ and CD8+ T cell responses became detectable only after clearance of HEV RNA [15].

In conclusion, we show that patients with autoimmune hepatitis have a higher prevalence of anti HEV antibodies than control populations. The underlying cause for this observation remains to be determined. In addition, we did not find any evidence for an increased prevalence of chronic hepatitis E in German individuals with autoimmune hepatitis receiving standard immunosuppressive medications. However in single cases HEV infections may occur and thus hepatitis E needs to be considered in the differential diagnosis of non-response to immunosuppressive medications in AIH.

Author Contributions

Conceived and designed the experiments: SP MPM HW RR BB TW. Performed the experiments: RR BB SP SW BZ HB KR. Analyzed the data: RES RT KW JS PVS AG. Contributed reagents/materials/analysis tools: AG JS RR BB. Wrote the paper: SP HW MPM TW KR HB.

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Figure 4 HEV replication cycle.

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Figure 6 HEV replication cycle.

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