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## Review

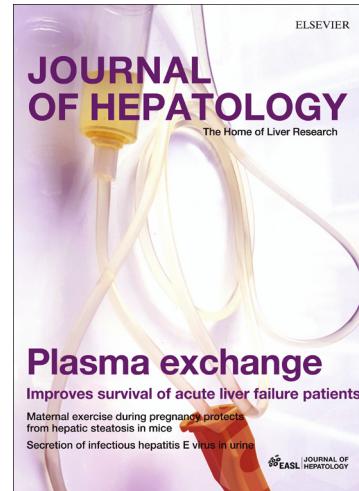
Update on Hepatitis E Virology:Implications for Clinical Practice

Yannick Debing, Darius Moradpour, Johan Neyts, Jérôme Gouttenoire

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## Update on Hepatitis E Virology:Implications for Clinical Practice

Yannick Debing<sup>1,†</sup>, Darius Moradpour<sup>2</sup>, Johan Neyts<sup>1</sup> and Jérôme Gouttenoire<sup>2\*</sup>

<sup>1</sup>Rega Institute for Medical Research, University of Leuven, Belgium and

<sup>2</sup>Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland

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**†Current address:** Center for Heart Failure Research, Cardiovascular Research Institute Maastricht, Maastricht University, The Netherlands

**Address for correspondence:** Dr. Jérôme Gouttenoire, Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, Rue du

Bugnon 48, CH-1011 Lausanne, Switzerland; Phone +41 21 314 07 49, Fax +41 21 314 40 95, E-mail: [Jerome.Gouttenoire@chuv.ch](mailto:Jerome.Gouttenoire@chuv.ch)

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## Summary

Hepatitis E virus (HEV) is a positive-strand RNA virus transmitted by the fecal-oral route. The 7.2-kb genome encodes three open reading frames (ORF) which are translated into (i) the ORF1 polyprotein, representing the viral replicase, (ii) the ORF2 protein, corresponding to the viral capsid, and (iii) the ORF3 protein, a small protein involved in particle secretion. Although HEV is a non-enveloped virus in bile and feces, it circulates in the bloodstream wrapped in cellular membranes. HEV genotypes 1 and 2 infect only humans and cause mainly waterborne outbreaks. HEV genotypes 3 and 4 are widely represented in the animal kingdom and are transmitted as a zoonosis mainly *via* contaminated meat. HEV infection is usually self-limited but may persist and cause chronic hepatitis in immunocompromised patients. Reduction of immunosuppressive treatment or antiviral therapy with ribavirin have proven effective in most patients with chronic hepatitis E but therapy failures have been reported. Alternative treatment options are needed, therefore. Infection with HEV may also cause a number of extrahepatic manifestations, especially neurologic complications. Progress in the understanding of the biology of HEV should contribute to improved control and treatment of HEV infection.

**Keywords:** antivirals | chronic hepatitis | positive-strand RNA virus | tissue tropism | zoonotic infection.

**Abbreviations:** 7mG, 7-methylguanylate; CSF, cerebrospinal fluid; gt, genotype; HCV, hepatitis C virus; HEV, hepatitis E virus; ESCRT, endosomal sorting complexes required for transport; IFN, interferon; IRF3, interferon regulatory factor 3; ISG, interferon-stimulated gene; NF- $\kappa$ B: nuclear factor- $\kappa$ B; ORF, open reading frame; PCP, papain-like cysteine protease; PEG-IFN- $\alpha$ , pegylated interferon- $\alpha$ ; PRR, pattern recognition receptor; RdRp, RNA-dependent RNA polymerase; RT-qPCR, reverse transcription quantitative PCR; RIG-I, retinoic acid-inducible gene I; TBK1, TANK-binding kinase 1; Tsg101, tumor susceptibility gene 101.

## Introduction

Hepatitis E virus (HEV) infection is among the most frequent causes of acute hepatitis worldwide, with an estimated 20 million infections and 70,000 deaths attributed to HEV genotypes 1 and 2 every year [1]. However, the majority of infections are thought to remain asymptomatic [2]. The virus has been recognized as a cause of water-borne hepatitis outbreaks in India not related to hepatitis A and B viruses in the early 1980ies [3, 4]. It was first visualized by immune electron microscopy in a feces sample from a human volunteer infected with stool extracts from presumed cases of epidemic non-A, non-B hepatitis in 1983 [5]. HEV was molecularly cloned in 1990, allowing the rapid development of serological tests and the investigation of its epidemiology [6, 7].

HEV has been classified as the sole member of the *Orthohepevirus* genus within the *Hepeviridae* family [8]. The recent development of advanced sequencing technology allowed the identification of novel HEV-related viruses in a variety of animals and led to a revised taxonomic classification of this family (Fig. 1) [9].

Twenty-five years after the identification of the HEV genome, basic and clinical virology research is gaining momentum due to the increased awareness and perceived importance of hepatitis E as a relevant public health issue [10, 11]. Indeed, previously unrecognized HEV infections in industrialized countries by genotypes 3 and 4 are now known to be zoonotically transmitted and to cause persistent infection in immunocompromised patients, especially transplant recipients [2, 12, 13]. Patients with chronic hepatitis E may rapidly develop liver cirrhosis. Moreover, the recent availability of cell culture models (e.g. [14, 15]) offers new opportunities for the study of HEV biology and the development of therapeutic and/or prophylactic strategies.

In this review article, we provide an overview on the molecular virology of hepatitis E and its implications for clinical practice. Current understanding of the HEV life cycle, the viral tissue tropism and host antiviral response as well as potential antivirals shall be discussed.

## Clinical course of hepatitis E

Irrespective of the viral genotype, HEV infection leads to a self-limiting illness lasting for few weeks, with a broad range of clinical manifestations ranging from an asymptomatic course to acute liver failure, resulting in fatality rates of 0.2-4%. In general, after a 2-to-6 week incubation period, liver enzyme elevation occurs and may be accompanied by symptoms such as abdominal pain, nausea and vomiting, anorexia, fever, and jaundice [2, 16].

## Acute infection

HEV strains infecting humans have been classified into 4 distinct genotypes belonging to a single serotype. Genotypes 1 and 2 are restricted to humans, are spread mainly through contaminated drinking water and represent main causes of water-borne outbreaks of hepatitis in developing regions (Fig. 1). The most severe course of disease is observed in pregnant women infected with HEV genotype 1, with high maternal, fetal and neonatal morbidity and mortality rates as high as 25% [17-19]. Capsid-based recombinant vaccines have proven their efficacy in large studies performed in Nepal and in China [20-23]. However, a vaccine has thus far been licensed only in China.

HEV genotypes 3 and 4 are now recognized as zoonotic agents with their main reservoir in pigs and game (Fig. 1). Autochthonous infection occurs mostly through the consumption of un(der)cooked meat [2]. HEV seroprevalence rates in developed regions range between 5 and 20% and peak at 52% in southwestern France [24]. Of note, we and others have found that middle-aged or elderly men are particularly prone to develop symptomatic autochthonous acute hepatitis E [2, 25].

### **Chronic infection**

Chronic HEV infections have been reported in immunocompromised patients such as organ transplant recipients and patients with HIV infection or hematological malignancies undergoing chemotherapy [12, 13, 26-29]. These chronic infections are caused by genotype 3 and possibly also genotype 4 and may rapidly evolve to cirrhosis and loss of a liver graft [12, 13].

### **Molecular organization of HEV**

HEV is a non-enveloped, small, icosahedral virus of about 27-34 nm in diameter [5, 30]. Infectious virions contain a 7.2-kb single-stranded, positive-sense RNA genome harboring 3 open reading frames (ORFs). These are translated into (i) the ORF1 protein, comprising the functional domains required for RNA replication, (ii) the ORF2 protein, corresponding to the viral capsid; and (iii) the ORF3 protein, a small, hitherto poorly characterized phosphoprotein involved in viral particle secretion (Fig. 2A). The genomic RNA of HEV has the typical features of a eukaryotic mRNA, including a 7-methylguanylate (7mG) cap at its 5' end and a poly-A stretch at the 3' end, followed and preceded, respectively, by short untranslated regions.

Two viral RNA species are generated during HEV genome replication, the full-length RNA of 7.2 kb and a subgenomic RNA of 2.2 kb (Fig. 2A) [31, 32]. The latter species starts a few nucleotides downstream of the ORF1 stop codon [31] and allows the expression of ORF3 and ORF2. The start codons of these two ORFs are separated by only 11 nucleotides and result in frameshifted products (Fig. 2A). The regulation of ORF2 and ORF3 expression remains poorly understood to date. As a consequence

of the overlap of the two ORFs, this sequence is highly conserved intra- and intergenotypically, allowing the establishment of a robust and pangenotypic PCR-based diagnostic test. This assay is now widely used to diagnose acute or chronic HEV infection [33].

## ORF1

ORF1, representing about 2/3 of the genome length, encodes the so-called HEV replicase. Domains specifically required for viral RNA replication have been computationally assigned based on sequence homology with other viruses [34]. These include methyltransferase (MeT), macro, RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) domains [35] (Fig. 2A). The MeT, Hel and RdRp have also been functionally studied. However, the functions of the other (predicted) domains, namely the Y, papain-like cysteine protease (PCP) and variable (V) domains, remain uncertain. Thus, the function of the putative PCP, which shows a weak homology with the Rubella virus protease [34], is a subject of debate (Fig. 2A). Indeed, positive-strand RNA viruses commonly encode proteases to process their polyproteins, and several reports suggested that this is the case also for the PCP and the ORF1 protein [36-40]. However, others have not found any evidence of HEV ORF1 processing [41-43]. Using a panel of ORF1 expression systems, including a recently developed HEV replicon [15, 44], we only observed the full-length form of ORF1 protein (Dao Thi VL, Ahola T, DM and JG, unpublished). An emerging hypothesis suggests that the PCP domain, rather than having protease activity, may display broad deubiquitination activity, including deISGylation of ISG15-modified proteins [45]. In this way, the PCP domain may prevent the proteasomal degradation of selected proteins, possibly those required for viral RNA replication. Clearly, further work is needed in this interesting area.

Similar to alphavirus replicase proteins, ORF1 exhibits virus-specific methyltransferase and guanylyltransferase activities, allowing the transfer of a methyl group to GTP, yielding m<sup>7</sup>GTP (7-methylguanosine), and the covalent coupling of the m<sup>7</sup>GTP product with the 5' end of the viral RNA, respectively [46]. However, as these observations were made with a construct comprising a large portion of ORF1, including the MeT, Y and PCP domains (ORF1 amino acids [aa] 1-979), one cannot yet formally attribute these enzymatic activities to the methytransferase domain only.

The HEV RNA helicase domain is capable of unwinding RNA duplexes with a 5' single-stranded overhang in the 5'-to-3' direction *in vitro* [47]. In addition, the same recombinant HEV helicase protein displays a 5'-nucleoside triphosphatase (NTPase) activity that is required for RNA capping [48]. Therefore, HEV, as other positive-strand RNA viruses including the hepatitis C virus (HCV), may have coupled these two functions in one protein to economize on coding capacity of the viral genome.

Macro domains, known as ADP-ribose-binding modules [49], can interact with

proteins modified by ADP-ribosylation. This modification is involved in the regulation of various cellular functions, such as transcription, chromatin organization, organelle assembly, protein degradation and DNA repair [50]. While several positive-strand RNA viruses, such as corona- and alphaviruses, encode a macro domain, its exact role in the viral life cycle remains unclear. *In vitro* characterization of the HEV macro domain revealed its binding to poly(ADP-ribose) and poly(A), possibly simultaneously [51], thereby suggesting the possible recruitment of poly(ADP-ribosyl)ated cellular factors to the replication complex while bound to the viral poly-A tail.

## **ORF2**

Given its antigenic properties as well as interest for diagnostic test and vaccine development, the capsid protein encoded by ORF2 is the best studied of the three HEV proteins. ORF2 encodes a 72-kDa protein of 660 aa which possesses an N-terminal signal peptide driving its secretion into the extracellular compartment. The ORF2 protein is N-glycosylated at three sites when expressed in mammalian cells [52, 53], i.e. Asn 132, Asn 310 and Asn 562 (Fig. 2A) [54]. During the viral life cycle, the ORF2 protein may interact with host factors such as heat shock protein 90 [55], glucose-regulated protein 78 (also known as BiP) [56], and heparin sulfate proteoglycans which may serve as initial attachment factors [57].

## **ORF3**

The ORF3 protein is a 13-kDa protein of 113 (genotype 3) or 114 aa (genotypes 1, 2 and 4). It is phosphorylated at the conserved serine residue 71 (Ser 70 in genotype 3) by the cellular mitogen-activated protein kinase (MAPK) [58]. A yeast two-hybrid study suggested homodimerization of ORF3 protein through a proline-rich C-terminal region [59]. Primary sequence analyses of the ORF3 protein did not reveal any domains homologous to other proteins or other distinguishing features, except for the presence of two hydrophobic N-terminal domains spanning aa 7 to 23 and aa 28 to 53. Early reports indicated that ORF3 protein associates, *via* the N-terminal hydrophobic domain [58], with the cytoskeleton and, more specifically, with microtubules [60]. The protein has also been observed at early and recycling endosomes [61] or multivesicular bodies (MVB) and has been implicated in HEV egress [62, 63]. Therefore, the subcellular localization, structure and function of the ORF3 protein remain to be fully explored.

## **HEV life cycle**

As a first contact with the target cell, HEV interacts, similar to many other viruses [64], with heparan sulfate proteoglycans, probably syndecans, allowing initial attachment of the virus [57] (Fig. 2B). The receptor(s) governing entry of the virus are

still unidentified. The downstream cascade of events includes clathrin-dependent endocytosis involving dynamin-2 and membrane cholesterol pathways [65]. Moreover, cytoskeleton remodeling is crucial for HEV endocytosis [65]. Post-entry steps, including viral genome uncoating and release, have not been addressed. However, recent data suggest a low-pH-independent mechanism [65] (Fig. 2B).

HEV RNA replication requires, first, translation of the viral replicase by the host translation machinery (Fig. 2B). As for all positive-strand RNA viruses, the mechanism of RNA replication involves the synthesis of a complementary negative-strand RNA by the HEV RdRp and the subsequent synthesis of genomic positive-strand RNA from this negative-strand RNA template. The HEV RNA helicase is expected to fulfill a crucial role in this process by unwinding the two RNA strands [47]. RNA capping likely results from the cooperation of the 5' triphosphatase activity, harbored by the helicase domain, followed by the transfer of a 7mG to the 5' end of newly synthesized genomes by the methyltransferase domain [46, 48].

The site of RNA replication within the host cell has not been identified yet. However, the ORF1 protein has been shown to be membrane-associated and to localize to an intermediate compartment between the endoplasmic reticulum and the Golgi, suggesting a localization within the early secretory pathway [43]. As the formation of a replication complex composed of viral proteins, replicating viral RNA and rearranged cellular membranes is a hallmark of positive-strand RNA viruses [66], one may hypothesize that HEV also induces specific membrane rearrangements which, however, have yet to be identified.

Further, subgenomic RNA is generated and capped to allow translation of the ORF2 and ORF3 proteins (Fig. 2B). Subgenomic RNA is synthesized by the RdRp. However, the mechanisms regulating genome-length vs. subgenomic RNA synthesis are unknown. The capsid protein assembles and packages the capped genomic RNA. Virtually no data are available on the assembly step. However, the spontaneous assembly of RNA and ORF2 protein into virus-like particles in insect cells argues in favor of a self-assembly process involving only a limited number of viral or host factors ([67]; reviewed in [68]). It implies that newly synthesized genomes must be present in close proximity of the capsid protein to allow virion formation. Investigation of the intraviral interactome revealed a number of protein-protein interactions which support the existence of (a) viral protein complex(es) [69].

Viral egress is believed to require the cellular secretory machinery together with the ORF3 protein. Several studies have demonstrated that a conserved PSAP motif in the ORF3 protein (aa 95-98 in genotype 3) is involved in the interaction with tumor susceptibility gene 101 (Tsg101), a component of the endosomal sorting complexes required for transport (ESCRT) pathway [62, 70, 71]. Moreover, inhibition of HEV release by dominant-negative mutants of the pathway confirmed that HEV hijacks the ESCRT machinery for virion release from infected cells [62]. Interestingly, abrogation of ORF3 protein expression in an infectious clone resulted in impaired particle

secretion *in vitro*, with intracellular accumulation of infectious virus with a high density [72]. Virus of comparable density has been observed in bile or feces while HEV circulating in the blood has a low density [62, 72, 73]. Density gradient investigation as well as ultrastructural analyses revealed the presence of host membranes enveloping the HEV particle in cells and culture supernatants, thereby confirming the biochemical evidence for secretion into the bloodstream of pseudo-enveloped HEV [74, 75].

Hence, while non-enveloped HEV is found in bile and feces, the virion found in blood appears to be wrapped by cellular membranes [62, 73]. Similar observations have recently been reported for a rat HEV strain, suggesting that this feature is conserved among hepeviruses [76]. Interestingly, the secretion of membrane-wrapped virions has also been reported for hepatitis A virus, a member of the *Picornaviridae* family ([77]; reviewed in [78]). While the mechanism underlying HEV membrane envelopment as well as the nature and the composition of the membrane are yet to be further defined, ORF3 protein appears to be at the heart of particle secretion and possibly formation. Importantly, ORF3 protein is present on the secreted membrane-wrapped virion, as demonstrated by capture of HEV particles by ORF3 antibodies in culture supernatant and serum but not in feces [75] (Fig. 2B).

It is tempting to speculate that membrane envelopment protects HEV against neutralizing antibodies when present in the serum, as supported by successful *in vitro* HEV infection in the presence of anti-ORF2 antibodies [75].

### **Interference of HEV with host antiviral defenses**

While HEV infection is almost always self-limited, persistent infection may be observed in immunocompromised patients. In these, the virus can persist in the liver in the absence of a competent adaptive immune response. Hence, this clinical observation would suggest that innate immunity alone does not suffice to clear viral infection but that a concerted action of innate and adaptive immunity is required. Like many other viruses, HEV may have developed strategies to subvert these host antiviral defenses. However, these strategies are poorly understood to date.

Viral infection usually elicits an immune response involving the production of type I and III interferon (IFN) after recognition of the viral RNA as a pathogen-associated molecular pattern by pattern recognition receptors (PRR) [79]. Signaling cascades downstream of PRR lead to the induction and production of type I and III IFN through the activation of key molecules such as TANK-binding kinase 1 (TBK1), interferon regulatory factor 3 (IRF3) and nuclear factor- $\kappa$ B (NF- $\kappa$ B). Secreted IFNs can then induce interferon-stimulated gene (ISG) transcription in other cells *via* the Jak-STAT signaling cascade (Fig. 3). Few studies tried to clarify the consequences of HEV infection on the host innate immune response. The ORF3 protein has been shown to enhance type I IFN production *via* a direct interaction with the PRR retinoic acid-

inducible gene I (RIG-I) (Fig. 3) [80]. Under similar experimental conditions, the same authors found that the ORF1 protein inhibits RIG-I signaling and prevents IFN- $\beta$  induction by de-ubiquitination of RIG-I and TBK1 [81]. According to this latter study, the de-ubiquitinating activity of the PCP domain but also the macro domain could be directly involved (Fig. 3). The observation that the virus may exert opposing effects on the host antiviral response *via* two different viral proteins is intriguing. In addition, transcriptional analyses of A549 human lung epithelial cells infected by HEV revealed an enhanced expression of proinflammatory cytokines, i.e. IL-6, IL-8 and RANTES, as well as activation of the IRF3 and NF- $\kappa$ B pathways (Fig. 3) [82], indicating that the control of antiviral responses by HEV is only partly effective.

To overcome the lack of complete blockade of the RNA sensing pathway, interference of HEV with IFN signaling or effector functions would be a plausible hypothesis. In general, after viral RNA sensing and activation of the downstream cascade, type I and III IFN is being produced and functions in a paracrine and autocrine manner. In the case of HEV infection, an inhibition of the IFN- $\alpha$ -induced phosphorylation of Stat1 in infected cells as well as a down-regulation of ISG expression have been observed [83]. In line with this observation, ISG expression induced by either type I, II or III IFNs has been found to be limited in cells replicating an infectious HEV clone [84]. The proposed mechanism involves the binding of ORF3 protein to Stat1 to restrict its phosphorylation and, thereby, its activation (Fig. 3) [83]. Taken together, the ORF3 protein may exert a dual and opposite effect: enhancing IFN- $\alpha$  production on the one hand and limiting IFN- $\alpha$  signaling and effector functions on the other. Independent confirmation of this function of ORF3 is still awaited, however.

As IFN- $\alpha$  showed strong antiviral activity *in vitro* and has proven its efficacy to clear the virus in patients with chronic hepatitis E [44, 84-86], we may hypothesize that IFNs play a crucial role in viral clearance mediated by the immune system [87]. Differences between HCV and HEV infections at the host transcriptional level have been investigated in the chimpanzee model, revealing a lower ISG induction in acute HEV infection as compared to acute HCV infection [88]. Such studies performed over the entire course of acute hepatitis E could not be easily conducted in humans. However, ISG levels may be more easily explored in patients with chronic hepatitis E. A preliminary study conducted on a limited number of whole-blood samples from chronically HEV-infected patients showed a modulation of about 30 ISGs. Thus, whereas IFN pathway activation is not sufficient to clear viral infection, basal activation of IFN signaling is detectable in these patients [89]. Investigation of ISG levels in chronic hepatitis E in the blood and in the liver should be explored to further understand the mechanisms of HEV persistence in immunocompromised patients.

#### **HEV tissue tropism and associated extrahepatic manifestations**

Although HEV is a primarily hepatotropic virus, it may also replicate to some extent in other tissues, as extrahepatic manifestations such as neurological symptoms, myositis as well as renal and hematologic complications have been observed in the context of hepatitis E [26, 90-92] (Fig. 4).

Experimental HEV infection in animals allowed the detection of negative-strand viral RNA, suggestive of ongoing viral replication, in the liver but also in the small intestine, colon and lymph nodes of pigs [93]. Furthermore, the presence of negative-strand RNA intermediate has been reported in the liver, kidney, small intestine, spleen and stomach in a rabbit HEV model [94]. Given the fecal-oral transmission of HEV, the virus is likely replicating first in the gastro-intestinal tract as a primary site of infection, wherefrom the virus can reach the bloodstream to infect other organs, similarly to what was observed for avian influenza virus H5N1 and rotavirus [95, 96].

Among the most notorious extrahepatic manifestations of HEV infection are neurological complications such as neuralgic amyotrophy (brachial neuritis, Parsonage-Turner syndrome) and Guillain-Barré syndrome [97]. Neurological manifestations have been observed in both acute and chronic hepatitis E, but the exact incidence and underlying pathogenic mechanisms are not clear yet [97]. It has been suggested that immune reactions triggered by HEV infection may play a role, e.g. by the development of antiganglioside antibodies through molecular mimicry [98]. A few studies have reported the presence of HEV RNA in the cerebrospinal fluid (CSF) of some but not all patients with neurological complications, usually at considerably lower titers than in the serum [99-101]. In addition, evidence for intrathecal antibody production has been reported (Silva M *et al.* Revised version submitted). Finally, the viral quasispecies was analyzed in the serum and CSF of a single kidney transplant recipient with chronic hepatitis E and neurological symptoms; HEV sequences in the CSF were found to be distinct from those in the serum in this patient [99]. Even though quasispecies compartmentalization requires confirmation at a larger scale, it suggests the existence of neurotropic HEV variants and possibly active viral replication in the central nervous system. Whereas we did not observe robust viral replication in stem cell-derived neural progenitor cells infected with genotype 3 HEV [102], the virus can efficiently replicate in neuroblastoma cells *in vitro* (V.L. Dao Thi, DM and JG, unpublished data).

Potential infection of the human placenta by HEV may explain intrauterine vertical transmission, the often severe course of hepatitis E in pregnant women and the associated obstetric complications (see above). Indeed, active HEV replication in the placenta of HEV-infected pregnant women has been suggested by the detection of both negative-strand viral RNA as well as ORF3 protein and has been associated with fetal loss or maternal acute liver failure [103]. In line with these findings, we have also observed HEV genotype 3 replication in the human placental cell line JEG3 *in vitro* (S. Drave, YD, JN and E. Steinmann; unpublished results).

The kidney tropism of HEV suggested by renal disorders [104, 105], has been recently confirmed by the detection of HEV in urine of acute and chronic HEV-infected patients and monkeys as well as by immunohistochemical evidence of infected cells in the kidney of one of these animals [106]. Other reported extrahepatic manifestations include acute pancreatitis and hematological manifestations [107], but no additional evidence is available regarding HEV tropism in these tissues.

Future research efforts regarding HEV tissue tropism should focus on (i) the identification of cell entry receptor(s), (ii) confirming viral replication in extrahepatic sites such as neural tissue and kidney, in both animal and human samples; (iii) determining whether specific HEV variants are responsible for neurotropism or other extrahepatic manifestations as well as delineating the responsible genetic determinants; and (iv) the development of *in vitro* and *in vivo* models for the most common extrahepatic manifestations to improve our understanding of the underlying mechanisms. Stem cell-derived models may provide attractive alternatives to cancer cell line-based culture models in the near future [102, 108].

### Treatment and antivirals

Soon after the discovery of chronic hepatitis E in immunocompromised patients, the first case reports were published describing the efficacy of ribavirin or pegylated IFN- $\alpha$  (PEG-IFN- $\alpha$ ) monotherapy or a combination of both to treat chronic hepatitis E (Table 1) [85, 109-111]. In the following years, ribavirin has become the drug of choice and its efficacy has been validated in larger studies [112], although a controlled trial is still lacking to date. Ribavirin was also found to be an effective treatment for severe acute hepatitis E [113, 114]. Both drugs were shown to inhibit HEV replication *in vitro*, in subgenomic replicon as well as in full-length infectious systems [115]. Interestingly, a moderate but significant synergistic effect was noted for the combination of ribavirin and IFN- $\alpha$  *in vitro*.

Depletion of intracellular GTP pools was shown to be a mechanism of action of ribavirin *in vitro* [115], although it remains to be established to what extent this and other potential mechanisms apply in the liver of infected patients [116]. Although ribavirin treatment results in viral clearance in most patients, cases of treatment failure have been reported, often linked to dose reductions because of anemia [112, 113, 117]. These cases either present viral recurrence after therapy cessation or development of an apparent “resistance” to ribavirin. A G1634R mutation in the RdRp domain of HEV ORF1 protein was detected at the time of treatment failure in two cases [117]. This mutation has also been observed in a third patient experiencing ribavirin treatment failure [118]. *In vitro* assessment of this mutation indicated a similar sensitivity to ribavirin but an increased fitness as compared to the wild-type, which may explain the failure of ribavirin treatment [117]. A recent study found an increased presence of the G1634R mutation in patients with ribavirin-treatment

failure (48%) compared to patients showing with a sustained virological response (31%), although this difference was not statistically significant [119]. The clinical implications of this finding and the potential use of the G1634R mutation as a prognostic marker for ribavirin treatment outcome remain to be explored.

Cases of ribavirin treatment failure in transplant patients, most of which cannot be treated with PEG-IFN- $\alpha$ , highlight the need for alternative treatment options for chronic hepatitis E. We have recently shown that the HCV polymerase inhibitor sofosbuvir inhibits RNA replication of HEV genotype 3 *in vitro* and that it has an additive antiviral effect when combined with ribavirin (Table 1) [44]. Sofosbuvir, a nucleotide analog with a pangenotypic inhibitory effect on the HCV RdRp [120], leads to sustained virological response in the majority of patients with chronic hepatitis C when combined with other antivirals [121]. The excellent tolerability of sofosbuvir, including in solid organ transplant recipients or cirrhotic patients [122], suggests this drug may be used as an add-on to ribavirin in the treatment of chronic hepatitis E. A clinical proof-of-concept study will be required to explore the antiviral potential of sofosbuvir in combination with ribavirin in chronic hepatitis E, especially in patients who fail to achieve HEV elimination with ribavirin alone. In the same study [44], we have reported that nucleoside analogs such as 2'-C-methyladenosine or 2'-C-methylcytidine can also inhibit HEV infection, albeit with lower potency, opening the door to the possible future development of more specific compounds derived from the same classes of inhibitors.

As solid organ transplant recipients represent the majority of patients with chronic hepatitis E, research focusing on the effect of immunosuppressive drugs and regimens on clearance of HEV infection is of prime importance [123]. For instance, analysis of a limited number of cases suggests that the use of the immunosuppressant mycophenolate mofetil is associated with HEV clearance [124] (Table 1). Patients receiving the calcineurin inhibitor tacrolimus have a higher risk of developing chronic hepatitis E than those receiving cyclosporine A [28]. The importance of the particular choice of immunosuppressive therapy is to some extent supported by *in vitro* data. Mycophenolic acid, the active component of mycophenolate mofetil, was found to exert potent *in vitro* anti-HEV activity, mediated by depletion of intracellular GTP pools [115, 125]. It remains questionable, however, whether such GTP depletion occurs *in vivo* and to what extent the direct antiviral activity is offset by mycophenolic acid's immunosuppressive effects. In addition, steroids were found not to influence HEV replication *in vitro*, while calcineurin and mTOR inhibitors both stimulate HEV replication [125, 126]. The proviral effect of mTOR inhibitors such as rapamycin and everolimus was found to be mediated by blocking an antiviral signaling pathway downstream of mTOR dependent on eIF4E-binding protein 1 [126], while the calcineurin inhibitors, cyclosporine A and tacrolimus, promote HEV replication through inhibition of cyclophilins A and B [125].

Although these findings provide interesting indications on what therapies may be beneficial or detrimental in preventing and treating chronic hepatitis E in transplant patients, they require verification *in vivo*, either in a suitable animal model or preferentially in sufficiently large well-designed clinical studies [127]. However, a recent study provided *in vivo* evidence of significantly higher HEV RNA level in patients with chronic hepatitis E receiving an mTOR inhibitor as compared to those receiving calcineurin inhibitors as immunosuppressive regimen [128]. In addition, the use of mycophenolic acid did not affect the response to ribavirin [128].

Interest to develop specific new HEV antivirals is, because of the limited potential return on investment, limited. Hence, research efforts may focus on (i) optimizing the current antiviral treatments (ribavirin and/or PEG-IFN- $\alpha$ ) as well as immunosuppressive regimens; (ii) the clinical evaluation of other drugs with proven *in vitro* inhibition of HEV replication, such as sofosbuvir; and finally (iii) determining the most adapted immunosuppressive regimens to prevent chronic hepatitis E in transplant recipients.

## Conclusions

HEV is a remarkable virus, with relatively simple genetic organization, a positive-strand RNA genome resembling cellular mRNAs, and only three ORFs and (poly)protein products. Given its efficient clearance by a competent immune system, HEV has to propagate rapidly. Therefore, HEV possesses characteristics of a virus employing the “hit-and-run” strategy, which favors its genetic evolution. This may explain the broad representation of HEV in the animal kingdom and the crossing of species barriers. To follow this strategy, the virus had to acquire a high stability in the environment for its survival and efficient propagation. A deeper understanding of the structure of the viral particle as well as the identification of the receptor(s) for HEV should shed new light on its zoonotic transmission and tissue tropism. Advances on current challenges, such as the epidemiology of autochthonous hepatitis E, the optimization and standardization of diagnostic assays, the pathogenesis and clinical management of extrahepatic manifestations, the prevention of HEV infection in populations at risk, and the treatment of chronic hepatitis E may be facilitated by parallel progress in the understanding of fundamental aspects of HEV biology and the clinical investigation of hepatitis E.

**Key point box**

- Hepatitis E virus (HEV) is an important cause of acute hepatitis in developing regions, with a high morbidity and mortality in pregnant women
- HEV can persist and cause chronic hepatitis in immunocompromised patients
- The HEV life cycle is only partly understood, yielding attractive research opportunities
- HEV tropism may not be restricted to the liver, possibly explaining some extrahepatic manifestations
- A number of approved drugs affect HEV replication *in vitro* or in patients with hepatitis E

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## Legend to figures

### **Figure 1. Phylogenetic relationship of hepeviruses identified in various hosts.**

Nucleotide sequences of 305 full-length hepatitis E virus genomes were retrieved from GenBank and aligned with ClustalW, followed by phylogenetic tree building using the neighbor-joining method (Geneious 7.1 software, Biomatters). While genotypes 1 and 2 (gt 1 and 2; yellow and orange, respectively) are restricted to humans and to endemic regions such as Asia, Africa and Mexico, genotypes 3 and 4 (gt 3 and 4; blue and green, respectively) are also found in a wide range of animal species. Genotype 3 is present worldwide in various hosts such as swine, wild boar, deer, mongoose and Japanese macaques. Genotype 4 is found mainly in China as well as Southeast Asia and infects swine, wild boar and sheep. Viral strains that have not been assigned to one of these 4 genotypes may also infect humans, as documented recently for camel HEV [129]. Moreover, more distant hepeviruses were identified in birds, bats, rats, ferrets and fish.

### **Figure 2. Genetic organization and life cycle of the hepatitis E virus (HEV). (A)**

Genetic organization of HEV. The 7.2-kb positive-strand RNA genome has a 5' 7-methylguanylate (7mG) cap and a 3' polyadenylated (poly-A) tail. It comprises three open reading frames (ORF). ORF1 encodes a polyprotein of about 190 kDa which harbors methyltransferase (MeT), Y, putative papain-like cysteine protease (PCP), variable (V), macro, RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp) domains. ORF2 and ORF3 are translated from a 2.2-kb subgenomic RNA generated during viral replication. The capsid protein encoded by ORF2 is N-glycosylated at 3 sites, i.e. Asn 132, Asn 310 and Asn 562, as denoted by grey diamonds. (B) The HEV life cycle includes the following steps: 1) viral attachment to heparin sulfate proteoglycans (red) and entry through as yet unidentified receptor(s) (burgundy); 2) clathrin-mediated endocytosis; 3) release of the viral positive-strand RNA genome into the cytosol; 4) translation to yield the ORF1 protein (orange); 5) replication through a negative-strand RNA intermediate (dark red) and synthesis of full-length as well as a 2.2-kb subgenomic RNAs; 6) translation of the subgenomic RNA to yield the ORF2 and ORF3 proteins (green and purple, respectively); and 7) packaging, assembly and release of newly formed virus. ORF3 protein is likely associated with intracellular membranes and may trigger virion release via the endosomal sorting complexes required for transport (ESCRT) pathway. Recent studies suggest that virus secreted into the bloodstream is associated with the ORF3 protein and wrapped by cellular membranes while virus secreted into the bile is nonenveloped.

**Figure 3. Interference of HEV with host antiviral responses.** Upon HEV infection and release of the viral genome into the cytoplasm, host antiviral defenses sense the viral RNA through RIG-I and signal *via* downstream cascades leading to type I and III interferon (IFN) production. Once translated, the HEV ORF1 protein has been reported to inhibit signaling *via* retinoic acid-inducible gene I (RIG-I) and to prevent IFN induction by deubiquitination of RIG-I and TANK-binding kinase 1 (TBK-1). At the same time, the HEV ORF3 protein may have an opposing effect by enhancing type I IFN production *via* direct interaction with RIG-I. Consequently, HEV infection leads to the activation of interferon regulatory factor 3 (IRF3) and nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathways, inducing the expression of IFNs and pro-inflammatory cytokines, i.e. IL-6, IL-8 and RANTES. IFNs activate the Jak-STAT pathway in a paracrine and autocrine manner, resulting in the induction of IFN-stimulated genes (ISGs). In the case of HEV infection, binding of the ORF3 protein to Stat1 has been reported to restrict its phosphorylation and the activation of the downstream cascade, thereby inhibiting ISG expression.

**Figure 4. Reported sites of HEV replication.** HEV infects and replicates primarily in the liver. However, studies performed in animal models reported HEV replication also in the small intestine, colon and lymph nodes as well as kidney, spleen and stomach. Furthermore, replication in the kidney has been recently suggested by the presence of HEV in the urine of patients with acute and chronic hepatitis E as well as experimentally infected monkeys. Among extrahepatic manifestations, neurological complications are the most frequent. HEV RNA has been found in the cerebrospinal fluid of some patients with such complications and evidence for intrathecal antibody production has been provided in one case, suggesting possible infection of the central nervous system. The most severe symptoms are observed in pregnant women, possibly related to the reported infection of placental tissue.

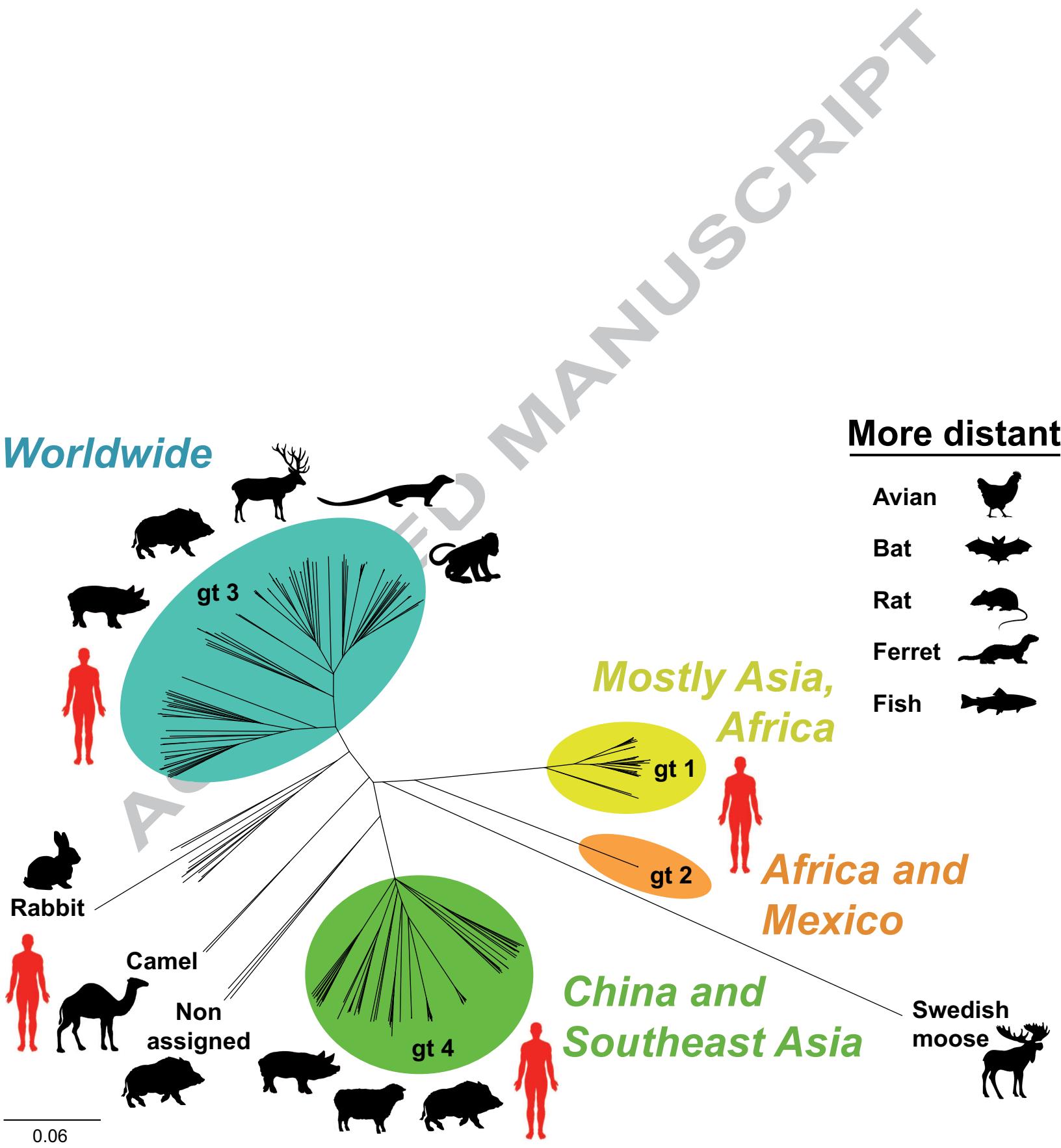
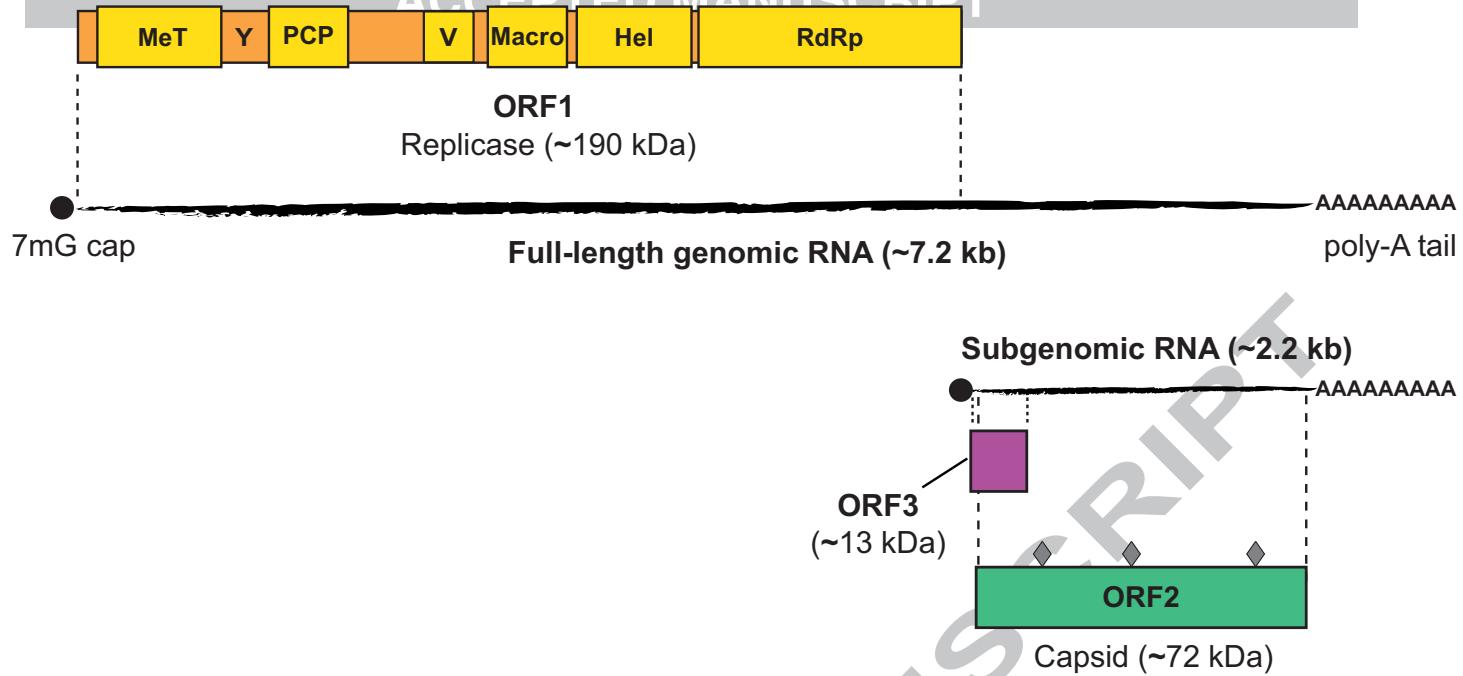
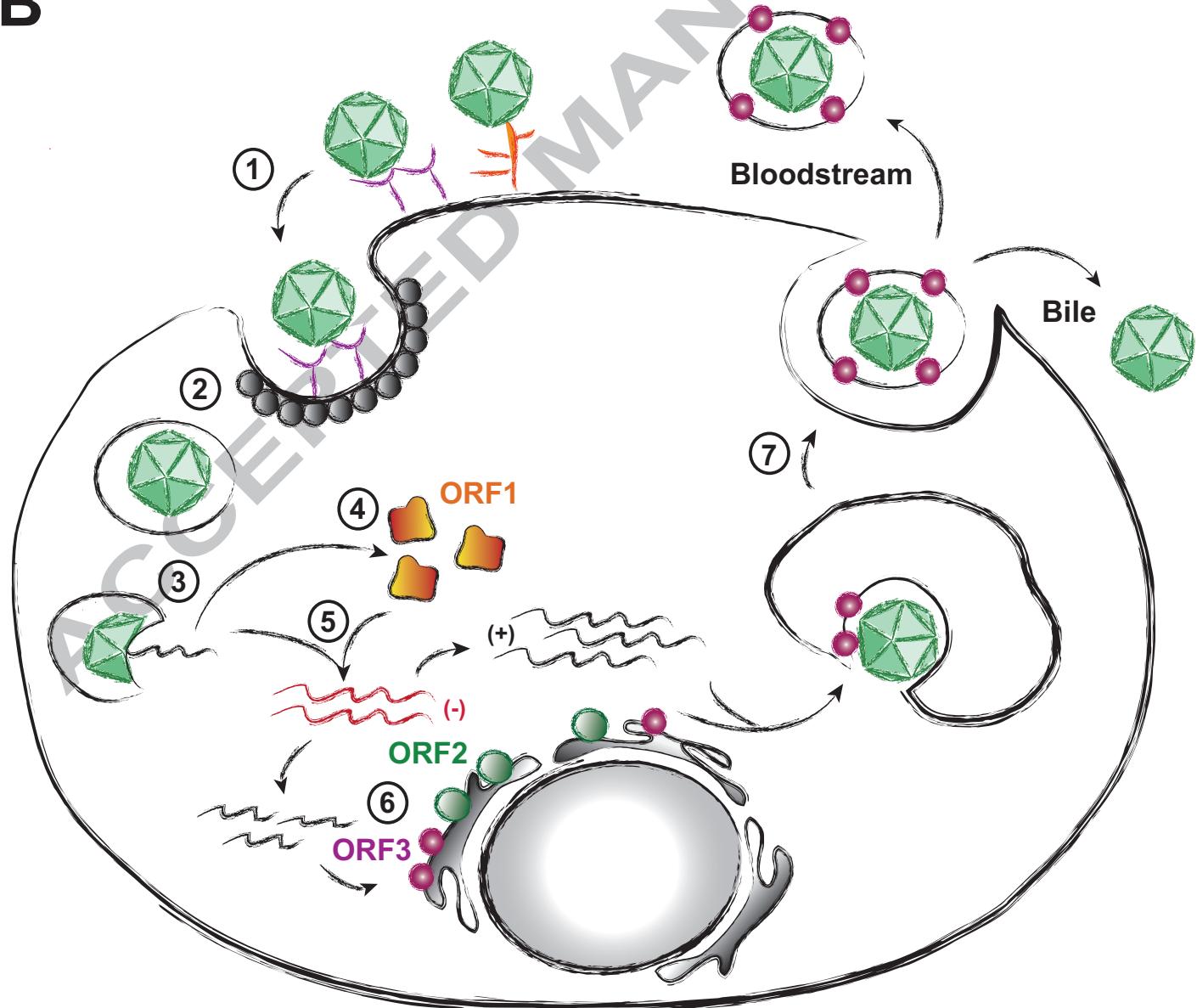
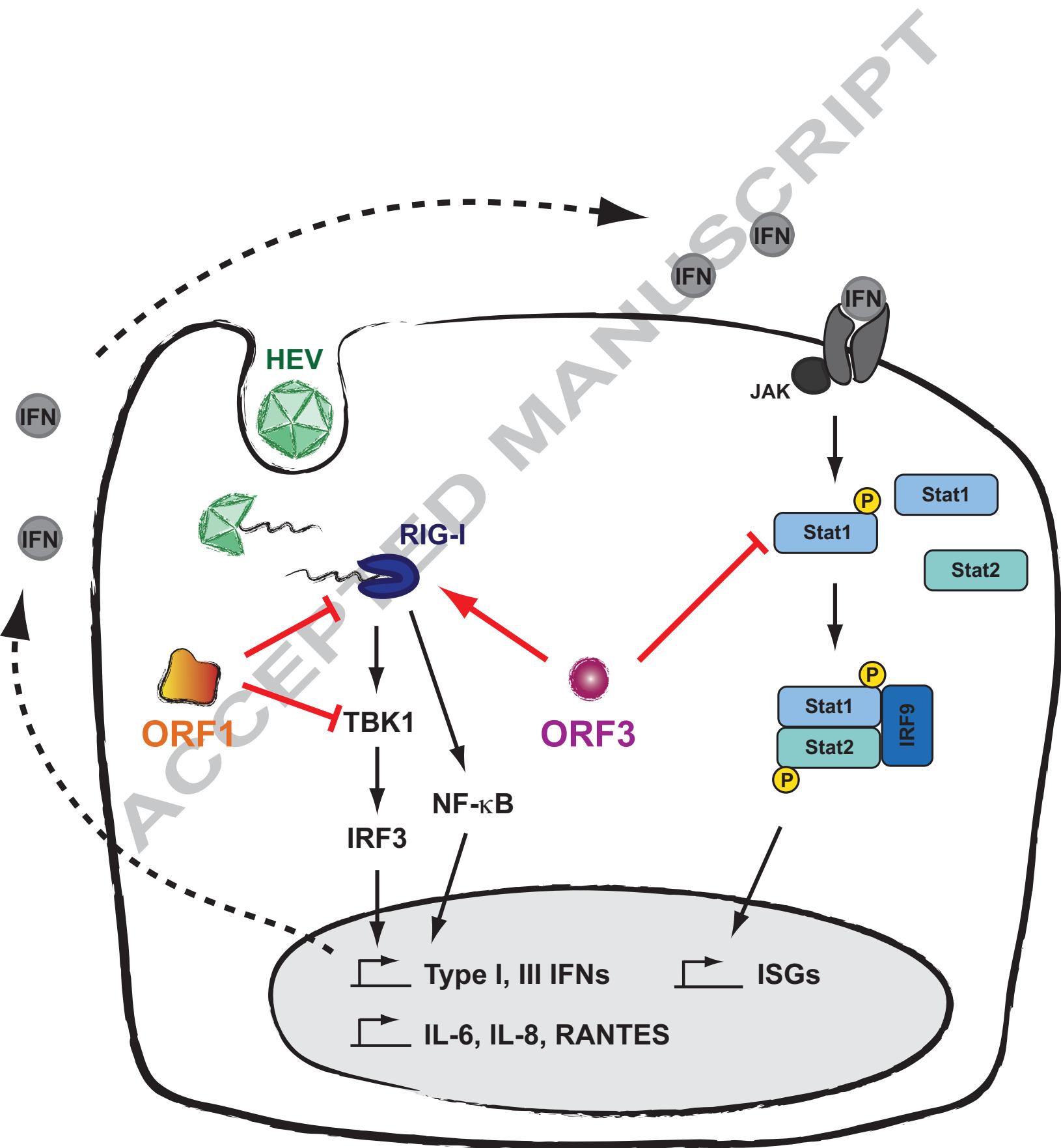


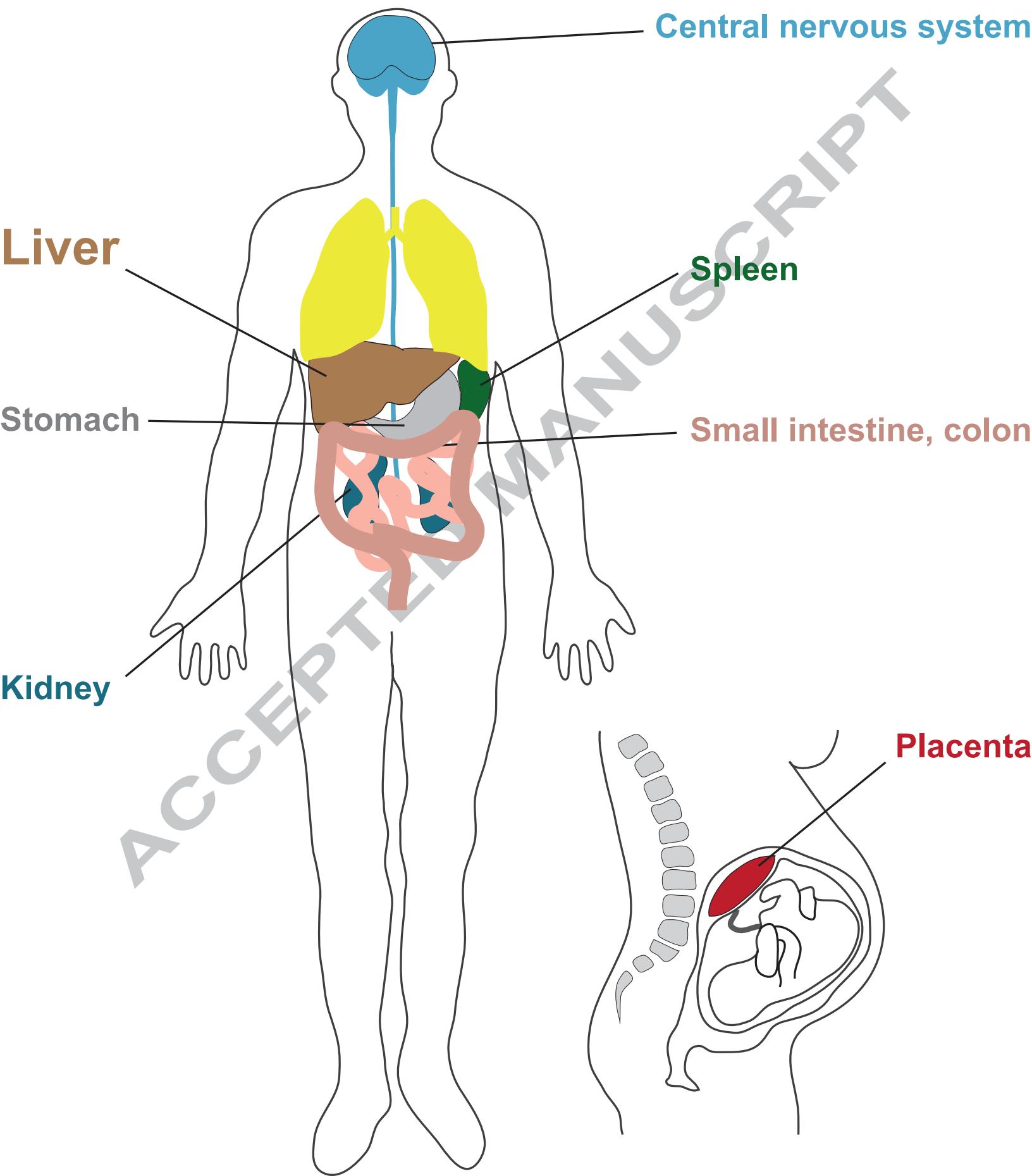
Figure 2

**A**

ACCEPTED MANUSCRIPT

**B**





Drug	<i>In vitro</i> effect	<i>In vivo</i> effect	Mechanism of action	References
Ribavirin	Inhibition of HEV replication	HEV clearance in chronic hepatitis E; occasional cases of treatment failure	Intracellular GTP depletion through inosine 5'-monophosphate dehydrogenase inhibition	[112, 115, 117]
PEG-IFN- $\alpha$	Inhibition of HEV replication	HEV clearance in chronic hepatitis E	Immune activation	[44, 85, 115]
Sofosbuvir	Inhibition of HEV replication <i>in vitro</i>	Unknown	Nucleotide analog; inhibition of the viral RNA-dependent RNA polymerase	[44]
Mycophenolic acid (including prodrug mycophenolate mofetil)	Inhibition of HEV replication	Unclear, possibly associated with HEV clearance in chronic hepatitis E	Intracellular GTP depletion through inosine 5'-monophosphate dehydrogenase inhibition; immune suppression	[124, 125, 127]
mTOR inhibitors (rapamycin, everolimus)	Stimulation of HEV replication	Higher HEV RNA levels in patients with chronic hepatitis E on mTOR inhibitors	Inhibition of an eIF4E binding protein 1-dependent antiviral signaling pathway downstream of mTOR	[126, 128]
Calcineurin inhibitors (cyclosporin A, tacrolimus)	Stimulation of HEV replication	Unknown; tacrolimus use associated with increased risk of viral persistence	Inhibition of cyclophilin A and B	[28, 125]

**Table 1 – Overview of approved drugs affecting hepatitis E virus (HEV) replication.** eIF4E, eukaryotic initiation factor 4E; GTP, guanosine triphosphate; mTOR, mammalian target of rapamycin; PEG-IFN- $\alpha$ , pegylated interferon- $\alpha$ .