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Hepatitis E virus treatment and ribavirin therapy: viral mechanisms of nonresponse

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Hepatitis E virus (HEV) can cause chronic infections in immunosuppressed patients with adverse clinical outcomes. Intervention strategies are limited with ribavirin (RBV) being the only main therapeutic option as off-label drug. Recent reports on RBV monotherapy failures show a coherence with the presence of certain single nucleotide variants (SNVs) and in-frame insertions in the hypervariable region of open reading frame 1 in the HEV genome. Importantly, some of the alterations were present in the viral population as minor variant before RBV administration. Individualized infection medicine by early detection of emerging viral variants in patients could improve treatment outcome and prognosis.

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The hepatitis E virus

Hepatitis E virus (HEV) infects approximately 20 million people each year, leading to nearly 70 000 deaths annually [1]. Recently, HEV was taxonomically reassigned to the family of *Hepeviridae*, which includes the two genera *Orthohepevirus* and *Piscihepevirus* [2]. The *Orthohepevirus A* species is subdivided into eight distinct genotypes (HEV genotype 1-8) of which genotypes 1-4, 7 are considered human pathogen. The fecal-orally transmitted genotypes 1 and 2, which are mainly found in low-income countries on the African continent, in Central America and South-East Asia, solely infect humans. Pregnant women harbor a high risk for a fatal outcome during HEV genotype 1 infection with mortality rates up to 30% in the last trimester [3]. In contrast, many animals serve as reservoir for the zoonotic genotypes 3, 4 and 7, especially domestic pigs, wild boars and dromedaries [4]. Having contact with these animals or consumption of contaminated meat-products are major risk factors for acquiring an HEV infection. The latter genotypes are responsible for most of infections in industrialized nations [1]. Acute HEV infections are usually self-limiting in immunocompetent individuals, but they can cause arthralgia, flu-like myalgia, vomiting and symptoms characteristic of hepatitis like jaundice and itching [1]. However, in vulnerable populations, such as immunosuppressed patients, for example, HIV-infected patients or solid organ transplantation (SOT) recipients HEV can progress to chronicity and can cause fulminant hepatitis in persons with preexisting liver injuries and during pregnancy [5]. Most chronic cases are caused by genotype 3 [6] and 4 [7], whereas high maternal mortality rates are associated with genotype 1 and 2 [8,9]. Of special note, HEV transmission via contaminated blood products has been reported in several articles [10–13]. As blood products are mainly required for serious clinical conditions, including pregnancy, liver disease, SOT and hematological neoplasm, hepatitis E infections can take more severe courses and lead to chronic hepatitis and cirrhosis [14]. Estimations for England assume 80,000–100,000 transfusion associated HEV infections in 2013 [15], 1,600–5,900 per year occur in Germany [14,16].

Treatment of hepatitis E virus infections

The only vaccine for HEV, 'Hecolin' or HEV 239, comprising of amino acids 368–606 of the genotype 1 open reading frame (ORF) 2 capsid protein, has only been approved in China, where it is available since October 2012. Although being very immunogenic and efficacious against genotype 4, its protective capacity against HEV genotype 3 infections is pending [17]. To date, there are no HEV-specific antiviral drugs and treatment for patients with chronic hepatitis E is only supportive. Chronic HEV infections are defined as HEV RNA persisting in the liver of immunosuppressed patients for three months. After this period, it is unlikely that patients achieve spontaneous viral clearance without therapeutic intervention [18]. The options to treat chronic HEV are as follows: first, reduction of immunosuppression, second, administration of pegylated IFN-α (pegIFN-α) and third, the use of ribavirin (RBV) [1,19]. The possibility to reduce immunosuppressive medication has to be evaluated first [1]. Studies reported clearance rates of up to 25% [20,21]. However, chances of rejection of the allograft are constituted by reduction of immunosuppression beyond a certain level [22,23]. Administration of pegIFN-α is a second option to treat chronic HEV. In a small patient cohort treatment durations varied between three and twelve months and

although 80% of the patients achieved sustained clearance of the viral RNA, considerable side effects were noted [24,25]. Noteworthy, besides known adverse events, IFN administration may also cause rejection in organ transplant recipients [23] and *in vitro* data suggests careful assessment of IFNs when treating HEV [26,27]. This is also due to higher doses needed to inhibit HEV replication compared to, for example HCV [27]. It was shown that the ORF1 polyprotein can antagonize type I IFN induction, whereas ORF3 was reported to inhibit IFN-α signaling [28–30]. The most commonly employed HEV treatment is the off-label use of RBV, either as monotherapy or in combination with pegIFN-α. Its efficacy in reducing viral loads in patients has been validated in large studies for acute as well as chronic infections [31•,32•,33]. Also in immunocompromised individuals, it can be safely applied and results in clearance of the viral RNA [34,35]. Furthermore, *in vitro* data suggests a moderate synergistic effect when combined with pegIFN-α [36]. Adversely, because of its teratogenic characteristics, RBV cannot be administered to pregnant women. There are several modes of action proposed for RBV and its antiviral effect against RNA viruses [37,38] and reviewed in the context of HEV [39]. Most important for the background of this article is a mutagenic effect caused by RBV, as the drug is incorporated into newly synthesized RNA genomes, ideally leading to viral extinction. This effect has been observed in several RNA virus populations [40–44], including HEV [45••,46••].

In summary, despite no licensed drugs available for HEV, there are still three treatment options currently available. These include a reduction of immunosuppression in SOT patients, the administration of pegIFN-α or RBV, as well as the combination of the mentioned. As outlined above, treatment failure can occur and in the case of RBV, these failures might be associated with the selection of viral fitness enhancing mutations, rendering the intra-host population supreme over the effect of lethal mutagenesis proposed for RBV.

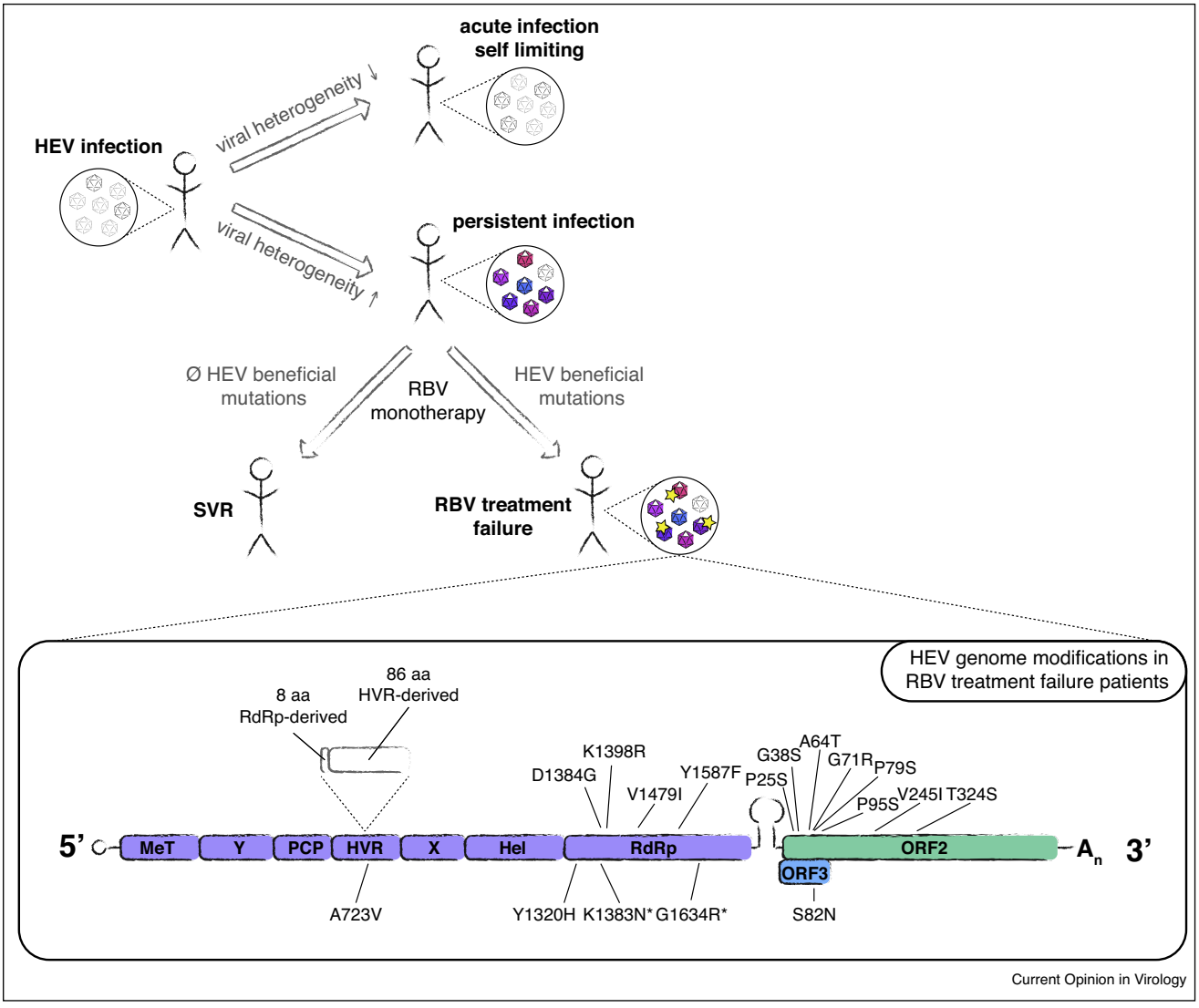
Hepatitis E virus intra-host population

RNA viruses, such as HEV, diversify into so-called intra-host populations, viral swarms or mutant clouds within infected individuals [47]. They are no mere clonal copies, but rather a heterogeneous population of virions with closely related non-identical genomes. Advantageously, these mutant clouds can rapidly adapt to changing environmental conditions, such as the administration of antiviral drugs, and more easily evade the pressure by the host's immune system [48••,49]. Highly diverse intra-host populations are the result of very high replication rates of RNA viruses and a RNA-dependent RNA polymerase (RdRp) lacking proof reading capacity. The greater the HEV heterogeneity, the more likely the infection progresses to chronicity [50,51•]. HEV and most other RNA virus populations replicate in close proximity to the so-called genomic error threshold. This threshold is defined as the maximum error rate at which the genetic information of the master sequence is still maintained and transmittable [47,48••]. As outlined above, broad-spectrum antiviral agents like RBV can cause increased mutation rates resulting in the extinction of the virus by lethal mutagenesis. The genomic information of the population cannot be passed on to the next generation of progeny virus anymore. The majority of the genomes are nonfunctional, a phenomenon referred to as error catastrophe [52]. However, during this process, the mutated viral intra-host populations can acquire variants accounting for decreased RBV sensitivity or altered replication fitness. This has recently also been shown to be the case for HEV RBV treatment failure [45••,53•,54]. These mutagenic effects of RBV on HEV and the viral population accumulating advantageous variants can be pictured as 'armed race': viral extinction versus selection of fitness-enhancing mutations [45••].

RBV treatment failure

In their clinical practice guidelines for HEV and SOT, the European Association for the Study of the Liver (EASL) recommends a strategic reduction of immunosuppression in patients with persistent HEV infection as first intervention strategy [55]. This already may facilitate viral clearance, however, the risk of graft rejection has to be considered thoroughly. Accordingly, a treatment with pegIFN is not recommended because of the risk of organ rejection. In line with the guidelines on treatment of HEV infection by the British Transplantation Society, RBV is the first choice of therapeutic drug intervention. Does an initial RBV monotherapy of three month not lead to a sustained viral response (SVR), that is, HEV RNA not being detectable in plasma and stool six month after treatment cessation, an additional six month of RBV monotherapy is indicated [55,56]. With continuous HEV RNA replication in the serum/plasma or a potential relapse, pegIFN can be administered in liver-transplant patients. For other SOT patients, no alternative therapy is available. Of note, there are several reports on RBV treatment failure in risk group patients (Figure 1). These either occurred due to a reduction of RBV doses because of severe anemia or due to insufficient duration of administration. Pischke *et al.* reported one patient dying of acute liver failure after experiencing a virological breakthrough associated with RBV dose reduction [33,57]. On the other hand, treatment failures have also been reported under continuous high dose RBV therapy. About every fourth SOT patient with persistent HEV experiences recurrence of the viral burden after the initial three month of RBV monotherapy [56]. These cases are often linked to RBV-associated modifications in the HEV genome, that is, the emergence of new single nucleotide variants (SNV) in the viral population altering the cloud's consensus amino acid sequence or insertions in the hypervariable region (HVR) of ORF1. Interestingly, relapser who were retreated for six month cleared the virus and achieved SVR, as shown in a retrospective study including 59 SOT recipients [34].

Figure 1



HEV genome modifications that cause RBV treatment failure in patients. A persistent HEV infection in solid organ transplant recipients is often associated with an increased viral heterogeneity. The more diversified viral population can acquire beneficial genome alterations before and during ribavirin (RBV) monotherapy. These single nucleotide variations (SNVs) or in-frame insertions can lead to RBV treatment failure. About 40% of chronically infected solid organ transplantation patients fail a first three months treatment and do not achieve a sustained virological response (SVR). HEV genome modifications described in RBV treatment failure patients, i.e. 17 SNVs and their respective location and one in-frame insertion in the hypervariable region of open reading frame 1 (ORF), are depicted in the box below. SNVs marked with an asterisk indicate variations described in both studies mentioned in the text. MeT – methyltransferase, Y – Y-domain, PCP – papain-like cysteine protease, X – X-domain, Hel – helicase, RdRp – RNA-dependent RNA polymerase. Amino acid positions of SNVs were annotated according to references NP_056779 (ORF1), NP_056788 (ORF2) and YP_003864075 (ORF3).

Studies describing the occurrence of HEV genome alterations in patients undergoing RBV monotherapy failure are rare. The analysis of the exact mechanisms of how these variations confer RBV resistance *in vivo* are limited, as still there is no robust HEV model system available yet. This fact massively hampers mechanistically molecular studies *in vitro*. In the following, we summarize the current knowledge about HEV mutations detected in patients undergoing RBV monotherapy and failed therapy.

Viral mechanisms of non-response

Recently, treatment failures were linked to the selection of a distinct HEV polymerase variant (G1634R) resulting in increased replication fitness (Table 1) (Figure 1) [53•]. In a subsequent study, 63 SOT patients with chronic

Table 1

HEV genome alterations

Type of genome modification	Changes in HEV genome[a]	Mechanism	Reference
SNV	A723V	Associated with RBV non-responsiveness in SOT patient	[46••]
	Y1320H	Increased HEV replication without affecting RBV sensitivity; compensatory mutation for the K1383N-induced fitness loss	[46••]
	K1383N	variant in the polymerase F1-motif; increased *in vitro* RBV sensitivity; possible increase in polymerase fidelity	[45••,46••]
	G1634R	Associated with RBV non-responsiveness in SOT patient; increased efficiency of viral replication	[45••,46••]
	Additional ORF1 RdRp variants: D1384G, K1398R, V1479I, Y1587F	Associated with RBV non-responsiveness in SOT patient; altered RBV sensitivity and viral fitness	[45••]
	ORF2 capsid variants: P25S, G38S, A64T, G71R, P79S, P95S, V245I, T324S	NA	[45••]
	ORF3 capsid variants: S82N	NA	[45••]
Insertion	120 nt (HVR- and RdRp derived)	Associated with RBV non-responsiveness in SOT patient	[46••]
	174 nt (human ribosomal subunit S17)	Increased viral replication; efficient growth in cell culture	[69•,70•]
	117 nt (human ribosomal subunit S19)	Growth advantage in cell culture	[71]
	186 nt (HVR- and RdRp derived)	Growth advantage in cell culture	[72]
	243–333 nt (HVR- and RdRp derived; fragment of a human tyrosine aminotransferase and HVR; inter-α-trypsin inhibitor)	more vigorous virus growth; provide potential acetylation, ubiquitination, and phosphorylation sites	[73]

[a] Amino acid positions of SNVs were annotated according to references NP_056779 (ORF1), NP_056788 (ORF2) and YP_003864075 (ORF3).

hepatitis E (genotype 3) were screened for the existence of this variant. The authors found that the presence of the 1634R variant at RBV initiation does not lead to absolute RBV resistance and although its proportion is increased in patients whose treatment failed, the presence of the 1634R variant did not compromise the response to a second RBV treatment [54]. In another article, four genotype 3 infected patients not achieving SVR after RBV monotherapy were longitudinally monitored and the emergence of nonsynonymous SNVs under treatment was elucidated (Figure 1) [45••]. Importantly, in the viral populations of three of the four patients, the G1634R mutation was identified. While in one individual the arginine at position 1634 was the predominant amino acid before treatment already, in the other two it was present in a minority of viral genomes and was positively selected under RBV therapy, suggesting that this SNV may have contributed to treatment failure. In tissue culture, this mutation increases replication fitness, while no changes in RBV sensitivity was noted (Table 1) [45••,53•]. Additionally, further RdRp SNVs that may affect viral replication efficiency or RBV sensitivity *in vivo* were reported (Figure 1) [45••]. Intrudingly, a variant in the polymerase F1-motif, K1383N, was described to emerge in two of the subjects with RBV treatment failure as well as in a further study, where Debing *et al.* describe one chronically HEV infected patient not achieving SVR under RBV [46••]. One individual furthermore carried an adjacent D1384G SNV [45••]. Especially the lysine motif is highly conserved in RdRp of RNA viruses and has been shown to be involved in GTP binding in Japanese encephalitis virus, with a postulated role in selecting the correct nucleotide and thus increasing the polymerase fidelity [58]. This could cause less RBV to be incorporated in the nascent RNA strand, conserving the genomic information of the viral population and reducing transition events [45••]. However, this increase in fidelity apparently causes fitness expense, as the K1383 N variant shows diminished replication *in vitro* (Table 1). Further polymerase SNVs identified in patients were Y1320H, G1634K, K1398R, V1479I, Y1587F [45••], as well as a A723V mutation in the HVR (Figure 1) [46••]. None of these decreased RBV sensitivity in tissue culture. Noteworthy, except the G1634R SNV, the mutations were only tested in the combination identified in the patients [45••]. Besides the polymerase domain being subject to positive selection of SNVs, also other parts of the ORF were screened for variants. In a study from Japan, a V239A mutation was identified in the helicase domain of genotype 3 HEV, which was associated with increased virulence probably by enhanced helicase activity [59]. The authors do not comment on treatment, nor are the viral populations monitored over time, narrowing the significance of the study. Of note, alterations are not limited to ORF1, but are selected in all three ORF. Eight partially transient variations of the viral capsid protein sequence occurring under RBV therapy and one ORF3 variation were diagnosed in the genotype 3 infected patient cohort (Figure 1) [45••]. In depth *in vitro* molecular characterization of these SNVs is hampered by the lack of an efficient infectious HEV tissue culture system. From studies using HEV reverse genetic systems one can speculate about decreased HEV replication and infectivity by disturbed viral genomic RNA packaging [60,61], abolished glycosylation of capsid protein and insufficient formation of HEV particles or abrogated ORF2 protein

dimerization [62]. Also reduced production or production of non-functional ORF2 and ORF3 proteins [63,64], as well as effects on HEV replication and infectivity by modifications of the *cis*-reactive element structure [65–68] are mechanisms described *in vitro*.

Besides mutations of single nucleotides altering the amino acid sequence, HEV can also undergo larger alterations of its genome, that is the insertion of genomic segments. These can either be host derived or be mere duplications of different parts of the virus' own RNA (Table 1). Different studies reported various insertion in the HEV genome *in vitro* and *in vivo*, however, only one study applied next generation sequencing methods to assess insertions in the viral genome *in vivo* with one patient experiencing RBV treatment failure [46••]. This is of importance as only in depth sequencing of the population can detect minor variants in the viral population. In this afore-mentioned study, an insertion of 120 nt comprising of HVR- and RdRp derived fragments was identified (Figure 1). It was detectable before treatment initiation as a minor variant and was selected to become dominant in the viral population. The emergence of this insertion is possibly linked with RBV monotherapy resistance. Comparably to the G1634R mutation, the insertion enhanced viral replication in cell culture [46••]. The exact function of the HEV HVR remains elusive, but the incorporation of in-frame insertions seems to be a common phenomenon in chronic HEV infection, independent of RBV administration. The most prominent example is a 58 amino acid (aa) insertion of the human ribosomal subunit S17 in the HVR of ORF1 in the cell culture adapted HEV strain Kernow-C1 p6 (Table 1) [69•,70•]. This recombination process had already taken place in the chronically genotype 3 infected patient, from which the virus was derived. After six passages in HepG2/C3A hepatoma cells, the mutant swarm predominantly carried this insertion. Subsequent reanalysis of the viral input revealed a minor fraction of the viral population with this rearrangement, arguing that the recombination had taken place in the infected host and that it is not an artifact of cell culture adaptation. This insertion enabled the virus to efficiently replicate in cell lines providing the most robust cell culture system to date [69•,70•]. Similar results are reported for a human/HEV recombinant virus isolated from feces of a liver-transplant patient chronically infected with a genotype 3 strain (Table 1) [71]. Here, the authors identified a 39 aa insertion of S19 ribosomal subunit integrated in the HVR. Interestingly, also in this case, the insertion was positively selected in cell culture, underlining the replication advantage conferred by these insertions. HEV can also insert parts of its own genome into the HVR of ORF1. When inoculating A549 lung carcinoma cells with feces from an immunosuppressed patient suffering from chronic HEV infection, Johne *et al.* discovered a 186 nt in-frame insertion derived from two parts of ORF1, HVR and RdRp, comparable to the HEV patient variant sequenced by Debing *et al.* (Table 1) [46••,72]. In another study, Lhomme *et al.* identified replication-boosting insertions of 243 −333 nt in three out of 27 genotype 3 strains isolated from chronically infected SOT patients (Table 1) [73]. Again, in one sample, the insertions consisted of parts of HVR and RdRp. The other two were derived from human genes, one of a fragment of a human tyrosine aminotransferase (TAT) gene followed by a HVR duplication, the other by a fragment of the human inter-α-trypsin inhibitor (ITI) gene [73]. These alterations introduce new ubiquitination, acetylation and phosphorylation sites, possibly influencing transcription and translation. In addition, the insertions could possibly affect the localization of parts of the HEV genome. Kenney and Meng identified and mapped two separate nuclear localization signals (NLSs) in the S17 insertion of the Kernow-C1 strain [74]. By nuclear relocation, the viral RNA would be shielded from pattern recognition receptors (PRR) in the cytosol, contingently dampening the host's immune response and enhancing establishment of chronicity. These examples highlight possible recombination processes in the HEV life cycle. How these insertions enable the virus to overcome the antiviral effect of RBV is so far not understood, as limited data on positive selection of HVR insertions in chronically infected individuals during RBV monotherapy are available. Potentially, a conferred increased replication leads to the accumulation of beneficial mutations before treatment. Interestingly, for other RNA viruses, host gene derived insertions are frequently reported, for example, 228 and 270 nt fragments of human gp120 are incorporated into the genome of bovine viral diarrhea virus (BVDV) [75] or parts of the ribosomal subunit S28 into the hemagglutinin gene of an influenza virus [76]. They were shown to increase viral pathogenicity. Besides distinct mechanisms of the viral RdRp to promote recombination between RNA molecules, like template-switching [77], there are also reports on nonreplicative RNA recombination. For poliovirus, crossover events between nonhomologous segments are common [78].

Conclusion

Modifications of the HEV genome are commonplace in intra-host populations in infected individuals, especially under RBV monotherapy. These include SNVs and large in-frame insertions of up to 300 nt. Several studies report on variants identified in patients or tissue culture. Most of the mutations and insertions described were detected using first generation sequencing methods, thus only changes in the consensus sequences of the viral swarms were discovered. The here illustrated studies used deep sequencing of longitudinal samples from chronically genotype 3 infected patients to monitor also low frequency variants under RBV therapy [45••,46••]. Their emergence in treatment failure scenarios confers resistance to the drug. Because of a lack of efficient model systems, the exact molecular mechanisms of these resistances are not fully elucidated to date. Certain SNVs as

well as the reported insertions result in an increased replication fitness of the virus [45••,46••]. The sheer number of new infectious virions in a patient could be the causative of overcoming the anti-HEV effect of RBV. No SNV or insertion is reported to decrease sensitivity to RBV in tissue culture experiments yet. Further studies to investigate the emergence of minor variants during RBV treatment, as well as their predictive capabilities in regard to treatment failure before administration, are needed. In addition, an efficient and robust full life cycle *in vitro* model is required for mechanistic studies. Lastly, the quest for new effective HEV-specific antiviral molecules must be promoted to supplement or even substitute RBV. Very recently, the nucleotide analog sofosbuvir was shown to display significant anti-HEV activity *in vitro* [79] and first data with different therapy outcomes are available from HEV infected patients [80–82].

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Declarations of interest

None.

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