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In-depth longitudinal study on hepatitis E virus evolution in chronic infection with ribavirin treatment failure

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ABSTRACT

Hepatitis E virus (HEV) can persist in immunocompromised patients, and ribavirin therapy may fail when resistance-associated variants emerge in the viral population. The evolutionary dynamics towards treatment failure remain poorly defined because comparative clinical and *in vitro* systems are scarce. Our study employed longitudinal deep sequencing of a chronic hepatitis E transplant recipient and two persistently infected cell cultures derived from the same patient HEV strain, demonstrating that cyclical ribavirin exposure increases synonymous and non-synonymous variant densities across the viral genome and selects both known and previously undescribed resistance-associated mutations. Variants identified in patient and culture cluster mainly in ORF1, identifying this region as the principal determinant of ribavirin treatment failure. These findings indicate that cyclical ribavirin pressure promotes HEV diversification and antiviral non-response, and support use of early, continuous, high-dose treatment when initial reduction of immunosuppression does not clear infection.

INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of hepatitis E, a widespread but often underrecognized healthcare concern, resulting in an estimated 19.47 million cases of acute hepatitis E globally in 2021 ¹. The ~7.2 kb long positive-sense single-stranded RNA virus genome encodes three major open reading frames (ORFs), flanked by short 5'- and 3'- untranslated regions. ORF1 encodes the non-structural polyprotein containing functional domains necessary for viral replication, whereas ORF2 encodes the viral capsid protein, and ORF3 encodes a small multifunctional phosphoprotein involved in viral egress and immune evasion. Human pathogenic HEV genotypes belong to the *Paslahepevirus* genus within the *Hepeviridae* family ². HEV-1 and HEV-2 are mainly transmitted via the fecal-oral route and responsible for large waterborne outbreaks in resource-limited endemic settings ³. In contrast, infections with HEV-3 and -4 are mainly transmitted in high-income countries, and are of

zoonotic nature, occurring primarily after consumption of contaminated animal products or close contact with natural host species such as domestic swine, wild boar, or deer ³. Parenteral transmissions via blood transfusions are an additional albeit rare source of infections ^{4, 5}. Additional zoonotic genotypes such as HEV-7 and HEV strains from rabbits and rats have also been reported to infect humans ^{6, 7, 8}. Although HEV infection in immunocompetent individuals is typically self-limiting, chronic HEV infection, first reported in 2008 and now defined as viremia persisting for longer than three months, can manifest in immunocompromised persons, such as solid organ and hematopoietic stem cell transplant patients, most often involving HEV-3 ^{9, 10}. The risk of rapid progression to liver cirrhosis in chronic hepatitis E patients was demonstrated in a large multicenter study, underlining the need for effective treatment ¹¹. However, no HEV-specific therapy exists yet. According to a recent meta-analysis, reduction of immunosuppression lead to viral clearance in up to 32% of patients ¹². Ribavirin (RBV) remains the main off-label therapeutic option when reduction of immunosuppression fails, with sustained virological response achieved in 76% of chronic hepatitis E patients ¹². Side-effects such as anemia are common, and non-response and viral relapses were reported in 6% and 18% of patients, respectively ¹². Pegylated interferon- α may be used in cases of RBV non-response, but is contraindicated in kidney, lung, and heart transplant patients due to the risk of graft rejection ¹³.

The host and viral mechanisms involved in the development and disease progression of chronic hepatitis E remain poorly understood. The use of immunosuppressives tacrolimus, rapamycin, or everolimus may increase the risk of developing persistent infection. Additionally, chronic patients exhibit inadequate HEV-specific CD8⁺ T cell responses ¹⁴. On the viral side, reports of intraviral and host transcript-derived insertions into the heterogeneous hypervariable region (HVR) of the viral ORF1 gene in strains collected from chronic HEV infections have increased in recent years ^{15, 16, 17, 18, 19, 20}. RNA viruses like HEV exist within hosts as a population of variants termed as quasispecies. The genomic differences within a quasispecies

74 arise due to the lack of proofreading of the viral RNA-dependent RNA polymerases (RdRp)
75 and permit viruses to quickly adapt to changing environmental pressures ²¹. Greater HEV
76 quasispecies heterogeneity has been associated with progression to persistent infection ²². RBV
77 increases mutational rates within the RNA genome, either leading to error catastrophe or
78 selection of beneficial variants ^{23, 24}. RBV treatment can lead to the emergence of resistance-
79 associated variants, particularly within the RNA-dependent RNA polymerase (RdRp) domain
80 of ORF1, contributing to treatment failure. Several mutations, such as G1634R, K1383N, and
81 D1384G, have been associated with non-response ^{16, 25}. More recently, resistance-associated
82 variants to the anti-HCV nucleoside analogue sofosbuvir have been reported, including
83 A1343V in the RdRp, which was shown to reduce drug susceptibility ^{26, 27}. These findings
84 underscore the need for viral genomic monitoring when exploring alternative or combination
85 therapies.

86 The dynamics of HEV quasispecies evolution under natural conditions and RBV
87 pressure have not yet been properly investigated, owing in part to the lack of study models
88 capable of mimicking chronic infection in humans. While it is well established that RBV
89 induces viral quasispecies diversification, longitudinal studies documenting viral evolution
90 under extended RBV exposure are rare. Especially *in vitro* models mimicking chronic infection
91 in humans are challenging to establish with currently only three HEV-3 strains used extensively,
92 Kernow-C1/p6, 83-2-27, and 47832c ^{28, 29, 30, 31}. In particular, the HEV-3c strain 47832c had
93 been isolated from an RBV-naïve patient and used to persistently infect the A549 lung
94 carcinoma cell line ³¹. Here, we present a unique and historically significant case of this same
95 kidney transplant recipient with chronic HEV infection, followed for over 12 years, who
96 underwent multiple RBV treatment cycles without sustained viral clearance. This individual
97 was among the first chronic HEV patients described and among the earliest treated with RBV.
98 Using deep sequencing, we characterized the viral evolutionary dynamics *in vivo* and compared
99 them to those observed in parallel *in vitro* cultures infected with the same patient-derived

47832c strain, providing unprecedented insight into the mechanisms underlying RBV resistance and persistence.

RESULTS

Clinical and laboratory monitoring of chronic hepatitis E patient

To investigate the evolutionary dynamics of HEV under antiviral pressure, we drew on a well-characterized clinical and *in vitro* model system. The source material was plasma from a kidney transplant recipient who developed chronic hepatitis E (CHE) during follow-up at the nephrology department of Charité Universitätsmedizin Berlin. From this sample, collected at an early stage of infection prior to any RBV therapy, we had previously recovered an HEV-3c strain, designated 47832. This isolate was subsequently used to establish a persistently HEV-infected A549 cell line, yielding the culture-adapted strain 47832c³¹. The latter harbors a distinct insertion in the hypervariable region (HVR) of ORF1, comprising two tandem duplications of viral sequence, a feature we have described in detail elsewhere³¹. For the current study, the 47832c-infected cell line provided a controlled platform to initiate two parallel long-term cell culture experiments designed to mimic antiviral treatment scenarios in CHE. Clinical parameters of the index patient including ALT, AST, GGT, creatinine, and hemoglobin were determined regularly. Courses of liver and kidney function, as well as reports on periodic anemia are depicted alongside the measured HEV plasma titers (Figure 1A-C). Liver enzymes became elevated at 14.8 months post-transplant (ALT=1200 IU/L; AST=438 IU/L; GGT=564 IU/L), prompting testing for viral hepatitis including HEV. HEV RNA was detected via qualitative PCR during the first RBV treatment regimen and with RT-qPCR starting at 41 months post-transplant. The patient first tested HEV-positive 16.6 months post-transplantation and given 400 mg/day (mg/d) RBV after remaining RNA-positive for 5 months, upon which viral titers became undetectable. HEV re-occurrence at 41 months post-transplant was confirmed via real-time quantitative PCR (qPCR) and RBV was continued at 44.1 months and

increased to 800 mg/d at 60.1 months post-transplant (Figure 1A-C). Sanger sequencing (performed by group of Nassim Kamar, Toulouse, France at timepoints indicated by blue numbers) confirmed the presence of RBV-resistant RdRp mutations K1383N, D1384G, V1479I, and G1634R (Figure 2A). Transaminase levels fell upon initiation of RBV therapy and subsequently normalized. Levels again rose upon re-occurrence of HEV titers and thereafter appeared to correlate with viral titers and antiviral treatment regimens (Figure 1A). Fibroscans were performed 60.1 and 98.4 months post-transplant and an increased liver stiffness (11.8 kPa vs. 7.2 kPa) corresponding to a fibrosis degree of ≥ 3 was detected. Kidney function remained compromised up until 41.8 months post-transplant following acute graft rejection up to Banff classification IIb and decreased with addition of rapamune to immunosuppressive regimen, but periodically increased above the cut-off thereafter, particularly during RBV treatment (Figure 1B). Hemoglobin levels appeared to persistently correlate with RBV treatment regimens, as well as during the recovery phase after transplantation. Cessation of RBV treatment led to rapid resolution of anemia. The difference in RBV dose (400 mg/d vs. 800 mg/d) did not appear to additionally decrease hemoglobin levels (Figure 1C). Treatment was terminated at 78.4 months post-transplant without viral clearance. Plasma samples at end of RBV treatment and 19 months after treatment (m76.5 and m95.2 post-transplant, respectively) were chosen for sequencing analysis of RBV-associated mutations. Titers at these timepoints were 4.04×10^5 IU/ml and 1.67×10^6 IU/ml, respectively.

Patient HEV codon variants

To monitor the dynamic changes in the HEV population and pinpoint occurrence of treatment-resistant variants, we performed Illumina sequencing of the full viral genome at time points before (76.5 months post-transplant = m76.5) and after (95.2 months post-transplant = m95.2) cessation of the 800 mg/d RBV treatment cycle. Amplified fragments covering near-complete HEV ORFs were sequenced, raw reads processed, and variants analyzed using an in-house

bioinformatics pipeline. Absolute numbers of detected synonymous (syn.) and non-synonymous (non-syn.) variants during the monitored time frame are visualized in Figure 2A. Patient HEV ORFs under RBV pressure (m76.5) contained 42, 269, and 202 syn. codon variants of $\geq 5\%$, $\geq 20\%$, and $\geq 50\%$ frequencies, respectively. After RBV treatment cessation (m95.2), numbers of $\geq 5\%$ and $\geq 50\%$ frequent codon variants increased to 177 and 274, respectively, while $\geq 20\%$ frequent ones decreased to 17. Similarly, $\geq 5\%$ and $\geq 50\%$ frequent non-syn. variants increased from 14 and 57 to 51 and 71, respectively, while $\geq 20\%$ frequent ones decreased from 69 to 6. Frequencies of non-syn. variants detected both under RBV pressure (m76.5) and after RBV cessation (m95.2), as well as described RBV treatment-associated variants (regardless of frequency) are depicted in Figure 2B. Forty-one mutant variants (compared to prototype 47832) were detected in ORF1 in m76.5, with 11 decreasing (two falling below the $\geq 5\%$ threshold by m95.2), 10 increasing, and 20 remaining stable at $\geq 5\%$ frequency upon RBV cessation. Of 10 ORF2 mutant variants, 4 fell below the threshold and 6 increased by m95.2. In ORF3, both detected variants increased to $\geq 5\%$ by m95.2. The most prominent change in amino acid class was towards polar/neutral residues with 46%, 30%, and 50% of detected ORF1, ORF2, and ORF3 mutant variants, respectively. Changes to unpolar/hydrophobic and basic residues were only observed in ORF1 with 7% and 2%, respectively, while acidic substitutions were only seen in 10% of detected ORF2 variants. Notable variants that were detected under RBV pressure (m76.5) and subsequently decreased or dropped below the $\geq 5\%$ threshold were V161I, V246I, A720T, insA824V, F809S, P825S, D916N, K1383N, D1384G, A1499V in ORF1, P25S, G36S, N562D, A606V in ORF2, and none in ORF3.

HEV titers in *in vitro* models

Three replicates of the persistently infected A549 cell line were treated with continuous RBV to assess inherent RBV sensitivity of the 47832c strain. In all three replicates, HEV titers under

RBV pressure fell rapidly and became undetectable by week 8, 10, and 11, respectively. Viral titer progression is depicted in Supplementary Figure S5.

Subsequently, to mimic the effect of clinical cycles of RBV treatment on the observed variant evolution in a tissue culture model, two persistently HEV-infected cell cultures were established (Figure 3) – each separated into untreated Control (Ctrl) and RBV-treated (RBV) lineages (experimental outline depicted in Supplementary Figure S6). RBV treatment was started at 5 weeks after seeding of cells in culture B, the virus was allowed for development of broader quasispecies distribution by a longer incubation time of 18 weeks before first RBV treatment in culture A. Culture A RBV lineage was subjected to five treatment cycles of increasing duration, with the final cycle being terminated based on the decision to end the experiment. Samples collected at w1, w18, w31, w34, w54, w60 were chosen for NGS analysis. Culture B RBV lineage was treated with RBV for four cycles of increasing duration. Cycle four was terminated for the same reasons as in culture A. Sample from w1, w5, w20, w23, w40, and w43 were selected for sequencing. Rational for sampling timepoints are (1) baseline, (2) timepoint of lineage split, (3) first week after RBV in first cycle of increasing duration, (4) timepoint of minimum titer in said cycle, (5) timepoint of maximum titer in final cycle, (6) termination timepoint of RBV cycles. Ctrl sampling follows RBV after split.

***In vitro* HEV variant dynamics**

Absolute numbers of detected syn. and non-syn. variants during the course of the experiment are visualized in Figure 3 (see also Supplementary Table S3). Briefly, all syn. and non-syn. variants found in cultures A and B w1 were considered background and subtracted from all subsequent analysis timepoints. The same variant thresholds as before were applied to *in vitro* samples. In both cultures, RBV treatment increased the absolute number of $\geq 5\%$ frequent syn. and non-syn. variants compared to the untreated Ctrl lineages by w31 (A)/w20 (B). A less prominent increase in $\geq 50\%$ frequent syn. and non-syn. variants was likewise detected over

time with RBV treatment, starting at w34 (A)/w23 (B). The $\geq 50\%$ frequent variants in Ctrl lineages increased starting at w34 (A)/w23 (B), albeit at lower numbers than under RBV pressure. Inversely, numbers of $\geq 20\%$ frequent syn. and non-syn. variants decreased over time in the Ctrl lineages of both cultures, with the exception of culture B syn. variants, which increased slightly and reached a maximum at w40. The overall numbers of syn. and non-syn. variants across the HEV coding regions were thus increased under RBV treatment (RBV) compared to untreated long-term culture (Ctrl).

Variant evolution in cell culture adaptation without RBV treatment

To differentiate variants evolving under RBV pressure from those arising due to cell culture adaptation, and to account for sporadic mutations, amino acid substitutions detected in at least two *in vitro* Ctrl timepoints were assessed (Figure 4). ORF1 harbored 20 non-RBV-specific variants, while 7 were detected in ORF2, and 4 in ORF3. Overall, no non-syn. variant was common to all 10 Ctrl timepoints after w1. 35% of detected non-RBV-specific variants were detected at 2 timepoints (n=11), 13% at 3 timepoints (n=4), 26% at 4 timepoints (n=8), 10% at 5 timepoints (n=3), 3% at 6 timepoints (n=1), 10% at 7 timepoints (n=3), and 3% at 9 timepoints (n=1). These substitutions were consequently not regarded in the context of RBV-associated viral evolution. The distribution of Ctrl-/RBV-specific variants within the HEV genome are depicted in Supplementary Figures S7 and S8.

HEV RBV-associated variants across patient and *in vitro* timepoints

To assess only non-syn. variants arising under RBV pressure, the aforementioned common Ctrl substitutions were disregarded, and only amino acid changes present exclusively in two or more patient or *in vitro* timepoints were selected and their frequencies compared (Figure 5). Thirty-four RBV-specific variants were detected in HEV ORF1, including 10 present in patient plasma during RBV treatment (m76.5). Shared patient/*in vitro* variants were **G557S**, **A720V/T**, **P823S**,

A836V/T, P831L/S, V967I, A1018V/T, A1499V/T, G1634R, and V1692A/L. Seventeen variants were detected in ORF2, with only 2 being shared between patient and *in vitro* models, namely **G36S** and **A575V/T**. In ORF3, 8 RBV-specific variants were observed, with none shared among patient and *in vitro*. Notable variants that only arose *in vitro* but became dominant ($\geq 50\%$ frequency) within the last RBV cycle included **E486K, A646T, insP831L, insN1637S, A1532T, and I1533V** in ORF1, **R2H** and **S587N** in ORF2, and **A16T** in ORF3. Similar to the patient-specific strain, changes towards polar/neutral residues were most common at 43% in ORF1, 39% in ORF2, and 40% in ORF3. Reversion to unpolar/hydrophobic residues occurred in 7% (ORF1), 11% (ORF2), and 0% (ORF3), while 5% basic substitution was seen only in ORF1 (n=2), and 6% acidic substitution in ORF2 (n=1).

DISCUSSION

When first line anti-HEV management – reduction of immunosuppression – is insufficient to clear chronic HEV infection, RBV is the recommended therapeutic option in transplant patients³². Treatment failure can occur, but the underlying dynamics and the role of viral variant evolution are yet not fully understood, even though certain amino acid substitutions in the HEV genome have been linked to RBV non-response^{16, 25}. In the present work, we provide a comprehensive longitudinal comparison of *in vivo* and *in vitro* viral evolution under RBV pressure, using two closely related systems: one of the earliest reported kidney transplant recipients with CHE who experienced persistent viremia and RBV treatment failure, and the persistently HEV-infected cell culture system derived from the same patient's viral population. Detailed longitudinal analyses of RBV's effects on HEV variant dynamics have been scarce. Prior studies have demonstrated that increased population heterogeneity in the polyproline region of the HVR, the Y-Domain, the RdRp, and the ORF2 favors development of chronicity^{22, 25, 33}. Our data expand this knowledge by showing that long-term RBV exposure drives a

broad spectrum of mutations, including known resistance-associated variants such as G1634R and K1383N, as well as additional ORF1 substitutions not previously linked to RBV failure. In both patient and RBV-treated *in vitro* cultures, viral replication persisted despite prolonged antiviral exposure, accompanied by a marked increase in the number of synonymous and non-synonymous variants. Upon RBV commencement, the number of codon sites with variant counts of $\geq 5\%$ initially increased dramatically but quickly plateaued. Intriguingly, upon RBV cessation in the patient, they increase further, suggesting an initial surge in low level mutation by RBV pressure but eventually transitioning into a purifying selection. This observation may explain the non-responsiveness of the *in vitro* cultures to periodic RBV administration, caused by a combination of population heterogeneity and purifying selection of dominant resistance-associated variants.

ORF1 RdRp substitutions appear central to RBV resistance: K1383N, D1384G, and G1634R were dominant in patient plasma, with G1634R also emerging in culture A. V1479I, previously linked to RBV resistance was selected in our patient and in culture B both with and without RBV pressure, suggesting non-specific association²⁵. Since only G1634R is known to enhance HEV replication and does not appear in culture B, resistance in that system likely involves other substitutions. Positions A1499, A1532, and I1533 (I1533V also present in culture A), have been previously described in a CHE transplant patient failing HEV clearance under combinatorial RBV/sofosbuvir treatment, while ORF2 variant G36S (common in both patient and *in vitro*) was detected in an immunocompromised patient^{34, 35}.

Interestingly, the two RBV-treated *in vitro* cultures displayed distinct variant profiles, with culture A more closely paralleling the patient's viral evolution. This may be due to a broader quasispecies diversification before RBV treatment in culture A, or by lack of an *in vivo* environment that allows more complex mutational profiles to emerge through compartmentalization, as confirmed by studies on neurological, renal, and seminal HEV manifestations^{36, 37, 38, 39}. Culture B-specific mutations including E486K and A1532T, in ORF1,

274 R2H in ORF2, and A16T in ORF3, which all became dominant during RBV resistance in the
275 last treatment cycle, should not be overlooked however, as these emerged from the same
276 patient-specific pre-treatment viral population. ORF2 translation is initiated from the first AUG,
277 placing R2H within the signal peptide directing glycosylated ORF2-dimer to the secretory
278 pathway ^{40, 41, 42}. A disruption of the signal peptide could lead to increased intracellular
279 accumulation and nascent virion production ^{40, 41}. Alongside the clinically described, but not
280 yet RBV-associated variants at A1499, A1532, and I1533, these are prime candidates for future
281 functional studies to assess their roles in RBV treatment response.

282 A recent report confirmed the accumulation of HEV variants under RBV treatment in chronic
283 hepatitis E ⁴³. The patient responded to treatment and did not develop canonical RBV-associated
284 mutations, but N562D in the ORF2 was similarly detected as in our patient ⁴³. N562 is a
285 glycosylation site of the capsid protein, with full-length glycosylated ORF2-dimer suggested to
286 act as a molecular decoy to exhaust antibody-mediated neutralization, possibly similar to the
287 recently described P79S variant ^{44, 45}. N562D has been described to efficiently dimerize with
288 no change in antibody recognition ⁴⁶. The exact function of N562D therefore remains unclear.

289 Our data shows that RBV pressure alone can select resistance-associated mutations, as
290 evidenced by shared dominant amino acid changes in the patient population as well as in the
291 culture, despite the latter lacking adaptive immunity.

292 Functionally, most amino acid changes reflected a shifted towards polar/neutral residues,
293 including RBV-associated variants K1383N and D1384G. Few changes towards basic or acidic
294 residues were observed, mainly G1634R, G36D and N562D (both acidic and in ORF2), and
295 E486K in ORF1. The role of such chemical changes in RBV response warrants investigation.

296 Clinically, monitoring of kidney, liver, and blood parameters in our patient revealed an apparent
297 correlation between high viral titer and elevated ALT, AST, and/or GGT, indicating liver
298 damage ^{47, 48}. RBV can decrease and even normalize levels of transaminases ⁴⁹. RBV's
299 hemolytic effect is well described and in line with the decreases in hemoglobin levels observed

during RBV administration without further deterioration after increasing doses from 400 mg/d to 800 mg/d⁵⁰. This suggests high initial RBV doses may be feasible in CHE kidney transplant patients, though individual risk–benefit assessment remains essential, as HEV can affect extrahepatic sites including the kidney⁵¹.

Our findings reinforce the urgent need for effective and HEV-specific antivirals. While sofosbuvir – an anti-HCV nucleoside analogue – once held promise, results have been disappointing, and resistance mutations such as A1343V further temper expectations^{26, 27, 34, 52}. Nonetheless, combinatorial therapies may still be promising. A recent report demonstrated the effectiveness of combined ledipasvir/sofosbuvir/ribavirin in a ribavirin-refractory chronic HEV patient with K1383N, D1384G, and G1634R mutations (same as our patient)⁵³. Early RBV administration may also shorten periods of viremia, giving the virus less time to adapt and diversify⁴⁹.

This study is distinguished by the rare combination of long-term clinical follow-up, parallel *in vitro* modeling, and retrospective analysis of one of the earliest documented CHE cases with RBV treatment failure. The integration of these complementary perspectives provides unprecedented insight into the evolutionary pathways of HEV under prolonged antiviral pressure, offering both historical context and forward-looking guidance for clinical management.

MATERIALS AND METHODS

Ethics approval

This study was approved by the local ethics committee of the Charité Universitätsmedizin Berlin, Germany (approval number EA1/367/16) and written informed consent was obtained from participating individuals. Patient samples were de-identified for this study. All experiments were performed in accordance with relevant guidelines and regulations.

Chronic hepatitis E patient and virus strain

A plasma sample from a kidney transplanted patient treated at the nephrology department of Charité Universitätsmedizin Berlin, Virchow-Campus, who developed chronic hepatitis E (CHE) virus infection was previously used to establish a persistently HEV-infected A549 cell line³¹. The original HEV-3c strain from the patient plasma was designated as 47832 and originated from an early infection timepoint before initial RBV treatment. The corresponding culture isolate was termed 47832c and contains an insertion in the HVR originating from two duplicated parts of the HEV ORF1-encoding region³¹. The 47832c-infected cell line was used in this study to establish two distinct cell cultures to imitate antiviral treatment in CHE. The patient received two rounds of 400 mg/d and one round of 800 mg/d RBV treatment but did not clear the virus. Plasma samples for virus sequencing were collected at 76.5 (m76.5) and 95.2 months (m95.2) post-transplant. Kidney function (creatinine) and hemoglobin levels were determined regularly prior and after kidney transplantation. Liver function (alanine and aspartate transaminases (ALT, AST), gamma-glutamyl transferase (GGT) was assessed regularly during chronic hepatitis E infection.

Patient and public involvement

Patients were not involved in the design and conduct of this research, since not appropriate to the retrospective nature of the study.

Cell culture

The persistently HEV 47832c-infected lung carcinoma A549 cell line was cultured as previously described³¹. In treatment cycles, 100 µM RBV (Sigma-Aldrich, USA) was supplemented upon passaging. For pre-testing, continuous RBV treatment was initiated after cell thawing. For cultures A (long-term intra-host evolution) and B (short-term intra-host evolution), treatment was interrupted when viral titers in supernatant reached 10⁴ to 10⁵ IU/ml

and continued after 3-4 weeks. Culture B received treatment after 5 weeks (w5) post-thawing. RBV treatment commenced after 18 weeks (w18) in culture A to allow a broader viral population diversification before treatment. Untreated Ctrl lineages (Ctrl) were split off from RBV-treated lineages (RBV) starting at first treatment cycles.

Determination of HEV titers

Viral RNA was extracted and HEV RNA titers quantified as previously described^{48, 54}. Viral genome equivalents/ml were converted into IU/ml using the WHO 1st International Standard for HEV RNA⁵⁵.

Amplification of HEV genome

Extracted HEV RNA from plasma and cultured supernatants was reverse transcribed as previously described¹⁸. For sequencing, the near-complete HEV genome was amplified (Supplementary Figure S4) in one (F5.2 for patient plasma¹⁸) or four fragments (F1.2 – F4.2) and an HVR-spanning fragment (HVR2) including the 186bp ORF1 insertion (culture). Nested PCR were performed with KAPA HiFi HotStart ReadyMix (Roche, Switzerland) for plasma-derived or Q5 High-Fidelity 2X Master Mix (New England Biolabs, USA) for culture-derived cDNA. Fragment lengths and nucleotide positions are summarized in Supplementary Table S1. Primer sequences and cycling conditions are listed in Supplementary Table S2. Fragments F1.2 through F4.2 were pooled for each culture timepoint at equimolarity. F1.2-F4.2 and F5.2 sequencing libraries were generated using Nextera XT DNA Library Preparation Kit (Illumina, USA) or NEBNext II Ultra chemistry (New England Biolabs, USA) for HVR2 fragment libraries.

Next Generation Sequencing of HEV genomes

Paired-end sequencing was performed on an Illumina HiSeq 2500 (2x250bp; F5.2), NextSeq550 (2x150bp; F1.2-F4.2) or MiSeq (2x150bp; HVR2) platform (Illumina, USA).

Near-full length genomes were assigned 5×10^6 paired-end reads, whereas F2.2 fragments alone or HVR2 fragments were assigned 1×10^6 and 5×10^4 reads, respectively. Raw data was processed using an in-house bioinformatics pipeline as previously described^{19, 56}. Processed reads from patient samples were mapped against the reference sequence of patient strain 47832 (KC618403), while those from *in vitro* samples were mapped against the cell culture-derived reference 47832c (KC618402) to account for differences in mutations acquired during cell culture adaptation. Codon variant frequency thresholds of $\geq 5\%$ ($< 20\%$), ensuring high confidence variant calling against the background of PCR amplification errors, $\geq 20\%$ ($< 50\%$), mimicking Sanger-like sensitivity, and $\geq 50\%$, indicating dominant codon variants, were chosen. Detected non-syn. codon variants were numbered according to 1a-Burma (M73218), as remains convention. Syn. and non-syn. variants from cultures A and B w1 were defined as background and subtracted from all subsequent Ctrl/RBV timepoints to analyze variants arising from prolonged adaptation or RBV treatment alone.

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Author contributions

DH, CPP, JH, RJ and CTB conceived and designed the study; DH and CPP performed the experiments; DH, BA, DT, and PK analyzed the data; DH drafted the manuscript; CPP, BA, DT, PK, PB, JK, JH, CTB and RJ revised and proof-read the manuscript. JH and CTB supervised the study. All authors contributed to the article and approved the submitted version.

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Competing interests

The authors declare no competing interests.

Data availability

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB110695 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB110695>).

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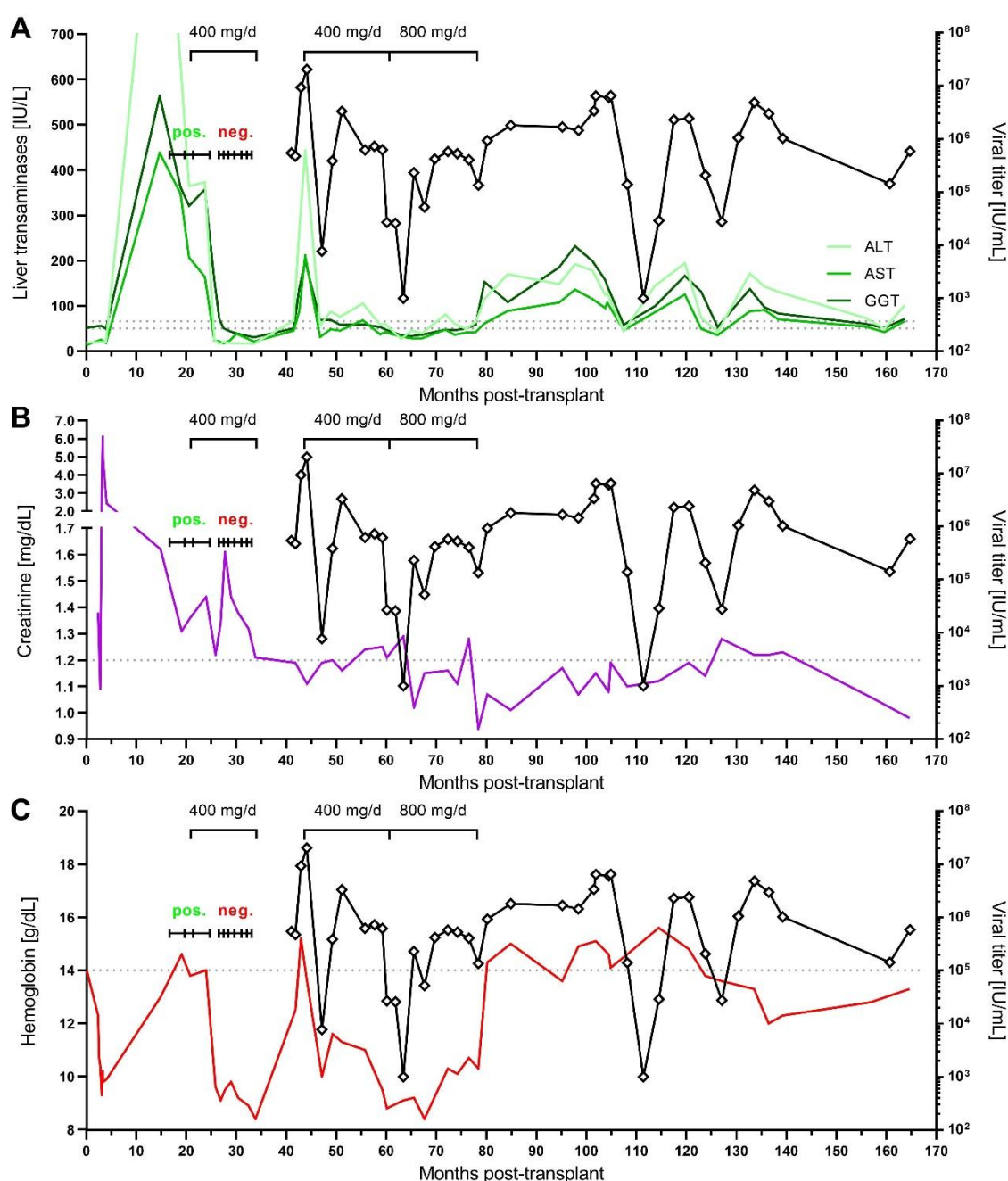
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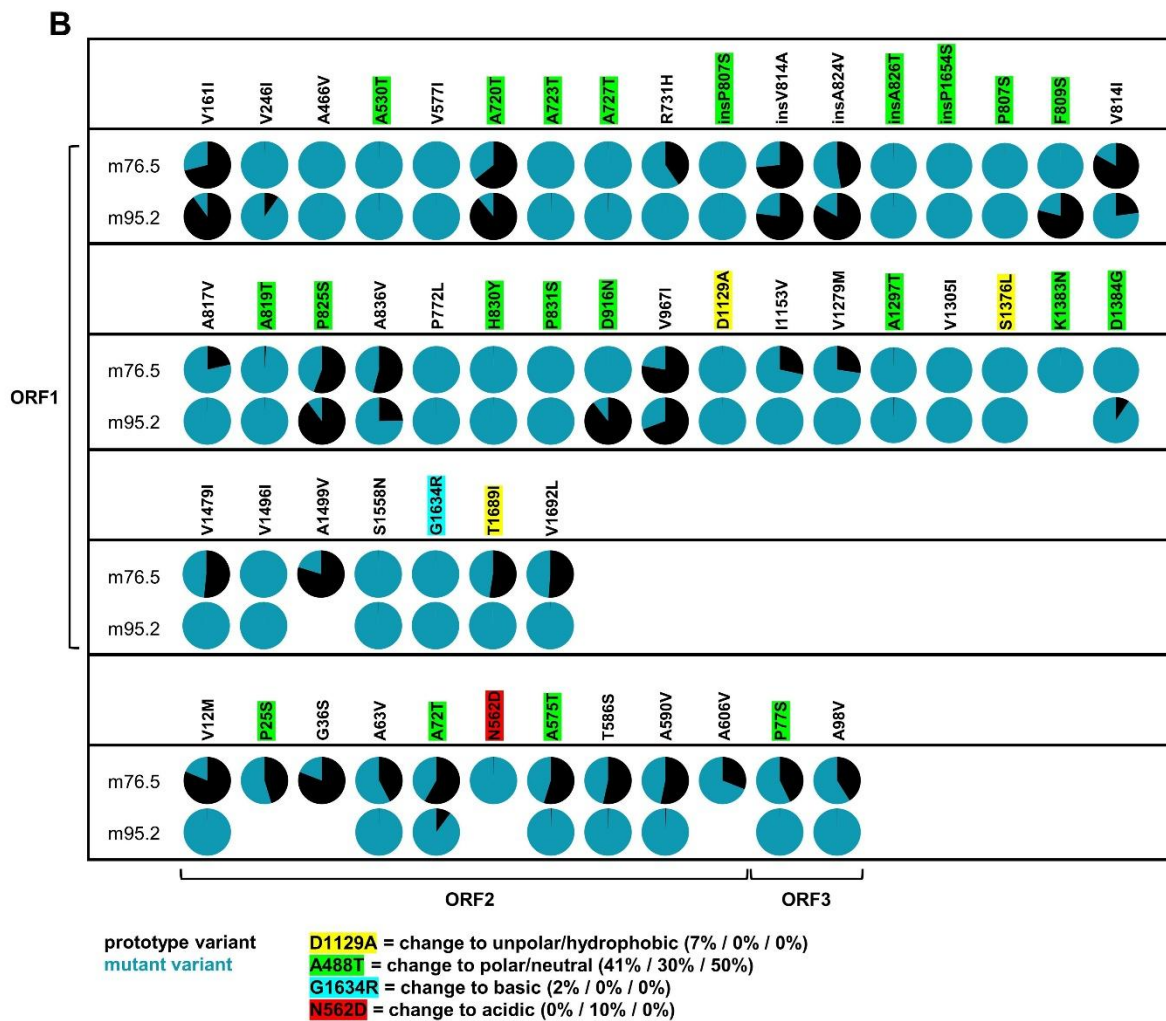
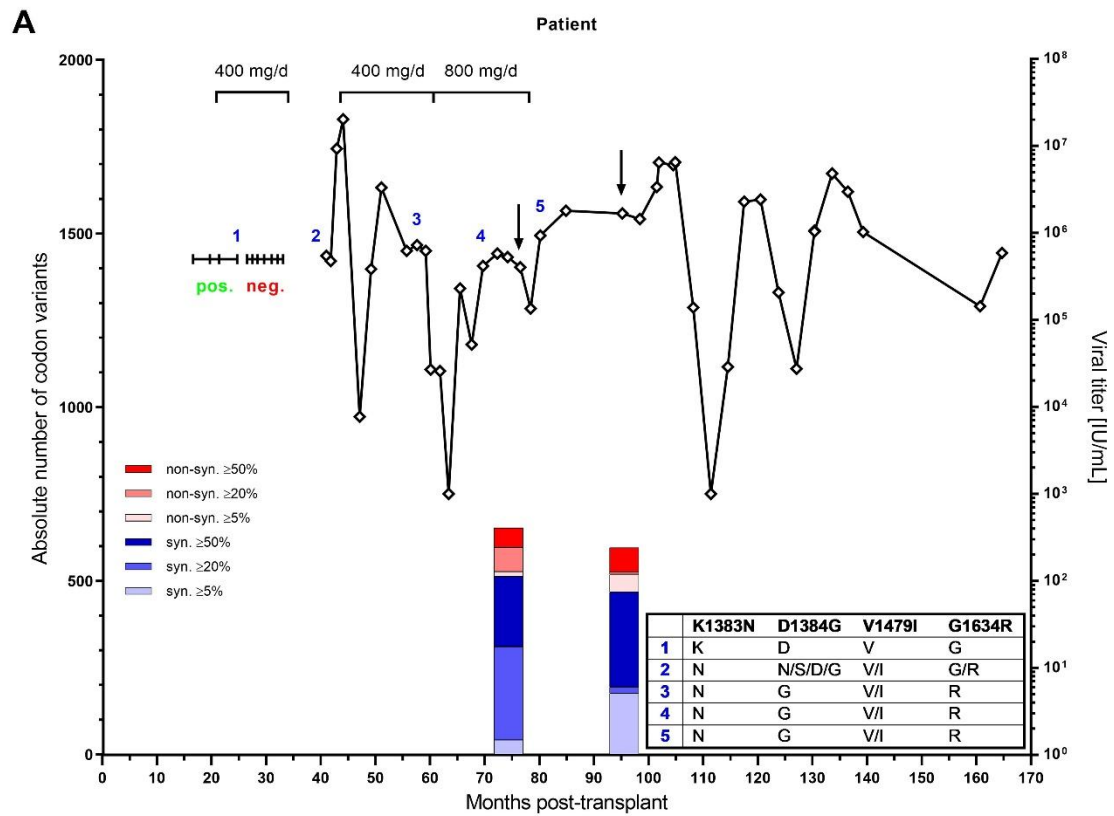
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FIGURES



597 **Figure 1.** Clinical parameters measured before and during chronic HEV infection. Blood levels
598 of liver transaminases (A), creatinine (B), and hemoglobin (C) depicted as colored lines with
599 reference value cut-offs (ALT, AST = 50 IU/L; GGT = 66 IU/L; creatinine = 1.2 mg/dL;
600 hemoglobin = 14 g/dL) as dotted grey lines. HEV plasma titers in IU/mL (black line) as
601 determined by RT-qPCR at different timepoints (in months) post-transplant (diamonds).
602 Duration and dose of RBV treatment cycles indicated in black brackets.



604 **Figure 2.** (A) Patient HEV plasma titers in IU/mL as determined by RT-qPCR at different
 605 timepoints (in months) post-transplant (diamonds). Positive (pos.) and negative (neg.)
 606 qualitative determination of HEV RNA before month 41 indicated by connected strokes.
 607 Duration and dose of RBV treatment cycles indicated in black brackets. Blue numbers indicate
 608 timepoints of Sanger sequencing of RdRp. Bottom right table summarizes RBV-associated
 609 codon variants detected via Sanger sequencing. Arrows indicate timepoints chosen for
 610 sequencing analysis. Absolute numbers of syn. (blue) and non-syn. (red) codon variants
 611 (stacked bars) across the entire HEV protein coding region in both analyzed timepoints. Codon
 612 variants counts within thresholds of $\geq 5\%$ (lower), $\geq 20\%$ (middle), and $\geq 50\%$ (upper bracket)
 613 denoted in each bar. (B) Patient-specific HEV variants detected under RBV pressure (m76.5)
 614 and after cessation (m95.2). Frequencies $\geq 5\%$ are depicted as pie charts with prototype 47832
 615 variants in black and mutant variants in blue. ORF1-, ORF2-, and ORF3-specific variants are
 616 denoted by brackets. Changes in amino acid class are denoted by color code.

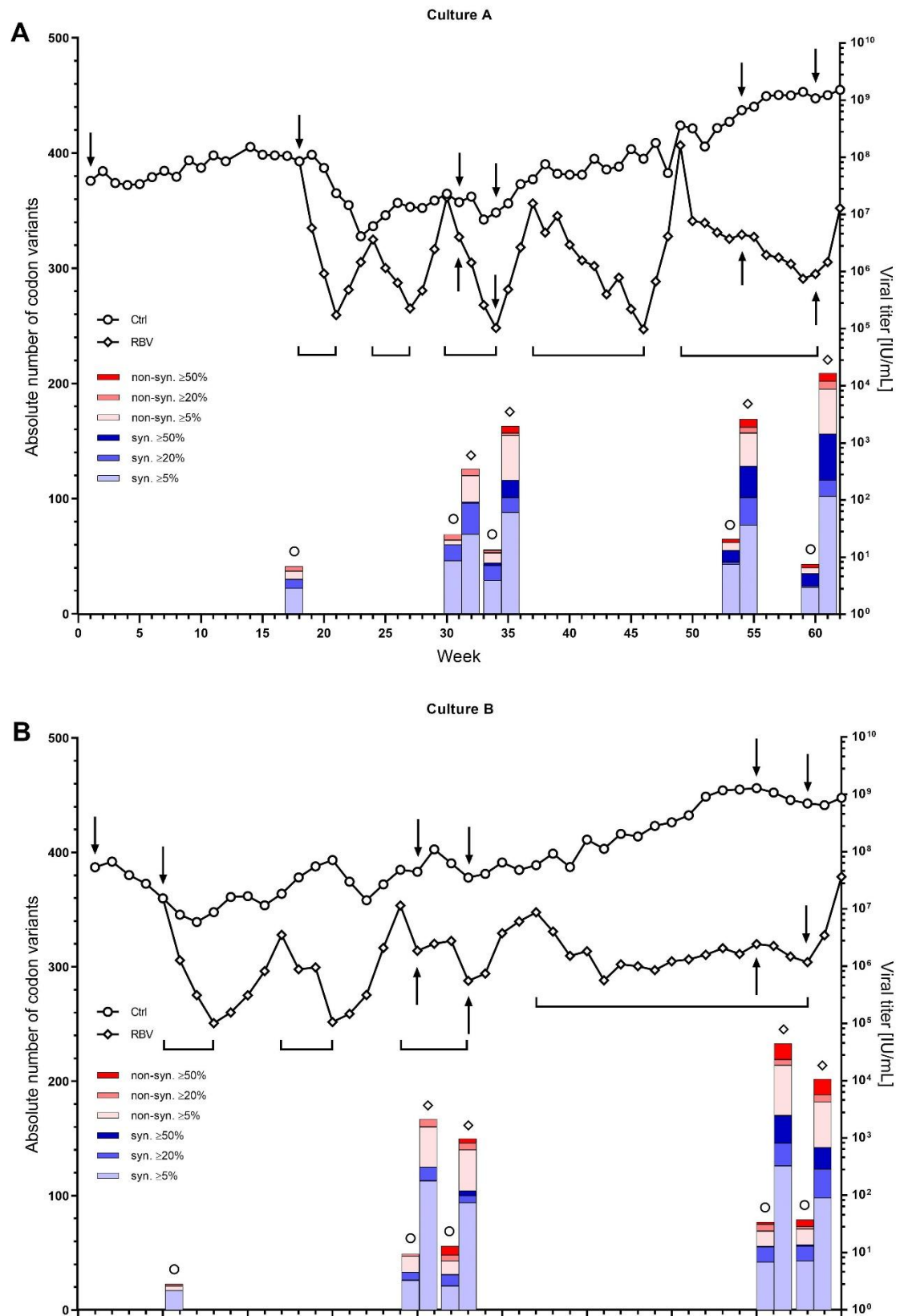


Figure 3. HEV viral titers in collected cell culture supernatant in IU/ml as determined by RT-qPCR in cultures A (A) and B (B). Timepoints in Ctrl lineages are depicted as circles.

Timepoints in RBV lineages are depicted as diamonds. Duration of RBV treatment cycles indicated by black brackets. Arrows indicate timepoints chosen for sequencing analysis. Absolute numbers of background-corrected syn. (blue) and non-syn. (red) codon variants (stacked bars) across the entire HEV protein coding region in both analyzed timepoints. Codon variants counts within thresholds of $\geq 5\%$ (lower), $\geq 20\%$ (middle), and $\geq 50\%$ (upper bracket) denoted in each bar.

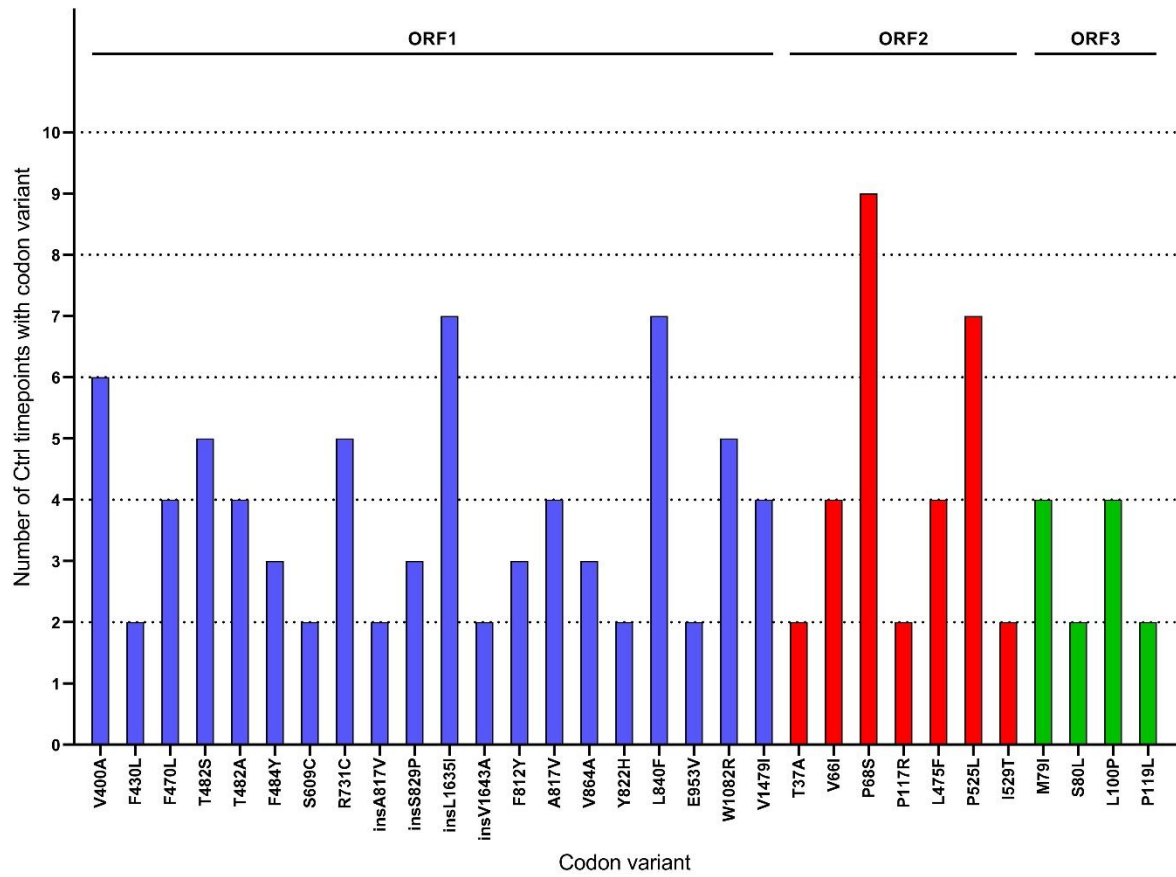


Figure 4. Non-syn. variants commonly detected in *in vitro* Ctrl lineages of both cultures. Bars indicate number of timepoints of detected variant with blue being within ORF1, red within ORF2, and green within ORF3.

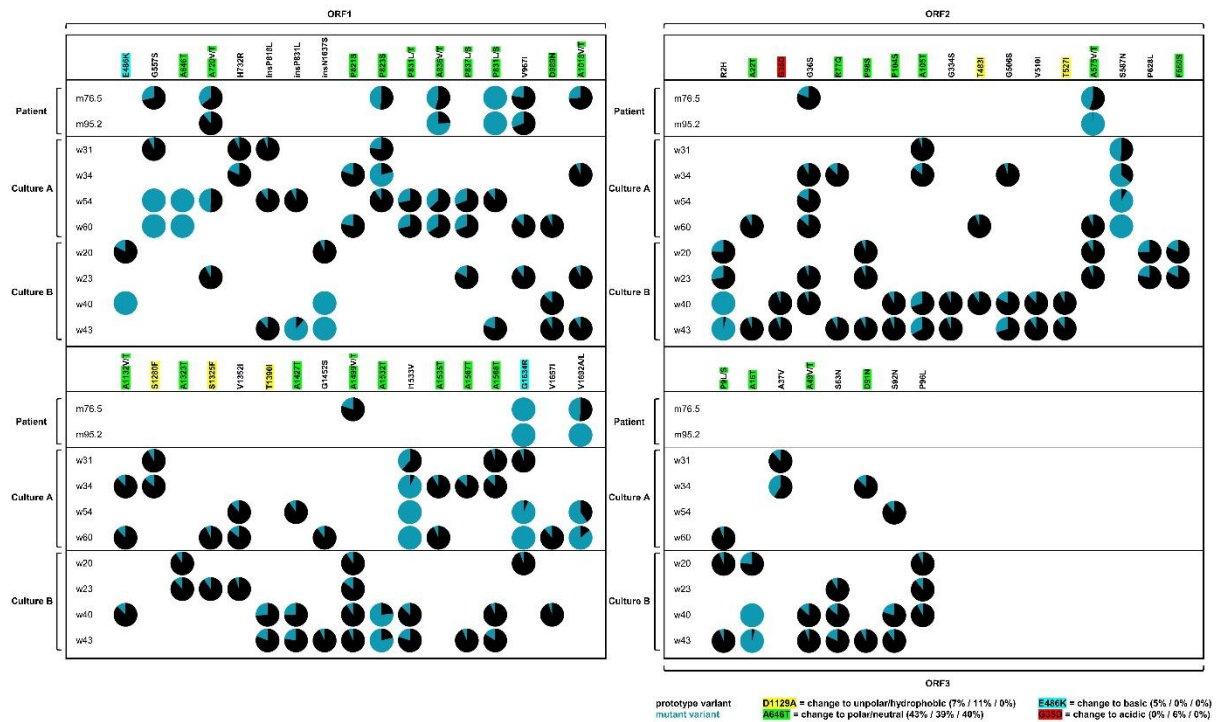


Figure 5. RBV-specific HEV variants detected in *in vitro* timepoints (Adapted; Naïve) compared to patient timepoints. Frequencies $\geq 5\%$ are depicted as pie charts with prototype 47832c variants in black and mutant variants in blue. ORF1-, ORF2-, and ORF3-specific variants are denoted by brackets. Changes in amino acid class are denoted by color code.

Supplementary Files

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- [HarmsetalHEVRibavirinsupplementarymaterial.pdf](#)