SUPPLEMENTARY MATERIAL AND METHODS

Amplification of HEV ORF1, ORF2 and ORF3 genome regions

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

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

Library preparation and Illumina sequencing

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

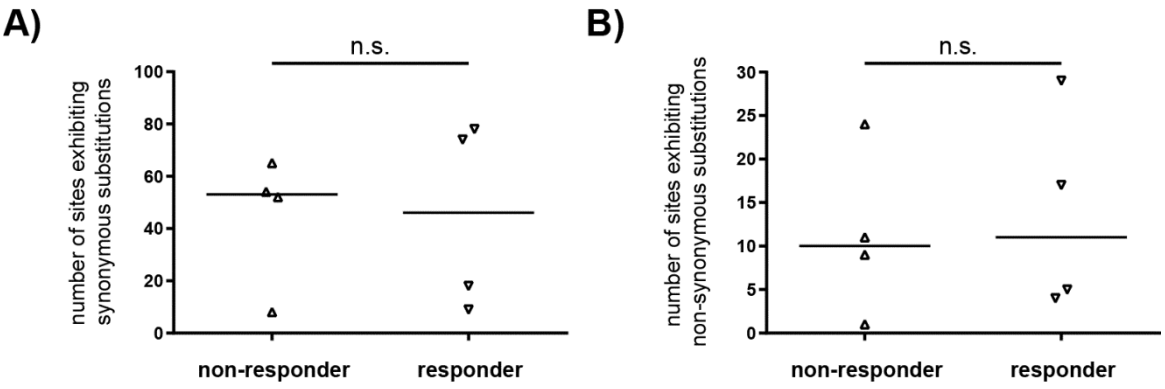
Evaluation of deep sequencing data

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

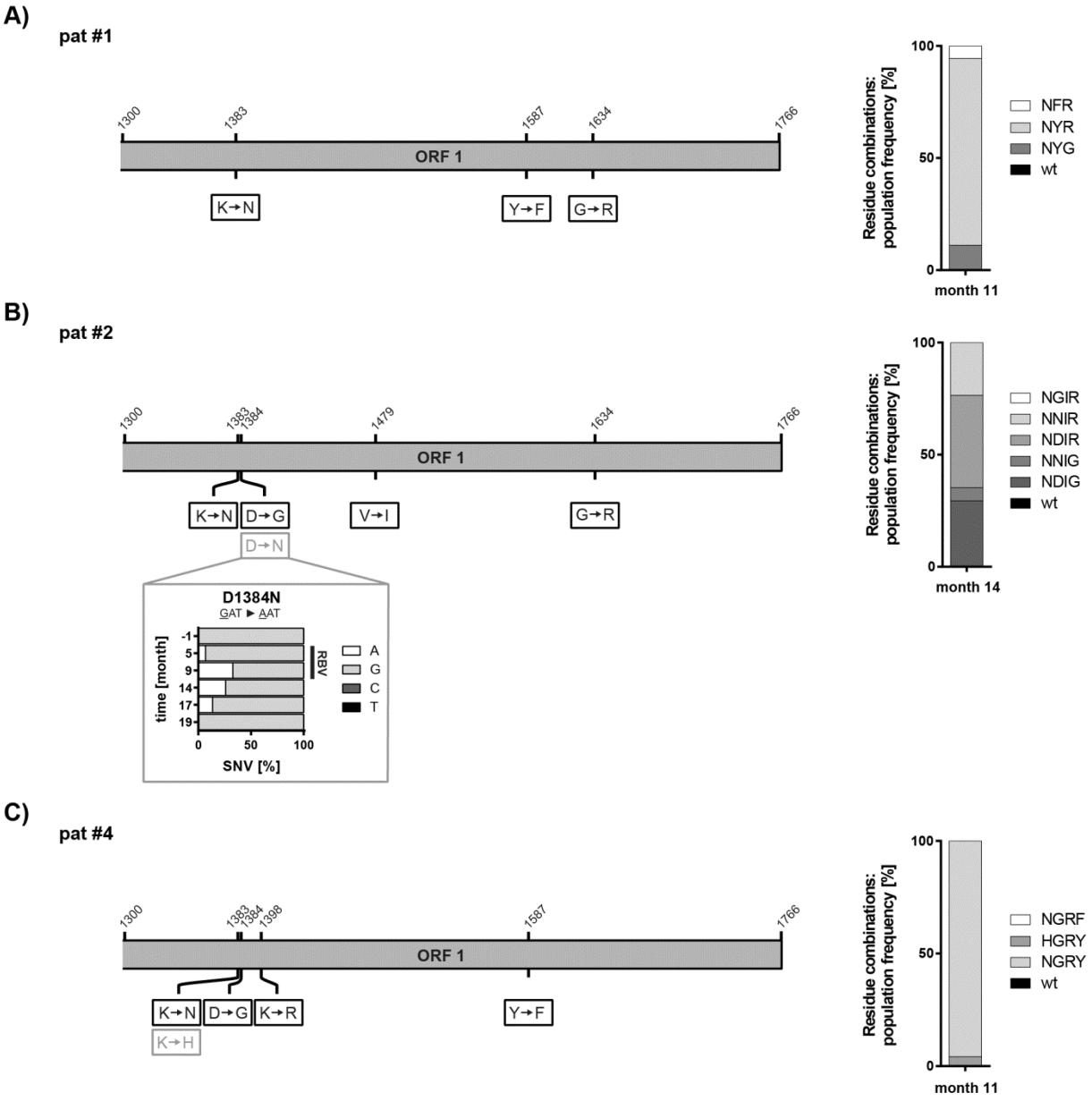
Clonal Sequencing

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

SUPPLEMENTARY FIGURES

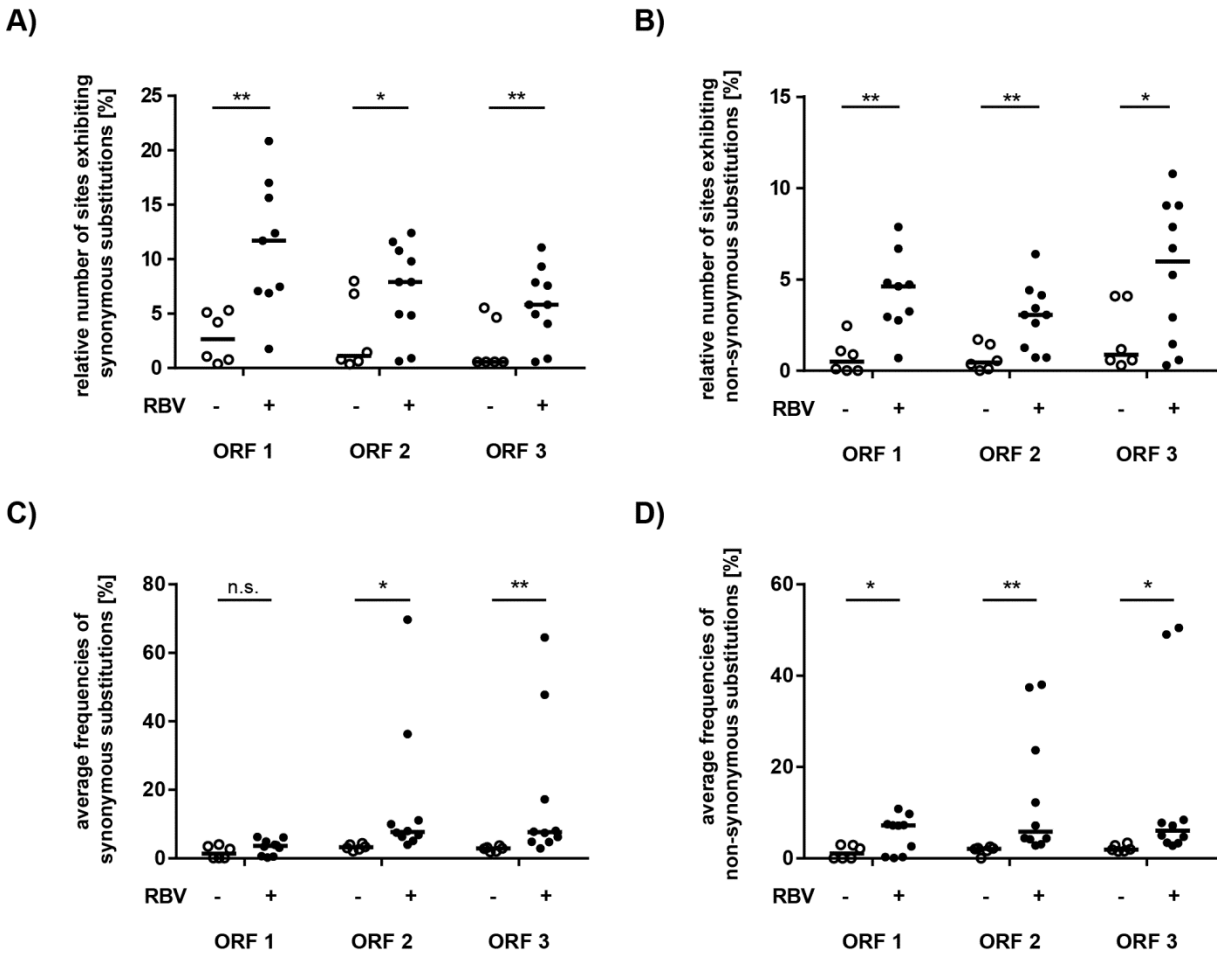


Supplement Figure 1: Comparison of the initial numbers of sites exhibiting nucleotide substitutions in the HEV ORF1 of chronically infected patients responding to RBV treatment vs. non-responder before the application of the first dose. Synonymous (**A**) and non-synonymous (**B**) nucleotide variants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

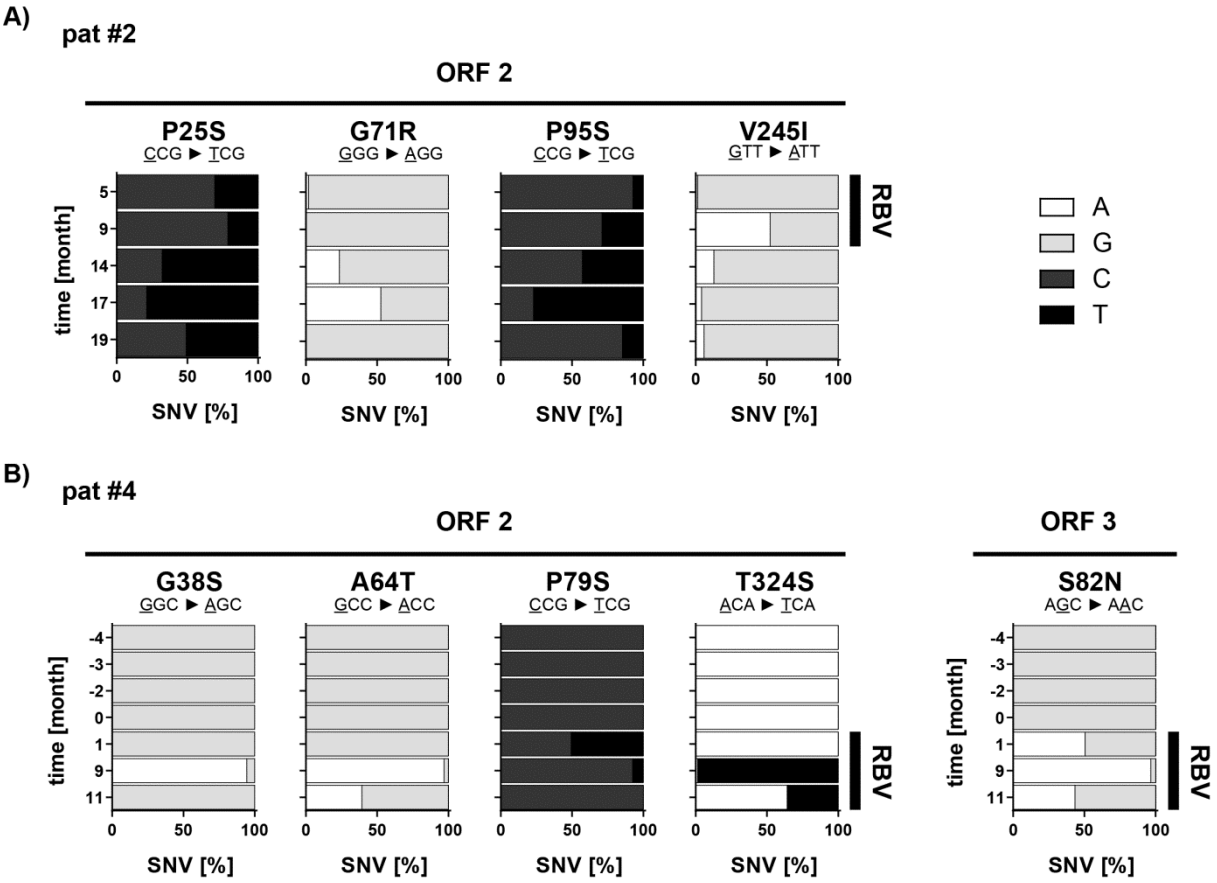


Supplement Figure 2: Linkage analysis of the identified mutations and their appearance in the viral populations. The single nucleotide variations (SNVs) identified in the three patients not achieving sustained virological responses after RBV treatment were analyzed in regards to their linkage and combinations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

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



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



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