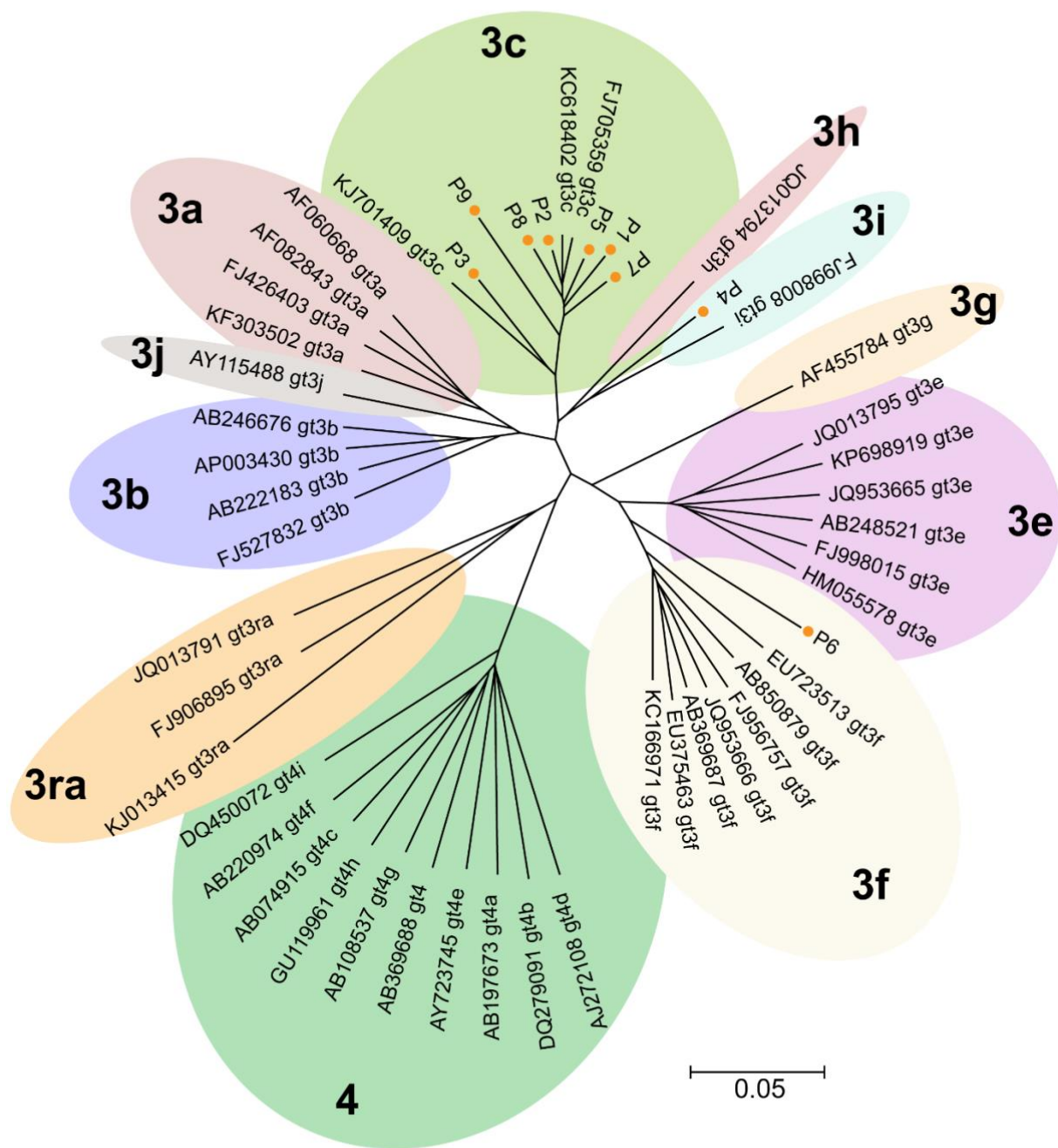


## Supplementary Information to

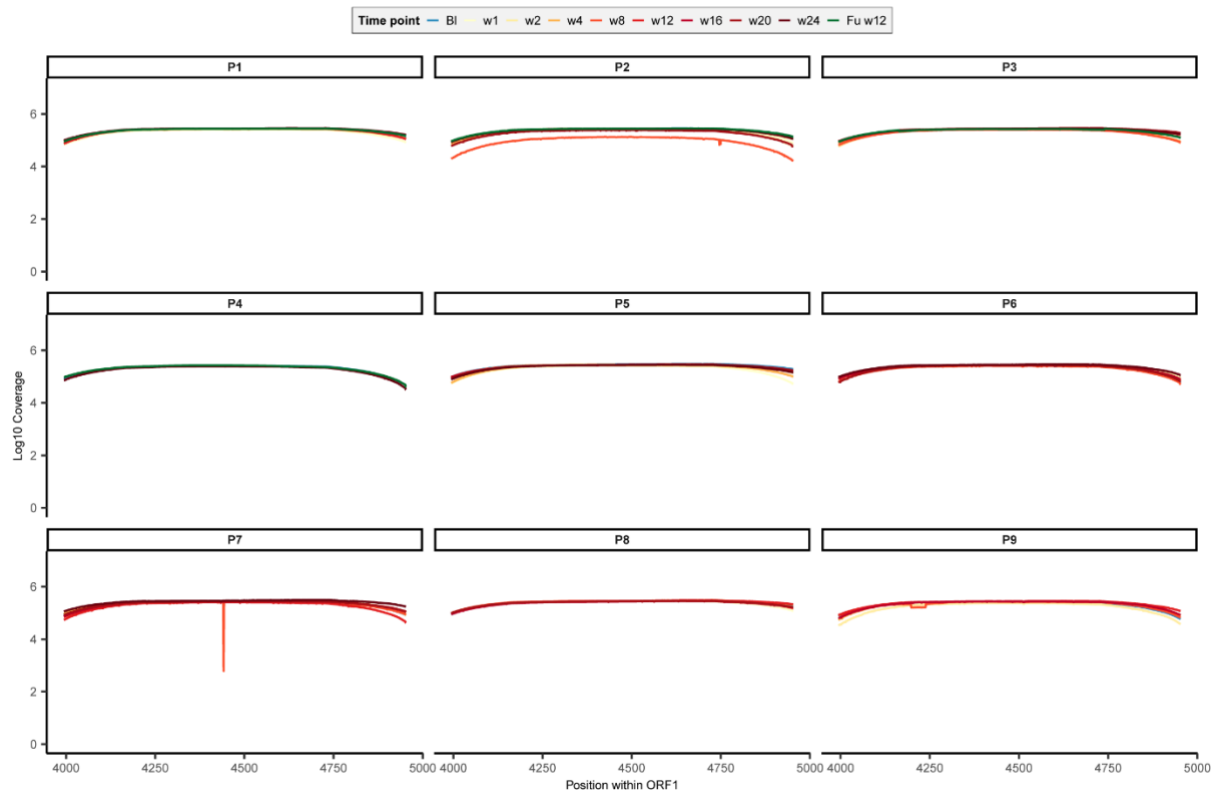
### **Emergence of resistance-associated variants during sofosbuvir treatment in chronically infected hepatitis E patients**

André Gömer<sup>1,\*</sup>, Mara Klöhn<sup>1,\*</sup>, Michelle Jagst<sup>1,2,\*</sup>, Maximilian Nocke<sup>1</sup>, Sven Pischke<sup>3,4</sup>, Thomas Horvatits<sup>3,4,5</sup>, Julian Schulze zur Wiesch<sup>3,4</sup>, Tobias Müller<sup>6</sup>, Svenja Hardtke<sup>7,8</sup>, Markus Cornberg<sup>7,9,10,11</sup>, Heiner Wedemeyer<sup>7,9,10</sup>, Patrick Behrendt<sup>9,10,12</sup>, Eike Steinmann<sup>1,13,\*†</sup>, Daniel Todt<sup>1,14,\*†</sup>



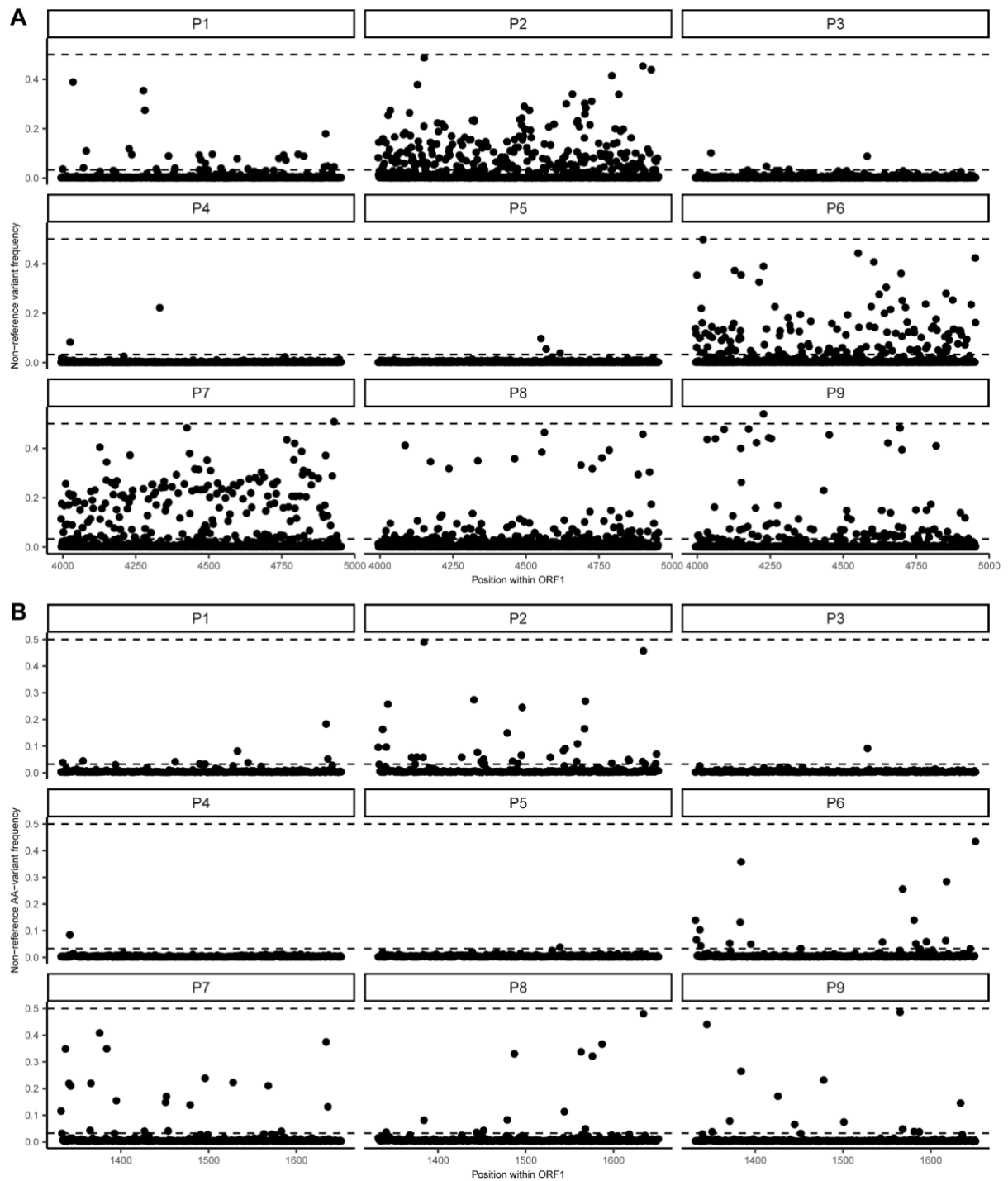
**Figure S1: Phylogenetic analysis of HEV genotypes in the patient cohort.**

Construction of a maximum-likelihood tree with haplotypes derived from the NGS data and NCBI deposited HEV sequences that depicts HEV subtypes.



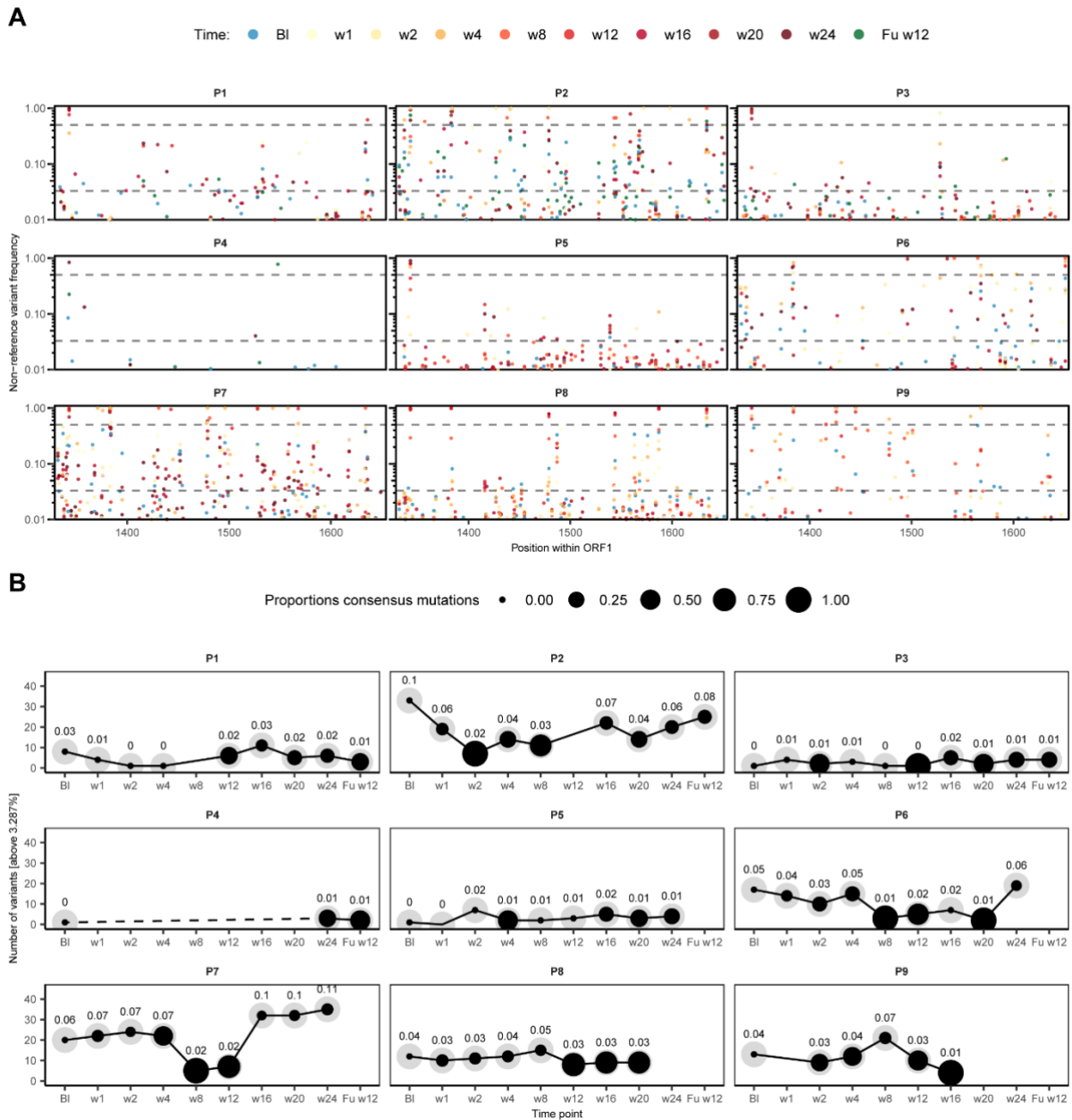
**Figure S2: Coverage statistics.**

Sequence coverage over the amplicon region for each patient (color coded) and each amplicon that was generated. Mean coverage 231,117 (sd: 56,587).



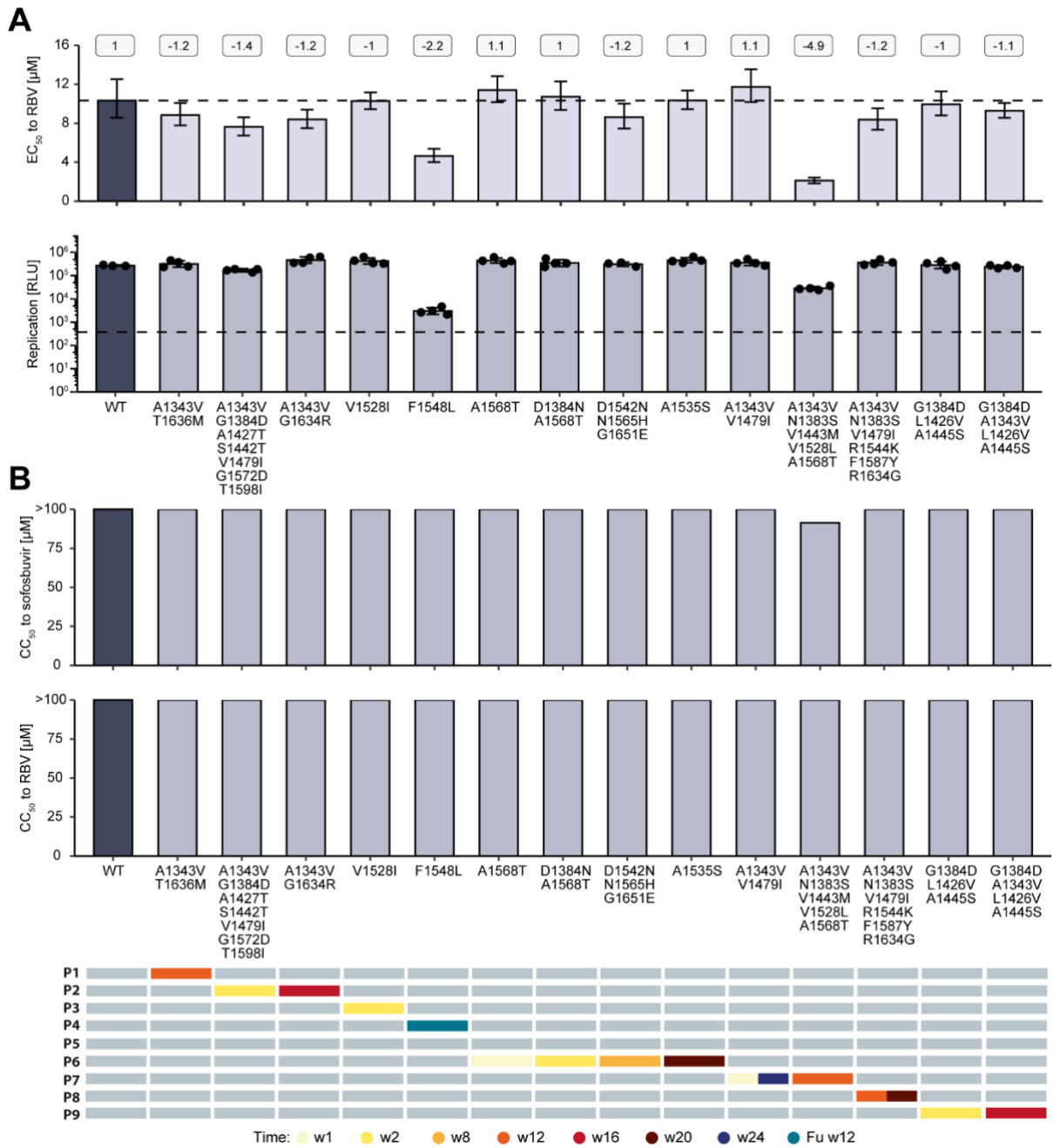
**Figure S3: Sequence diversity at baseline.**

A) Nucleotide diversity at baseline for each patient. All variant frequencies were below 50%, except in P7, position 936, and P9, position 234. For these positions, non-reference variant frequencies were at 50.8% and 53.9%, respectively. The major nucleotides present for P7 at position 936 were: A: 0.5%, C: 46%, T: 49%, G: 47%, and for P9 at position 234 were: A: 16%, C: 37.9%, T: 46%, G: 0.05%. B) At the amino acid level, all variants were below the consensus threshold.



**Figure S4: Effects of sofosbuvir on viral amino acid sequence diversity.**

A) Frequency of non-reference variants within the RdRp of HEV ORF1 compared to the time of treatment initiation (baseline). The cutoff for biological relevance (lower dashed line) was set at 3.287%, according to Illumina error rates. The top dashed line (50%) indicates consensus sequence mutations. Each dot represents a variant at a given time point which is color coded, see Legend. B) Quantification of variants for each patient and time point that occur above the error cutoff. The size ratio of the dot indicates the percentage of consensus sequence changes compared to the total number of mutations. Labels indicate size normalized variation.



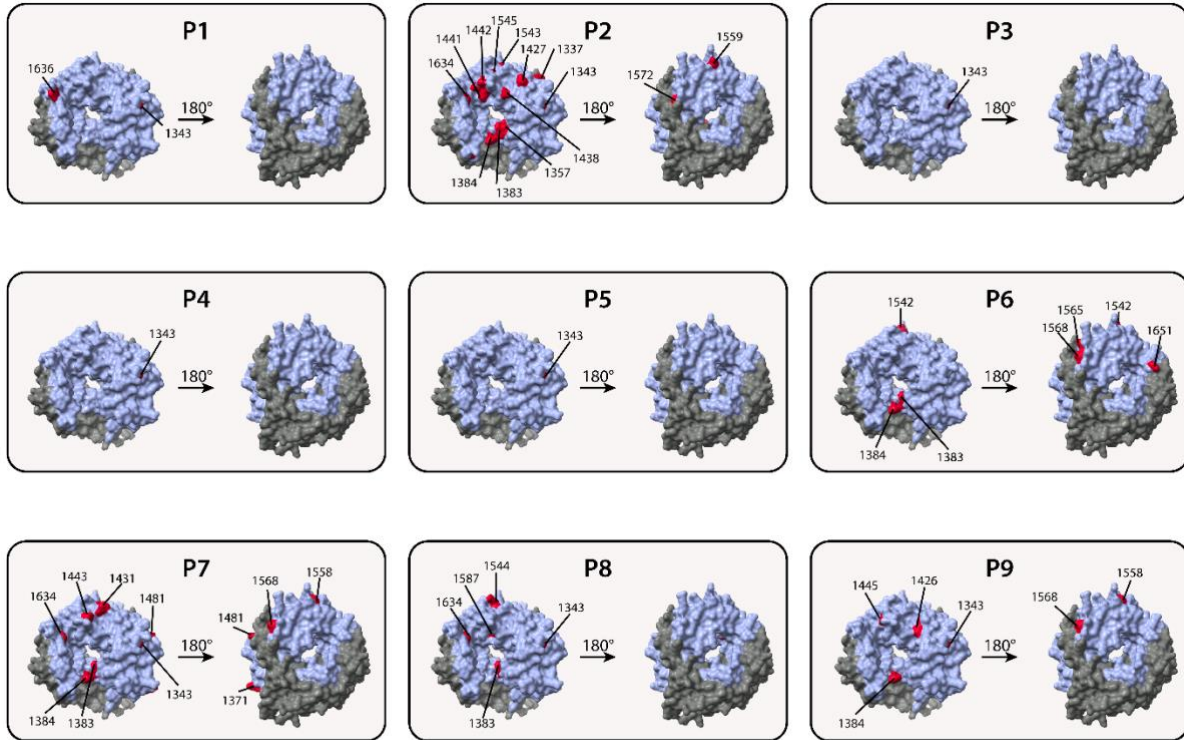
**Figure S5: Impact of patient-derived on sofosbuvir and ribavirin sensitivity and replication.**

A) Variant sensitivity (EC<sub>50</sub>) and replication capacity (relative light units) of HepG2 cells harboring patient-derived under ribavirin treatment. Dashed lines represent wild-type control (top panel) and replication-deficient control (GAA, bottom panel). Boxes above graph depict fold changes compared to WT. Top panel error bars = 95 % confidence interval, bottom panel error bars = ± SD. B) Cell viability was determined for patient-derived variants by MTT assay and plotted as half-maximum cytotoxic concentration (CC<sub>50</sub>) as determined by the four-parameter logistic model. n ≥ 3.



**Figure S6: Codon frequency evolution of A1343V.**

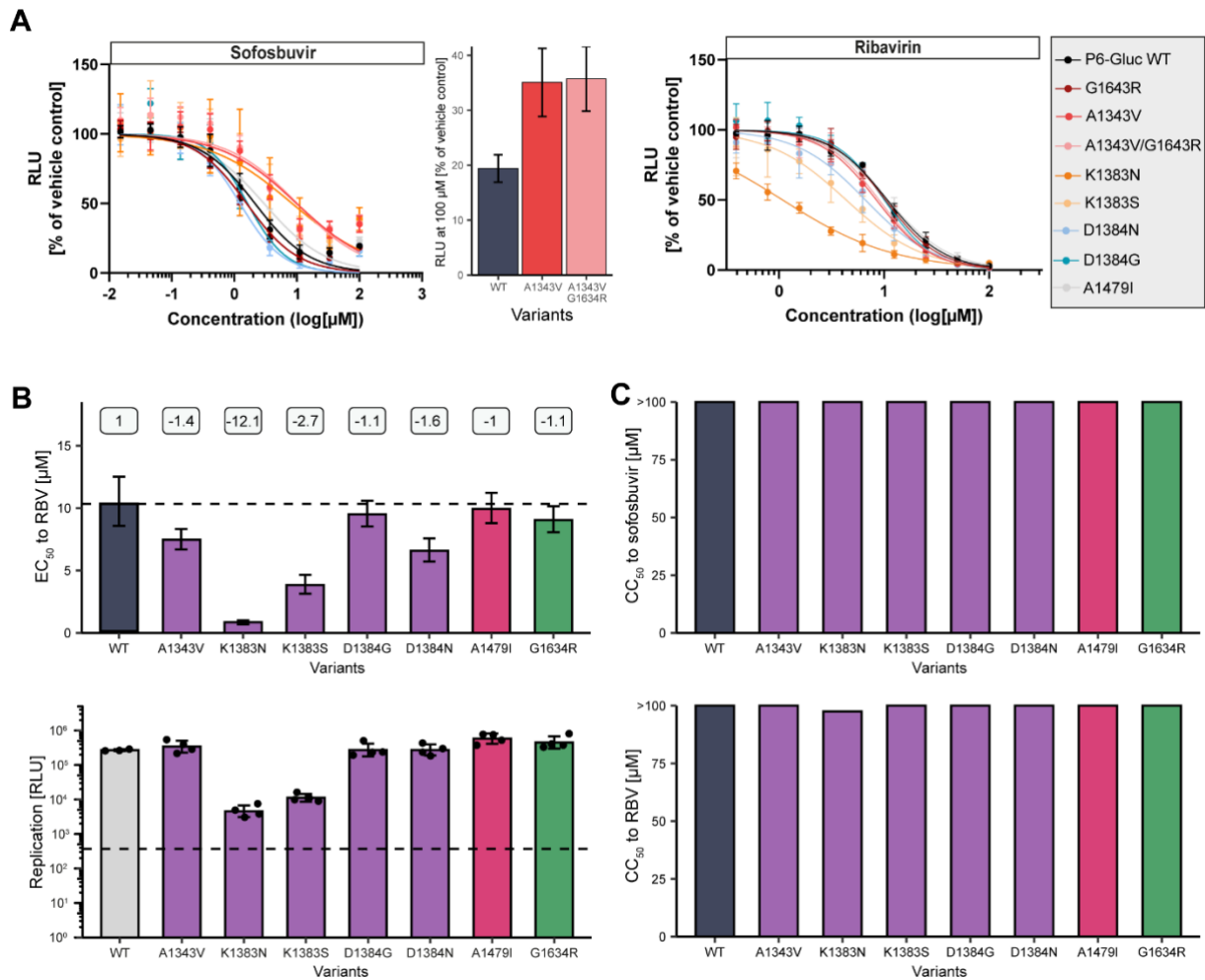
Frequency of the codon encoding the amino acid at position 1343. GCT, GCC, and GCG encode for alanine, while a transition (C->T) at the second codon position translates for valine (GTN). Letter height in bits indicates the frequency of each nucleotide at the respective codon position.



**Figure S7: Localization of patient-derived consensus substitution on the RdRp structure model.**

P6-based alpha-fold model of HEV RdRp. Models are shown from two sides (rotated 180° at y plane). Blue shows all residues that were included in the amplicon and grey highlights those that were not included. Residues that had consensus sequence changes were highlighted in red.

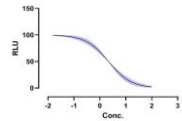
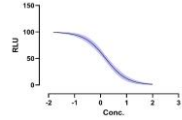
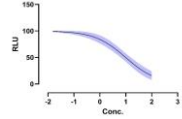
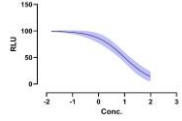
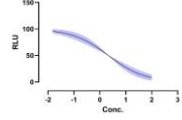
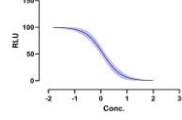
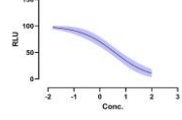
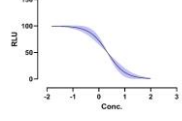


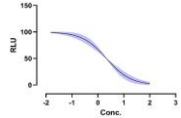
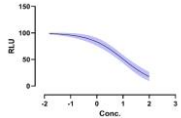
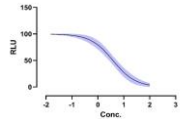
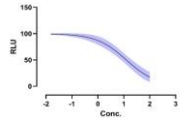
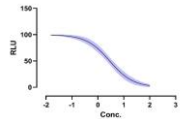
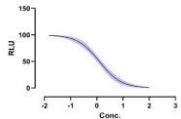
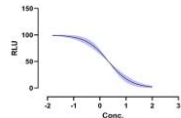
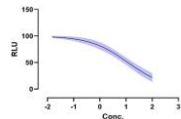
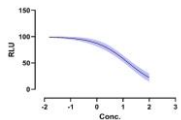


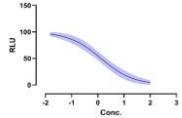
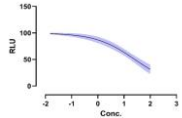
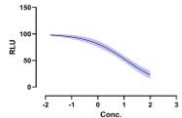
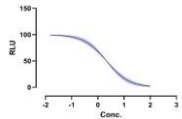
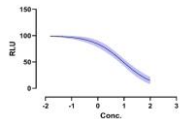
**Figure S8: Impact of single amino acid variants on sofosbuvir and ribavirin sensitivity and replication.**

(A) Dose-response curves of single amino acid (G1634R, A1343V, K1383N, K1383S, D1384N, D1384S) variants, p6-Gluc WT and the A1343V/G1634R variant were plotted and adjusted in GraphPad Prism to the non-linear fit regression model (left and right panel). Percent of relative light units at maximum sofosbuvir concentration (100  $\mu$ M) (middle panel). B) Sensitivity (EC<sub>50</sub>) and replication capacity (relative light units) of variants in HepG2 cells under ribavirin treatment. Dashed lines represent wild-type control (B, top panel) and replication-deficient control (GAA, bottom panel). Boxes above graph depict fold changes compared to WT. Top panel error bars = 95 % confidence interval, bottom panel error bars =  $\pm$  SD. C) Cell viability was determined for single amino acid variants by MTT assay and plotted as half-maximum cytotoxic concentration (CC<sub>50</sub>) as determined by the four-parameter logistic model. n  $\geq$  3.

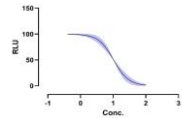
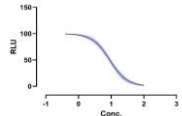
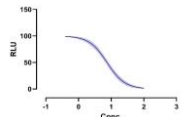
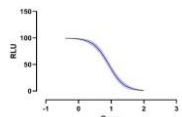
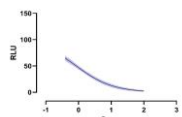
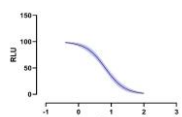
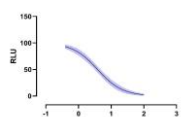
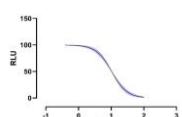
**Table S1: Dose-response parameters for sofosbuvir treatment.** EC<sub>50</sub>, EC<sub>10</sub>, EC<sub>90</sub> and CC<sub>50</sub> values with confidence intervals (CI). Significance of EC<sub>50</sub> and replication data indicated by p values. F = degree of freedom, DRC = dose-response curves.

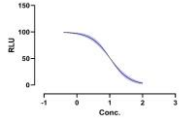
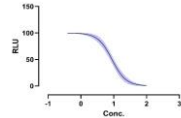
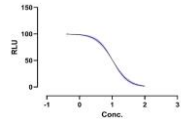
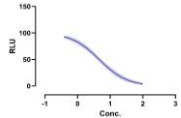
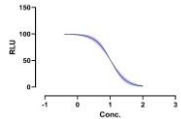
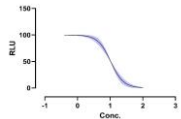
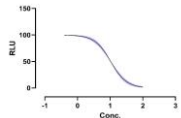
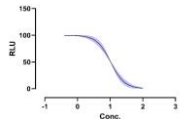
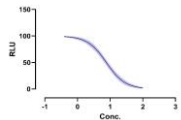
		EC50		EC10		EC90		CC50	test against wt		replication [RLU]		DRC curves
		value	CI	value	CI	value	CI	value	p-value	F (DFn, DFd)	fold-change to WT	p-values	
sofosbuvir	P6-Gluc WT	2.138	1.667 to 2.775	0.239	0.1429 to 0.4072	19.13	11.22 to 32.01	>100	NA	NA	1.0	NA	
	G1634R	1.575	1.286 to 1.939	0.1993	0.1305 to 0.3088	12.44	8.026 to 19.00	>100	0.0055	8.784 (1, 34)	1.6	0.6264	
	A1343V	9.913	6.474 to 15.84	0.481	0.2019 to 1.159	204.3	NA	>100	<0.0001	61.37 (1, 34)	1.5	0.8024	
	A1343V/G1634R	10.03	6.051 to 17.59	0.6298	0.2408 to 1.676	159.6	NA	>100	<0.0001	46.23 (1, 34)	1.8	0.1337	
	K1383N	3.525	2.123 to 6.135	0.08513	0.02898 to 0.2549	146	NA	>100	0.0532	4.120 (1, 25)	-25.3	<0,0001	
	D1384N	1.235	0.9867 to 1.551	0.2237	0.1414 to 0.3604	6.816	4.231 to 10.78	>100	<0.0001	22.66 (1, 34)	1.4	0.9367	
	K1383S	3.783	2.261 to 6.706	0.1253	0.04305 to 0.3739	114.2	NA	>100	0.0306	5.256 (1, 25)	-14.6	<0,0001	
	D1384G	1.625	1.201 to 2.247	0.3388	0.1894 to 0.6220	7.791	4.233 to 13.96	>100	0.0924	2.998 (1, 34)	1.1	0.9999	

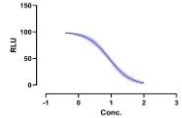
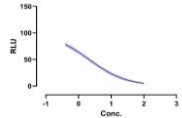
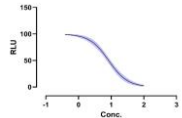
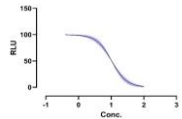
A1479I	2.978	2.092 to 4.348	0.2452	0.1200 to 0.5145	36.17	17.16 to 74.12	>100	0.0653	3.627 (1, 34)	2.6	0.0468	
T1636M (p1_w12)	10.48	6.680 to 17.26	0.4127	0.1639 to 1.045	266.1	NA	>100	<0.0001	59.57 (1, 34)	1.6	0.3689	
V1528I (p3_w2)	3.835	2.770 to 5.478	0.4259	0.2248 to 0.8272	34.54	17.73 to 65.55	>100	0.0006	14.13 (1, 34)	1.5	0.4634	
F1548L (p4_Fuw12)	12.58	7.866 to 21.12	0.671	0.2629 to 1.724	235.9	NA	>100	<0.0001	68.73 (1, 34)	-48.9	<0,0001	
A1568T (p6_w1)	2.865	2.185 to 3.814	0.2942	0.1695 to 0.5208	27.9	15.73 to 48.49	>100	0.0347	4.841 (1, 34)	2.0	0.0341	
A1568T/D1384N (p6_w2)	1.248	1.008 to 1.553	0.1556	0.09950 to 0.2471	10.01	6.301 to 15.65	>100	<0.0001	23.62 (1, 34)	1.8	0.1064	
A1535S (p6_w20)	2.602	1.918 to 3.600	0.2695	0.1459 to 0.5099	25.11	13.26 to 46.45	>100	0.2034	1.682 (1, 34)	1.9	0.4368	
V1479I/ A1343V (p7_w1,24)	11.78	7.523 to 19.50	0.301	0.1158 to 0.7766	460.9	NA	>100	<0.0001	68.67 (1, 34)	1.5	0.3689	
P2_w2	16.24	11.31 to 24.05	0.6792	0.3166 to 1.452	388.3	NA	>100	<0.0001	151.2 (1, 34)	-1.5	0.2731	

P6_w8	1.332	0.8516 to 2.094	0.0591	0.02331 to 0.1549	30.02	11.43 to 76.19	>100	0.0412	4.504 (1, 34)	1.6	0.4009	
P7_w12	25.93	16.60 to 43.78	0.5948	0.2288 to 1.488	1131	NA	91.41	<0.0001	161.4 (1, 34)	-6.1	<0,0001	
P8_w12, 20	12.42	8.420 to 19.07	0.2871	0.1235 to 0.6627	537.2	NA	>100	<0.0001	96.14 (1, 34)	1.5	0.4346	
P9_w2	2.196	1.769 to 2.747	0.2516	0.1601 to 0.4012	19.18	12.02 to 30.14	>100	0.8039	0.06261 (1, 34)	1.5	0.3714	
P9_w16	9.392	6.577 to 13.77	0.4925	0.2366 to 1.037	179.1	NA	>100	<0.0001	80.99 (1, 34)	1.1	0.9996	

**Table S2: Dose-response parameters for ribavirin treatment.** EC<sub>50</sub>, EC<sub>10</sub>, EC<sub>90</sub> and CC<sub>50</sub> values with confidence intervals (CI). Significance of EC<sub>50</sub> and replication data indicated by p values. F = degree of freedom, DRC = dose-response curves.

		EC50		EC10		EC90		CC50	test against wt		replication [RLU]		DRC curves
		value	CI	value	CI	value	CI	value	p-value	F (DFn, DFd)	fold-change to WT	p-values	
ribavirin	P6-Gluc WT	10.34	8.564 to 12.52	3.197	2.198 to 4.728	33.46	22.63 to 48.68	>100	NA	NA	1.0	NA	
	G1634R	9.048	8.064 to 10.15	2.393	1.880 to 3.070	34.2	26.66 to 43.56	>100	0.0233	5.645 (1, 34)	1.8	0.2825	
	A1343V	7.462	6.685 to 8.327	1.887	1.497 to 2.395	29.51	23.25 to 37.21	>100	<0.0001	36.43 (1, 34)	1.3	0.9054	
	A1343V/G1634R	8.394	7.502 to 9.393	2.545	2.012 to 3.242	27.69	21.74 to 35.02	>100	0.0006	14.25 (1, 34)	1.8	0.0798	
	K1383N	0.8556	0.7070 to 1.014	0.05092	NA	14.38	9.944 to 21.04	97.54	<0.0001	863.1 (1, 34)	-56.0	<0.0001	
	D1384N	6.588	5.720 to 7.577	1.473	1.098 to 1.995	29.47	21.75 to 39.55	>100	<0.0001	42.86 (1, 34)	1.1	>0,9999	
	K1383S	3.833	3.142 to 4.656	0.5599	-infinity to 0.8626	26.24	17.13 to 39.72	>100	<0.0001	103.0 (1, 34)	-23.6	<0.0001	
	D1384G	9.502	8.533 to 10.59	2.935	2.345 to 3.698	30.76	24.41 to 38.49	>100	0.1174	2.581 (1, 34)	1.1	>0,9999	

A1479I	9.93	8.782 to 11.22	2.267	1.748 to 2.961	43.5	33.27 to 56.44	>100	0.4948	0.4764 (1, 34)	2.2	0.0464	
T1636M (p1_w12)	8.843	7.784 to 10.07	2.997	2.313 to 3.921	26.1	19.95 to 33.81	>100	0.0192	6.045 (1, 34)	1.2	0.9908	
V1528I (p3_w2)	10.27	9.440 to 11.17	3.031	2.537 to 3.638	34.77	28.97 to 41.55	>100	0.8441	0.03926 (1, 34)	1.6	0.2259	
F1548L (p4_Fuw12)	4.632	3.998 to 5.371	0.5501	0.3970 to 0.7679	39	28.06 to 53.86	>100	<0.0001	115.5 (1, 25)	-86.3	<0,0001	
A1568T (p6_w1)	11.41	10.16 to 12.83	3.427	2.689 to 4.401	38.02	29.60 to 48.45	>100	0.0965	2.922 (1, 34)	1.7	0.1522	
A1568T/D1384N (p6_w2)	10.72	9.358 to 12.30	3.944	2.998 to 5.245	29.11	21.91 to 38.26	>100	0.6089	0.2666 (1, 34)	1.3	0.8415	
A1535S (p6_w20)	10.35	9.442 to 11.35	3.002	2.475 to 3.659	35.66	29.26 to 43.25	>100	0.9922	9.636e-005 (1, 34)	1.7	0.108	
V1479I/ A1343V (p7_w1,24)	11.73	10.17 to 13.55	3.946	2.955 to 5.330	34.87	25.82 to 46.56	>100	0.083	3.189 (1, 34)	1.3	0.7509	
P2_w2	7.617	6.741 to 8.613	1.767	1.366 to 2.304	32.83	25.17 to 42.49	>100	<0.0001	25.17 (1, 34)	-1.6	0.1754	

P6_w8	8.632	7.457 to 9.998	1.66	1.219 to 2.280	44.89	32.63 to 61.19	>100	0.017	6.297 (1, 34)	1.2	0.999	
P7_w12	2.097	1.814 to 2.413	0.118	NA	37.26	26.79 to 51.89	>100	<0.0001	485.4 (1, 34)	-9.4	<0,0001	
P8_w12, 20	8.367	7.340 to 9.531	1.928	1.465 to 2.559	36.32	27.35 to 47.82	>100	0.0022	11.00 (1, 34)	1.4	0.7059	
P9_w2	9.945	8.793 to 11.26	3.109	2.409 to 4.049	31.81	24.43 to 41.05	>100	0.5148	0.4333 (1, 34)	1.1	0.9997	
P9_w16	9.284	8.555 to 10.08	3.047	2.565 to 3.637	28.29	23.70 to 33.60	>100	0.0112	7.204 (1, 34)	-1.1	0.999	