Highly replicating hepatitis C virus variants emerge in immunosuppressed patients causing severe disease

Paul Rothhaar, Christian Heuss, Margaret Tulessin, Zhiqing Wang, Ha Gyu-Thomas Seong, Colin Förster, Jocelyn Quistrebert, Haiting Chai, Marvin Reineke, Louise Benning, Jonathan Honegger, Maike Hofmann, Robert Thimme, Jörg Timm, STOPHCV investigators, Paul Schnitzler, Uta Merle, Naglaa H. Shoukry, Julie Bruneau, Chaturaka Rodrigo, Andrew Lloyd, Rowena A. Bull, M. Azim Ansari, Carolin Mogler, John McLauchlan, Xavier Forns, Sofía Pérez-del-Pulgar, Volker Lohmann

Supplementary Figure S1-S9

Supplementary Table S1-S3

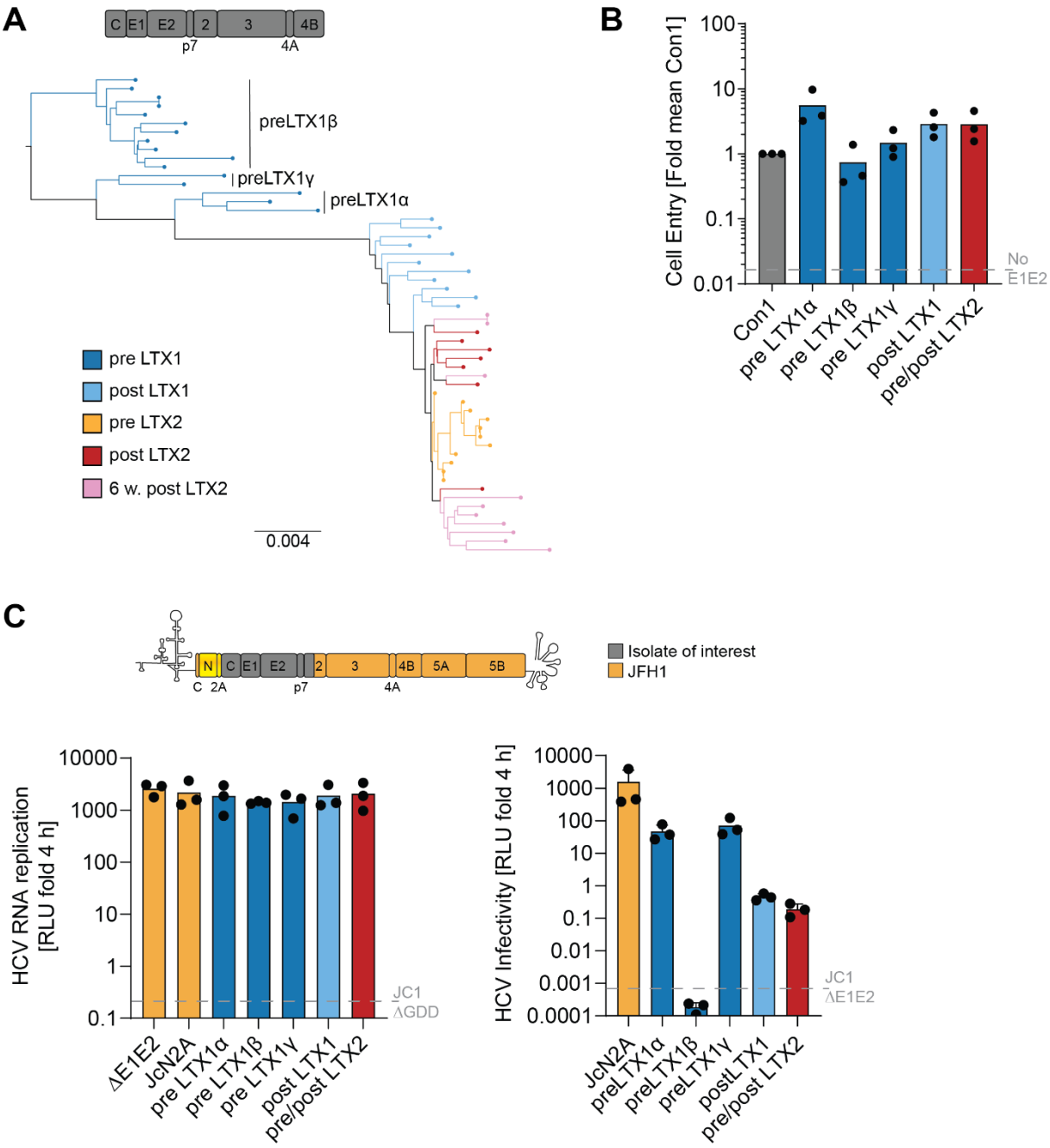


Fig. S1: Phenotypic characterization of the evolution of the assembly module in the GLT1 patient. A) Phylogenetic tree for the first PCR fragment which includes the whole assembly module. B) HCVpp bearing HCV envelope proteins of the indicated isolates on their surface were added to Huh7-Lunet CD81 cells. After 72 h, cells were lysed, and infectivity was determined based on a luciferase reporter in the HCVpp. Measurements were first normalised to the input determined via SG-PERT and then normalised to the Con1 reference isolate. Particles without envelope proteins (No E1E2) served as negative control. C) Huh7-Lunet CD81 cells were transfected with the chimeric full-length HCV reporter construct depicted in the upper panel. It combines the sequence of the isolate of interest up to the C3 junction site in NS2 with the remaining part of the JFH1 isolate[89]. RNA replication was determined at 72 hours post transfection (left panel) and the supernatant was used to infect naïve Huh7-Lunet CD81 cells. At 72 hours post infection, cells were lysed, and infectivity was determined via luciferase measurement (right panel). A replication deficient JC1 variant (JC1 ΔGDD) and a JC1 variant lacking envelope proteins (JC1 ΔE1E2) were used as negative controls for replication and infection respectively. B&C) Data

are from three independent biological replicates measured in technical duplicates. Each dot depicts the result of one replicate and the bar indicates the mean of all replicates.

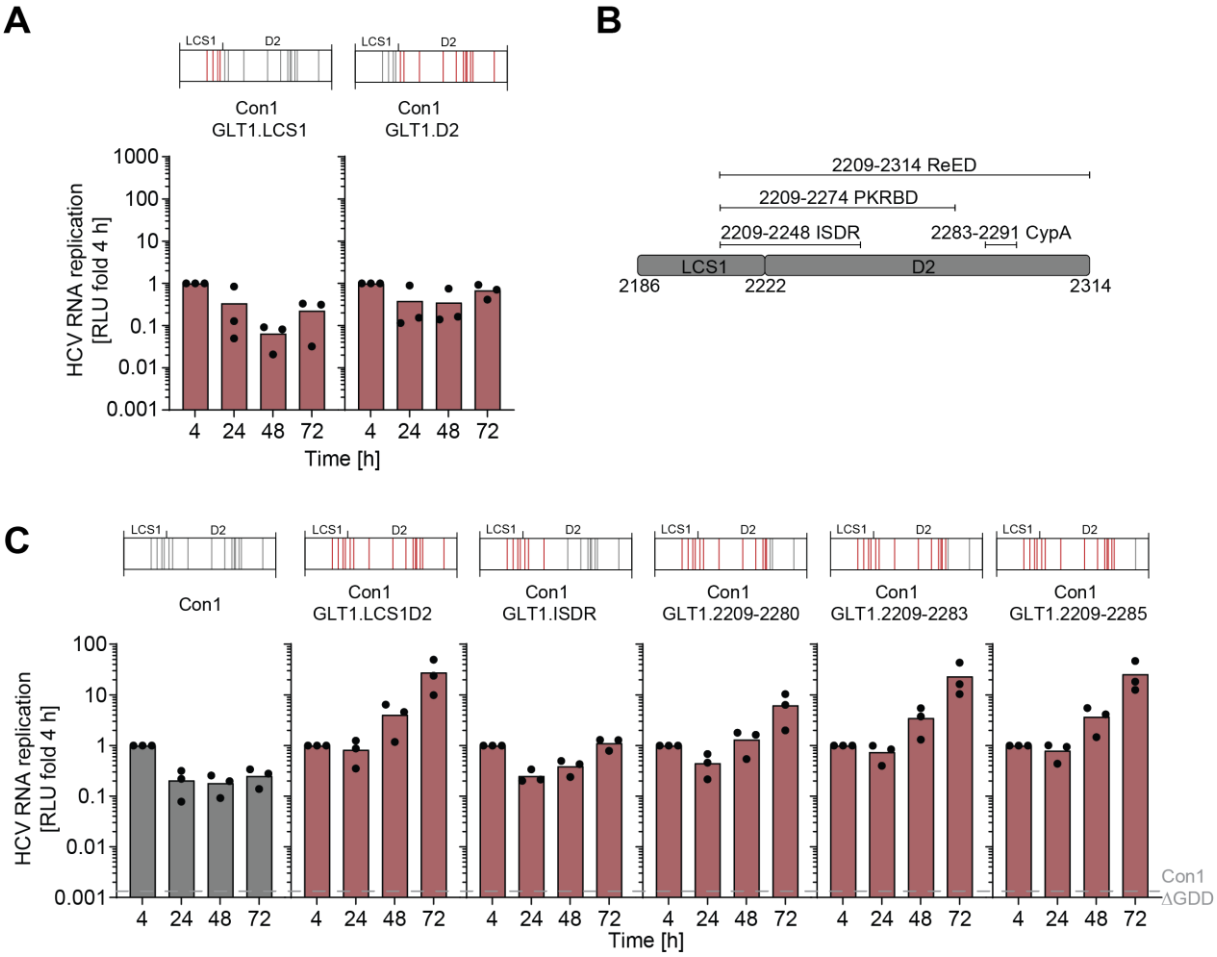


Fig. S2: Mapping of relevant residues in the ReED. A&C) SGRs of the indicated constructs were electroporated into Huh7-Lunet SEC14L2 cells, luciferase activity in cell lysates (RLU) was quantified as a correlate of RNA replication efficiency at the given time points and normalised to 4 h to account for differences in transfection efficiency. Con1 ΔGDD served as a replication deficient negative control. Data are from three independent biological replicates measured in technical duplicates. Each dot depicts the result of one replicate and the bar indicates the mean of all replicates. B) Schematic illustrating LCS1D2, informing about the location of the ReED, the ISDR[18], the PKR binding domain (PKRBD)[24] and the Cyclophilin A (CypA) binding site[23].

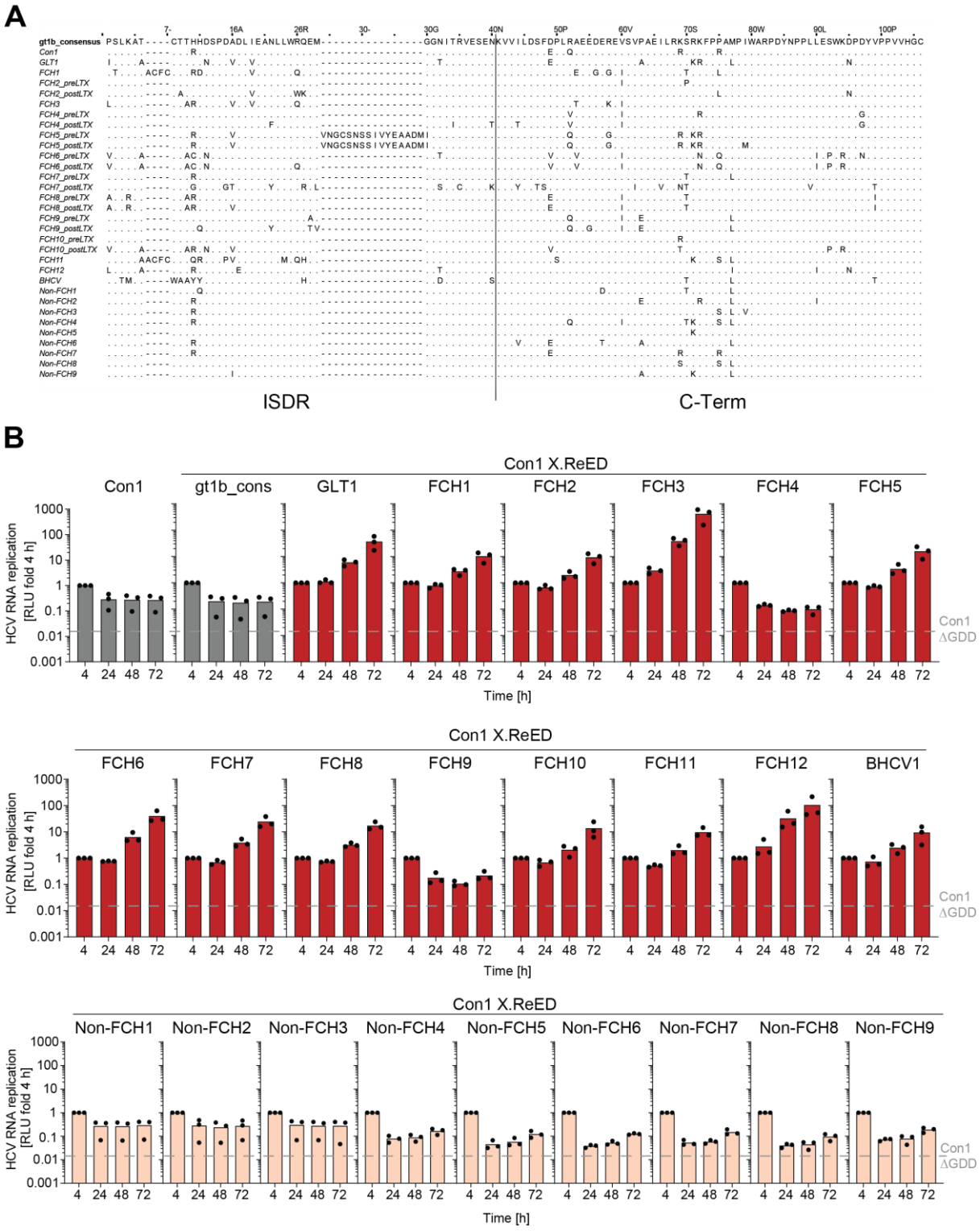
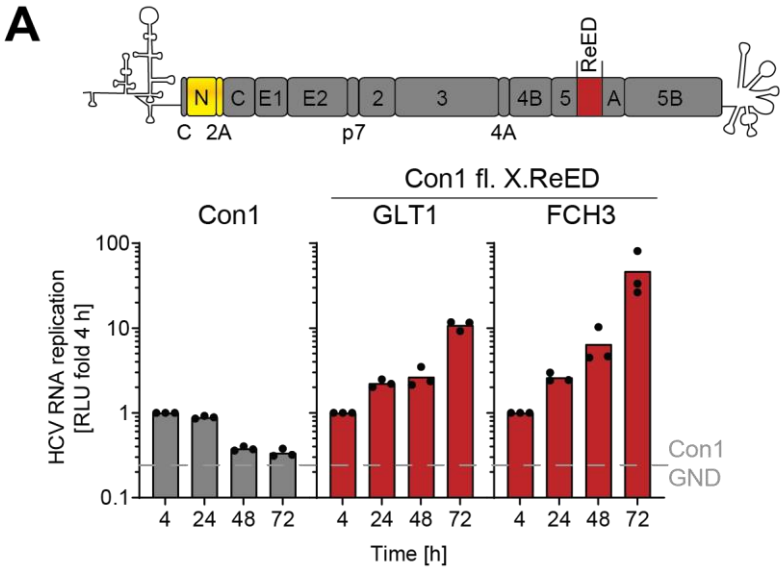


Fig. S3: Replication kinetics of SGRs from the FCH cohort. A) Amino acid alignment of all ReED sequences from the FCH cohort, using the genotype 1b consensus ReED as a reference. Dots indicate an amino acid being identical to the gt1b consensus. Note that also pre LTX sequences are shown when available. B) SGRs of the indicated constructs were electroporated into Huh7-Lunet SEC14L2 cells, luciferase activity in cell lysates (RLU) was quantified as a correlate of RNA replication efficiency at the given time points and normalised to 4 h to account for differences in transfection efficiency. Con1 ΔGDD served as a replication deficient negative control. Data are

from three independent biological replicates measured in technical duplicates. Each dot depicts the result of one replicate and the bar indicates the mean of all replicates.



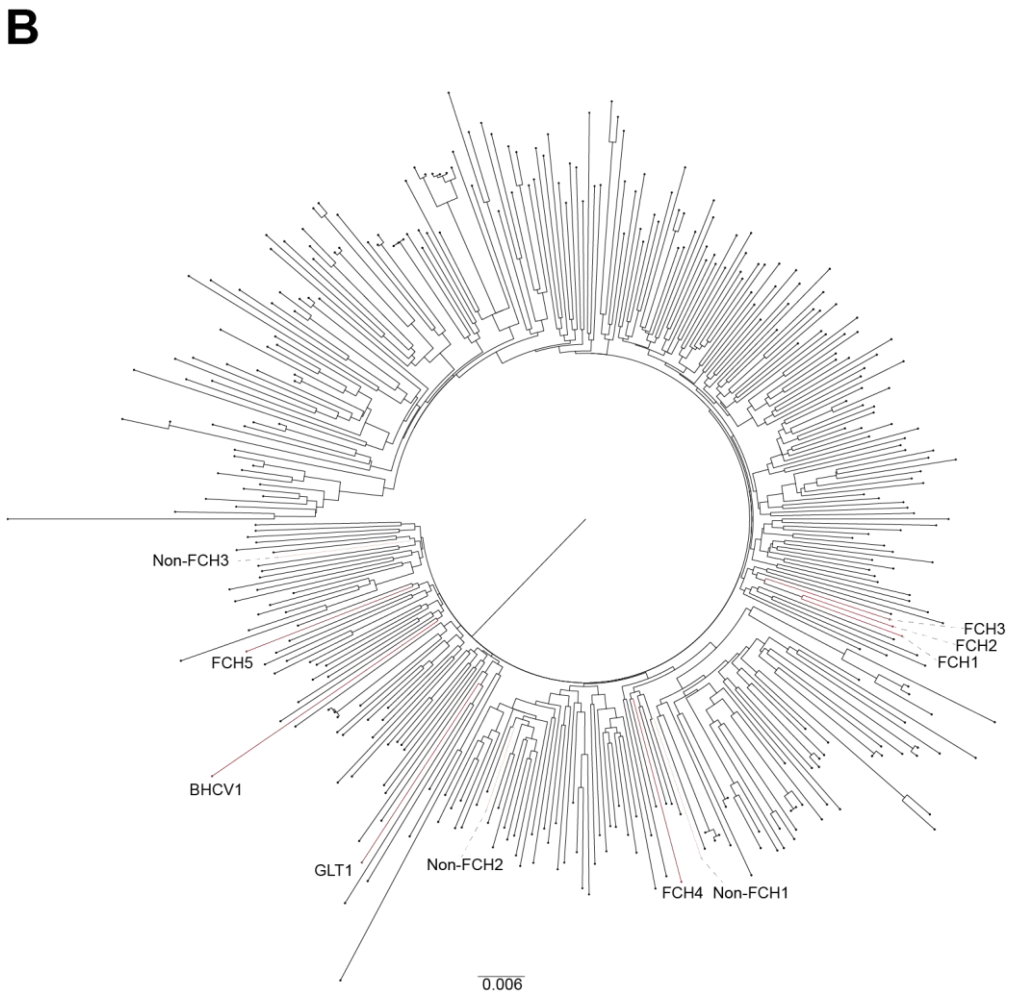


Fig. S4: Full-length and phylogenetic studies on the FCH cohort. A) Full-length replicons of the indicated constructs were electroporated into Huh7-Lunet SEC14L2 cells, luciferase activity in cell lysates (RLU) was quantified as a correlate of RNA replication efficiency at the given time points and normalised to 4 h to account for differences in transfection efficiency. Con1 GND

served as replication deficient negative controls. Data are from three independent biological replicates measured in technical duplicates. Each dot depicts the result of one replicate and the bar indicates the mean of all replicates. B) Phylogenetic tree of gt1b isolates retrieved from ViPR[74]. Highlighted are the 7 FCH (red) and 3 non-FCH (apricot) patients for which complete replicase sequences post LTX were available. Accession numbers: FCH1 - MK092096, FCH2 - MK092098, FCH3 - MK092100, FCH4 - MK092102, FCH5 - MK092104, BHCV1 - HQ719473, GLT1 - OM222702, Non-FCH1 - MK092106, Non-FCH2 - MK092108, Non-FCH3 - MK092110.

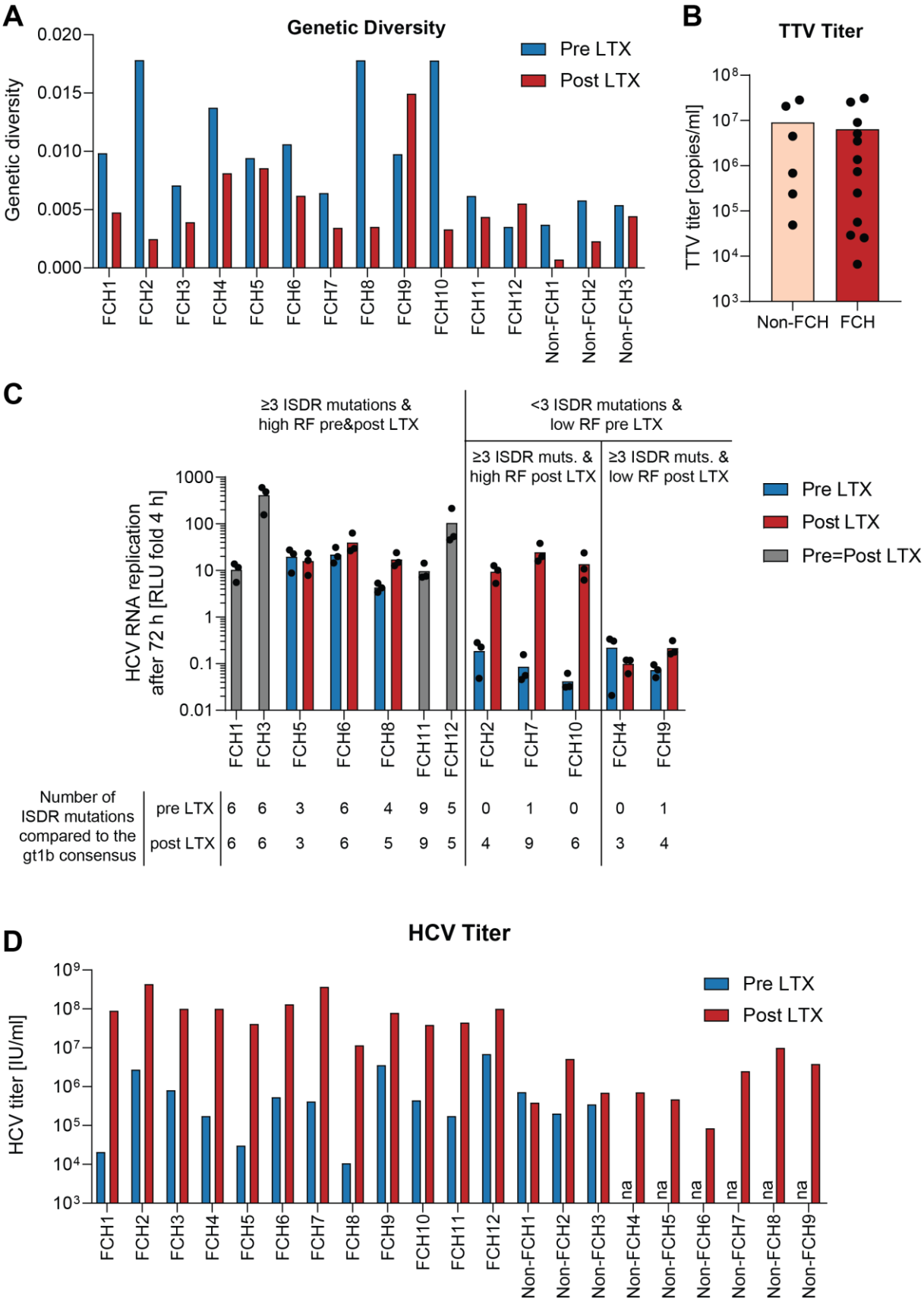


Fig. S5: Patient characteristics and evolution of RF in the FCH cohort. A&D) Data on genetic diversity based on NGS analysis of NS5B (A) and HCV serum titers pre and post LTX for FCH1-12 and non-FCH1-3 (D) were previously published in bulk[26] and are here stratified by patient. No genetic diversity data and no pre LTX titers were available for non-FCH4-9. B) Torque teno virus

(TTV) titers in post LTX patient serum were determined via qPCR. C) For the determination of ISDR mutations (lower panel), an insertion was counted as one mutation irrespective of its length. SGRs of the indicated constructs were electroporated into Huh7-Lunet SEC14L2 cells, luciferase activity in cell lysates (RLU) was quantified as a correlate of RNA replication efficiency and normalised to 4 h to account for differences in transfection efficiency. Displayed is the time point 72 h post electroporation, data for the post LTX samples is taken from (Fig. S3B). Data are from three independent biological replicates measured in technical duplicates. Each dot depicts the result of one replicate and the bar indicates the mean of all replicates.

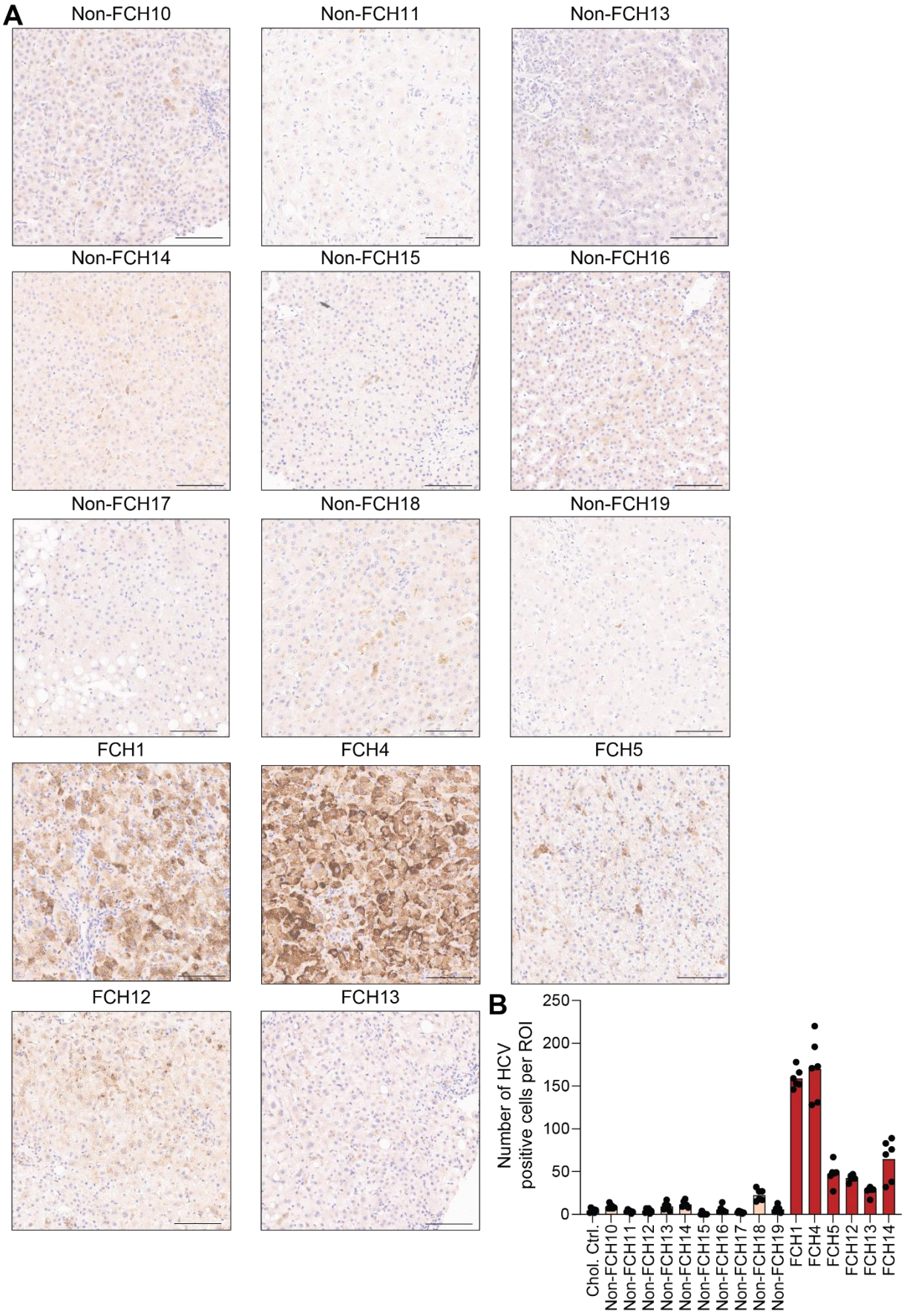


Fig. S6: Representative images of IHC stained liver tissue sections. A) An HCV NS5A specific antibody was used for staining. Representative images showing one quantified ROI per patient for

the patients not displayed in Fig. 3D. Scale bars indicate 100 µm. B) Quantification displayed in Fig. 3D stratified by patient. Chol. Ctrl. represents an HCV negative cholestatic liver. Note that only patients FCH1, 4, 5 and 12 overlap with the patients characterised in Fig. 3A-C due to limited availability of samples.

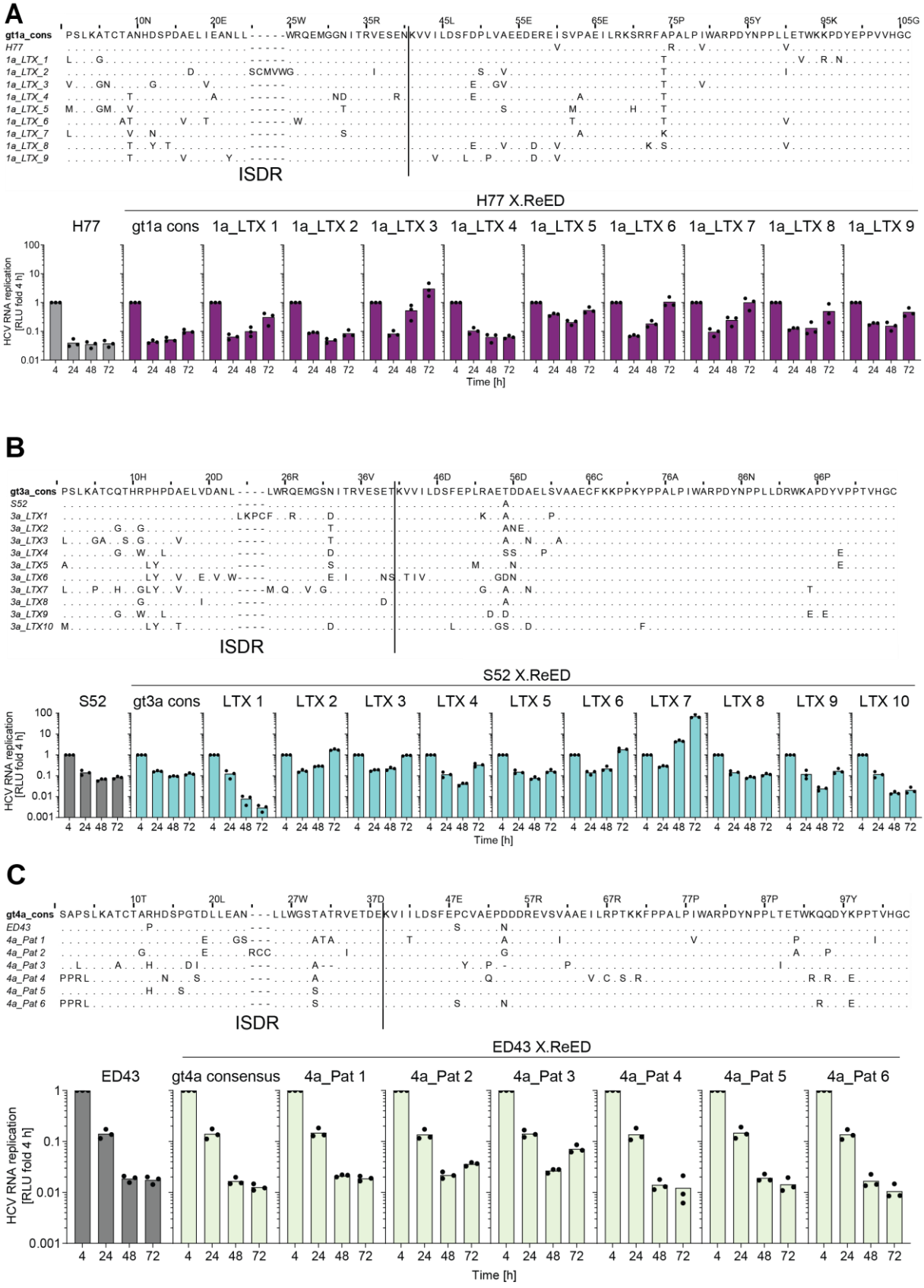


Fig. S7: Replication kinetics of chimeric SGRs from gts 1a, 3a and 4a. Upper panels depict amino acid sequence alignments of patient derived ReEDs compared to either the gt1a (A), gt3a (B) or gt4a (C) consensus. Dots indicate an amino acid being identical to the gt1b consensus. Lower panels show replication data. SGRs of the indicated constructs were electroporated into

Huh7-Lunet SEC14L2 cells, luciferase activity in cell lysates (RLU) was quantified as a correlate of RNA replication efficiency at the given time points and normalised to 4 h to account for differences in transfection efficiency. H77 ΔGDD/ Con1 ΔGDD served as a replication deficient negative control. Data are from three independent biological replicates measured in technical duplicates. Each dot depicts the result of one replicate and the bar indicates the mean of all replicates.

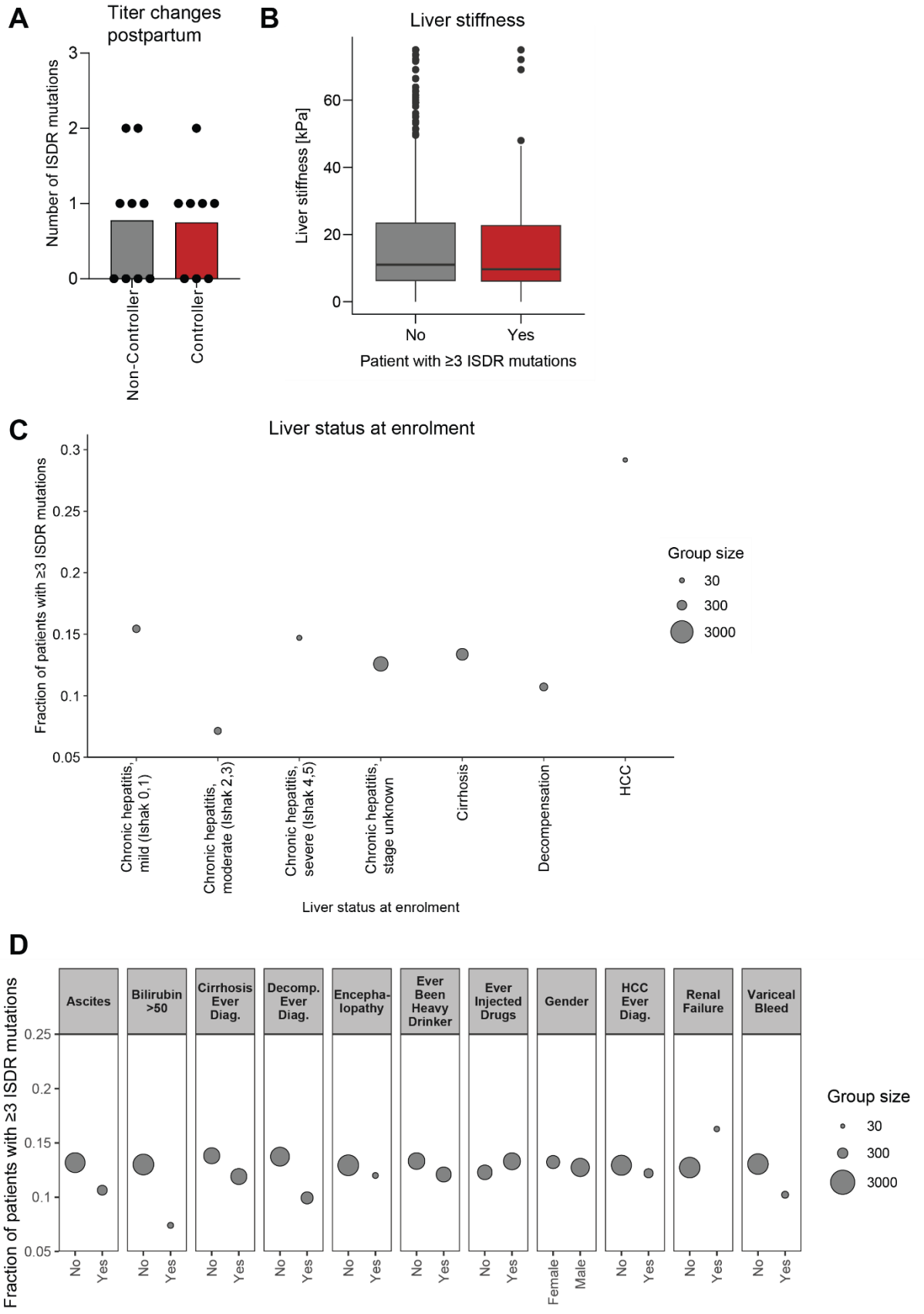


Fig. S8: Sequence signatures of high RF in various clinical contexts. A) HCV serum titers for each patient were determined in the third trimester of pregnancy and 3 months postpartum. If the HCV titers dropped by more than 1 log postpartum, patients were considered controllers[37].

Combined data of 10 gt1a, 1 gt1b, 1 gt2a, 1 gt2b and 4 gt3a patients. For each patient the number of ISDR amino acid differences compared to the gt specific consensus was determined. No dominant amino acid changes in the ISDR between the two time points was detected in any of the patients thus the results represent both the third trimester of pregnancy and 3 months postpartum time points. B-D) Data based on the HCV Research UK cohort[32]. Data for gts 1a, 1b and 3a were pooled. For each patient's ISDR sequence, the number of amino acid differences compared to the gt specific consensus sequence was determined. If an ISDR had 3 or more mutations, the patient was considered to be infected with a potential high replicator. Post LTX patients were excluded from the analysis. B) Fraction of high replicators in the context of liver stiffness determined via Fibroscan. 112 potential high replicators were compared to 768 potential low replicators. C&D) Fraction of high replicators in the context of liver status at the time point of sequencing (C) or a variety of other contexts (D). Statistical significance was determined using a two-sided Student's t-test (B) or a Fisher's exact test (C&D). In none of the cases, a significant enrichment of potential high replicators was detected.

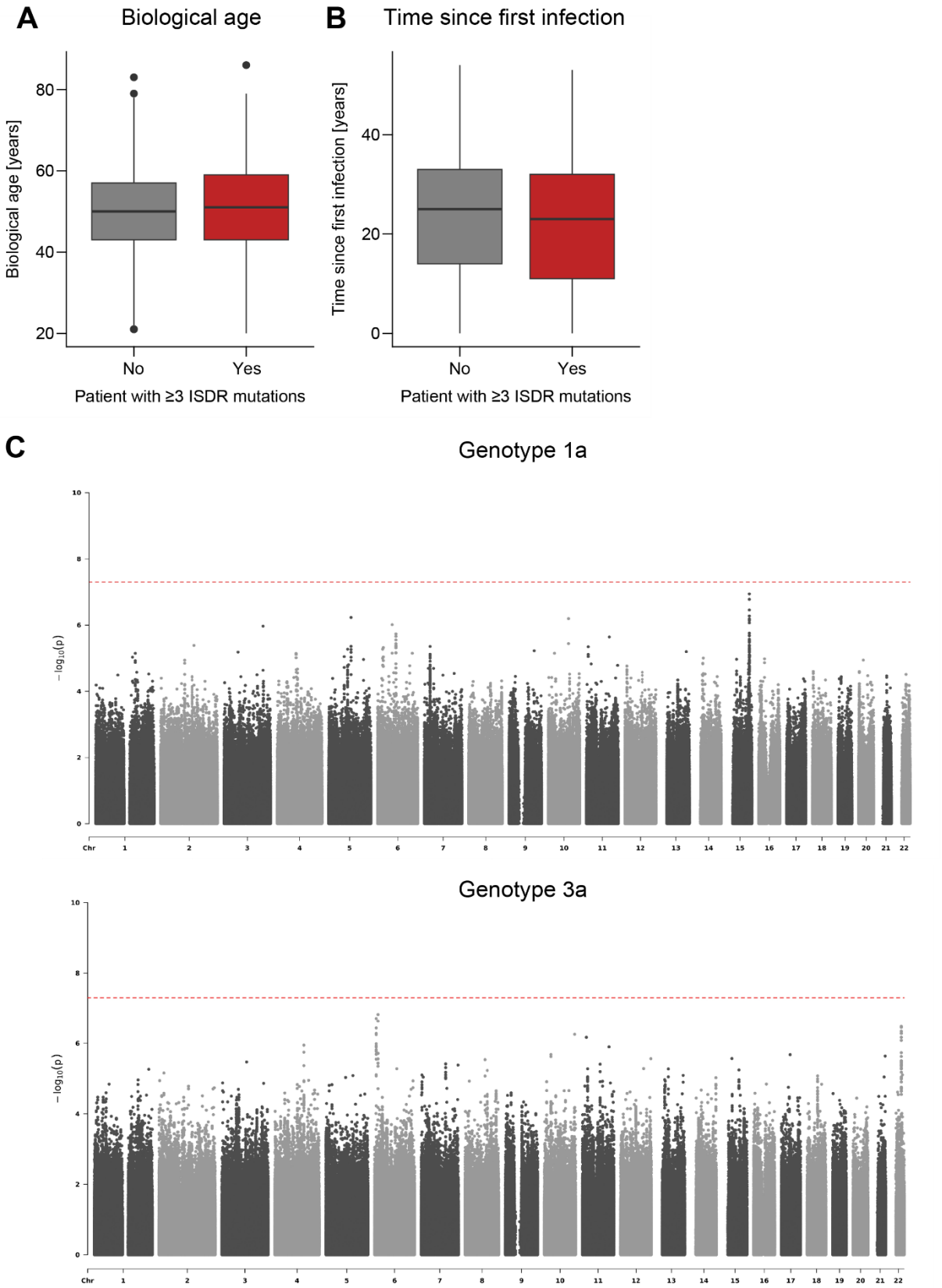


Fig. S9: Genetic and temporal determinants of high RF. A) Fraction of high replicators in the context of biological age. 273 potential high replicators were compared to 1841 potential low replicators. B) Fraction of high replicators in the context of time since first infection. 130 potential high replicators were compared to 843 potential low replicators. A&B) Data based on the HCV

Research UK cohort[32]. Statistical significance was determined using a two-sided Student's t-test. In none of the cases, a significant enrichment of potential high replicators was detected. C) Association between SNPs and patients being infected by a potential high replicator. 1347 gt1a and 1181 gt3a patients were analysed. The dashed red line indicates the threshold for statistical significance determined via logistic regression.

Table S1: Oligo nucleotides used and generated during this project.

Name	Sequence (5'-3')	Description	Gt
A_9416	CAGGATGGCCTATTGGCCTGGAG	cDNA generation[21]	1b
S_59	TGTCTTCACGCAGAAAGCGTCTAG	Nested PCR from cDNA to amplify N-terminal half of HCV genome[21]	1b
S_93	AAGCGTCTAGCCATGGCGTT		1b
A_5982	TGACCAGGTCCTCGGTGGAG		1b
A_6103	GCTATCAGCCGGTTCATCCACTGC		1b
S_4540	GTAACGAGCTCGCCGCGCAGCTGTC	Nested PCR from cDNA to amplify C-terminal half of HCV genome[21]	1b
S_5120	GTGCGCCAGGGCTCAGGCTCCACC		1b
A_9364	GGAGCAGGTAGATGCCTACCCCTAC		1b
A_9386	TTAGCTCCCCGTTCATCGGTTGG		1b
S_6767	ATTCCAGGTCGGGCTCAA	Nested PCR from cDNA to amplify ReED	1b
S_6843	ACTTCCATGCTCACCGACCC		1b
A_7501	AGAGGGGGCATGGAGGAGTA		1b
A_7552	ACGGTAGACCAAGACCCGTC		1b
A9400	GGCCGGAGTGTTTACCCCAACCTTC	cDNA generation[90]	1a
S6583_1a	GCGCTGTGGAGGGTGTCTGC	Nested PCR from cDNA to amplify ReED	1a
S6853_1a	TGACGTCCATGCTCACTG		1a
A7416_1a	CGGAAGTTGAGGAGCTGCC		1a
A7538_1a	AGATCCGGATCCCCAGGCTCC		1a
A9429_3a	ATGGAGTGTTATCCTACCAGC	cDNA generation	3a
S6872_3a	TGACCTCGATGTTGAGAGACC	Nested PCR from cDNA to amplify ReED	3a
S6826_3a	ATAGGATCTCAACTCCCCTGTG		3a
A7488_3a	CTTGGAAGTAGTGCTGGACTG		3a
A7622_3a	ACGCTCTGCTCCTCGCTGTC		3a

Table S2: Patient characteristics of the gt1b post LTX patients. na = not available.

Name	Source	Date sequenced pre LTX sample	LTX Date	Date sequenced post LTX sample	Date biopsy post LTX	Accession No.	Note
FCH1	Barcelona	17.03.11	17.03.11	02.06.11	02.05.11	MK092096/7	
FCH2	Barcelona	21.02.12	21.02.12	19.04.12	na	MK092098/9	
FCH3	Barcelona	09.10.12	09.10.12	12.11.12	na	MK092100/1	
FCH4	Barcelona	22.11.13	22.11.13	18.02.14	12.02.14	MK092102/3	
FCH5	Barcelona	01.01.14	01.01.14	31.03.14	06.02.14	MK092104/5	

FCH6	Barcelona	04.12.09	04.12.09	26.02.10	na		
FCH7	Barcelona	22.02.12	22.02.12	11.06.12	na		
FCH8	Barcelona	13.03.12	13.03.12	13.06.12	na		
FCH9	Barcelona	25.03.12	25.03.12	14.05.12	na		
FCH10	Barcelona	30.04.13	30.04.13	05.08.13	na		
FCH11	Barcelona	24.07.13	24.07.13	29.08.13	na		
FCH12	Barcelona	10.10.13	10.10.13	29.01.14	09.12.13		
Non-FCH1	Barcelona	05.01.01	05.01.01	29.03.01	na	MK092106/7	
Non-FCH2	Barcelona	13.04.07	13.04.07	20.06.07	na	MK092108/9	
Non-FCH3	Barcelona	09.09.01	09.09.01	16.11.01	na	MK092110/1	
Non-FCH4	Heidelberg	na	12.11.09	10.01.12	na		Previous LTX on 09.08.07
Non-FCH5	Heidelberg	na	29.03.12	24.08.12	na		
Non-FCH6	Heidelberg	na	08.10.11	13.11.12	na		Previous LTX on 28.09.11
Non-FCH7	Freiburg	na	12.2011	07.11.12	na		
Non-FCH8	Essen	na	03.12.12	03.06.13	na		
Non-FCH9	Essen	na	05.12.12	05.06.13	na		

Table S3: DNA plasmids used and generated during this project.

Name	Description
pFK	Plasmid containing a T7 promotor for *in vitro* transcription
pFK i341 NS3-3' Con1	Bicistronic SGR of the prototype gt1b wildtype isolate[13]
pFK i341 NS3-3' Con1 ΔGDD	Replication deficient negative control
pFK i341 NS3-3' GLT1	gt1b wildtype isolate[21]
pFK JC1	Prototype isolate for infection experiments[89]
pFK JcN2A	JC1 with a Nluc reporter[91]
pFK JcN2A ΔGDD	Replication deficient negative control
pFK JcN2A ΔE1E2	Infection deficient negative control
pFK i389 NS3-3' GLT1	Monocistronic GLT1 SGR
pFK i389 NS3-3' preLTX1α	Monocistronic SGRs of the different timepoints of the GLT1 patient
pFK i389 NS3-3' preLTX1β	
pFK i389 NS3-3' postLTX1	
pFK i389 NS3-3' 6 w postLTX2	
pFK i389 preLTX1α G5A.ReED	Chimeras of the pre LTX timepoints harbouring the GLT1.ReED or ISDR
pFK i389 preLTX1β G5A.ISDR	
pFK i389 preLTX1β G5A.ReED	
pFK i389 N2A preLTX1α/C3JFH1	

pFK i389 N2A preLTX1β/C3JFH1	Chimeras of the indicated assembly module and the JFH1 replicase to assess particle production
pFK i389 N2A preLTX1γ/C3JFH1	
pFK i389 N2A postLTX1/C3JFH1	
pFK i341 NS3-3' Con1 gt1b.ReED	Con1 based chimera harbouring the gt1b consensus ReED
pFK i341 NS3-3' Con1 FCH1.ReED	Con1 based chimeras harbouring patient derived ReEDs from time points pre or post LTX
pFK i341 NS3-3' Con1 FCH2_pre.ReED	
pFK i341 NS3-3' Con1 FCH2_post.ReED	
pFK i341 NS3-3' Con1 FCH3.ReED	
pFK i341 NS3-3' Con1 FCH4_pre.ReED	
pFK i341 NS3-3' Con1 FCH4_post.ReED	
pFK i341 NS3-3' Con1 FCH5_pre.ReED	
pFK i341 NS3-3' Con1 FCH5_post.ReED	
pFK i341 NS3-3' Con1 BHCV1.ReED	
pFK i341 NS3-3' Con1 Non-FCH1.ReED	
pFK i341 NS3-3' Con1 Non-FCH2.ReED	
pFK i341 NS3-3' Con1 Non-FCH3.ReED	
pFK i341 NS3-3' Con1 FCH6_pre.ReED	
pFK i341 NS3-3' Con1 FCH6_post.ReED	
pFK i341 NS3-3' Con1 FCH7_pre.ReED	
pFK i341 NS3-3' Con1 FCH7_post.ReED	
pFK i341 NS3-3' Con1 FCH8_pre.ReED	
pFK i341 NS3-3' Con1 FCH8_post.ReED	
pFK i341 NS3-3' Con1 FCH9_pre.ReED	
pFK i341 NS3-3' Con1 FCH9_post.ReED	
pFK i341 NS3-3' Con1 FCH10_pre.ReED	
pFK i341 NS3-3' Con1 FCH10_post.ReED	
pFK i341 NS3-3' Con1 FCH11.ReED	
pFK i341 NS3-3' Con1 FCH12.ReED	
pFK i341 NS3-3' Con1 Non-FCH4.ReED	
pFK i341 NS3-3' Con1 Non-FCH5.ReED	
pFK i341 NS3-3' Con1 Non-FCH6.ReED	
pFK i341 NS3-3' Con1 Non-FCH7.ReED	
pFK i341 NS3-3' Con1 Non-FCH8.ReED	
pFK i341 NS3-3' Con1 Non-FCH9.ReED	
pFK i341 NS3-3' Con1 EC1.ReED	
pFK i341 NS3-3' GLT1 Con1.ReED	GLT1 based chomera harbouring the Con1 ReED
pFK N2A Con1 fl. ΔGDD	Replication deficient negative control
pFK N2A Con1 fl.	Con1 WT or Con1 based chimeric replicons to assess replication of full-length constructs
pFK N2A Con1 fl. GLT1.ReED	
pFK N2A Con1 fl. FCH3.ReED	
pFK i341 NS3-3' FCH3	SGR of the consensus sequence of patient FCH3
pFK i341 NS3-3' FCH4	SGR of the consensus sequence of patient FCH4
pFK i341 NS3-3' H77	Prototype gt1a isolate[92]
pFK i341 NS3-3' H77 1a_LTX1.ReED	H77 based chimeras harbouring patient derived ReEDs
pFK i341 NS3-3' H77 1a_LTX2.ReED	
pFK i341 NS3-3' H77 1a_LTX3.ReED	
pFK i341 NS3-3' H77 1a_LTX4.ReED	

pFK i341 NS3-3' H77 1a_LTX5.ReED	
pFK i341 NS3-3' H77 1a_LTX6.ReED	
pFK i341 NS3-3' H77 1a_LTX7.ReED	
pFK i341 NS3-3' H77 1a_LTX8.ReED	
pFK i341 NS3-3' H77 1a_LTX9.ReED	
pFK i341 NS3-3' H77 gt1a_cons.ReED	H77 based chimera harbouring the gt1a consensus ReED
pFK i341 NS3-3' Con1 GLT1.ISDR	Chimeras for mapping of the ReED between Con1 and GLT1
pFK i341 NS3-3' Con1 G.2209-2280	
pFK i341 NS3-3' Con1 G.2209-2283	
pFK i341 NS3-3' Con1 G.2209-2285	
pFK i341 NS3-3' Con1 G.2209-2303	
pUC	Plasmid containing a T7 promotor for *in vitro* transcription
pUC i389 NS3-3' BHCV1	SGR of the consensus sequence of patient BHCV1[27]
pUC i387 NS3-3' S52	Prototype gt3a isolate[35]
pUC i387 NS3-3' S52 gt3a_cons.ReED	S52 based chimera harbouring the gt3a consensus ReED
pUC i387 NS3-3' S52 UK1.ReED	S52 based chimeras harbouring patient derived ReEDs
pUC i387 NS3-3' S52 UK2.ReED	
pUC i387 NS3-3' S52 UK3.ReED	
pUC i387 NS3-3' S52 UK4.ReED	
pUC i387 NS3-3' S52 UK5.ReED	
pUC i387 NS3-3' S52 UK6.ReED	
pUC i387 NS3-3' S52 UK7.ReED	
pUC i387 NS3-3' S52 UK8.ReED	
pUC i387 NS3-3' S52 UK9.ReED	
pUC i387 NS3-3' S52 UK10.ReED	
pUC i387 NS3-3' ED43	Prototype gt4a isolate[35]
pUC i387 NS3-3' ED43 4a_cons.ReED	ED43 based chimera harbouring the gt4a consensus ReED
pUC i387 NS3-3' ED43 Pat1.ReED	ED43 based chimeras harbouring patient derived ReEDs
pUC i387 NS3-3' ED43 Pat2.ReED	
pUC i387 NS3-3' ED43 Pat3.ReED	
pUC i387 NS3-3' ED43 Pat4.ReED	
pUC i387 NS3-3' ED43 Pat5.ReED	
pUC i387 NS3-3' ED43 Pat6.ReED	
Other	

pCMV DR 8.74	Production of HCVpp
pCMV Luc	
pczVSV-Gwt	
pcDNA3cE1E2Con1	
pcDNA3cE1E2 preLTX1α	
pcDNA3cE1E2 preLTX1β	
pcDNA3cE1E2 preLTX1γ	
pcDNA3cE1E2 postLTX1	
pcDNA3cE1E2 GLT1	