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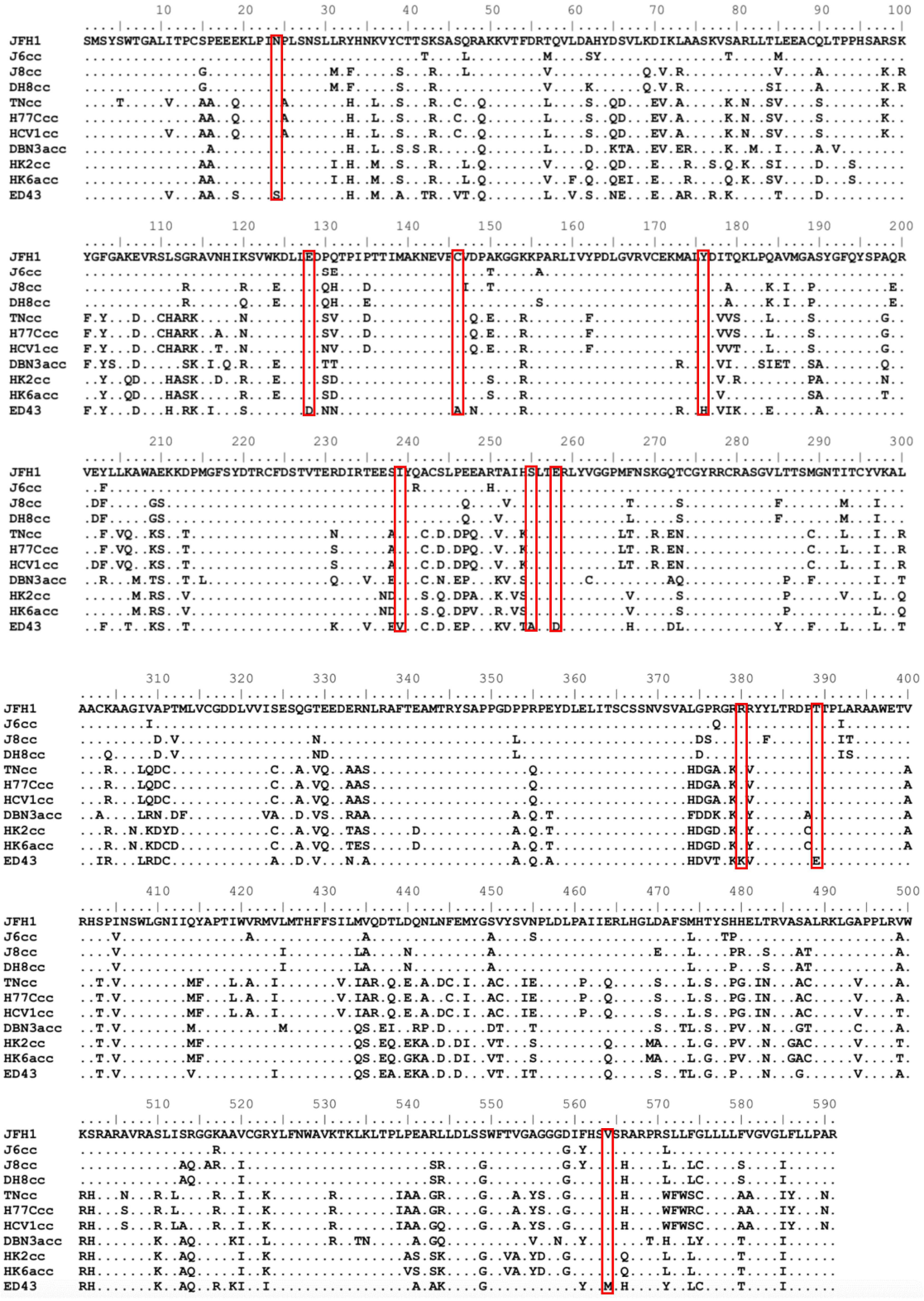
Hepatitis C virus genome-wide analysis for development of efficient culture systems and unravelling of antiviral resistance in genotype 4

Long V. Pham[1], Martin Schou Pedersen[1,2], Ulrik Fahnøe[1], Carlota Fernandez Antunez[1], Daryl Humes[1], Kristian Schønning[2,3], Santseharay Ramirez[1] and Jens Bukh[1]

Affiliation: [1]Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark, [2]Department of Clinical Microbiology, Hvidovre Hospital, Hvidovre, Denmark, and [3]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

A


JFH1
J6cc
J8cc
DH8cc
TNcc
H77Ccc
HCV1cc
DBN3acc
HK2cc
HK6acc
ED43

B

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                10        20        30        40        50        60        70        80        90        100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  MSTNPKPQRKTKRNTNRRPMDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRGRRQPIPKARRPEGRSWAQPGYPWPLYGNEGCGWAGWLLSP
ED43cc          ..................................................................................................

                110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  RGSRPSWGPNDPRRRSRNLGKVIDTLTCGFADLMGYIPLVGAPVGGVARALAHGVRALEDGINYATGNLPGCSFSIFLLALLSCLTVPASAVNYRNVSGI
ED43cc          ..................................................................................................

                210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  YHVTNDCPNSSIVYEADHHILHLPGCVPCVREGNQSRCWVALTPTVAAPYIGAPLESLRSHVDLMVGAATVCSGLYIGDLCGGLFLVGQMFSFRPRRHWT
ED43cc          ..............................................................G...................................

                310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  TQDCNCSIYTGHITGHRMAWDMMMNWSPTTTLVLAQVMRIPTTLVDLLSGGHWGVLVGVAYFSMQANWAKVILVLFLFAGVDAETHVSGAAVGRSTAGLA
ED43cc          ..................................................................................................

                410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  NLFSSGSKQNLQLINSNGSWHINRTALNCNDSLNTGFLASLFYTHKFNSSGCSERLACCKSLDSYGQGWGPLGVANISGSSDDRPYCWHYAPRPCGIVPA
ED43cc          ..........................................................R.......................................

                510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  SSVCGPVYCFTPSPVVVGTTDHVGVPTYTWGENETDVFLLNSTRPPHGAWFGCVWMNSTGFTKTCGAPPCEVNTNNGTWHCPTDCFRKHPETTYAKCGSG
ED43cc          ..................................................................................................

                610       620       630       640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  PWITPRCLIDYPYRLWHFPCTANFSVFNIRTFVGGIEHRMQAACNWTRGEVCGLEHRDRVELSPLLLTTTAWQILPCSFTTLPALSTGLIHLHQNIVDVQ
ED43cc          ..................................................................................................

                710       720       730       740       750       760       770       780       790       800
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  YLYGVGSAVVSWALKWEYVVLAFLLLADARVSACLWMMFMVSQVEAALSNLININAASAAGAQGFWYAILFICIVWHVKGRFPAAAAYAACGLWPLFLLL
ED43cc          ..................................................................................................

                810       820       830       840       850       860       870       880       890       900
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  LMLPERAYAYDQEVAGSLGGAIVVMLTILTLSPHYKLWLARGLWWIQYFIARTEAVLHVYIPSFNVRGPRDSVIVLAVLVCPHLVFDITKYLLAILGPLH
ED43cc          ........................A.....................C.....................................................

                910       920       930       940       950       960       970       980       990       1000
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  ILQASLLRIPYFVRAQALVKICSLLRGVVYGKYFQMVVLKAGALTGTYIYDHLTPMSDWAATGLRDLAVALEPVVFTPMEKKVIVWGADTAACGDIIRGL
ED43cc          ..................................................................................................

                1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  PVSARLGNEILLGPADTETSKGWRLIAPITAYAQQTRGLFSTIVTSLTGRDTNENCGEVQVLSTATQSFLGTAVNGVMWTVYHGAGAKTISGPKGPVNQM
ED43cc          ..................................................................................................

                1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  YTNVDQDLVGWPAPPGVRSLAPCTCGSADLYLVTRHADVIPVRRRGDTRGALLSPRPISTLKGSSGGPLLCPMGHAAGIFRAAVCTRGVAKAVDFVPVES
ED43cc          ..................................................................................................

                1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  LETTMRSPVFTDNSTPPAVPQTYQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGVYMSKAYGIDPNIRSGVRTITTGAPITYSTYGKFL
ED43cc          ..................................................................................V.................

                1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  ADGGCSGGAYDIIICDECHSTDSTTILGIGTVLDQAETAGVRLTVLATATPPGSVTTPHSNIEEVALPTTGEIPFYGKAIPLELIKGGRHLIFCHSKKKC
ED43cc          ..................................................................................................

                1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  DELARQLTSLGLNAVAYYRGLDVSVIPTSGDVVVCATDALMTGFTGDFDSVIDCNTSVIQTVDFSLDPTFSIETTTVPQDAVSRSQRRGRTGRGRLGTYR
ED43cc          ..........................................................GM......................................

                1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ED43-consensus  YVTPGERPSGMFDTAVLCECYDAGCAWYELTPAETTTRLKAYFDTPGLPVCQDHLEFWESVFTGLTHIDGHFLSQTKQSGENFPYLVAYQATVCAKALAP
ED43cc          ..................................................................L...............................
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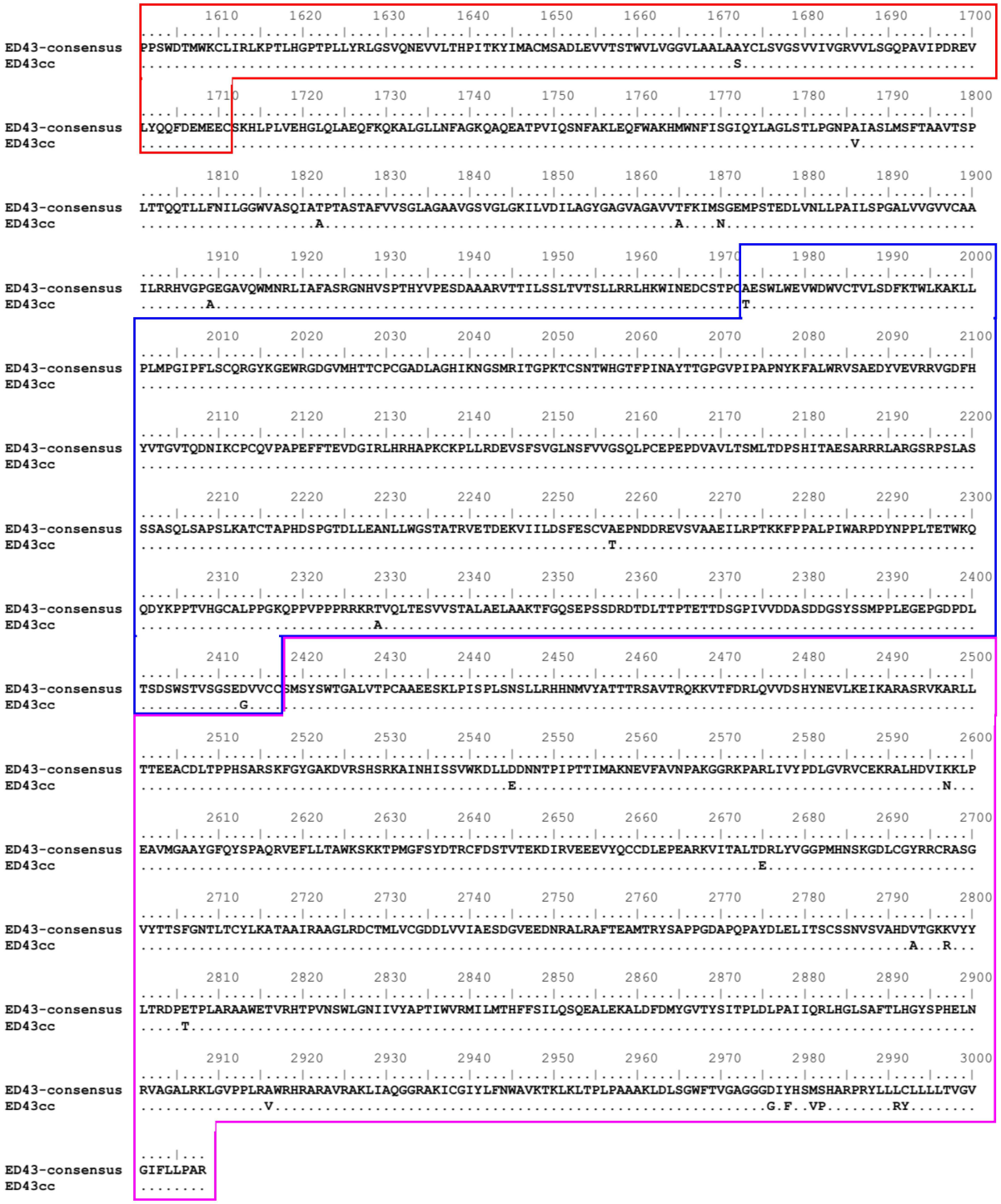
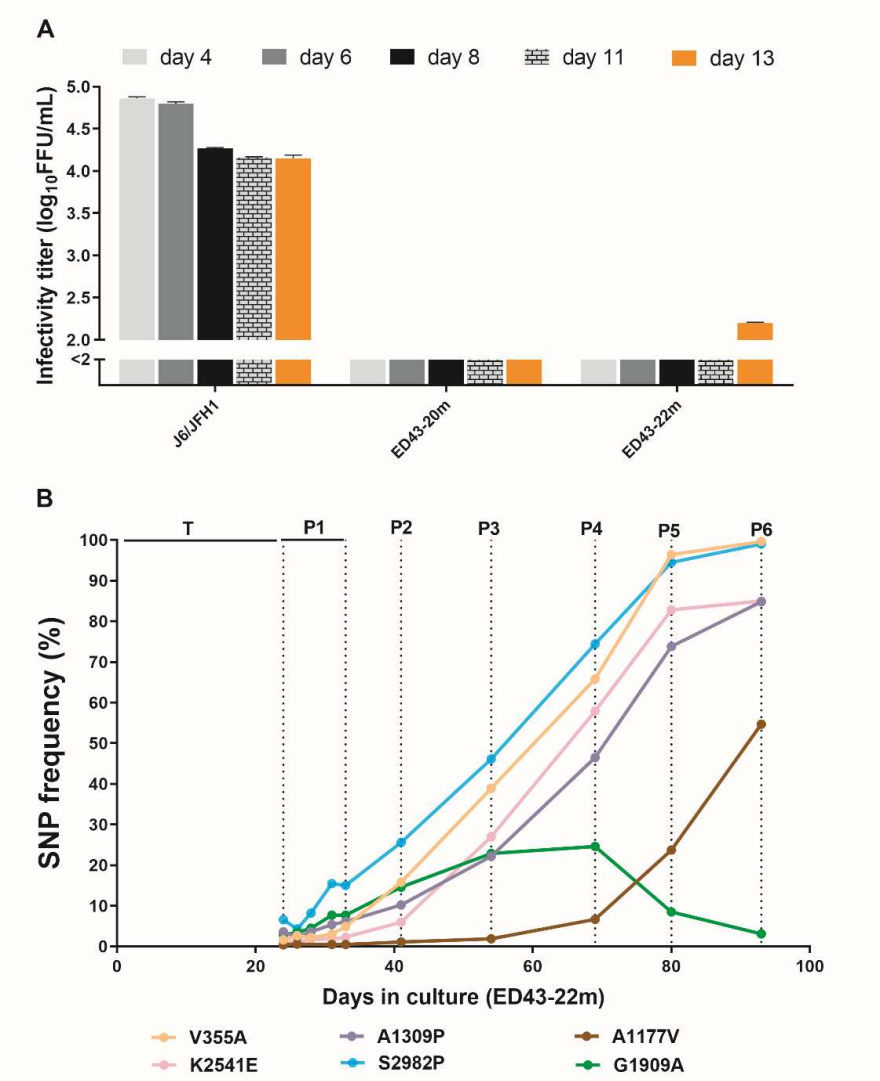
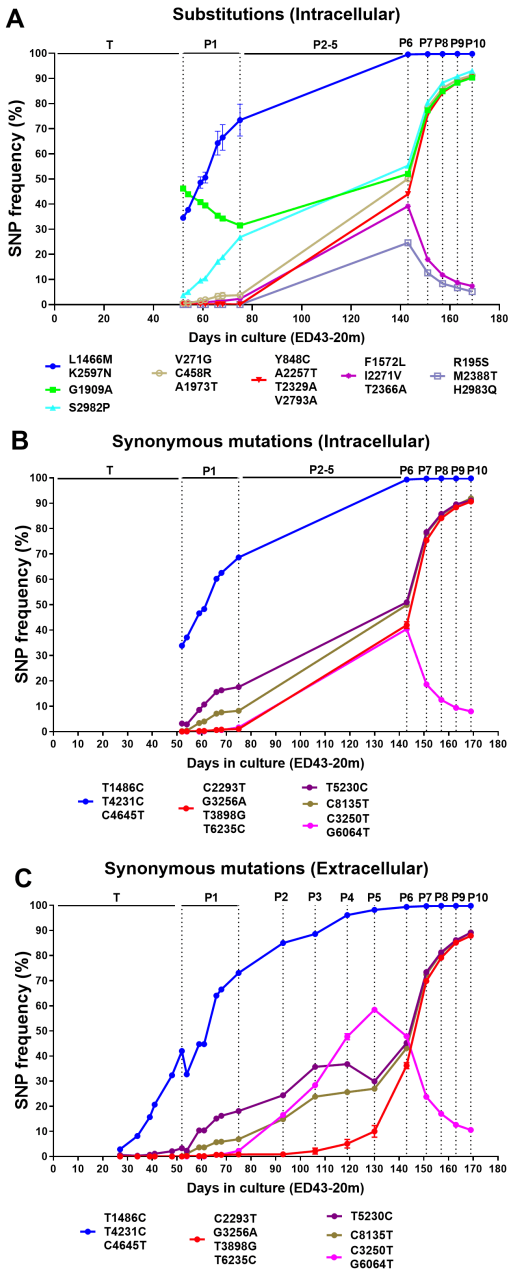

1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
ED43-consensus PPSWDTMWKCLIRLKPTLHGPTPLLYRLGSVQNEVVLTHPITKYIMACMSADLEVVTSTWVLVGGVLAALAAYCLSVGSVVIVGRVVLSGQPAVIPDREV
ED43cc .....S.....
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
ED43-consensus LYQQFDEMEECSKHLPLVEHGLQLAEQFKQKALGLLNFAGKQAQEATPVIQSNFAKLEQFWAKHMWNFISGIQYLAGLSTLPGNPAIASLMSFTAAVTSP
ED43cc .....V.....
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
ED43-consensus LTTQQTLLFNILGGWVASQIATPTASTAFVVSGLAGAAVGSVGLGKILVDILAGYGAGVAGAVVTFKIMSGEMPSTEDLVNLLPAILSPGALVVGVVCAA
ED43cc .....A.....A....N.....
1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
ED43-consensus ILRRHVGPGEGAVQWMNRLIAFASRGNHVSPTHYVPESDAAARVTTILSSLTVTSLLRRLHKWINEDCSTPCAESWLWEVWDWVCTVLSDFKTWLKAKLL
ED43cc .....A.....T.....
2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
ED43-consensus PLMPGIPFLSCQRGYKGEWRGDGVMHTTCPCGADLAGHIKNGSMRITGPKTCSNTWHGTFPINAYTTGPGVPIPAPNYKFALWRVSAEDYVEVRRVGDFH
ED43cc .....
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
ED43-consensus YVTGVTQDNIKCPCQVPAPEFFTEVDGIRLHRHAPKCKPLLRDEVSFSVGLNSFVVGSQLPCEPEPDVAVLTSMLTDPSHITAESARRRLARGSRPSLAS
ED43cc .....
2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
ED43-consensus SSASQLSAPSLKATCTAPHDSPGTDLLEANLLWGSTATRVETDEKVIILDSFESCVAEPNDDREVSVAAEILRPTKKFPPALPIWARPDYNPPLTETWKQ
ED43cc .....T.....
2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
ED43-consensus QDYKPPTVHGCALPPGKQPPVPPPRRKRTVQLTESVVSTALAELAAKTFGQSEPSSDRDTDLTTPTETTDSGPIVVDDASDDGSYSSMPPLEGEPGDPDL
ED43cc .....A.....
2410 2420 2430 2440 2450 2460 2470 2480 2490 2500
ED43-consensus TSDSWSTVSGSEDVVCCSMSYSWTGALVTPCAAEESKLPISPLSNSLLRHHNMVYATTTRSAVTRQKKVTFDRLQVVDSHYNEVLKEIKARASRVKARLL
ED43cc .....G.....
2510 2520 2530 2540 2550 2560 2570 2580 2590 2600
ED43-consensus TTEEACDLTPPHSARSKFGYGAKDVRSHSRKAINHISSVWKDLLDDNNTPIPTTIMAKNEVFAVNPAKGGRKPARLIVYPDLGVRVCEKRALHDVIKKLP
ED43cc .....E.....N...
2610 2620 2630 2640 2650 2660 2670 2680 2690 2700
ED43-consensus EAVMGAAYGFQYSPAQRVEFLLTAWKSKKTPMGFSYDTRCFDSTVTEKDIRVEEEVYQCCDLEPEARKVITALTDRLYVGGPMHNSKGDLCGYRRCRASG
ED43cc .....E.....
2710 2720 2730 2740 2750 2760 2770 2780 2790 2800
ED43-consensus VYTTSFGNTLTCYLKATAAIRAAGLRDCTMLVCGDDLVVIAESDGVEEDNRALRAFTEAMTRYSAPPGDAPQPAYDLELITSCSSNVSVAHDVTGKKVYY
ED43cc .....A...R...
2810 2820 2830 2840 2850 2860 2870 2880 2890 2900
ED43-consensus LTRDPETPLARAAWETVRHTPVNSWLGNIIVYAPTIWVRMILMTHFFSILQSQEALEKALDFDMYGVTYSITPLDLPAIIQRLHGLSAFTLHGYSPHELN
ED43cc .....T.....
2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
ED43-consensus RVAGALRKLGVPPLRAWRHRARAVRAKLIAQGGRAKICGIYLFNWAVKTKLKLTPLPAAAKLDLSGWFTVGAGGGDIYHSMSHARPRYLLLCLLLLTVGV
ED43cc .....V.....G.F..VP.....RY.....
ED43-consensus GIFLLPAR
ED43cc .....

Supplemental Figure 1. Aligment of different HCV sequences. (A) Alignment of HCV NS5B genotypes 1-4 and 6. NS5B sequences from JFH1cc (2a)(1), J6cc (2a)(2), J8cc (2b)(2), DH8cc (2b)(3), TNcc (1a)(4), H77Ccc (1a)(5), HCV1cc (1a)(5), DBN3acc (3a)(6), HK2cc (6a)(7), and HK6acc (6a)(7) viruses were aligned with corresponding sequence of ED43 (4a)(8). Red boxes indicate residues of ED43, which are different from JFH1 and all other culture strains included from our prior studies. **(B)** Alignment of HCV sequences from ED43 consensus clone and ED43cc. The polyprotein sequence of ED43 consensus (8) was aligned with the corresponding sequence of ED43cc virus. The red, blue, and pink boxes indicate the NS3/4A (aa 1027-1711), NS5A (aa 1973-2417), and NS5B (aa 2418-3008) sequences, respectively.

Pham LV, *et al. Gut* 2022; 71:627–642. doi: 10.1136/gutjnl-2020-323585



Supplemental Figure 2. Viability of the recombinant ED43-22m following transfection of Huh7.5 cells and emergence of substitutions during virus passages. ED43-22m originated from ED43-20m with the addition of substitutions L1466M(NS3) and K2597N(NS5B), the first dominant changes emerging during passage of ED43-20m viruses (see Figure 1B). (**A**) The infectivity titers were determined by FFU assays and shown by mean of triplicates ± SEM (y-axis). J6/JFH1 was included as a control. Y-axis break indicates cut-off of the assay. (**B**) NGS analysis of recovered viruses. Only substitutions that developed in > 20% of the virus population at any time points are shown. Samples were not collected during the transfection (T). Black dashed lines showed samples taken during the indicated cell-free passages of supernatant viruses (P1, 2, 3, etc.).



Supplemental Figure 3. Evolution of substitutions and synonymous mutations in culture adaptation of ED43 full-length recombinant ED43-20m, determined by NGS. Only SNPs with frequencies of >20% at any time points are shown. For SNPs that emerged with similar patterns, means ± SEM are shown instead. Black dashed lines showed samples taken during the indicated passages (P1, 2, 3, etc.). (**A,B**)

SNP frequencies of substitutions (panel A) and synonymous mutations (panel B), determined from intracellular viral RNA. The numbers refer to amino acid positions of the ED43 polyprotein (panel A) or nucleotide positions of the ED43 genome (panel B). Samples were not collected during the transfection (T) and serial passages P2-5. (**C**) SNP frequency of synonymous mutations determined from extracellular viral RNAs. The numbers refer to nucleotide positions of the ED43 genome. Samples were not collected during the transfection (T) from days 0 to 27. See also Figure 1B.

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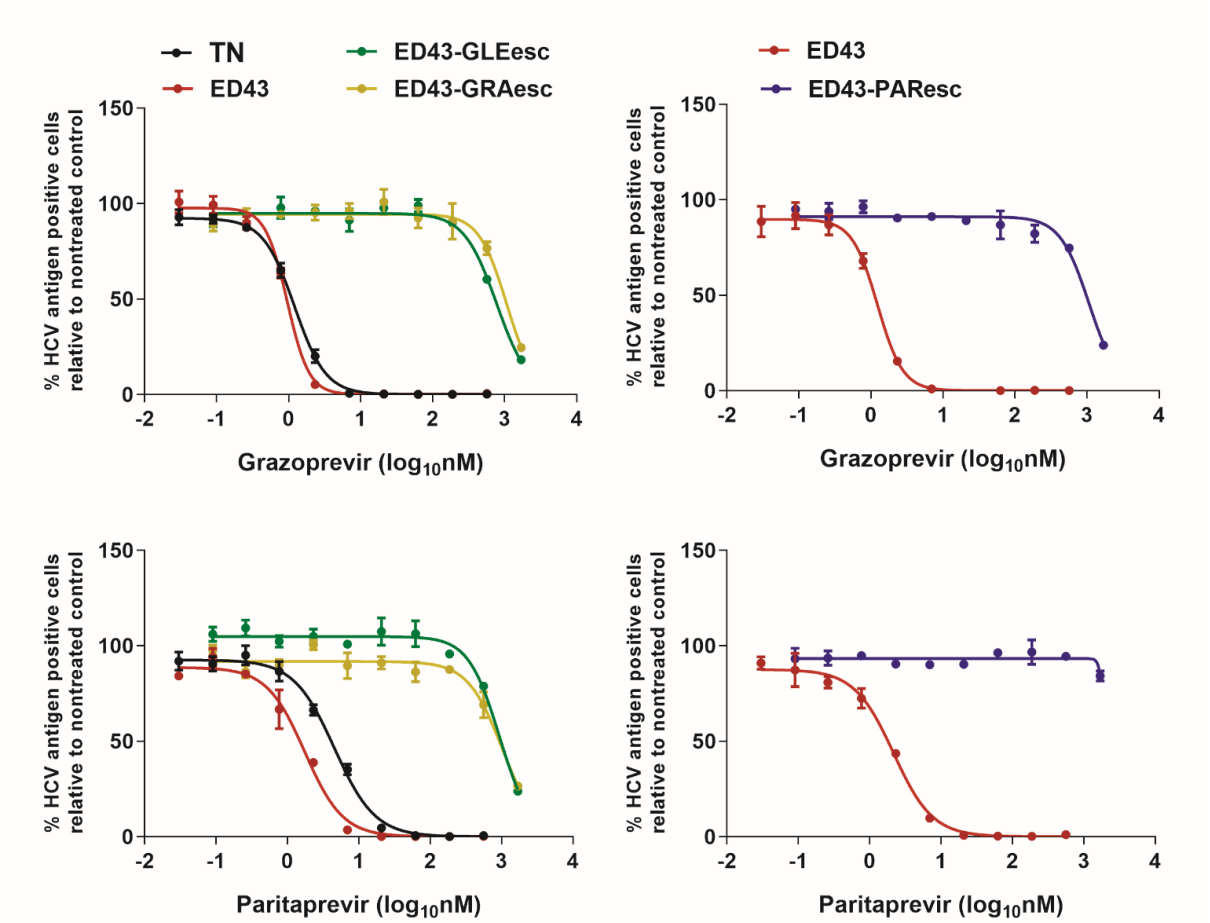
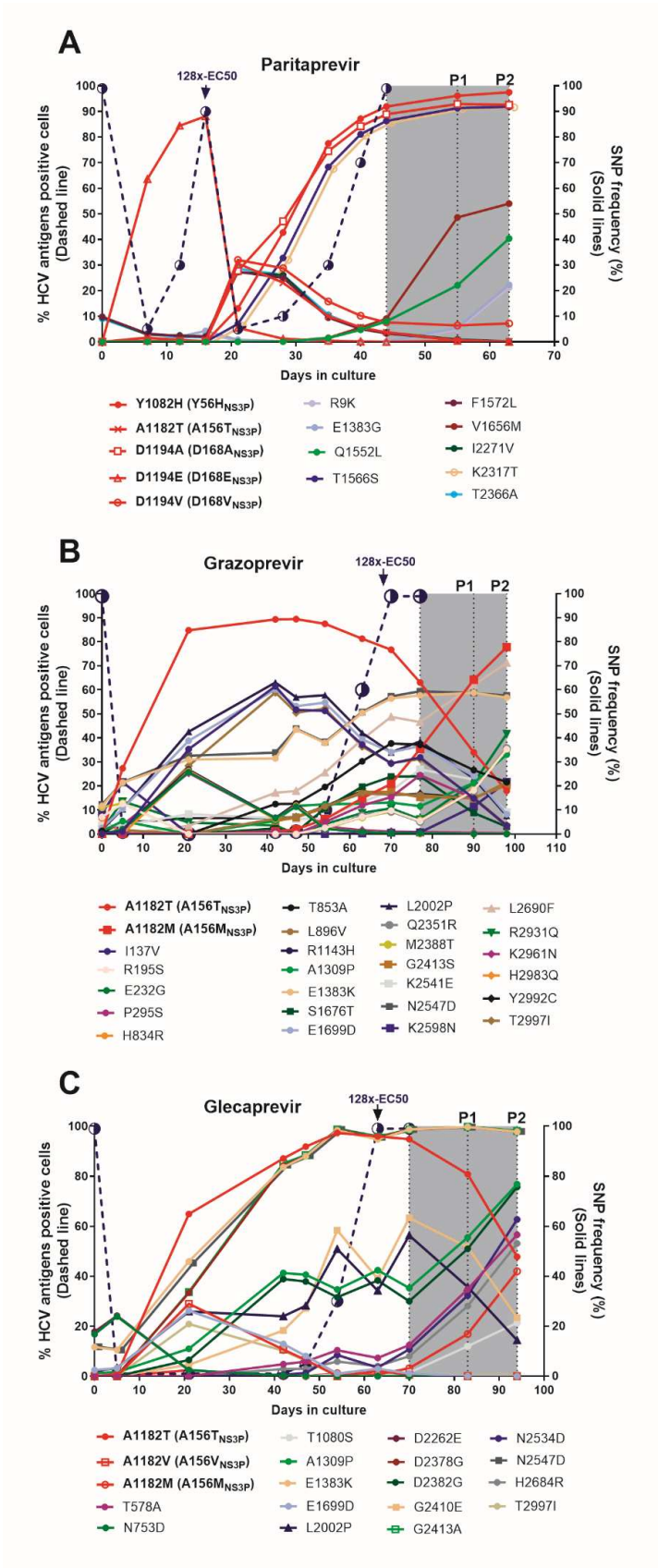
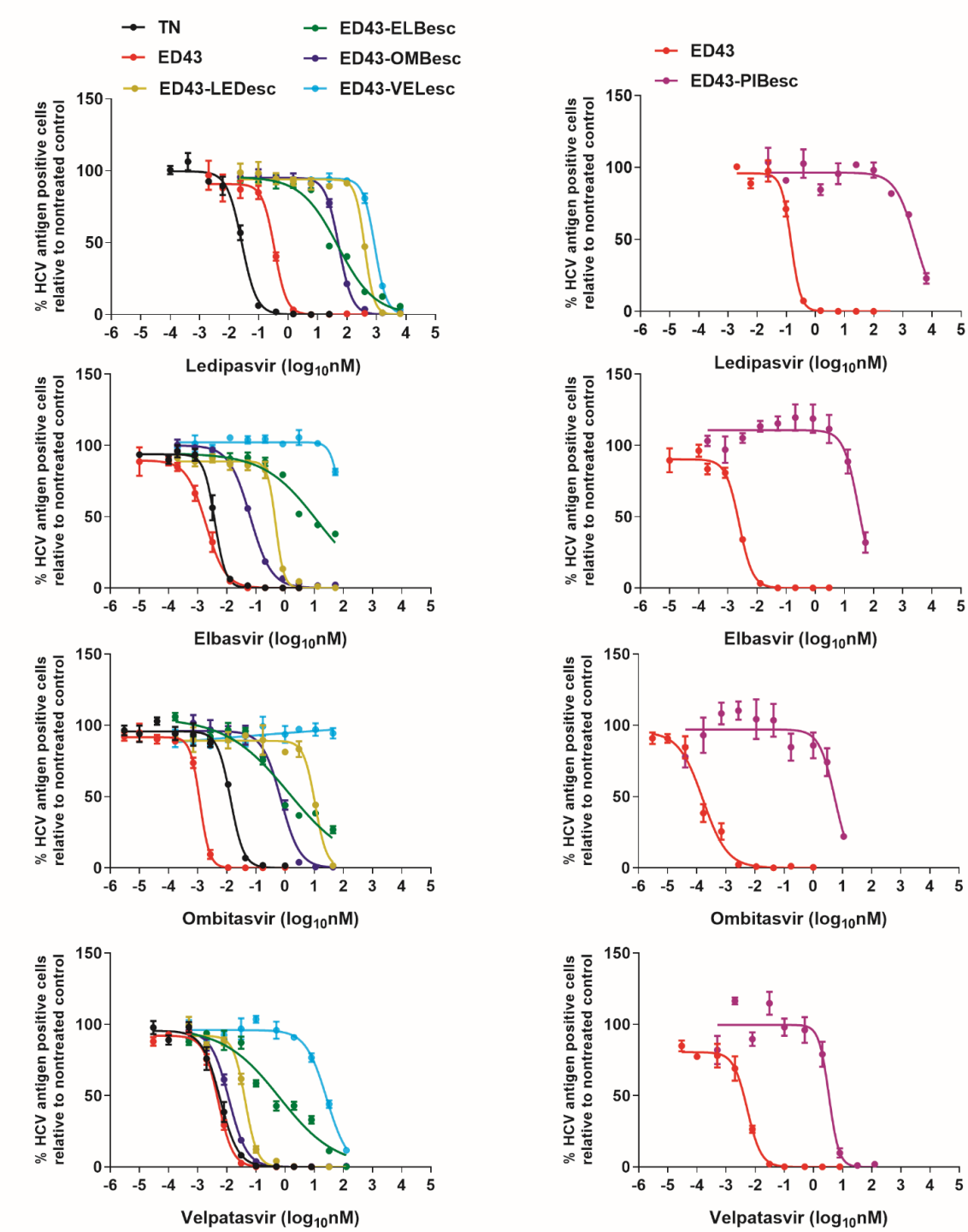


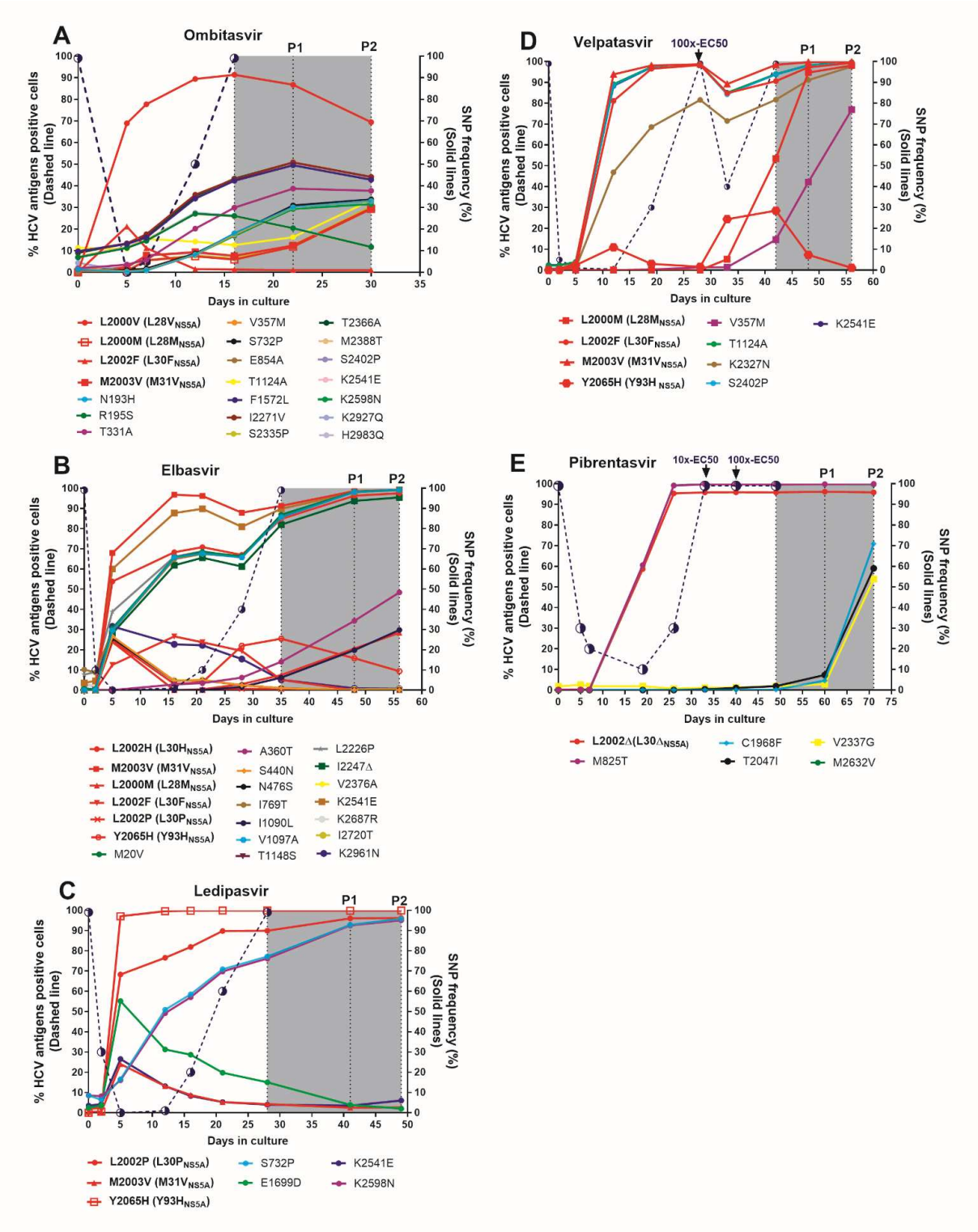
Supplemental Figure 4: Efficacy of protease inhibitors (PIs) against HCV genotype 4a (ED43) full-length culture viruses. Huh7.5 cells were seeded on 96-well plates overnight, then infected with the indicated viruses for 24 hours. The cells were subsequently treated with specific inhibitors for an additional 48 hours before analysis as described (7, 9). Values are means of triplicates ±SEM. The original ED43 virus was used in these 2 experiments as a control. TN (1a) virus was included for comparison. See also Figure 2D,E.


A
Paritaprevir
128x-EC50
P1
P2
% HCV antigens positive cells (Dashed line)
SNP frequency (%) (Solid lines)
Days in culture
Y1082H (Y56H$_{NS3P}$)
A1182T (A156T$_{NS3P}$)
D1194A (D168A$_{NS3P}$)
D1194E (D168E$_{NS3P}$)
D1194V (D168V$_{NS3P}$)
R9K
E1383G
Q1552L
T1566S
F1572L
V1656M
I2271V
K2317T
T2366A
B
Grazoprevir
128x-EC50
P1
P2
% HCV antigens positive cells (Dashed line)
SNP frequency (%) (Solid lines)
Days in culture
A1182T (A156T$_{NS3P}$)
A1182M (A156M$_{NS3P}$)
I137V
R195S
E232G
P295S
H834R
T853A
L896V
R1143H
A1309P
E1383K
S1676T
E1699D
L2002P
Q2351R
M2388T
G2413S
K2541E
N2547D
K2598N
L2690F
R2931Q
K2961N
H2983Q
Y2992C
T2997I
C
Glecaprevir
128x-EC50
P1
P2
% HCV antigens positive cells (Dashed line)
SNP frequency (%) (Solid lines)
Days in culture
A1182T (A156T$_{NS3P}$)
A1182V (A156V$_{NS3P}$)
A1182M (A156M$_{NS3P}$)
T578A
N753D
T1080S
A1309P
E1383K
E1699D
L2002P
D2262E
D2378G
D2382G
G2410E
G2413A
N2534D
N2547D
H2684R
T2997I

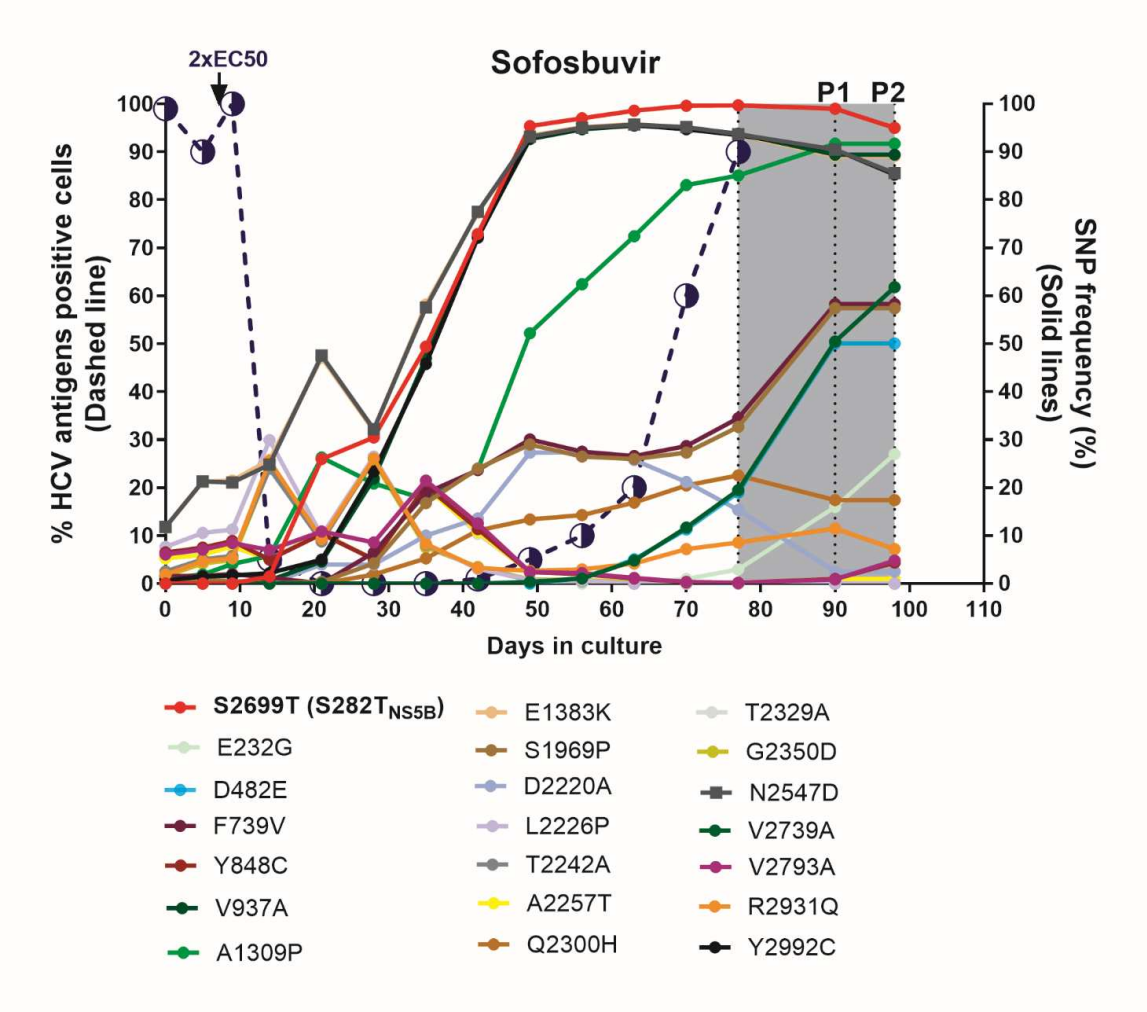
Supplemental Figure 5. NGS analysis of complete ORFs of ED43 escape viruses from treatments with protease inhibitors. (**A-C**) The frequencies of non-synonymous mutations in ORFs of the escape viruses under treatments with paritaprevir (A), grazoprevir (B) and glecaprevir (C), were analyzed by NGS as described (7). Only SNPs forming less than 20% of the genome population at day 0 that then emerged to represent more than 20% at least one-time point during treatment are shown. The putative RASs are shown in red with protein-specific numbers (in parentheses). Dashed line indicates HCV-antigen positive cells during the treatment. Shaded backgrounds indicate 1st- and 2nd passages without drugs (drug-free) using the samples from the last timepoint in each treatment experiment. See also Figure 2A-C.


TN
ED43
ED43-LEDesc
ED43-ELBesc
ED43-OMBesc
ED43-VELesc
ED43
ED43-PIBesc
% HCV antigen positive cells relative to nontreated control
Ledipasvir (log10nM)
Elbasvir (log10nM)
Ombitasvir (log10nM)
Velpatasvir (log10nM)

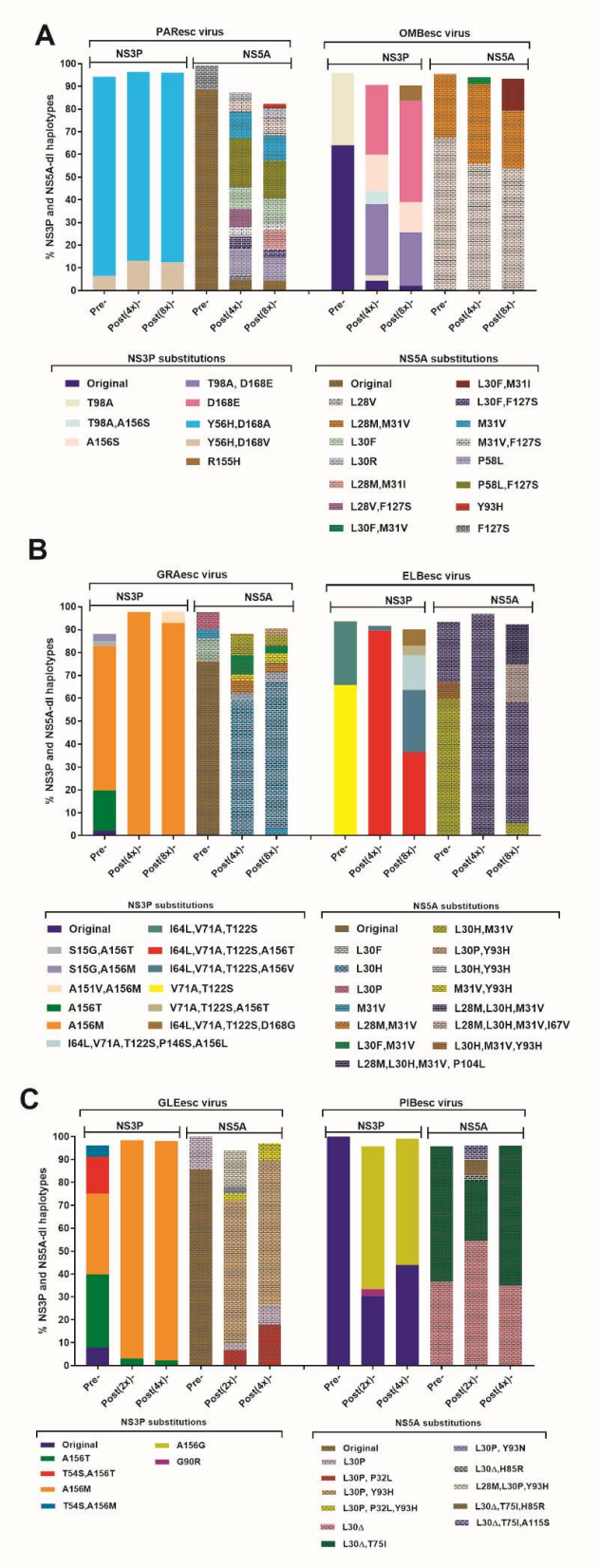
Supplemental Figure 6: Efficacy of NS5A inhibitors against HCV genotype 4a (ED43) viruses. Huhu7.5 cells were seeded on 96-well plates overnight, then infected with the indicated viruses for 24 hours. The cells were subsequently treated with specific inhibitors for an additional 48 hours before analysis as described (7, 9). Values are means of triplicates ±SEM. The original ED43 virus was used as a control. TN (1a) virus was included for comparison. See also Figure 3F,G.


A
Ombitasvir
P1
P2
% HCV antigens positive cells (Dashed line)
SNP frequency (%) (Solid lines)
Days in culture
L2000V (L28V$_{NS5A}$)
L2000M (L28M$_{NS5A}$)
L2002F (L30F$_{NS5A}$)
M2003V (M31V$_{NS5A}$)
N193H
R195S
T331A
V357M
S732P
E854A
T1124A
F1572L
I2271V
S2335P
T2366A
M2388T
S2402P
K2541E
K2598N
K2927Q
H2983Q
D
Velpatasvir
100x-EC50
L2000M (L28M$_{NS5A}$)
L2002F (L30F$_{NS5A}$)
M2003V (M31V$_{NS5A}$)
Y2065H (Y93H$_{NS5A}$)
V357M
T1124A
K2327N
S2402P
K2541E
B
Elbasvir
L2002H (L30H$_{NS5A}$)
M2003V (M31V$_{NS5A}$)
L2000M (L28M$_{NS5A}$)
L2002F (L30F$_{NS5A}$)
L2002P (L30P$_{NS5A}$)
Y2065H (Y93H$_{NS5A}$)
M20V
A360T
S440N
N476S
I769T
I1090L
V1097A
T1148S
L2226P
I2247Δ
V2376A
K2541E
K2687R
I2720T
K2961N
E
Pibrentasvir
10x-EC50
100x-EC50
L2002Δ(L30Δ$_{NS5A}$)
M825T
C1968F
T2047I
V2337G
M2632V
C
Ledipasvir
L2002P (L30P$_{NS5A}$)
M2003V (M31V$_{NS5A}$)
Y2065H (Y93H$_{NS5A}$)
S732P
E1699D
K2541E
K2598N

Supplemental Figure 7. NGS analysis of complete ORFs of ED43 escape viruses from treatments with NS5A inhibitors. (**A-E**) The frequencies of non-synonymous mutations in ORFs of the escape viruses under treatments with ombitasvir (A), elbasvir (B), ledipasvir (C), velpatasvir (D) and pibrentasvir (E), were analyzed by NGS as described (7). Only SNPs forming less than 20% of the genome population at day 0 that then emerged to represent more than 20% at least one-time point during treatment are shown. The putative RASs are shown in red with protein-specific numbers (in parentheses). Dashed line indicates HCV antigen positive cells during the treatment. Shaded backgrounds indicate 1st- and 2nd passages without drugs (drug-free) using the samples from the last timepoint in each treatment experiment. See also Figure 3A-E.

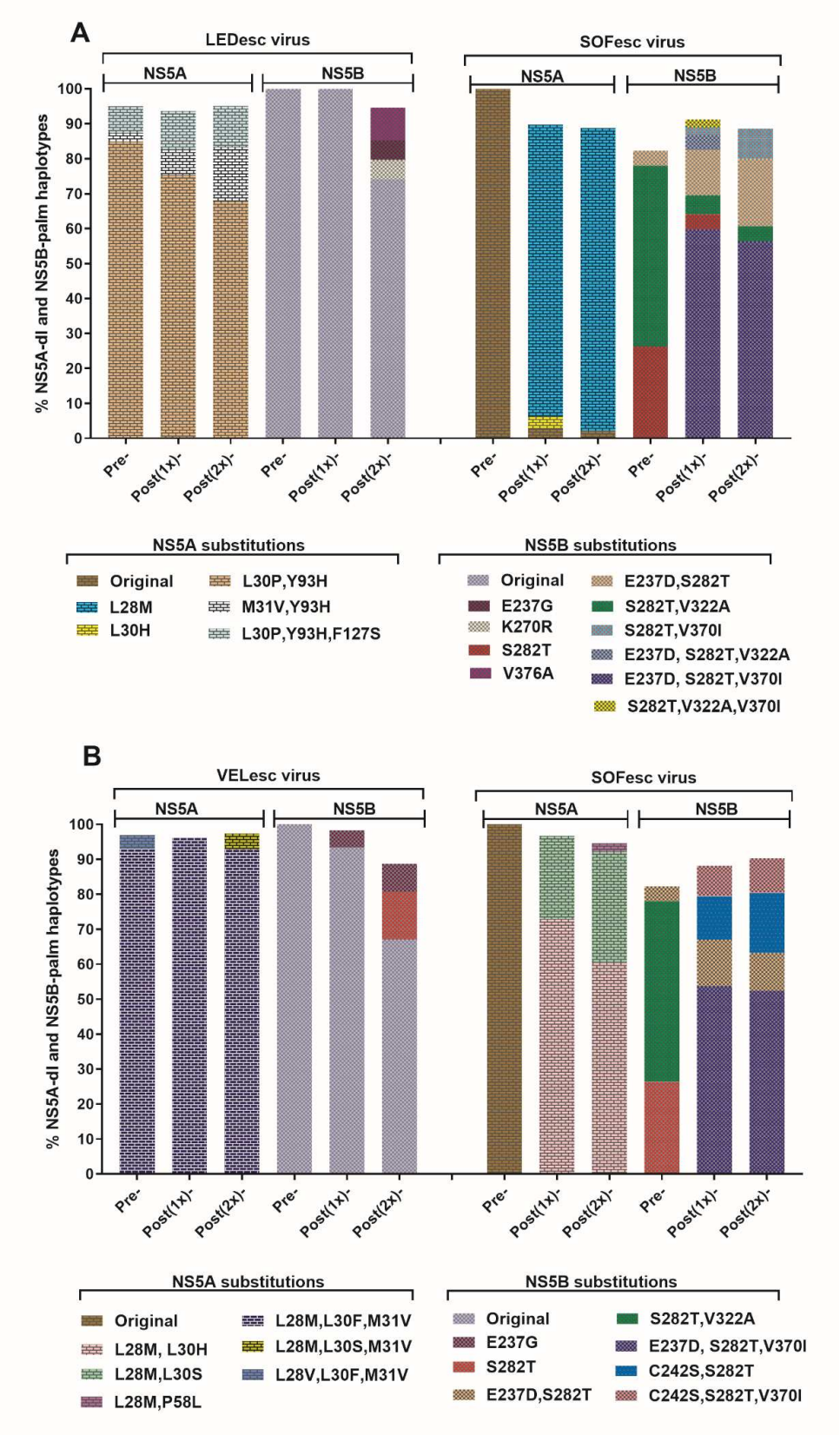


Supplemental Figure 8. NGS analysis of complete ORFs of ED43 escape viruses from treatments with sofosbuvir. The frequencies of non-synonymous mutations in ORFs of the escape viruses under treatment with sofosbuvir, were analyzed by NGS as described (7). Only SNPs forming less than 20% of the genome population at day 0 that then emerged to represent more than 20% at least one-time point during treatment are shown. The putative RASs are shown in red with protein-specific numbers (in parentheses). Dashed line indicates HCV-antigen positive cells during the treatment. Shaded backgrounds indicate 1st- and 2nd passages without drugs (drug-free) using the samples from the last timepoint in each treatment experiment. See also Figure 4A.

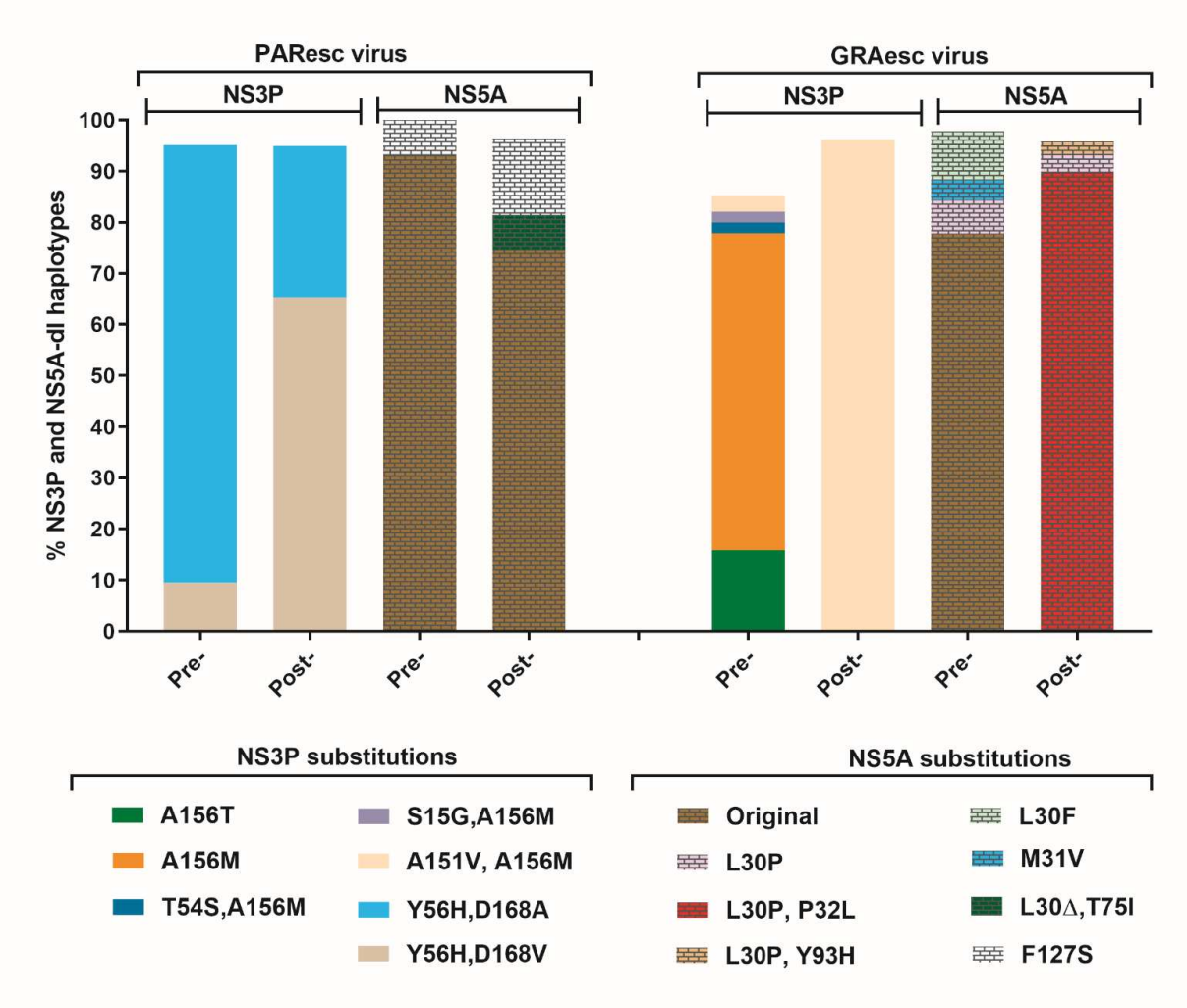

A
PAResc virus
OMBesc virus
NS3P
NS5A
% NS3P and NS5A-dl haplotypes
Pre-
Post(4x)-
Post(8x)-
NS3P substitutions
Original
T98A
T98A,A156S
A156S
T98A, D168E
D168E
Y56H,D168A
Y56H,D168V
R155H
NS5A substitutions
Original
L28V
L28M,M31V
L30F
L30R
L28M,M31I
L28V,F127S
L30F,M31V
L30F,M31I
L30F,F127S
M31V
M31V,F127S
P58L
P58L,F127S
Y93H
F127S
B
GRAesc virus
ELBesc virus
NS3P substitutions
Original
S15G,A156T
S15G,A156M
A151V,A156M
A156T
A156M
I64L,V71A,T122S,P146S,A156L
I64L,V71A,T122S
I64L,V71A,T122S,A156T
I64L,V71A,T122S,A156V
V71A,T122S
V71A,T122S,A156T
I64L,V71A,T122S,D168G
NS5A substitutions
Original
L30F
L30H
L30P
M31V
L28M,M31V
L30F,M31V
L28M,L30H,M31V, P104L
L30H,M31V
L30P,Y93H
L30H,Y93H
M31V,Y93H
L28M,L30H,M31V
L28M,L30H,M31V,I67V
L30H,M31V,Y93H
C
GLEesc virus
PIBesc virus
Post(2x)-
Post(4x)-
NS3P substitutions
Original
A156T
T54S,A156T
A156M
T54S,A156M
A156G
G90R
NS5A substitutions
Original
L30P
L30P, P32L
L30P, Y93H
L30P, P32L,Y93H
L30Δ
L30Δ,T75I
L30P, Y93N
L30Δ,H85R
L28M,L30P,Y93H
L30Δ,T75I,H85R
L30Δ,T75I,A115S

Supplemental Figure 9: Distributions of haplotypes in viruses after treatment with DAA combinations containing PIs and NS5A inhibitors. (**A-C**) Linkage analysis showed distributions of haplotypes in viruses that escaped from treatments with paritaprevir/ombitasvir (A), grazoprevir/elbasvir (B), and glecaprevir/pibrentasvir (C). For each combination, 2 different concentrations of PIs were used as outlined in Figure 5. Only haplotypes accounting for ≥2% of the viral population are shown. PAR, OMB, GRA, ELB, GLE, and PIB: paritaprevir, ombitasvir, grazoprevir, elbasvir, glecaprevir, and pibrentasvir, respectively. PAResc, GRAesc, GLEesc, OMBesc, ELBesc, and PIBesc: the virus that escaped from single treatments with paritaprevir, grazoprevir, glecaprevir (as shown in Figure 2), ombitasvir, elbasvir, and pibrentasvir (as shown in Figure 3), respectively. See also Figure 5.

Pham LV, *et al. Gut* 2022; 71:627–642. doi: 10.1136/gutjnl-2020-323585


A
LEDesc virus
SOFesc virus
NS5A
NS5B
% NS5A-dl and NS5B-palm haplotypes
Pre-
Post(1x)-
Post(2x)-
NS5A substitutions
Original
L28M
L30H
L30P,Y93H
M31V,Y93H
L30P,Y93H,F127S
NS5B substitutions
Original
E237G
K270R
S282T
V376A
E237D,S282T
S282T,V322A
S282T,V370I
E237D, S282T,V322A
E237D, S282T,V370I
S282T,V322A,V370I
B
VELesc virus
SOFesc virus
L28M, L30H
L28M,L30S
L28M,P58L
L28M,L30F,M31V
L28M,L30S,M31V
L28V,L30F,M31V
C242S,S282T
C242S,S282T,V370I

Supplemental Figure 10: Distributions of haplotypes in viruses after treatment with DAA combinations containing NS5A inhibitors and sofosbuvir. (**A,B**) Linkage analysis showed distributions of viral haplotypes after treatments with ledipasvir/sofosbuvir (A) and velpatasvir/sofosbuvir (B). For each DAA combination, the concentrations of 5x-EC_{50} of NS5A inhibitors were used in combination with either 1x- or 2x-EC_{50} of sofosbuvir. For details, see legend of Supplemental Figure 9. LED, VEL, and SOF: ledipasvir, velpatasvir, and sofosbuvir, respectively. LEDesc, VELesc, and SOFesc: the virus that escaped from single treatments with ledipasvir, velpatasvir (as shown in Figure 3), and sofosbuvir (as shown in Figure 4), respectively. See also Figure 6.



Supplemental Figure 11. Distributions of viral haplotypes after treatment with DAA combination glecaprevir/pibrentasvir. The concentrations of 4x-EC_{50} of glecaprevir in combination with 5x-EC_{50} of pibrentasvir were used for treatments. For details, see Supplemental Figure 9 legend. GRAesc, PAResc: the virus escaped from single treatments with grazoprevir, paritaprevir (as shown in Figure 2), respectively. See also Figure 7.

Supplemental Table 1. Sanger sequencing analysis of recovered ED43(C5A) viruses.

		HCV gene	NS2	NS3	NS3	NS3	NS4A	NS4B	NS4B	NS4B	NS4B	NS5A
		ED43 nucleotide position	2819	4211	4733	5054	5354	5697	5804	5933	5949	7578
		Original nucleotide	**A**	**A**	**A**	**T**	**G**	**C**	**A**	**A**	**G**	**A**
Recombinant virus	**Second passage titer (day)[a]**	**Synonymous mutation**										
ED43(C5A)-2m	**4.0 (13)**	**T547C, T4303T/C, T5365A, T7255C, T7495T/C**	**G/a**	**A/G**	**.**	**.**	**T**	**T**	**G**	**G**	**G/A**	**.**
ED43(C5A)-7m	**4.1 (13)**	**None**	**G**	**G**	**.**	**T/c/a**	**T**	**T**	**G**	**G**	**A**	**.**
ED43(C5A)-9m	**4.2 (11)**	**None**	**G**	**G**	**G**	**.**	**T**	**T**	**G**	**G**	**A**	**G**
		ED43 amino acid position	827	1291	1465	1572	1672	1786	1822	1865	1870	2413
		H77 amino acid position	827	1291	1465	1572	1672	1786	1822	1865	1870	2416
		Amino acid change	**T-A**	**I-V**	**S-G**	**F-L/I[b]**	**A-S**	**A-V**	**T-A**	**T-A**	**S-N**	**D-G**

Note: Nucleotide changes resulting in amino acid substitutions are shown. Letters with shaded background indicate the engineered mutations. Acquired mutations are indicated with only capital letters (complete nucleotide changes), or capital/capital letters (50/50 quasispecies), or capital/lower letters (major/minor change). “Dots” indicate identical nucleotides with the original sequence. "None”; no synonymous mutations were found in recovered viruses. See also Figure 1A.

[a]The infectivity titers are shown as $\log_{10}$FFU/mL.

[b]Nucleotide change T to C and T to A results in amino acid substitution F to L and F to I, respectively.

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