Characteristics of hepatitis C virus resistance in an international cohort after a decade of direct-acting antivirals

Anita Y.M. Howe, Chaturaka Rodrigo, Evan Cunningham, Mark W. Douglas, Julia Dietz, Jason Grebely, Stephanie Popping, Javier Alejandro Sfalcin, Milosz Parczewski, Christoph Sarrazin, Adolfo de Salazar, Ana Fuentes, Murat Sayan, Josep Quer, Midori Kjellin, Hege Kileng, Orna Mor, Johan Lennerstrand, Slim Fourati, Velia Chiara di Maio, Vladimir Chulanov, Jean-Michel Pawlotsky, P. Richard Harrigan, Francesca Ceccherini-Silberstein, Federico Garcia, and SHARED Collaborators

Table of contents

Supplementary patients and methods

M1. RAS prevalence, DAA class resistance, and RAS patterns

Based on the 2020 EASL Recommendations on Treatment of Hepatitis C, RAS were examined at the following positions: 36, 41, 43, 54, 55, 56, 80, 122, 155, 156, 158, 166, 168, 170 and 175 within NS3; 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92, 93 within NS5A; 150, 159, 206, 282, 316, 320, 321 within NS5B for sofosbuvir (SOF); and 314, 316, 368, 395, 411, 414, 445, 446, 448, 553 - 559, 561 and 565 within NS5B for dasabuvir (DSV). Patients with detectable GT-specific RAS variants in each target gene were counted and normalized to the total number of patients for the RAS prevalence analysis. Patients were categorized into the respective DAA class for the class resistance analysis if a substitution was detected at any of the listed amino acid positions within the respective gene. For the NS5A RAS patterns evaluation, individual substitutions within NS5A were parsed and tallied at all positions according to the Recommendations.

M2. Potential NS5A substitutions associated with virologic failure

The substitution frequency of an amino acid position was defined by summing the samples with substitutions and dividing by the number of samples with sequences available at that position. The sFC at each position was calculated by subtracting the substitution frequency after-treatment from that before-treatment. The background drifts were determined by taking an average of the sFCs across the first 200 amino acids within NS5A. A "genetic drift corridor" using two standard deviations from the background drifts was created to exclude positions with usual genetic fluctuations. Amino acid positions with sFC beyond the "genetic drift corridor" were considered positions of interest. Two broad categories of the sFC based on the direction of the change were observed: a "positive" sFC represented an increase in the substitution levels (or decrease in the reference amino acid levels) after treatment; a "negative" sFC represented a decrease in the substitution levels (or increase in the reference amino acids) after treatment.

The *P*-value derived from Fisher's Exact test between the before- and after-treatment groups at each amino acid position was generated in a pairwise comparison. A Benjamini-Hochberg method and the Bonferroni correction were employed to determine the minimum alpha values for multiple comparisons. Bonferroni correction attempts to limit even a single false

positive (type I error rate) at the expense of increasing the false negatives (type II error rate). For 200 comparisons with Bonferroni correction, a *P*-value <0.00025 was considered significant. The Benjamini-Hochberg statistic sets the proportion of false positives among results identified as significant (the false discovery rate, q). In this study, we considered a 15% false discovery rate ($q \leq 0.15$) acceptable. According to the Benjamini-Hochberg procedure, the *P*-values were ranked from smallest to highest for all comparisons and designated P_i. The q-value for each *P*i was calculated as P_i*(i/m), where i = rank of *P*-value and m = total number of comparisons. Amino acid positions with a *P*-value associated with a $q < 0.15$ were considered positions of interest.

Individual amino acids were parsed within the positions of interest, and odds ratios were calculated to identify specific amino acids responsible for the frequency change after treatment. Only amino acid substitutions observed in two or more patients were included in the analysis.

Supplementary results and discussion

R1. Prevalence of RAS across different HCV genotypes

RAS against NS3-targeting DAAs

The natural and post-treatment prevalence of NS3 RAS prevalence was estimated from 531 PI-naive and 552 DAA failures, respectively The brefore/after treatment prevalence of RAS in NS3 varied among different GTs: 44% (94/216) / 64% (99/155) in GT1a, 48% (74/155) / 61% (178/291) in GT1b, 100% (2/2) / 50% (1/2) in GT1-other, 17% (1/6) / 71% (5/7) in GT2, 17% (25/143) / 61% (14/23) in GT3, 11% (3/27) / 35 % (18/51) in GT4, and 100% (3/3) / 100% (2/2) in GT6 (Figure 1 in the manuscript). Overall, an increase of RAS prevalence (14 – 55%) was seen following DAA treatment.

In GT1a, Q80K (37/155, 24%) and R155 (31/155, 20%) represented the most common RAS after failing PI-treatments, whereas Y56F (86/291, 30%) was frequently seen in GT1b. Over 90% of the GT1a and GT1b patients in these analyses received the first-generation PIs; the RAS prevalence for the newer PIs remained undetermined. In GT3, about half of the patients (10/23) were treated with the first-generation PIs, and the other half (13/23) received glecaprevir or voxilaprevir-containing regimens. A156G or D168R/K/L, highly resistant to all PIs, were detected in ~50% (11/23) of all GT3 PI-failures. Six glecaprevir- or voxilaprevir-treated patients

harbored a single V170I substitution after failing the treatments. Unfortunately, *in vitro* drug susceptibility of this variant in GT3 was not available to ascertain its biological relevance. Mutations at amino acids 168 and 156 were also common in GT4; nearly all GT4 virologic failures with detectable RAS harbored D168V/E and/or A156T in this GT.

RAS against NS5A-targeting DAAs

A similar analysis was conducted in the NS5AI-naïve (n = 1597) and NS5AI-treated (n = 1487) patients for the NS5A RAS prevalence. The natural prevalence of NS5A in the untreated patients varied among GTs: 18% (123/685) in GT1a, 38% (98/255) in GT1b, 100% (6/6) in GT1-other, 100% (65/65) in GT2, 17% (82/472) in GT3, 86% (91/106) in GT4, none in the one GT5 patient, and 86% (6/7) in GT6 (Figure 1 in the manuscript). Following treatment failure, 74 – 100% of these patients had one or more RASs in the NS5A region: 74% (304/412) in GT1a, 88% (383/435) in GT1b, 100% (19/19) in GT1-other, 100% (23/23) in GT2, 74% (362/488) in GT3, 94% (97/103) in GT4, and 86% (6/7) in GT6 (Figure 1 in the manuscript). In GTs 1a, 1b, and 3, failing NS5AI-treatment resulted in a 50 – 57% increase of NS5A RAS. About 60% (845/1226) of all NS5A RAS selections were attributed to LDV- or DCV-based regimens, the two most prescribed NS5AIs in these patients (Figure S1).

A detailed characterization of the NS5A RAS in different GTs was described in the NS5A RAS patterns section in the manuscript.

RAS against NS5B-targeting DAAs

In marked contrast to the rapid selection of RAS in NS3 and NS5A, the RAS prevalence in the SOF-naive and SOF-treated patients was low (Figure 1 in the manuscript). The overall NS5B_SOF RAS prevalence in treatment naïve (46/314, 14%) and SOF-exposed virologic failure (223/999, 22%) was significantly lower ($P < .001$) than those in the NS3 (untreated: 202/552, 36% and treated: 317/531, 60%) and NS5A (untreated: 471/1597, 20% and treated: 1194/1487, 80%). The natural NS5B_SOF RAS prevalence were 2% (2/108) in GT1a, 44% (16/36) in GT1b, none in GT1-other (0/1) and GT2 (0/18), 20% (27/132) in GT3, 5% (1/19) in GT4. Following SOF-treatment failure, the NS5B RAS prevalence was: 3% in GT1a (7/223), 47% (115/246) in GT1b, 4% (1/28) in GT2, 21% (86/406) in GT3, 18% (14/78) in GT4, and

none in GT1-other (0/16), GT5 (0/1) and GT6 (0/1) (Figure 1 in the manuscript). There was no major difference in the NS5B_SOF before and after SOF treatment.

In GT1a, 4/223 (2%) SOF-failures had a single S282T mutation. In GT1b, only one virologic failure 1/115 (0.9%) harbored S282T. All GT1b patients with detectable RAS had L159F and/or C316N within NS5B; however, these mutations conferred only a low resistance level to SOF in HCV GT1b replicons.[1] There was only one GT2 SOF-exposed patient with detectable RAS (1/28, 4%), and S282G was detected in this patient. In GT3, 6/406 (2%) SOF-failures selected a single S282T/R, and 120/406 (30%) had A150V/I/S/T with or without K206E/Q; all three RAS are resistant to SOF *in vitro*.[2] While listed only for GT3 in the 2020 EASL Recommendations, A150V and K206E/Q were frequently detected in virologic failures infected with the other GTs (Tables S4 - S5); however, their link to drug resistance has not been established *in vitro*. In contrast to the GTs 1a, 1b, 2, and 3 SOF-exposed patients, 14/78 (18%) GT4 patients who failed SOF-treatments selected S282T mutation ($P < 0.001$). To date, S282T is the only RAS associated with reduced SOF susceptibility (2- to 19-fold) in all GTs.[3] In the registration trials, only one out of ~2500 patients selected S282T.[4] In this instance, HCV variants harboring S282T reverted to wild-type shortly after treatment was discontinued suggesting that this variant is replicative unfit. Considering that most of the SHARED sequences came from real-world clinics where samples were collected infrequently, the detection of S282T at such high prevalence suggests that this substitution could be more stable in a GT4 background.

Dasabuvir is a component of a triple-DAA combination with OMB and PAR/r for treating GT1, often with the addition of RBV. The natural prevalence of NS5B_DSV was 0% in GT1a but 39% in GT1b. The benefit of multiple DAAs in the DSV-containing cocktail was evident from the low RAS prevalence in the GT1a (22/65, 34%) and GT1b (20/48, 42%) patients who failed DSV-containing regimens (Figure 1 in manuscript). Most of the GT1a DSV-failures selected NS5B S556G (14/65, 22%), while GT1b selected C316N (15/48, 31%) with or without S556G (10/48, 21%).

The above sections summarized the key RAS variants in different GTs based on the 2020 EASL Recommendations; however, many previously unrecognized RAS variants were observed in our patient cohort. These newly discovered RAS variants are listed in Table S2.

R2. Novel substitutions potentially associated with virologic failure in NS5A

The number of NS5A sequences before/after treatment used for the analyses were: 697/424 for GT1a, 262/451 for GT1b, 479/497 for GT3, and 108/108 for GT4.

In GT1a, known RAS at amino acids 30 (sFC = 0.46, $P << .0001$) , 31 (sFC = 0.17, $P << .0001$), and 93 (sFC = 0.18, $P << .0001$) were identified by both approaches (Table 2, Figure 5 in manuscript); amino acid 28 was identified only by the pairwise comparison ($P << .0001$). Previously unrecognized substitutions at amino acids 48 (sFC = -0.12, $P << .0001$) and 78 (sFC = -0.11, $P << .0001$) were identified by both methods, while amino acid 73 ($P = 0.007$) was identified by the pairwise comparison only. Consistent with the results described in the previous section, M28T/V, Q30H/K/R/E, L31M/V, and Y93H/N/C were the key amino acid subsitutions observed following treatment failure (Table 2 in manuscript). Within the newly identified amino acid positions, R48K (OR = 0.5, 95% CI 0.4 – 0.7), R73K (OR = 0.3 95% CI 0.1 – 0.9), and R78K (OR = 0.6, 95% CI 0.5 – 0.8) were negatively associated with NS5AI treatment. All three positions had a mixture of R (reference) and K before treatment and the level of the R increased after treatment. Since replicons containing the reference amino acids were sensitive to NS5AIs, it is unlikely that moving "towards" the reference was linked to drug resistance, and instead are probably due to adaptive changes.

There were no new positions identified in GT1b. Known RAS at amino acids 31 (sFC = 0.38, P << .0001) and 93 (sFC = 0.63, P << .0001) were the only two positions identified by both methods. Amino acid 28 (P << .0001) was identified by the pairwise comparison but not by the sFC method. L28M, L31I/M/V, and Y93H were the key amino acids identified at these three positions.

In GT3, amino acid 93 (sFC = 0.54, P << .0001) and a previously undescribed substitution at amino acid 14 (sFC = 0.11, P < .0001) were identified by both approaches. Amino acid 13 (P = .002) was identified by the pairwise comparison, and amino acids 159 (sFC = -0.17) and 171 (sFC = 0.15) were identified by the sFC approach. In addition to Y93H , C13S (OR = 3.3, 95% CI 1.4 – 8.2), S14T (OR = 1.5, 95% CI 1.2 – 2.0), and E171D (OR = 13.9, 95% CI 1.7 – 114.2) were more frequently detected in patients following NS5AI failure. A decreased frequency of H159Q (OR = 0.3, 95% CI 0.1 – 0.9) was observed after treatment. The newly identified S14T was detected in 35% (29/83) of virologic failures previously considered having

no detectable RAS. Interestingly, all virologic failure samples with C13S (n = 21) co-existed with S14T.

In addition to the known RAS at amino acids 28 (sFC = 0.31, P << .0001) and 93 (sFC = 0.2, p << .0001), several previously unrecognized positions were identified by both methods in GT4; these include amino acids 6 (sFC = 0.14, p < .004), 17 (sFC = 0.18, P << .0001), 83 (sFC = 0.12, P = .0003), 117 (sFC = -0.13, P = .0009). Additional substitutions at positions 30 (sFC = 0.13), 56 (sFC = 0.13), and 37 (sFC = -0.13) had a notable frequency shift after treatment but did not meet the acceptance criteria in the pairwise comparison. In addition to the known RAS, L28S/V, Y93H/C and L30R, the odds of having W6R (OR = 4.0, 95% CI 1.3 – 12.7), S17T (OR = 34.3, 95% CI 2.0 – 583.3), T56R (OR = 18.5, 95% CI 1.1 – 325.4), and T83V (OR = 12.3, 95% CI 1.6 – 96.9) were higher in patients after exposure to NS5AIs. In contrast, the likelihood of having L37F (OR = 0.5, 95% CI 0.3 – 0.9) and D117E (OR = 0.1, 95% CI 0.02 – 0.5) were lower after treatment compared to pre-treatment.

Among the substitutions identified in GT4, L30R was highly prevalent before and after treatment in GT4d (Figure 3 in manuscript). GT4a and GT4d are the two predominant subtypes of GT4 circulating in Africa, the Middle East, and recently in Southern Europe.[5] A recent survey of 573 GT4 patients who participated in clinical trials of approved DAAs showed that the SVR rates for GT4a ranged from 96% to 100%, but for GT4d were only 81% to 100%.[6] Among the 12 virologic failures in this study, seven were infected with 4d, two with 4r, one each with 4b and 4a, and an unknown GT4 subtype. It is tempting to speculate that L30R, presented as a natural polymorphism in GT4d, might precipitate virologic failure in this subtype. Nevertheless, detailed reverse genetics and in vitro-in vivo correlations are required to establish if these new substitutions have reduced drug susceptibility or compensatory functions.

Table S1. Genotype-specific reference sequences recommended by the US Foods and Drugs Administration

Genotype	Reference strain	GenBank accession id	Length (bp)	Nucleotide position NS3-4A	Nucleotide position NS5A	Nucleotide position NS5B
GT 1a	H77	NC_004102	9646	3420-5474	6258-7601	7602-9377
GT 1b	Con1	AJ238799	9030	3420-5474	6258-7598	7599-9374
GT 2	JFH-1	AB047639	9678	3431-5485	6269-7666	7667-9442
GT 3	S52	GU814263	9555	3436-5490	6274-7629	7630-9402
GT 4	ED43	GU814265	9497	3419-5473	6257-7591	7592-9364
GT 5	SA13	AF064490	9408	3328-5382	6166-7515	7516-9291
GT 6	EUHK2	Y12083	9340	3374-5428	6212-7564	7565-9340

Table S2. Newly observed resistance-associated substitutions variants in the SHARED cohort

NS3 RAS						
GT1a	**GT1b**	**GT2**	**GT3**	**GT4**	**GT5**	**GT6**
V36A/C/F/G/L/M Q41R/**N/H/L** F43I/L/S/V T54A/S V55I/**A** Y56H Q80K/L/R/**H** S122G/N/R/**I** R155G/I/K/M/Q/S/T/V A156G/P/S/T/V V158I/**M/L** D168A/C/E/F/G/H/I/K/L/N/Q/R/T/V/Y I/V170T/V	V36A/C/G/L/M/**I** Q41R/**H** F43I/S/V T54A/C/G/S V55A Y56H/L/F Q80K/L/R S122A/D/G/I/N/R/T/**C** R155C/G/I/K/L/Q/M/S/T/W A156G/P/S/T/V/**D** V158I D168A/C/E/F/G/H/I/K/L/N/Q/R/T/V/Y I/V170T/V M175L	F43V V55A/I Y56H/F A156L/M/T/V D168A/E/F/G/H/N/S/T/V/Y	Q41K/**R** Y56H Q80K/R R155K A156G/P/T/V A166S/T/Y Q168H/K/L/R	Q41K Y56H Q80R R155C/K/**Q** A156G/H/K/L/S/T/V Q168A/E/G/H/T/V/**N**	R155K A156T/V D168A/E/H/K/R/V/Y	V36I Q41K/R Y56H L80K/Q S122T A156T/V D168A/E/G/H/V/Y/**C** I170V
NS5A RAS						
GT1a	**GT1b**	**GT2**	**GT3**	**GT4**	**GT5**	**GT6**
K24E/Q/R/T/**N/S** K26E/**Q/R** M28A/G/S/T/V/**M** P29R/**H** Q30C/D/E/G/H/K/L/N/R/T/Y/del30/**A** L31I/F/M/P/V/**C** P32L/S/del32 S38F/**Y/H/W/P** H58C/D/L/P/R/**Q/Y/S/N/G** A92K/T/**P** Y93C/F/H/L/N/R/S/T/W	Q24K/**R/H/A** L28A/M/T/**I/V/F** P29S/del29/**L** R30G/H/P/Q/S/**K/V** L31F/I/M/V/W/**E** P32F/L/S/del32 P58A/D/L/S/R/T/**G** Q/E62D/**R/H/K/** A92E/K/T/V/**G/M/S** Y93C/H/N/R/S/T/**F**	T24A/S L/F28C/S P29S L30H/S L31I/M/V C92R/S/T/W Y93F/N/H	S24F/**T/G/A/P** M28T/K/**L/G/V/I** A30D/E/K/S/**T/V/R/G** L31F/I/M/P/V S62L/**T/P/M/Q/A/V/I/E/D** E92K Y93H/N/S/**F**	L28M/S/T/V L30F/G/H/R/S/**T/A/C/Q** M/L31I/V T58A/P/S Y93C/H/N/S/R/W	L28I Q30H L31F/I/V P32L	Q24H/**K** F/L28A/I/L/M/T/V R30E/H/N/S/**A** L31I/M/V P32A/L/Q/R/S T58A/G/H/N/S E92T T93A/H/N/S/**Y**
NS5B_SOF RAS						
GT1a	**GT1b**	**GT2**	**GT3**	**GT4**	**GT5**	**GT6**

L159F S282G/R/T/**C** C316H/R L320I/F/V V321A/**F**	L159F S282G/R/T/**C** C316F/H/N V321I	L159F S282G/R/T	A150V/E/**T/R/I/S/L/G** L159F/**P** K206E/**R/Q/N/T/G/H** S282G/R/T/**C** V321A/**G/V**	S282C/G/R/T V321A/**I/L**	S282G/R/T	S282G/R/T

NS5B_DSV RAS	
GT1a	**GT1b**
L314H	C316H/N/Y/W
C316Y	S368T
A395G	N411S
M414I/T/V	M414I/T/V
E446K/Q/**T**	C445F/Y
Y448C/H	Y448C/H
A553T/V	A553V
G554S	G554S
Y555H	S556G/R
S556G/R/**N**	G558R
G557R	D559G/N
G558R	
D559G/N	
Y561H/N	
S565F	

Resistance-associated substitutions (RAS) from the 2020 EASL Recommendations on Treatment of Hepatitis C were black.
Newly observed RAS variants (not listed in the 2020 Recommendations) from the virologic failures in the SHARED cohort were highlighted in red.

Table S3. Cohort characteristics of the representative subgroup and all virologic failures in SHARED

Characteristics Number of patients, n	Representative VF subgroup* n = 730	VFs in the SHARED cohort n = 1894
Gender, n (%)	721 (99%)	1819 (96%)
Male, n (%)	570 (79%)	1410 (78%)
Age, n (%)	670 (92%)	1803 (95%)
Age in 2021, median (IQR)	59 (53 - 65)	59 (52 - 65)
Coinfection[1], n (%)	523 (72%)	971 (51%)
HIV-HCV, n (%)	93 (13%)	159 (16%)
HBV-HCV, n (%)	12 (2%)	38 (4%)
Fibrosis, n (%)	312 (43%)	741 (39%)
F0-F1	58 (19%)	162 (22%)
F2	46 (15%)	115 (16%)
F3	55 (18%)	107 (14%)
F4	153 (49%)	357 (48%)
Cirrhosis, n (%)	534 (73%)	1227 (85%)
yes, n (%)	295 (55%)	641 (52%)
Hepatocellular carcinoma, n (%)	37 (5%)	138 (7%)
yes, n (%)	11 (30%)	24 (17%)
Genotype[2], n (%)	730 (100%)	1894 (100%)
GT1a, n (%)	218 (30%)	513 (27%)
GT1b, n (%)	259 (35%)	619 (33%)
GT1-other, n (%)	9 (1%)	22 (1%)
GT 2, n (%)	12 (2%)	39 (2%)
GT3, n (%)	133 (18%)	566 (30%)
GT4, n (%)	97 (13%)	127 (7%)
GT6, n (%)	2 (0.3%)	8 (0.4%)
DAA Treatment[3], n (%)	730 (100%)	1894 (100%)
NS5AI + NI, n (%)	419 (57%)	1236 (65%)
NS5AI + PI, n (%)	84 (12%)	190 (10%)
PI + NI, n (%)	81 (11%)	132 (7%)
NS5AI + PI + NI or NNI, n (%)	115 (16%)	202 (11%)
other, n (%)	31 (4%)	134 (7%)
Treatment history[4], n (%)	457 (62%)	1299 (69%)
treatment naïve, n (%)	297 (65%)	860 (66%)
treatment experienced, n (%)	160 (35%)	439 (34%)
prior PEG/RBV, n (%)	55 (34%)	232 (53%)

prior DAA, n (%)	34 (21%)	84 (19%)
unknown, n (%)	71 (44%)	123 (28%)

* Each patient in the representative VF subgroup had all three drug target genes sequences (NS3, NS5A, and NS5B).

1. HIV- and HBV-coinfection with HCV were not mutually exclusive; 3 (0.4%) and 3 (0.2%) participants were infected with HIV and HBV in addition to HCV in the representative VF subgroup and the VFs in the SHARED cohort, respectively.

2: Genotypes were derived from the HCV NS5A, NS3, or NS5B sequences.

3: DAA treatment associated with the HCV sequence examined.

4: Treatment history at the time when DAA treatment was administered.

VF, virologic failures; IQR, interquartile range; HIV, human immunodeficiency virus; HBV, hepatitis B virus; HCV, hepatitis C virus; GT, genotype; DAA, direct-acting antivirals; NS5AI, NS5A inhibitor; PI, protease inhibitor, NI, nucleoside (sofosbuvir); NNI, non-nucleoside (dasabuvir); other, pegylated interferon +/- ribavirin +/- DAA including boceprevir, telaprevir; PEG, pegylated interferon; RBV, ribavirin.

Table S4. Key NS5A RAS patterns in NS5AI-exposed virologic failures

	NS5A RAS pattern*	Number of samples	Percentage
GT1a n = 424	no RAS	98	23%
	30R	50	12%
	30H 93H	21	5%
	31M	15	4%
	93N	15	4%
	30R 31M	10	2%
	28V 30R	9	2%
	93H	8	2%
	30H	8	2%
	31V	7	2%
	30E	7	2%
GT1b n = 451	31M 93H	75	17%
	93H	73	16%
	no RAS	51	11%
	31V 93H	19	4%
	28M 93H	18	4%
	30Q 93H	16	4%
	31M	14	3%
	31I 93H	11	2%
	58S 93H	11	2%
	30Q 31M 93H	9	2%
GT1-other n = 20	31M 58P 62D	1	5%
	24K 30H 93H	1	5%
	30H 58Y 62D	1	5%
	24G/S 31M 58P 62Q	2	10%
	24R 58P 62Q 92T	1	5%
	28T 31M 58P 62D	1	5%
	28F 30S 31M 58P 62G	1	5%
	24G 30K 31M 58P 62Q	2	10%
	24A 30R 31M 58P 62L	1	5%
	28I 30S 31M 58P 62Q	1	5%
	24R 31M 58P 62Q 92T	1	5%
	28L 30S 31V 58P 62Q 93N	4	20%
	24Q 28L 30Q/R 31M 58P 62Q 93H	3	15%
GT2 n = 23	31M	6	26%
	24S 28C	4	17%

	31M 62S	3	13%
	24S 28C 92S	2	9%
	31M 58A	1	4%
	24S 28C 58S	1	4%
	24S	1	4%
	24A 28C 31M	1	4%
	31M 62T 92S	1	4%
	24S 28C 31M	1	4%
	24S 31M	1	4%
	24S 28L	1	4%
GT3 n = 497	93H	112	23%
	no RAS	83	17%
	62T 93H	42	8%
	62L 93H	35	7%
	62T	24	5%
	30K	17	3%
	93H/Y	11	2%
	30S 93H	11	2%
	30K 93H	10	2%
	30K 62L	9	2%
	62I 93H	9	2%
GT4 n = 108	30R 62E	15	14%
	28M 30R 62E	9	8%
	30R 58T 62E	8	7%
	62E	5	5%
	28S 30R 62E	5	5%
	30R 62E 93C	3	3%
	28M 62Q 93H	2	2%
	30R 31V 62E	2	2%
	28V 30R 58T 62E	2	2%
	28V 30R 31L 62S	2	2%
	28V 30R 62T	2	2%
	28V 30R 62N	2	2%
	28S 30H 31I 62E	2	2%
	30R 62Q	2	2%
	30H 62E	2	2%
	30R 62E 93H	2	2%
GT6 n = 7	28L 31M 58P 62Q 93H/Y	1	14%
	24K 28V 30A 58G	1	14%
	24K 28V 30S 58P 62M	1	14%
	24K 28V 30S 58P 62N	1	14%

	24K 28V 30A 58P 62E	1	14%
	no RAS	1	14%
	24K 28A 30A 58P 92T	1	14%

*only RAS patterns existed in ≥ 2% virologic failure population were presented

Table S5. Key NS3 RAS patterns in PI-exposed virologic failures

	NS3 RAS pattern*	Number of samples	Percentage
GT1a n = 163	no RAS	46	28%
	80K	9	6%
	155K	9	6%
	80K 155K	5	3%
	170V	5	3%
	56H 168A	3	2%
	54S	3	2%
	80L	3	2%
	80L 155K	3	2%
GT1b n = 299	no RAS	83	28%
	56F	35	12%
	170I	29	10%
	56F 170I	14	5%
	122T	9	3%
	168V	5	2%
	56H 168V	5	2%
GT1-other n = 2	80K	1	50%
	170V 175M	1	50%
GT2 n = 7	56F 122R	2	29%
	155P/R/S/W 166S/F	1	14%
	56F 122R 158M 168V	1	14%
	36V 56F 122R 168V	1	14%
	no RAS	1	14%
	56H 168V	1	14%
GT3 n = 23	170I	9	39%
	56H 168R 170I	3	13%
	166S 168K 170I	1	4%
	168R 170I	1	4%
	168L 170I	1	4%
	158I/V 168R 170I	1	4%
	41Q/R 156A/G 168Q/L 170I	1	4%
	156G 166S 170I	1	4%
	80K 156G 166S 170I	1	4%
	80R 166S 170I	1	4%
	80K 170I	1	4%

	166S 170I	1	4%
	80K/Q 156A/G 166T/A 170I	1	4%
GT4 n = 53	no RAS	29	55%
	168V	4	8%
	168E	3	6%
	80R 168E	2	4%
	156T	2	4%
	170A	2	4%
	166X 168V	1	2%
	122S	1	2%
	156T/A/S 170A	1	2%
	156V	1	2%
	56H 168V	1	2%
	55A/V 155Q 156T/I/A/V 168N	1	2%
	36X 41X 43X 122S	1	2%
	168E 170I	1	2%
	56H 168A	1	2%
	43Y/S	1	2%
	168A	1	2%
GT6 n = 2	80Q 122T 158F 166A 170V	1	50%
	36I 56H 80Q 122T 166A 168C 170V	1	50%

*Only RAS patterns existed in $\geq$ 2% virologic failure population were presented

Table S6. Key NS5B-SOF RAS patterns in NI-exposed (sofosbuvir) virologic failures

	NS5B_SOF RAS pattern*	Number of samples	Percentage
GT1a n = 232	no RAS	205	88%
	206K	10	4%
	206R	4	2%
GT1b n = 254	no RAS	134	53%
	316N	46	18%
	159F	23	9%
	159F 316N	20	8%
	159F 206K 316N	9	4%
	159F 206K	6	2%
	316H 321I	4	2%
GT1-other n = 16	no RAS	13	81%
	206K	1	6%
	206N	1	6%
	321I	1	6%
GT2 n = 30	no RAS	20	67%
	206Q	4	13%
	150T 206Q	3	10%
	150I	1	3%
	150T 282S/G	1	3%
	316H 321I	1	3%
GT3 n = 419	no RAS	282	67%
	150V	54	13%
	150T	12	3%
	206Q	10	2%
	150V 206E	9	2%
	206E	8	2%
GT4 n = 83	no RAS	38	46%
	150E 206N	16	19%
	282T	3	4%
	282T/S/C	3	4%
	150E 206S	2	2%
	206A 282T	2	2%
	206N	2	2%
	150E 206K	2	2%
	206K	2	2%
	282T/S	2	2%
GT6 n = 1	no RAS	1	100%

*Only RAS patterns existed in ≥ 2% virologic failure population were presented

Table S7. Key NS5B_DSV RAS patterns in NNI-exposed (dasabuvir) virologic failures

	N5B_DSV RAS pattern*	Number of samples	Percentage
GT1a n = 75	no RAS	46	61%
	556G	14	19%
	557R	2	3%
	316Y	2	3%
GT1b n = 51	no RAS	30	56%
	316N	8	15%
	316N 556G	5	9%
	556G	2	4%
	316N 414V	2	4%
	556S/G	2	4%
	556R	1	2%
	448H	1	2%
	316N/S/Y/C	1	2%
GT1-other n = 1	316H 368A	1	100%

*Only RAS patterns existed in $\geq$ 2% virologic failure population were presented

Fig. S1.

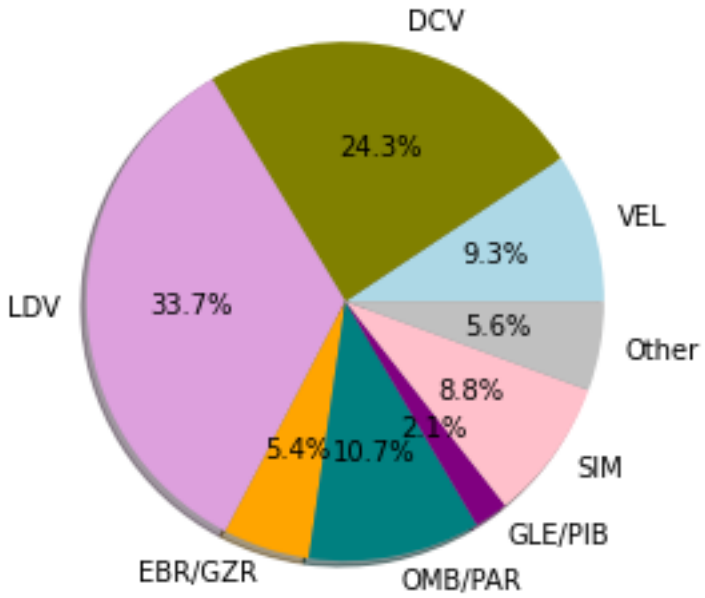
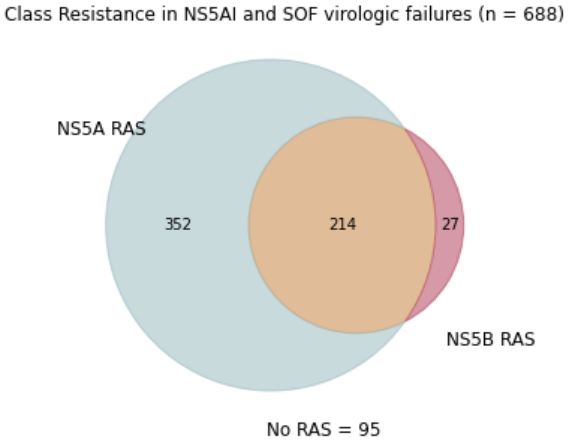


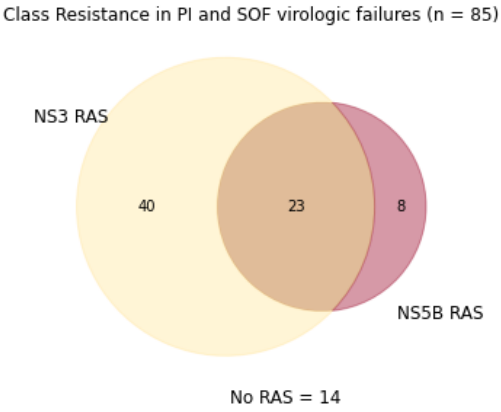
Fig. S1. Distribution of direct-acting antiviral regimens administered in virologic failures. There were a total of 1894 virologic failures in this cohort. Regimens containing a combination of direct-acting antivirals are labeled by the NS5A inhibitor and/or protease inhibitor: DCV (DCV/SOF±RBV, DCV/ASV, DCV/ASV/PEG/RBV, DCV/PEG/RBV); VEL (VEL/SOF±RBV); other (PEG/RBV/BOC, PEG/RBV/TVR, SOF/RBV, VEL/SOF/VOX); SIM (SIM/SOF±RBV, SIM/PEG/RBV, SIM/DCV±RBV, SIM/DCV); GLP/PIB; OMB/PAR (OMB/PAR/RIT/DAS±RBV, OMB/PAR/RIT±RBV); EBR/GZR (EBR/GZR±RBV, EBR/GZR/SOF); LDV (LDV/SOF±RBV, LDV/PEG/RBV). ASV, asunaprevir; BOC, boceprevir; DSV, dasabuvir; DCV, daclatasvir; EBR, elbasvir; GLE, glecaprevir; GZR, grazoprevir; LDV, ledipasvir; OMB, ombitasvir; PAR/r, paritaprevir/ritonavir; PEG, pegylated interferon; PIB, pibrentasvir; RBV, ribavirin; SIM, simeprevir; SOF, sofosbuvir; TVR, telaprevir; VEL, velpatasvir; VOX, voxilaprevir.

Fig. S2

(A) NS5AI + SOF



(B) PI + SOF



(C) PI + NS5AI

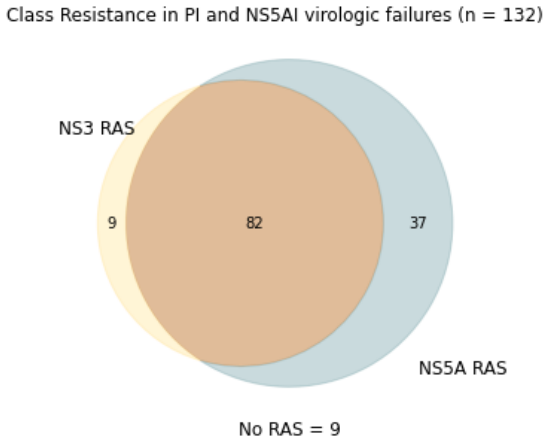


Fig. S2. Class resistance in patients treated with dual direct-acting antiviral combinations. Patients who failed an (A) NS5AI + SOF, (B) PI + SOF, or (C) PI + NS5AI were selected for the DAA class resistance analyses. Amino acid substitutions at all positions of the respective genes listed in the 2020 EASL Recommendations on Treatment of Hepatitis C were included for the evaluation. PI RAS, NS5AI RAS, and NI RAS are substitutions detected in NS3, NS5A and NS5B after treatment failure from the PI, NS5AI, and NI (SOF) drug classes, respectively. Each circle represents the number of patients with detectable RAS selected by each drug class: yellow, PIs; turquoise, NS5AIs; and red, NI. The size of the circle was proportional to the number of patients with detectable RAS. The intersecting regions represent dual- or triple- DAA class resistance. PI, protease inhibitor; NS5AI, NS5A inhibitor; NI, nucleoside inhibitor; SOF, sofosbuvir; DAA, direct-acting antiviral, and RAS, resistance-associated substitutions.

Supplementary references

1. Lam AM, Espiritu C, Bansal S, Micolochick Steuer HM, Niu C, Zennou V, Keilman M, et al. Genotype and subtype profiling of PSI-7977 as a nucleotide inhibitor of hepatitis C virus. Antimicrobial agents and chemotherapy 2012;56:3359-3368.
2. Wing PAC, Jones M, Cheung M, DaSilva S, Bamford C, Jason Lee WY, Aranday-Cortes E, et al. Amino Acid Substitutions in Genotype 3a Hepatitis C Virus Polymerase Protein Affect Responses to Sofosbuvir. Gastroenterology 2019;157:692-704 e699.
3. Xu S, Doehle B, Rajyaguru S, Han B, Barauskas O, Feng J, Perry J, et al. In vitro selection of resistance to sofosbuvir in HCV replicons of genotype 1 to 6. Antiviral therapy 2017;22:587-597.
4. Hedskog C, Dvory-Sobol H, Gontcharova V, Martin R, Ouyang W, Han B, Gane E, et al. Evolution of the HCV viral population from a patient with S282T detected at relapse after sofosbuvir monotherapy. Journal of viral hepatitis 2015;22:871-881.
5. Kamal SM, Nasser IA. Hepatitis C genotype 4: What we know and what we don't yet know. Hepatology 2008;47:1371-1383.
6. Boyd S, Harrington P, Komatsu T, Naeger L, Chan-Tack K, Murray J, Birnkrant D, et al. HCV genotype 4, 5 and 6: Distribution of viral subtypes and sustained virologic response rates in clinical trials of approved direct-acting antiviral regimens. Journal of viral hepatitis 2018;25:969-975.