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BASIC AND TRANSLATIONAL—LIVER

Prevalence of Resistance-Associated Substitutions in HCV NS5A, NS5B, or NS3 and Outcomes of Treatment With Ledipasvir and Sofosbuvir

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BACKGROUND & AIMS: We evaluated the effects of baseline hepatitis C virus (HCV) NS5A, NS5B, and NS3 resistance-associated substitutions (RASs) on response to the combination of ledipasvir and sofosbuvir, with or without ribavirin, in patients with HCV genotype 1 infection. **METHODS:** We analyzed data from 2144 participants in phase 2 and 3 studies of patients with HCV genotype 1a or b infection who received the combination of ledipasvir (90 mg) and sofosbuvir (400 mg) (ledipasvir/sofosbuvir) once daily, with or without ribavirin twice daily. Population and/or deep sequence analyses of the HCV NS3, NS5A, and NS5B genes were performed on blood samples collected at baseline. **RESULTS:** Overall, 16.0% of patients had detectable baseline RASs in NS5A. Among patients with HCV genotype 1b infection, there was no significant effect of baseline RASs in NS5A on sustained viral response 12 weeks after the end of treatment (SVR12) with ledipasvir/sofosbuvir and only a small effect in patients with HCV genotype 1a infection. RASs in NS5A that increased the half-maximal effective concentration to ledipasvir by more than 100-fold reduced the rate of SVR12 in treatment-naive patients given ledipasvir/sofosbuvir for 8 weeks ($P = .011$), but not for 12 weeks. These same baseline NS5A RASs reduced the percentage of treatment-experienced patients who achieved an SVR12 to 12 weeks (but not 24 weeks) ledipasvir/sofosbuvir ($P < .001$). These RASs had a small effect in patients given ledipasvir/sofosbuvir in combination with ribavirin for 12 weeks. Overall, 2.5% of patients had baseline NS5B nucleotide inhibitor RASs (L159F, N142T, S282G, or L320S) and all achieved an SVR12. Of patients previously treated with protease inhibitors, 53.7% had RASs in NS3 and 96.5% achieved an SVR12. **CONCLUSIONS:** Baseline RASs in NS5A have minimal effects on patient responses to ledipasvir/sofosbuvir therapy. When these RASs do have effects, they could be largely overcome by extending treatment duration or through treatment intensification.

Keywords: Direct-Acting Antivirals; ION-1; ION-2; ION-3.

Development of direct-acting antivirals (DAAs) in recent years has enhanced sustained virologic response (SVR) rates dramatically in hepatitis C virus (HCV) genotype 1 chronic infected patients.[1] In the phase 3 ION-1, ION-2, and ION-3 studies,[2–4] and the phase 2 LONESTAR study,[5] treatment-naive and -experienced HCV genotype 1–infected patients with and without cirrhosis who received 8, 12, or 24 weeks of the fixed-dose combination of NS5A inhibitor ledipasvir[6] and the nucleoside analog sofosbuvir (ledipasvir/sofosbuvir) with or without ribavirin achieved SVR rates of 94%–99%.

Despite the high rate of SVR 12 weeks after treatment (SVR12), because the high-rate replication and poor fidelity of the HCV-RNA–dependent polymerase leads to heterogeneous virus populations in infected patients, it is possible that the subpopulation of patients with pre-existing mutations that confer in vitro resistance to sofosbuvir or ledipasvir may influence outcome.[7] Such pre-existing mutations may exist at low levels in untreated patients, and emerge under the selective pressure of DAAs.[7,8]

For ledipasvir, in vitro and in vivo resistance are associated primarily with substitutions at genotype 1a residues K24, M28, Q30, L31, P32, H58, and Y93, and genotype 1b residues L31, P58, A92, and Y93.[9,10] Resistance to sofosbuvir is conferred by the S282T substitution in NS5B.[11] S282T first was described as the major resistance-associated substitution (RAS) for other nucleotide inhibitors (NIs).[12] In addition, the combination of S96T and N142T has been observed after in vitro selection with the NI

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Abbreviations used in this paper: **DAAs, direct-acting antiviral agents; HCV, hepatitis C virus; LiPA, Line Probe Assay; NI, nucleotide inhibitor; PI, protease inhibitor; RAS, resistance-associated substitution; SVR, sustained virologic response; SVR12, sustained virologic response 12 weeks after treatment.**

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R1479.[13] M289I/L/V were selected in vitro by various NIs,[11,13] and a combination of L159F and L320F was observed in 1 patient who had a partial response during treatment with mericitabine.[14,15] A comprehensive analysis of all substitutions in NS5B among all sofosbuvir-treated patients in the phase 2 and 3 studies identified 2 treatment-emergent substitutions, L159F and V321A, using deep sequencing (cut-off limit, 1%).[15,16]

The impact of HCV baseline RASs on SVR may depend on the susceptibility/fitness of the RASs, the patient population, the specific regimen, and treatment duration. For example, the efficacy of simeprevir in combination with sofosbuvir can be reduced significantly in patients infected with HCV genotype 1a with a NS3 Q80K polymorphism. The rates of sustained virologic response in treatment-naive patients treated for 8 weeks with and without Q80K detected by population sequencing (cut-off value, ~15%) were 73% and 84%.[17] SVR rates for patients with cirrhosis and a 12-week treatment duration were 74% with the Q80K polymorphism, compared with 92% without the polymorphism.[18]

Pre-existing RASs clearly influence virologic outcomes for the combination of the protease inhibitor (PI) asunaprevir with the NS5A inhibitor daclatasvir, which is an approved treatment in Japan. Although the overall SVR rate in the pivotal trial was 84%, the SVR rates for patients with baseline L31 or Y93 substitutions were between 38% and 41%.[19] In contrast, there was no apparent impact of baseline NS5A RASs on virologic response in patients treated with sofosbuvir + daclatasvir in a small phase 2b study.[20] These results, however, may have been a consequence of the small number of patients who relapsed, limiting the ability to evaluate the impact of NS5A RASs on outcome.

In this analysis, the baseline prevalence and effects of NS5A inhibitor, NI, and PI RASs on virologic response to ledipasvir and sofosbuvir with and without ribavirin in a large number of patients (n = 2144) from multiple studies from the ledipasvir/sofosbuvir phase 2/3 development program were investigated.

Materials and Methods

Ethics Statement

All studies were conducted in accordance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements. All patients provided written informed consent.

Study Design

Detailed descriptions of studies ION-1 (n = 865), ION-2 (n = 440), ION-3 (n = 647), LONESTAR (n = 100), and ELECTRON (n = 92) have been published[2–5,21] and are described briefly later. Patients had chronic HCV genotype 1 (1a or 1b) infection (with the exception of 1 genotype 1c, 1 genotype 1a/1b, 2 genotype 1h, 1 genotype 1l, 2 genotype 4a, and 2 genotype 1h patients) and received a fixed-dose combination tablet containing 90 mg of ledipasvir and 400 mg of sofosbuvir, administered orally once daily. Ribavirin was administered orally twice daily, with the dose determined according to body weight. ION-1 and ION-3 patients were treatment-naive, and ION-2 patients did not have a SVR after prior treatment with either pegylated interferon, ribavirin, or a PI or pegylated interferon and ribavirin. ION-1 and ION-2 included patients with cirrhosis, and patients received ledipasvir/sofosbuvir ± ribavirin for 12 or 24 weeks. ION-3 excluded patients with cirrhosis, and patients received ledipasvir/sofosbuvir ± ribavirin for 8 weeks or ledipasvir/sofosbuvir for 12 weeks. In LONESTAR, treatment-naive, noncirrhotic patients received ledipasvir/sofosbuvir ± ribavirin for 8 weeks, or ledipasvir/sofosbuvir for 12 weeks. Patients with and without cirrhosis who had failed a previous PI regimen received ledipasvir/sofosbuvir ± ribavirin for 12 weeks. Patients in ELECTRON received ledipasvir/sofosbuvir ± ribavirin for 12 weeks, or ledipasvir/sofosbuvir + ribavirin for 6 weeks (treatment-naive patients).

Laboratory Assessments

HCV RNA was determined at a central laboratory using the Roche High-Pure-System, COBAS TaqMan v.2 assay (Roche Molecular Diagnostics, Pleasanton, CA) with a lower limit of quantitation of 25 IU/mL. HCV genotype was determined using the Versant HCV Genotype 2.0 assay (Line Probe Assay [LiPA]) or by TRUGENE (both Siemens, Munich, Germany). The genotype results from LiPA and the TRUGENE assay were confirmed or refined by direct sequencing results from the viral NS5A, NS5B, and NS3, if available. Thirty-five LiPA/TRUGENE subtype assignments were refined or corrected.

Sequencing Analyses

Resistance testing was performed on available baseline plasma samples with a HCV RNA level of 1000 IU/mL or greater. For the NS5A gene, at the beginning of the studies only population sequencing was performed for patients who were enrolled in ION-1 part A and the initial group of patients in the ELECTRON study. Deep sequencing then was performed for all patients who were enrolled in ION-1 part B, ION-2 and ION-3, LONESTAR, and the later group of patients enrolled in the ELECTRON study. Overall NS5A population (n = 237) or deep (n = 1907) sequencing was performed at baseline for all enrolled patients in the phase 2/3 studies (ION-1, ION-2, ION-3, LONESTAR, and ELECTRON arms 12–13, 16, 17, 20, and 21). Baseline NS5B sequencing was performed successfully for a subset of patients by population (n = 64) and deep (n = 1628) sequencing. Baseline NS3 deep sequencing was performed successfully for all treatment-experienced patients (n = 467). Population sequencing (Sanger method) of the full-length HCV NS5A coding region was performed by the DDL Diagnostic Laboratory (Rijswijk, The Netherlands) or Monogram Biosciences (South San Francisco, CA). The sensitivity for detection of resistant substitutions using population sequencing is approximately 10%–20%.[22] Substitutions are reported as differences compared with a genotype-specific reference strain: genotype 1b Con1 (AJ238799); genotype 1a H77 (Genbank accession number: NC_004102). Deep sequencing was performed by Monogram Biosciences using an Illumina MiSeq deep sequencing platform (Illumina, San Diego, CA), or NS5A polymerase chain reaction amplicons generated by DDL were subjected to deep sequencing at Wuxi AppTec (Shanghai, China). Internally developed software (Gilead Sciences, Foster City, CA) was used to process and align sequencing data to identify the substitutions present at levels greater than 1%

(percentage total of reads). Substitutions at RAS positions were analyzed using 1%, 5%, 10%, 15%, and 20% cut-off values. The presence of baseline RASs was established by comparison with wild-type reference sequences (1a-H77 for genotype 1a samples and 1b-Con-1 for genotype 1b samples). RASs from clinical trials were summarized recently by the HCV Drug Resistance Advisory Group group.[22] For patients with genotype 1a HCV infection, NS5A RASs were defined as the following substitutions at the following positions: K24G/N/R, M28A/G/T, Q30E/G/H/L/K/R/T, L31I/F/M/V, P32L, S38F, H58D, A92K/T, and Y93C/F/H/N/S (ledipasvir-specific RASs). For patients with genotype 1b HCV infection, NS5A RASs were defined as the following substitutions at the following positions: L31F/I/M/V, P32L, P58D, A92K, and Y93C/H/N/S (ledipasvir-specific RASs). NS5B NI substitutions that are reported here included any substitutions that had a change from the corresponding genotype-specific reference at NS5B positions 96, 142, 159, 282, 289, 320, and 321 (nucleotide class–specific RASs). RASs at residues associated with resistance to PIs including substitutions at positions V36, F43, T54, V55, Q80, S122, R155, A156, D168, and M175 of the NS3 protease gene were included in the analysis (NS3 protease inhibitor class RASs). Patients who were lost to follow-up evaluation before the SVR12 visit or had early termination (n = 29) were excluded from the SVR12 analysis.

Drug Susceptibility Analyses

Resistance mutations were introduced into the genotype 1a or genotype 1b replicon by site-directed mutagenesis and tested in transient transfections as previously described.[23] Briefly, NS5A mutations were introduced into a plasmid encoding the PI-hRluc replicon using a QuikChange II XL mutagenesis kit, following the manufacturer's instructions (Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequencing. Replicon RNAs were transcribed in vitro from replicon-encoding plasmids using a MEGAscript kit (Ambion, Austin, TX). RNA was transfected into Huh-lunet cells using the method described by Lohmann et al.[24] Briefly, cells were trypsinized and washed twice with phosphate-buffered saline. A suspension of cells was mixed with RNA and subjected to electroporation. Cells were transferred into 40 mL of prewarmed culture medium and then seeded into 96-well plates (100 μL/well). Compounds were diluted in 100% dimethyl sulfoxide and added to cells. Cells were treated for 3 days, after which culture media was removed, cells were lysed, and *Renilla* luciferase activity was quantified using a commercially available assay (Promega, Madison, WI) and a Top Count instrument (Perkin Elmer, Waltham, MA). The median effective concentration values were calculated as the compound concentration at which a 50% reduction in the level of *Renilla* reporter activity was observed when compared with control samples with dimethyl sulfoxide. Dose-response curves and median effective concentration values were generated using the GraphPad Prism software package (GraphPad Software, La Jolla, CA) by nonlinear regression analysis. The replication level of either reference strains (1b-Con1 or 1a-H77) or chimeric replicons derived transiently from clinical isolates was determined as the ratio of the *Renilla* luciferase signal at day 4 to that at 4 hours after electroporation, to normalize for transfection efficiency. The replication capacity of each replicon was expressed as their normalized replication efficiency compared with that of the reference strain (1b-Con1 or 1a-H77) within the same experiment.

Results

Prevalence of NS5A RASs and Association With Treatment Outcome

NS5A population sequencing or deep sequencing was attempted for all patients who participated in the studies and was successful for 233 of 237 by population sequencing and 1904 of 1907 by deep sequencing. Twenty-nine patients were excluded from further outcome analyses because of either early study drug termination or loss to follow-up evaluation before the SVR12 visit, resulting in a final analysis population of 2108 patients (1575 patients with genotype 1a, 525 patients with genotype 1b, 2 patients with genotype 1h, 2 patients with genotype 4a, 2 patients with genotype 1c, 1 patient with genotype 1l, and 1 patients with recombinant genotype 1a+genotype 1b).

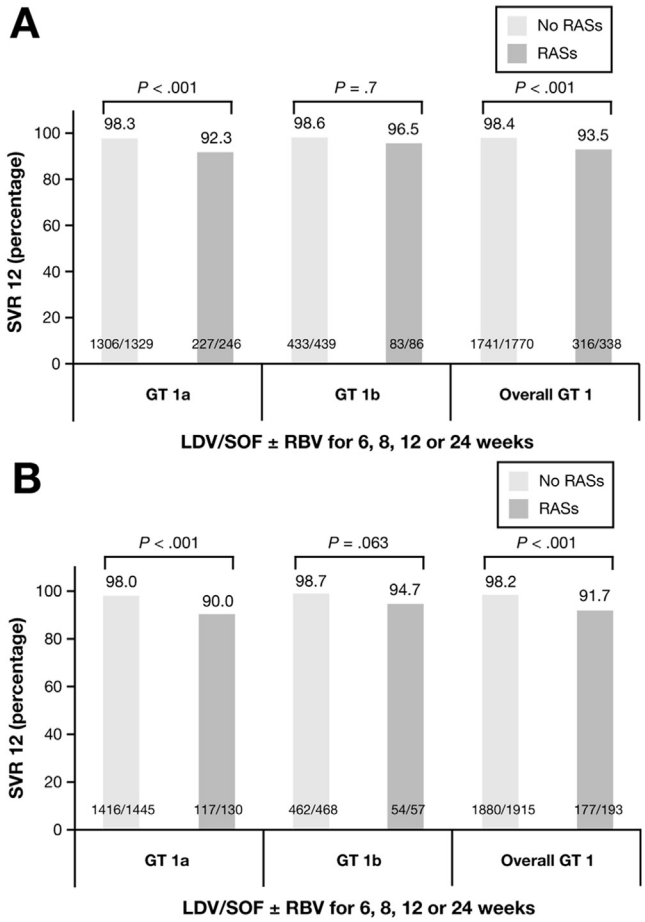


Figure 1. Prevalence of NS5A RASs and treatment response. Patient baseline sequences generated by population and deep sequencing were pooled for treatment-naive and treatment-experienced patients who were treated with ledipasvir/sofosbuvir ± ribavirin for 6, 8, 12, and 24 weeks. (*A*) SVR12 rates in patients with or without baseline NS5A RAS (using a 1% cut-off value for deep sequencing and population sequencing with a substitution detection of ~15%). (*B*) SVR12 rates in patients with or without baseline NS5A RASs (using a 15% cut-off value for deep sequencing and population sequencing with a substitution detection of ~15%). GT, genotype; LDV, ledipasvir; RBV, ribavirin; SOF, sofosbuvir.

Table 1. NS5A Resistance-Associated Substitutions Classified by Level of Resistance to Ledipasvir and RASs Among Patients With NS5A RASs Who Did Not Achieve SVR12

	Level of resistance to ledipasvir		
Genotype	2.5- to 100-fold	100- to 1000-fold	>1000-fold
GT1a	K24R, Q30L, Q30T, K24G, K24N, A92T, Y93F, M28T, S38F	Q30H, Q30G, Q30R, L31I, L31M, L31V, P32L	M28A, M28G, Q30E, Q30K, H58D, Y93C, Y93H, Y93N, Y93S
GT1b	L31M, P32L, L31I, L31V	P58D	A92K, Y93H

	Patients who did not achieve SVR12 with RAS			
Treatment	Baseline NS5A RAS	Prior treatment status	HCV genotype	Level of resistance to LDV
Ledipasvir/sofosbuvir 8 weeks	L31M (19.25%)	TN	1a	100- to 1000-fold
	L31M (25.45%)	TN	1a	100- to 1000-fold
	Y93N (15.37%)	TN	1a	>1000-fold
	Q30Y (2.04%), Q30H (1.16%), Y93H (3.60%)	TN	1a	>1000-fold
	M28T (93.52%), M28A (6.09%)	TN	1a	>1000-fold
Ledipasvir/sofosbuvir 12 weeks	L31M (>99%)	TN	1a	100- to 1000-fold
	Q30H (>99%)	TE	1a	100- to 1000-fold
	M28T (1.03%), Q30R (>99%), L31M (>99%)	TE	1a	100- to 1000-fold
	Y93H (59.82%)	TE	1b	>1000-fold
	Q30H (98.76%), Y93H (98.07%)	TE	1a	>1000-fold
	Q30H (>99%), Y93H (>99%)	TE	1a	>1000-fold
	Q30R (1.43%), Y93N (97.60%)	TE	1a	>1000-fold
	Y93F (10.81%), Y93N (1.71%)	TN	1a	>1000-fold
Ledipasvir/sofosbuvir 24 weeks	Y93H (94.07%)	TN	1b	>1000-fold
Ledipasvir/sofosbuvir+ribavirin 8 weeks	L31M (1.12%)	TN	1a	100- to 1000-fold
	Y93N (>99%)	TN	1a	>1000-fold
	Y93C (8.65%)	TN	1a	>1000-fold
	Y93H (63.83%)	TN	1b	>1000-fold
	Q30R (71.06%), Q30H (28.84%), Y93H (24.58%)	TN	1a	>1000-fold
Ledipasvir/sofosbuvir+ribavirin 12 weeks	L31M (>99%)	TE	1a	100- to 1000-fold
	Y93H (1.20%)	TE	1a	>1000-fold
Ledipasvir/sofosbuvir+ribavirin 24 weeks[a]	K24R (1.06%), Q30R (2.61%)	TE	1a	100- to 1000-fold

GT, genotype; LDV, ledipasvir; TE, treatment experienced; TN, treatment naive.
[a]Patient experienced a breakthrough owing to documented noncompliance during the dosing period.

In the pooled analysis of population and deep sequencing data from the phase 2 and 3 studies, 338 of 2108 (16.0%) patients were identified as having baseline NS5A RASs by population (cut-off value, ~15%) or deep sequencing (cut-off value, 1%), irrespective of subtype (246 of 1575, 15.6% genotype 1a; 86 of 525, 16.4% genotype 1b; and 8 patients with other genotypes). Of the 338 patients with baseline NS5A RASs, 316 (93.5%) patients achieved SVR12 after 6, 8, 12, or 24 weeks of treatment with ledipasvir/sofosbuvir with or without ribavirin, compared with 1741 of 1770 (98.4%) patients with no NS5A RASs ($P < .001$). The reduction in SVR rates appears to be driven predominantly by patients with genotype 1a NS5A RASs; the SVR12 rates in genotype 1b patients with baseline NS5A RASs were 96.5%, compared with 98.6% for patients without NS5A RASs ($P = .7$), whereas SVR12 rates for genotype 1a patients were 92.3% for those with NS5A RASs compared with 98.3% for patients without NS5A RASs ($P < .001$) (Figure 1*A*). Slightly lower treatment response rates of 90% were observed for genotype 1a patients with NS5A RASs using a 15% deep sequencing cut-off value (Figure 1*B*).

Treatment Outcomes by Level of RAS Resistance and Prior Treatment Status

NS5A RASs were classified by level of resistance to ledipasvir (Table 1). Patients with NS5A RASs were classified according to their prior treatment status. Among treatment-naive genotype 1 patients in the ledipasvir/sofosbuvir group, 102 of 887 (11.5%) had at least 1 RAS that conferred more than 100-fold resistance to ledipasvir. A significant reduction in the SVR rate (Figure 2*A*) was seen among treatment-naive patients with NS5A RASs conferring more than 100-fold ledipasvir resistance who received only 8 weeks of ledipasvir/sofosbuvir therapy (82.8%; $P = .011$).

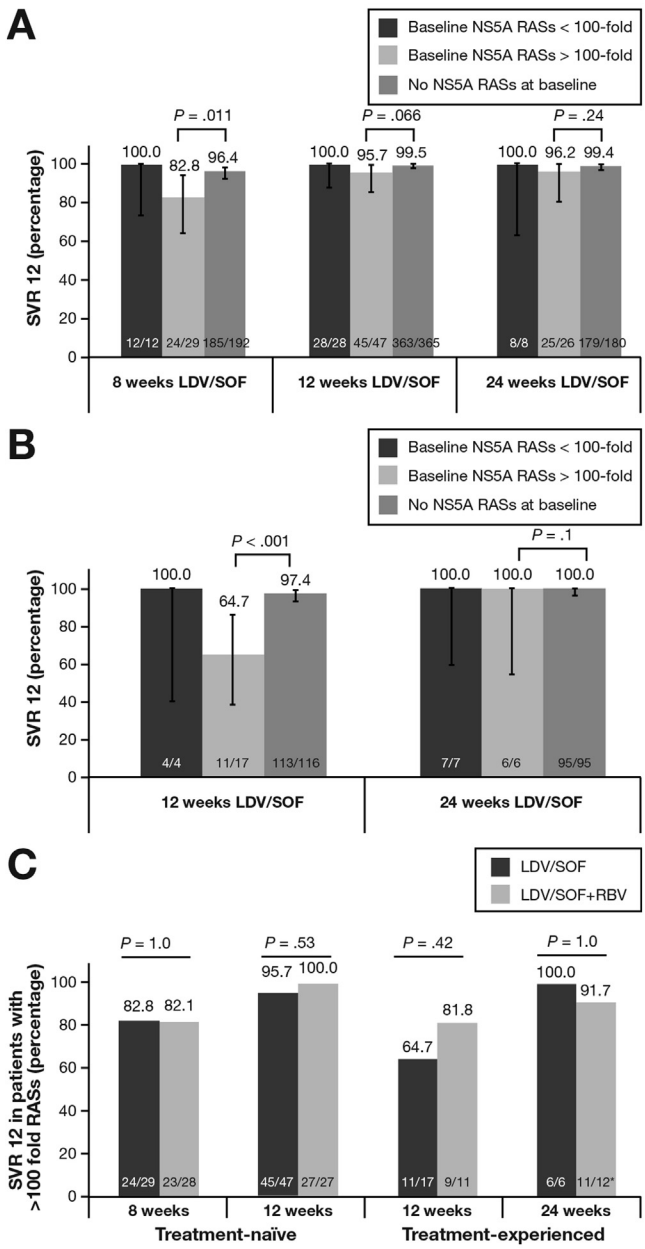


Figure 2. SVR12 by level of NS5A RASs in patients treated with ledipasvir/sofosbuvir. Patient baseline sequences generated by population and deep sequencing were pooled (using a 1% cut-off value for deep sequencing and population sequencing with a substitution detection of ~15%). (*A*) Treatment-naive. Three of 5 failures who had baseline NS5A RASs with more than 100-fold resistance to ledipasvir had a baseline viral load greater than 6×10^6 IU/mL. (*B*) Treatment-experienced. (*C*) SVR12 for patients with NS5A RASs with more than 100-fold resistance to ledipasvir in treatment-naive patients treated for 8 or 12 weeks and treatment-experienced patients treated for 12 or 24 weeks with and without ribavirin. *One patient experienced breakthrough as a result of documented noncompliance during the dosing period. LDV, ledipasvir; SOF, sofosbuvir; RBV, ribavirin.

A significant reduction in SVR based on the presence of high-level baseline NS5A RASs was not observed among treatment-naive patients treated for 12 or 24 weeks with ledipasvir/sofosbuvir, and all patients with NS5A RASs conferring less than 100-fold ledipasvir resistance achieved SVR12. Furthermore, of the 5 patients who did not achieve SVR12 after 8 weeks of ledipasvir/sofosbuvir therapy, 3 had high (>6 million IU/mL) HCV-RNA levels at baseline and 3 had at least 1 NS5A RAS conferring more than 100-fold resistance to ledipasvir at a frequency of more than 15% at baseline (Table 1). Similar SVR rates (82.1%) were observed for treatment-naive patients with NS5A RASs conferring more than 100-fold ledipasvir resistance who received 8 weeks of ledipasvir/sofosbuvir+ribavirin therapy (Figure 2*C*), which also included 4 of 5 virologic failure patients with HCV-RNA levels greater than 6 million IU/mL.

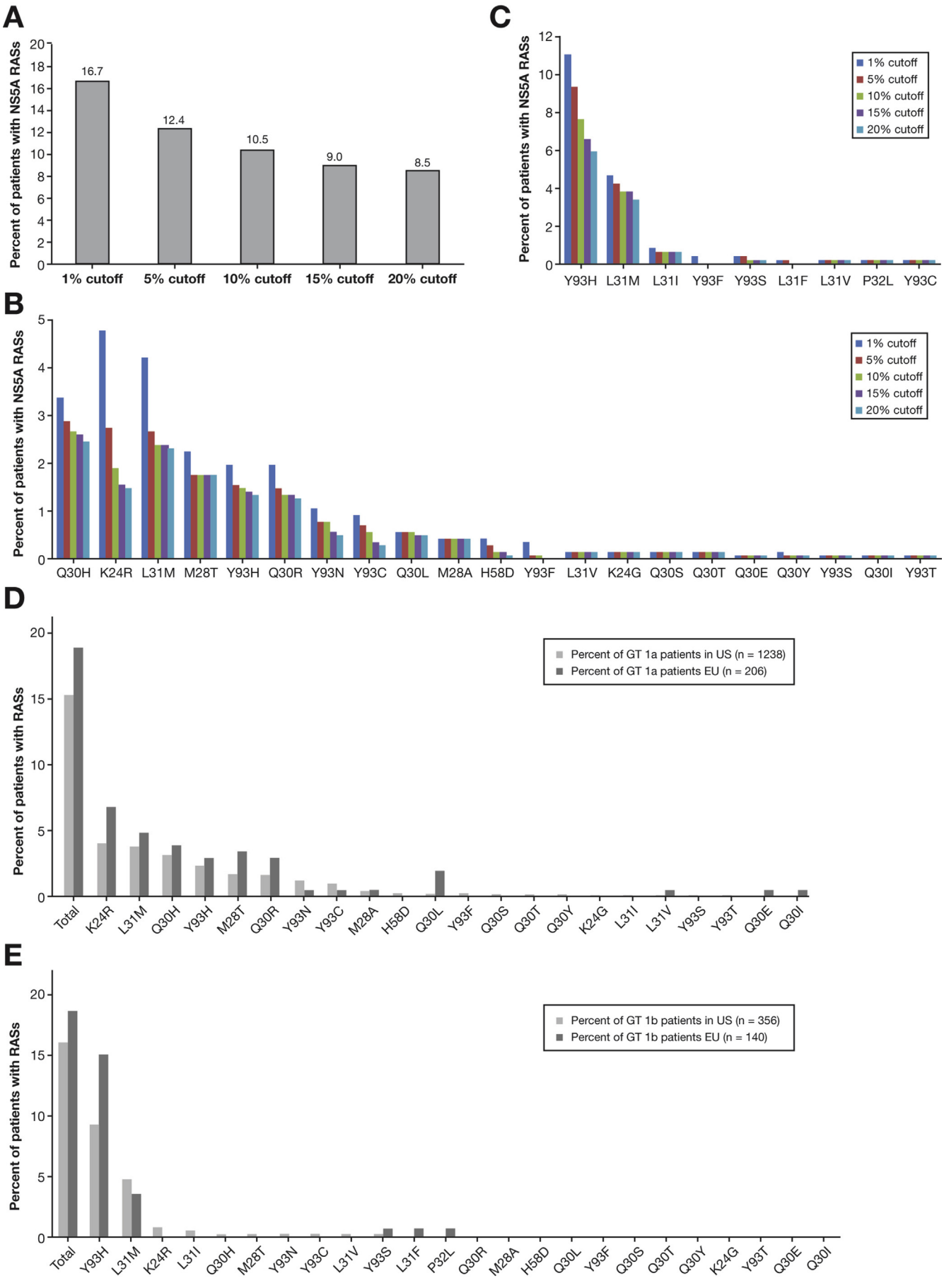
Among treatment-experienced patients, SVR rates were 97.4%–100% for those without baseline RASs or with RASs conferring less than 100-fold resistance to ledipasvir treated for 12 or 24 weeks with ledipasvir/sofosbuvir (Figure 2*B*). Those treatment-experienced patients with baseline NS5A RASs with more than 100-fold-resistance to ledipasvir who were treated for 12 weeks had a significantly lower SVR12 rate (64.7%; 11 of 17) than those without baseline RASs (97.4%; 113 of 116) or those with high-level RASs treated for 24 weeks (100%; 6 of 6). Of the 6 patients with RASs who did not achieve SVR12 after 12 weeks of treatment, all had at least 1 RAS conferring more than 100-fold resistance to ledipasvir at a frequency of more than 15% at baseline and 4 had multiple high-level NS5A RASs (Table 1).

Relative to 100% SVR for 24 weeks of ledipasvir/sofosbuvir treatment shown in Figure 2*B*, reduced SVR rates (81.8%) also were observed among treatment-experienced patients with NS5A RASs conferring more than 100-fold ledipasvir resistance who received 12 weeks of ledipasvir/sofosbuvir+ribavirin therapy (Figure 2*C*); however, the overall SVR rate was numerically higher than that observed for 12 weeks of ledipasvir/sofosbuvir without ribavirin (64.7%). No significant differences were observed among treatment-naive or treatment-experienced patients with NS5A RASs conferring more than 100-fold ledipasvir resistance who received ledipasvir/sofosbuvir with or without ribavirin for 8 or 12 weeks (treatment-naive) or 24 weeks (treatment-experienced) (Figure 2*C*).

Prevalence and Geographic Distribution of Specific NS5A RASs

The prevalence of specific ledipasvir RASs detected at baseline was evaluated using different deep sequencing assay cut-off values: 1%, 5%, 10%, 15%, and 20%. Of the 1030 patients who had successful deep sequencing and were treated with ledipasvir/sofosbuvir for 8, 12, and 24 weeks, 16.7%, 12.4%, 10.5%, 9.0%, and 8.5% had specific ledipasvir NS5A RASs with 1%, 5%, 10%, 15%, and 20% cut-off values, respectively (Figure 3*A*). Only slight differences in SVR rates were seen for the different cut-off values and SVR12 rates ranged from 87.1% to 91.9% (Supplementary Figure 1).

For genotype 1a patients, the majority of patients harbored a single ledipasvir RAS (data not shown). The most frequent NS5A RASs with the 1% cut-off limit were


A
Percent of patients with NS5A RASs
16.7
12.4
10.5
9.0
8.5
1% cutoff
5% cutoff
10% cutoff
15% cutoff
20% cutoff
C
Percent of patients with NS5A RASs
1% cutoff
5% cutoff
10% cutoff
15% cutoff
20% cutoff
Y93H L31M L31I Y93F Y93S L31F L31V P32L Y93C
B
Percent of patients with NS5A RASs
1% cutoff
5% cutoff
10% cutoff
15% cutoff
20% cutoff
Q30H K24R L31M M28T Y93H Q30R Y93N Y93C Q30L M28A H58D Y93F L31V K24G Q30S Q30T Q30E Q30Y Y93S Q30I Y93T
D
Percent of patients with RASs
Percent of GT 1a patients in US (n = 1238)
Percent of GT 1a patients EU (n = 206)
Total K24R L31M Q30H Y93H M28T Q30R Y93N Y93C M28A H58D Q30L Y93F Q30S Q30T Q30Y K24G L31I L31V Y93S Y93T Q30E Q30I
E
Percent of patients with RASs
Percent of GT 1b patients in US (n = 356)
Percent of GT 1b patients EU (n = 140)
Total Y93H L31M K24R L31I Q30H M28T Y93N Y93C L31V Y93S L31F P32L Q30R M28A H58D Q30L Y93F Q30S Q30T Q30Y K24G Y93T Q30E Q30I

K24R > L31M > Q30H > M28T > Y93H > Q30R. At 5%, 10%, 15%, and 20% cut-off limits, Q30H and L31M were the most frequent RASs in genotype 1a patients (Figure 3*B*). For all cut-off limits, the most frequent NS5A RASs in genotype 1b patients were Y93H and L31M (Figure 3*C*), and almost all patients harbored a single NS5A RAS.

A comparison of the prevalence of NS5A RASs between patients in the United States and the EU shows only small differences in the geographic distribution of baseline RASs (Figure 3*D* and *E*). Overall, approximately 15.0% of genotype 1a HCV-infected patients in the United States harbored NS5A RASs compared with approximately 20.9% in the European Union. For genotype 1b, approximately 15.5% of US patients and 17.1% of EU patients had baseline NS5A RASs. Similar frequencies were observed for single RASs with high levels of resistance in patients infected with genotype 1a virus including L31M, Q30H/R, and Y93H. For patients with genotype 1b infection, 9.3% and 15.0% of the patients had Y93H in the United States and European Union, respectively.

Relationship of Specific Substitution and Treatment Outcome

The relationship of baseline NS5A RASs to the treatment outcome in patients treated with ledipasvir/sofosbuvir was evaluated for the most common NS5A RASs: K24R, M28T, Q30H, Q30R, L31M, and Y93H. The SVR rates ranged from 80% to 93.3% and 75% to 88.2% for NS5A RASs that confer more than 100-fold resistance to ledipasvir (Q30H, Q30R, L31M in genotype 1a, and Y93H in genotype 1a and 1b) using 1%–15% deep sequencing cut-off values, respectively (Figure 4*A*). Slightly lower SVR rates were observed using the 15% cut-off limit compared with the 1% cut-off limit for these RASs. For K24R, which confers 3.7-fold resistance to ledipasvir, the SVR rate was 100% independent of deep sequencing cut-off values. For M28T, which confers 61-fold resistance to ledipasvir, the SVR rate also was reduced, however, the 2 patients with virologic failure with M28T also had other NS5A RASs conferring more than 100-fold resistance (Table 1). In the case of L31M, reduced SVR rates were observed only in patients with genotype 1a infection, consistent with only 3.4-fold reduced susceptibility for this RAS in the background of genotype 1b.

Overall, the relapse rate increased with the number of RASs. Of patients with no baseline RAS, 1.6% (28 of 1786) experienced virologic failure, compared with incidences of 4.9%, 10.2%, and 15.8% virologic relapse with 1 RAS, 2 RASs, and 3 or more RASs, respectively (Figure 4*B*). Moreover, the prevalence of baseline RASs decreased with the number of RASs, in which 1 RAS, 2 RASs, and 3 or more RASs had a prevalence of 12.6%, 2.3%, and 0.9%, respectively.

Forty-nine patients experienced virologic relapse in the phase 2/3 studies with sofosbuvir/ledipasvir regimens. Of these, 21 (43%) had NS5A RASs at baseline: 18 had genotype 1a infection and 3 had genotype 1b infection. For the 18 genotype 1a patients who did not achieve SVR12, 8 had double and triple substitutions that conferred more than 100-fold-resistance to ledipasvir (Table 1). For the 3 genotype 1b patients who did not achieve SVR12 with NS5A RASs, Y93H was detected as a dominant substitution. The overall SVR12 rate for genotype 1b with Y93H was 93.3% and 88.2% using 1% and 15% deep sequencing cut-off limits, respectively. Of the 21 patients with baseline RASs who relapsed, 71% (15 of 21) had at least one RAS conferring more than 100-fold-resistance to ledipasvir at a frequency of more than 15% at baseline (Table 1).

The relationship of the baseline NS5A mutant viral load to the treatment outcome was evaluated for each individual NS5A RAS. The baseline mutant viral load for the NS5A RASs was calculated by multiplying the total HCV viral load by the percentage of the specific NS5A RAS observed at baseline. These NS5A-specific baseline viral loads of NS5A RASs at positions 24, 28, 30, 31, and 93 were compared between patients achieving SVR12 and those experiencing virologic failure (Figure 4*C*). Although there was a small trend of high mutant viral loads for Y93 and Q30 and virologic failure, many patients with these substitutions and the same mutant viral load achieved SVR12 and overall there was no significant effect.

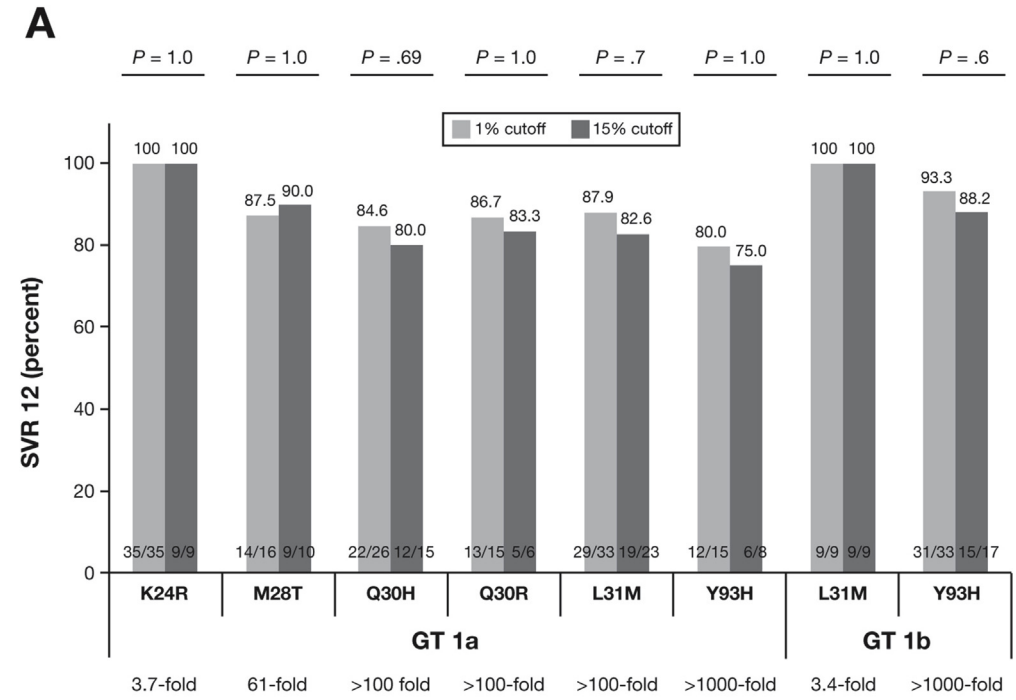
Baseline NS5B NI Substitutions

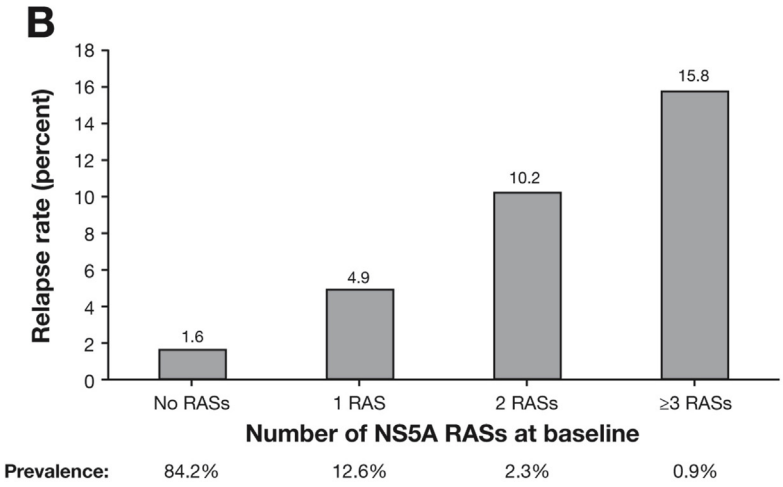
Baseline NS5B sequencing was attempted for a subset of patients from the ION-1 study and all patients from the LONESTAR, ELECTRON, ION-2, and ION-3 studies. Successful NS5B sequencing was obtained for 1692 patients (1291 genotype 1a, 395 genotype 1b, and 6 other), including deep sequencing results from 1628 patients. The NS5B RAS S282T was not detected in any patient using a 1% cut-off value for deep sequencing (Table 2). A total of 41 sequenced patients had other NI RASs at baseline (36 with L159F and 5 with N142T); all 41 of these patients achieved SVR12 (Table 2). In addition, 1 patient had S282G and another patient had L320S, 2 substitutions at 2 residues associated with NI resistance; both patients achieved SVR12.

Baseline NS3 Substitutions

NS3 deep sequencing results were obtained for 467 patients from LONESTAR and ION-2 patients (372 genotype

Figure 3. Prevalence of NS5A RASs by deep sequencing cut-off limit and geographic region. Substitution analyses were conducted on deep sequencing data (population sequences were not included). (*A*) Prevalence of NS5A RAS by 1%, 5%, 10%, 15%, and 20% deep sequencing cut-off limit in patients treated with ledipasvir/sofosbuvir (n = 1040). (*B*) Prevalence of specific NS5A RASs in patients with genotype 1a infection by deep sequencing cut-off limits. (*C*) Prevalence of specific NS5A RASs in patients with genotype 1b infection by deep sequencing cut-off limits. (*D*) Prevalence of NS5A RASs in patients with genotype 1a infection in the United States and the European Union. (*E*) Prevalence of NS5A RASs in patients with genotype 1b infection in the United States and the European Union. GT, genotype.





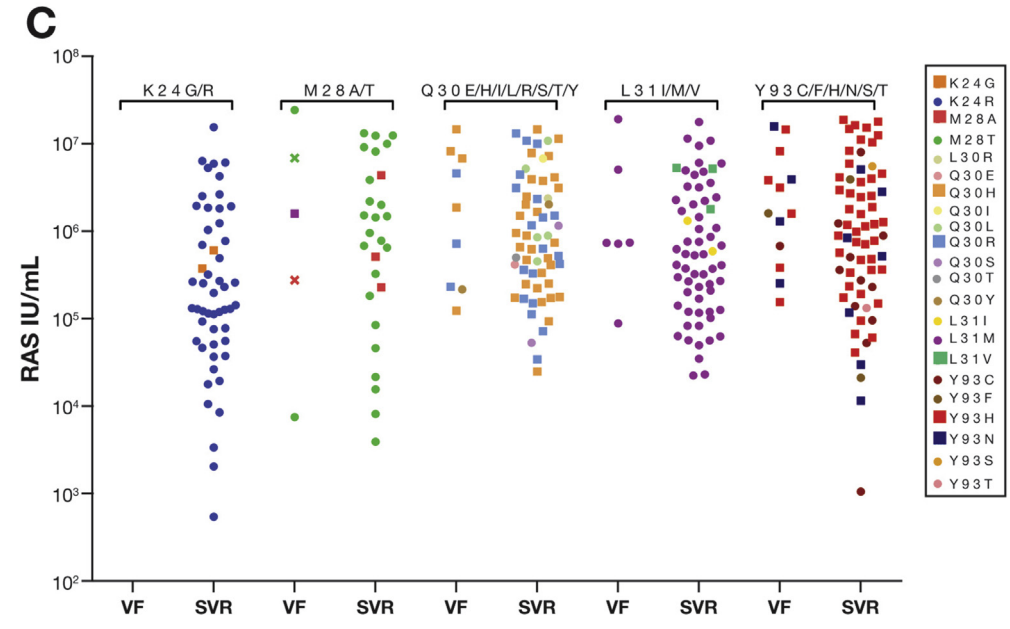


Figure 4. Treatment outcome in patients with NS5A RASs. Substitution analyses were conducted on deep sequencing data (population sequences were not included). (*A*) SVR12 by specific baseline NS5A RASs and cut-off limits (1% and 15%) in patients treated with ledipasvir/sofosbuvir. (*B*) Relapse rate in patients with 1, 2, or 3 or more NS5A RASs. The prevalence of 1 RAS, 2 RASs, and 3 or more RASs was 12.6%, 2.3%, and 0.9%, respectively. (*C*) Treatment outcome for all patients with baseline NS5A RAS by baseline RAS viral load. The baseline viral load for the NS5A RASs was calculated by multiplying the total HCV viral load by the percentage of the specific NS5A RAS observed at baseline and compared in patients achieving SVR12 and those experiencing virologic failure. GT, genotype; VF, virologic failure.

Table 2. SVR Rates in Patients With Baseline NS5B NI or NS3 PI RASs

Patients with baseline NS5B NI RASs			
NS5B RASs	Genotype	Patients with RASs, n (%)	SVR12 for patients with RASs (%)
L159F (NI)	GT1b (n = 36)	36/1692 (2.1)	36/36 (100)
N142T (NI)	GT1b (n = 4), GT1a (n = 1)	5/1692 (0.3)	5/5 (100)
S282G[a]	GT1a (n = 1)	1/1692 (0.1)	1/1 (100)
L320S[a]	GT1a (n = 1)	1/1692 (0.1)	1/1 (100)
Total RASs	GT1b (n = 40), GT1a (n = 3)	43/1692 (2.5)	43/43 (100)

Patients with baseline NS3 PI RASs				
Prior treatment	No. of patients with PI RASs, n[b] (%)	SVR12 for patients with PI RASs, n (%)	Patients with Q80 variants, n (%)	SVR12 for patients with Q80 variants, n (%)
Pegylated interferon +ribavirin+ protease inhibitor	141/265 (53.2)	139/141 (98.6)	110/265[c] (41.5)	107/110 (97.3)
Pegylated interferon +ribavirin	23/202 (11.4)	21/22 (95.5)	93/202[d] (46.0)	90/93 (96.8)

NOTE. Variant analyses were conducted at a 1% cut-off limit.
GT, genotype.
[a]Substitutions observed at RAS sites.
[b]RASs associated with resistance to protease inhibitors observed at baseline at positions V36, T54, V55, R155, A156, D168, I/V170, and M175L of the NS3 protease gene.
[c]Of the 110 patients with Q80 substitutions, 57 had a Q80 substitution and another NS3 RAS.
[d]Of the 93 patients with Q80 substitutions, 8 had a Q80 substitution and another NS3 RAS.

1a and 95 genotype 1b). Of these patients, 265 previously were treated with PI-containing regimens. Baseline NS3 RASs were detected in 141 of the 265 (53.2%) patients, of whom 139 (98.6%) achieved SVR12 (Table 2). For the patients who were PI treatment-naive (previous pegylated interferon+ribavirin treatment failures), 23 of 202 (11.4%) had baseline NS3 RASs and 95.5% achieved SVR12. In addition, Q80 polymorphisms were observed in 93 of 202 (46.0%) PI treatment-naive patients and 110 of 265 (41.5%) PI treatment-experienced patients, of whom 96.8% and 97.3% of patients achieved SVR12, respectively.

Discussion

Ledipasvir/sofosbuvir is an effective, simple, and safe single-tablet regimen for the treatment of genotype 1 chronic HCV, with SVR rates of 94%–99% in phase 3 clinical trials. This study describes the prevalence of pre-existing NS5A, NS5B NI, and NS3 RASs in patients infected with HCV genotype 1 in the phase 2 and 3 clinical trials as well as the impact of these RASs on treatment outcome. Overall, the presence of pre-existing RASs in the NS5A gene had no significant impact on treatment outcome in genotype 1b–infected patients, and a minimal impact on treatment outcome in genotype 1a–infected patients with SVR rates greater than 90%. The presence of pre-existing RASs in the NS5B and NS3 genes had no impact on treatment outcome.

In this analysis, the percentage of patients with baseline NS5A RASs ranged from 8.5% when a cut-off value of 20% was used, to a high of 16.7% when a cut-off value of 1% was used. This percentage of patients harboring NS5A RASs is similar to that reported by other studies,[25,26] when one takes into consideration the method (deep vs population sequencing) and the cut-off value used to determine the presence of a substitution. Overall, the treatment responses were similar regardless of the specific cut-off value used in the analysis, with slightly lower responses observed using the 15% cut-off value vs a 1% cut-off value. Thus, population sequencing would be sufficient to detect most clinically meaningful baseline RASs. However, of the 21 virologic relapse patients with baseline ledipasvir NS5A RASs, 5 had RASs at frequencies below the detection limit of population-based sequencing (15%).

Zeuzem et al[27] conducted a large study that investigated the prevalence of baseline NS5A RASs in genotype 1 patients and the effect on treatment response and included more than 5000 patients from 21 countries across HCV Gilead clinical trials from 2010 to 2015. The analysis included data from ledipasvir/sofosbuvir ± ribavirin–treated patients only when used according to recommended treatment guidelines and showed that baseline NS5A RASs have no clinically meaningful impact on treatment outcome with ledipasvir/sofosbuvir when used according to recommended guidelines in the vast majority of patient populations. Our analysis included data from the 5 phase 2 and 3 registrational clinical trials, including all treatment groups that supported the Gilead regulatory filings for ledipasvir/sofosbuvir (ledipasvir 90 mg/sofosbuvir 400 mg) and also included patients who were treated with investigational regimens. This allows an understanding of the influence of different treatment durations and the addition of ribavirin on the importance of RASs with respect to virologic treatment response.

Further assessment of the effects of baseline NS5A RASs and treatment outcome showed that reduced SVR rates in treatment-naive patients was limited to those with NS5A RASs conferring more than 100-fold ledipasvir resistance (Q30H/R, L31M in genotype 1a, or Y93H) who received 8 weeks of ledipasvir/sofosbuvir therapy, with 5 of 29 patients failing to achieve SVR12. For the 5 patients with virologic failure, 3 had NS5A RASs conferring more than 100-fold resistance to ledipasvir at a frequency of more than 15% of the viral population at baseline. Moreover, 3 of these 5 patients had a baseline viral load greater than 6 million IU/mL and, per current treatment guidelines, a treatment course of 12 weeks is recommended. Among treatment-naive patients who received 12 weeks of ledipasvir/sofosbuvir, no treatment outcome differences were observed based on the presence or absence of NS5A RASs. All patients with NS5A RASs conferring less than 100-fold ledipasvir resistance achieved SVR12.

Among treatment-experienced patients, a lower SVR12 rate was observed for those who had baseline NS5A RASs associated with more than 100-fold-resistance to ledipasvir and were treated for 12 weeks without ribavirin. Six of these 17 patients did not achieve SVR12. Of these 6 patients, all had at least 1 NS5A RAS conferring more than 100-fold resistance to ledipasvir at a frequency of more than 15% at baseline, and 4 of 6 had multiple high-level ledipasvir RASs. Treatment-experienced genotype 1a patients with pre-existing NS5A RASs that confer a more than 100-fold resistance to ledipasvir represented 6.9% (17 of 245) of the patients in this analysis. However, as recommended by treatment guidelines, all treatment-experienced patients with baseline RASs treated for 24 weeks with ledipasvir/sofosbuvir achieved SVR12.

The addition of ribavirin to 12 weeks of ledipasvir/sofosbuvir resulted in an improved SVR12 rate in treatment-experienced patients with NS5A RASs associated with more than 100-fold resistance to ledipasvir, relative to 12 weeks of ledipasvir/sofosbuvir without ribavirin; however, the SVR12 rate still was numerically lower than that observed with 24 weeks of therapy. This observation stands in contrast to data from the SIRIUS trial,[28] in which treatment-experienced cirrhotic patients were randomized to 24 weeks of ledipasvir/sofosbuvir or 12 weeks of ledipasvir/sofosbuvir + ribavirin. All of the patients (8 of 8) with NS5A RASs conferring more than 100-fold resistance to ledipasvir treated for 12 weeks with ledipasvir/sofosbuvir + ribavirin achieved SVR12; conversely, 7 of 9 (78%) patients with NS5A RASs conferring more than 100-fold resistance to ledipasvir treated for 24 weeks with ledipasvir/sofosbuvir achieved SVR12. These data suggest that for treatment-experienced patients with NS5A RASs, 12 weeks of ledipasvir/sofosbuvir + ribavirin provides similar effectiveness compared with ledipasvir/sofosbuvir for 24 weeks.

For the specific NS5A RASs Q30H/R, L31M in genotype 1a, and Y93H in genotype 1a and 1b which were detected most frequently and that confer more than 100-fold-resistance to ledipasvir, SVR rates ranged from 80% to 93.3% and 75% to 88.2% using 1% and 15% deep sequencing cut-off values, respectively. Overall, slightly lower SVR rates were observed using the 15% cut-off value compared with the 1% cut-off value. The number of RASs harbored within the virus seems to be a predictor of treatment failure. An increasing rate of virologic relapse was observed in patients without baseline NS5A RASs (1.6%) to patients with 1, 2, or at least 3 RASs (4.9%, 10.2%, and 15.8%, respectively). However, the prevalence of patients with 1, 2, or at least 3 pre-existing NS5A RASs decreased from 12.6% to 2.3% and 0.9%, respectively. This observation is in line with a higher relapse rate observed in patients who received sofosbuvir plus ledipasvir after failure with a ledipasvir-containing regimen and the presence of multiple NS5A RASs.[29]

Baseline NS3 RASs were detected in 53.2% of patients who were treated previously with PI-containing regimens, of whom 98.6% achieved SVR12. In addition, Q80 polymorphisms were observed in 46.0% of PI treatment-naive patients and in 41.5% of PI treatment-experienced patients, of whom 96.8% and 97.3% of patients achieved SVR12, respectively. Taken together, no association between any NS3 RAS and treatment outcome was observed in patients treated with ledipasvir/sofosbuvir, which is consistent with the lack of cross-resistance between PIs and either ledipasvir or sofosbuvir in vitro. In addition, the NS5B NI RAS S282T was not detected in any patient at baseline. Of the 2.5% of patients with other NI RASs at baseline, all achieved SVR12, including 1 patient with S282G.

In summary, high SVR rates were achieved in the presence of baseline HCV NS5A RASs upon treatment with ledipasvir/sofosbuvir in the majority of patient populations. NS5A RASs corresponding to more than 100-fold resistance to ledipasvir together with a shortened treatment duration of 8 weeks in treatment-naive patients or 12 weeks in treatment-experienced patients were associated with reduced SVR rates. Most of these patients were not treated according to current treatment guidelines. In the majority of these patients, at least 1 NS5A RAS conferring more than 100-fold resistance to ledipasvir was detected at a frequency of more than 15% at baseline, which could have been detected by population sequencing. The effect of these RASs may be overcome by extension of treatment duration to 12 and 24 weeks, respectively, or the addition of ribavirin or another DAA in treatment-experienced cirrhotic patients. Given the low magnitude of effect of baseline NS5A RASs in genotype 1 patients, routine baseline NS5A RAS testing before ledipasvir/sofosbuvir therapy does not appear to be clinically warranted. This is supported further by the high rates of SVR observed in postmarketing real-world cohorts of patients treated outside of clinical trials with ledipasvir/sofosbuvir regimens in which the SVR rate has been more than 90% across multiple diverse patient cohorts.[30,31]

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2016.06.002.

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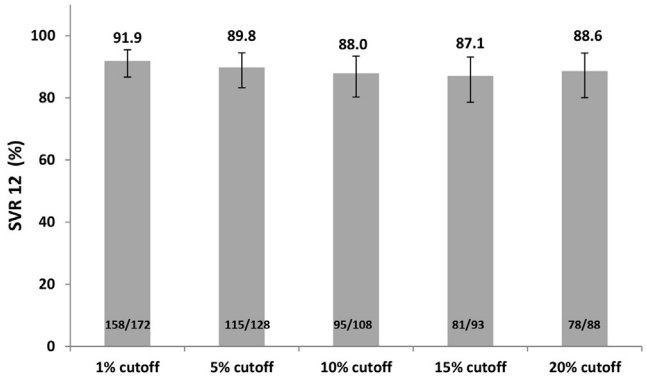

Supplementary Figure 1. SVR12 for patients treated with ledipasvir/sofosbuvir with NS5A RASs by deep sequencing cut-off limits. Substitution analyses were conducted on deep sequencing data (population sequences were not included).