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

Short Title: Ledipasvir/Sofosbuvir Baseline RASs and Treatment Outcomes

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Abbreviations: DAAs, direct-acting antiviral agents; 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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Abstract

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 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 50 to ledipasvir by more than 100-fold (Q30H/R, L31M in genotype 1a HCV, and Y93H) reduced the rate of SVR12 in treatment-naïve patients given ledipasvir/sofosbuvir for 8 weeks, (P=.011), but not 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 patients' response to ledipasvir/sofosbuvir therapy. When these RASs do have effects, they could be largely overcome by extending treatment duration or through treatment intensification.

KEY WORDS: direct-acting antivirals; ION-1; ION-2; ION-3

Introduction

Development of direct-acting antivirals (DAAs) in recent years has dramatically enhanced sustained virologic response (SVR) rates in 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 naïve and experienced HCV genotype 1 infected patients with and without liver 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 percent to 99 percent.

Despite these high SVR12 rates, 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 primarily associated 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 was first 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 following in vitro selection with the NI 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 (cutoff 1 percent).[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 significantly reduced in patients infected with HCV genotype 1a with an NS3 Q80K polymorphism. Rates of sustained virologic response in treatment naïve patients treated for 8 weeks with and without Q80K detected by population sequencing (~15 percent cutoff) are 73 percent and 84 percent.[17] SVR rates for patients with cirrhosis and 12 weeks treatment duration are 74 percent with the Q80K polymorphism, compared to 92 percent without the polymorphism. [18]

Pre-existing RASs clearly influence virologic outcomes for the combination of the PI asunaprevir with the NS5A inhibitor daclatasvir, which is an approved treatment in Japan. While the overall SVR rate in the pivotal trial was 84 percent, the SVR rates for patients with baseline L31 or Y93 substitutions were between 38-41 percent.[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.

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 below. Patients had chronic HCV genotype 1 (1a or 1b) infection (with exception of 1 genotype 1c, 1 genotype 1a/1b, 2 genotype 1h, 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-naïve, 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-naïve, non-cirrhotic 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, group 21).

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 VERSANT® HCV Genotype 2.0 assay (LiPA) or by TRUGENE® (both Siemens, Munich, Germany). The genotype results from LiPA and TRUGENE assay were confirmed or refined by direct sequencing results from the viral NS5A and 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 HCV RNA ≥1000 IU/mL. For the NS5A gene, at the beginning of the studies only population sequencing was performed for patients that were enrolled in ION-1 part A and the initial group of patients in the ELECTRON study. Deep sequencing was then performed for all patients that 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, and 20-21). Baseline NS5B sequencing was successfully performed for a subset of patients by population (n=64) and deep (n=1628) sequencing. Baseline NS3 deep sequencing was successfully performed for all treatment-experienced patient (n=467). Population sequencing (Sanger method) of the full-length HCV

NS5A coding region was performed by 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 percent to 20 percent. [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 Illumina MiSeq deep sequencing platform (Illumina, San Diego, CA), or NS5A PCR amplicons generated by DDL were subjected to deep sequencing at Wuxi AppTec (Shanghai, China). Internally developed software (Gilead Sciences) was used to process and align sequencing data to identify the substitutions present at levels above 1 percent (percent total of reads). Substitutions at RAS positions were analyzed using 1 percent, 5 percent, 10 percent, 15 percent, and 20 percent cutoffs. 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 recently summarized by the HCV Drug Resistance Advisory Group (DRAG) 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 position 96, 142, 159, 282, 289, 320, and 321 (nucleotide class specific RASs). RASs at residues associated with resistance to PIs including substitutions at position 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 prior to 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 of Lohmann et al.[24] Briefly, cells were trypsinized and washed twice with PBS. A suspension of cells was mixed with RNA and subjected to electroporation. Cells were transferred into 40 ml of pre-warmed culture medium and then seeded into 96-well plates (100 μL/well). Compounds were diluted in 100 percent DMSO and added to cells. Cells were treated for 3 days, after which culture media were 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). EC_{50} values were calculated as the compound concentration at which a 50 percent reduction in the level of *Renilla* reporter activity was observed when compared with control samples with DMSO. Dose-response curves and EC_{50} values were generated using 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 h post-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 due to either early study drug termination or lost to follow-up prior to the SVR12 visit resulting in a final analysis population of 2108 patients (1575 patients with genotype 1a, 525 genotype 1b, 2 genotype 1h, 2 genotype 4a, 2 genotype 1c, 1 genotype 1l, and 1 recombinant genotype 1a+genotype 1b patient).

In the pooled analysis of population and deep sequencing data from the Phase 2 and 3 studies, 338 of 2108 (16.0 percent) patients were identified as having baseline NS5A RASs by population (~15 percent cutoff) or deep sequencing (using 1 percent cutoff), irrespective of subtype (246 of 1575, 15.6 percent genotype 1a, 86 of 525, 16.4 percent genotype 1b, and 8 patients with other genotypes). Of the 338 patients with baseline NS5A RASs, 316 (93.5 percent) patients achieved SVR12 following 6, 8, 12, or 24 weeks of treatment with ledipasvir/sofosbuvir with or without ribavirin, compared with 1741 of 1770 (98.4 percent) patients with no NS5A RASs ($p<0.001$). 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 percent, compared with 98.6 percent for patients without NS5A RASs ($p=0.7$), while SVR12 rates for genotype 1a patients were 92.3 percent for those with NS5A RASs compared with 98.3 percent for patients without NS5A RASs ($p<0.001$) (Figure 1a). Slightly lower treatment response rates of 90 percent were observed for genotype 1a patients with NS5A

RASs using a 15 percent deep sequencing cutoff (Figure 1b).

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-naïve genotype 1 patients in the ledipasvir/sofosbuvir group, 102/887 (11.5 percent) had at least 1 RAS that conferred >100-fold-resistance to ledipasvir. A significant reduction in the SVR rate (Figure 2a) was seen among treatment-naive patients with NS5A RASs conferring >100-fold ledipasvir resistance who received only 8 weeks of ledipasvir/sofosbuvir therapy (82.8 percent; p=0.011). A significant reduction in SVR based on the presence of high-level baseline NS5A RASs was not observed among treatment-naïve patients treated for 12 or 24 weeks with ledipasvir/sofosbuvir, and all patients with NS5A RASs conferring <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 one NS5A RAS conferring >100-fold-resistance to ledipasvir at a frequency of >15 percent at baseline (Table 1). Similar SVR rates (82.1 percent) were observed for treatment-naïve patients with NS5A RASs conferring >100-fold ledipasvir resistance who received 8 weeks of ledipasvir/sofosbuvir+ribavirin therapy (Figure 2c), which also included 4/5 virologic failure patients with HCV RNA >6 million IU/mL.

Among treatment-experienced patients SVR rates were 97.4 to 100 percent for those without baseline RASs or with RASs conferring <100-fold resistance to ledipasvir treated for 12 or 24 weeks with ledipasvir/sofosbuvir (Figure 2b). Those treatment-experience patients with baseline

NS5A RASs with >100-fold-resistance to ledipasvir who were treated for 12 weeks had a significantly lower SVR12 rate (64.7 percent; 11/17) than those without baseline RASs (97.4 percent; 113/116) or those with high-level RASs treated for 24 weeks (100 percent; 6/6). Of the 6 patients with RASs who did not achieve SVR12 after 12 weeks of treatment, all had at least one RAS conferring >100-fold-resistance to ledipasvir at a frequency of >15 percent at baseline and 4 had multiple high-level NS5A RASs (Table 1).

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

Prevalence and geographical distribution of specific NS5A RASs

The prevalence of specific ledipasvir RASs detected at baseline was evaluated using different deep sequencing assay cutoffs: 1 percent, 5 percent, 10 percent, 15 percent, and 20 percent. Of the 1030 patients who had successful deep sequencing and were treated with ledipasvir/sofosbuvir for 8, 12, and 24 weeks, 16.7 percent, 12.4 percent, 10.5 percent, 9.0 percent, and 8.5 percent had specific ledipasvir NS5A RASs with 1 percent, 5 percent, 10

percent, 15 percent, and 20 percent cutoffs, respectively (Figure 3a). Only slight differences in SVR rates were seen for the different cutoffs and SVR12 rates ranged from 87.1 percent to 91.9 percent (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 percent cutoff were K24R>L31M>Q30H>M28T>Y93H>Q30R. At 5 percent, 10 percent, 15 percent, and 20 percent cutoffs, Q30H and L31M were the most frequent RASs in genotype 1a patients (Figure 3b). For all cutoffs, the most frequent NS5A RASs in genotype 1b patients were Y93H and L31M (Figure 3c), and almost all patients harbored a single NS5A RAS.

A comparison of the prevalence of NS5A RASs between patients in the US and the EU shows only small differences in the geographic distribution of baseline RASs (Figure 3d and 3e). Overall, approximately 15.0 percent of genotype 1a HCV-infected patients in the US harbored NS5A RASs compared with approximately 20.9 percent in the EU. For genotype 1b, approximately 15.5 percent of US patients and 17.1 percent 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 percent and 15.0 percent of the patients had Y93H in the US and EU, 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 80-93.3 percent and 75-88.2 percent for

NS5A RASs that confer >100-fold-resistance to ledipasvir (Q30H, Q30R, L31M in genotype 1a and Y93H) using 1 percent and 15 percent deep sequencing cutoffs, respectively (Figure 4a). Slightly lower SVR rates were observed using the 15 percent cutoff compared to the 1 percent cutoff for these RASs. For K24R, which confers 3.7-fold resistance to ledipasvir, the SVR rate was 100 percent independent of deep sequencing cutoff. For M28T, which confers 61-fold resistance to ledipasvir, the SVR rate was also reduced, however the 2 patients with virologic failure with M28T also had other NS5A RASs conferring >100-fold resistance (Table 1). In the case of L31M, reduced SVR rates were only observed 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 percent (28/1786) experienced virologic failure, compared with incidences of 4.9 percent, 10.2 percent and 15.8 percent virologic relapse with 1 RAS, 2 RASs and ≥3 RASs, respectively (Figure 4b). Moreover, the prevalence of baseline RASs decreased with the number of RASs, in which 1 RAS, 2 RASs and ≥3 RASs had a prevalence of 12.6 percent, 2.3 percent and 0.9 percent, respectively.

Forty-nine patients experienced virologic relapse in the Phase 2/3 studies with sofosbuvir/ledipasvir regimens. Of these, 21 (43 percent) 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 >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 percent and 88.2 percent using 1 percent and 15 percent deep sequencing

cutoffs, respectively. Of the 21 patients with baseline RASs who relapsed, 71 percent (15/21) had at least one RAS conferring >100-fold-resistance to ledipasvir at a frequency of >15 percent 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 4c). 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 and 395 genotype 1b, 6 other), including deep sequencing results from 1628 patients. The NS5B RAS S282T was not detected in any patient using a 1 percent cutoff 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, two substitutions at two 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 1a, 95 genotype 1b). Of these patients, 265 were previously treated with PI-containing regimens. Baseline NS3 RASs were detected in 141 of the 265 (53.2 percent) patients, of which 139 (98.6 percent) achieved SVR12 (Table 2). For the patients who were PI treatment-naïve (previous pegylated interferon+ribavirin treatment failures), 23 of 202 (11.4 percent) had baseline NS3 RASs and 95.5 percent achieved SVR12. Additionally, Q80 polymorphisms were observed in 93 of 202 (46.0 percent) PI treatment-naïve patients and 110 of 265 (41.5 percent) PI treatment-experienced patients, of which 96.8 percent and 97.3 percent 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 percent 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 >90 percent. 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 having baseline NS5A RASs ranged from 8.5 percent, when a cutoff of 20 percent was used, to a high of 16.7 percent, when a cutoff of 1 percent 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 versus population sequencing) and the cutoff used to determine the presence of a substitution. Overall, the treatment responses were similar regardless of the specific cutoff used in the analysis, with slightly lower responses observed using the 15 percent cutoff versus a 1 percent cutoff. 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 these RASs at frequencies below the detection limit of population-based sequencing (15 percent).

Zeuzem et al., conducted a larger study that investigated the prevalence of baseline NS5A RASs in genotype 1 patients and the effect on treatment response and included more than 5,000 patients from 21 countries across HCV Gilead clinical trials from 2010 to 2015 (Zeuzem et al, Hepatology, volume 62: p 254A, Abstract 91, 2016). 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 Harvoni and also included patients that 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 demonstrated that reduced SVR rates in treatment-naive patients was limited to those with NS5A RASs conferring >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 >100-fold-resistance to ledipasvir at a frequency of >15 percent of the viral population at baseline. Moreover, 3 of these 5 patients had a baseline viral load > 6 million IU/ml and per current treatment guidelines, a treatment course of 12 weeks is recommended. Among treatment-naïve 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 <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 >100-fold-resistance to ledipasvir and were treated for 12 weeks without ribavirin. Six out of these 17 patients did not achieve SVR12. Of these 6 patients,

all had at least one NS5A RAS conferring >100-fold-resistance to ledipasvir at a frequency of >15 percent at baseline and 4/6 had multiple high-level ledipasvir RASs. Treatment-experienced genotype 1a patients with pre-existing NS5A RASs that confer a >100 fold resistance to ledipasvir represented 6.9 percent (17/245) 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 >100-fold-resistance to ledipasvir, relative to 12 weeks of ledipasvir/sofosbuvir without ribavirin; however, the SVR12 rate was still numerically lower than that observed with 24 weeks of therapy. This observation stands in contrast to data from the SIRIUS trial [27], in which treatment-experienced cirrhotic patients, were randomized to 24 weeks of ledipasvir/sofosbuvir or 12 weeks of ledipasvir/sofosbuvir+ribavirin. All the patients (8/8) with NS5A RASs conferring >100-fold-resistance to ledipasvir treated for 12 weeks with ledipasvir/sofosbuvir+ribavirin achieved SVR12; conversely, 7/9 (78 percent) of patients with NS5A RASs conferring >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 to ledipasvir/sofosbuvir for 24 weeks.

For the specific NS5A RASs Q30H/R, L31M in genotype 1a and Y93H that confer >100-fold-resistance to ledipasvir, SVR rates ranged from 80-93.3 percent and 75-88.2 using 1 and 15 percent deep sequencing cutoffs, respectively. Overall, slightly lower SVR rates were observed using the 15 percent cutoff compared to the 1 percent cutoff. 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 percent) to patients with 1, 2 or at least 3 RASs (4.9, 10.2 and 15.8 percent, respectively). However, the prevalence of patients with 1, 2 or at least 3 pre-existing NS5A RASs decreased from 12.6 percent to 2.3 percent and 0.9 percent. This observation is in line with a higher relapse rate observed in patients who received sofosbuvir plus ledipasvir after failure to a ledipasvir-containing regimen and the presence of multiple NS5A RASs. [28]

Baseline NS3 RASs were detected in 53.2 percent of patients who were previously treated with PI-containing regimens, of whom 98.6 percent achieved SVR12. Additionally, Q80 polymorphisms were observed in 46.0 percent of PI treatment-naïve patients and 41.5 percent of PI treatment-experienced patients, of whom 96.8 percent and 97.3 percent 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 percent 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 >100-fold-resistance to ledipasvir together with a shortened treatment duration of 8 weeks in treatment-naïve 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 one NS5A RAS conferring >100-fold-resistance to ledipasvir was detected at a frequency of >15 percent 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 prior to ledipasvir/sofosbuvir therapy does not appear to be clinically warranted. This is further supported by the high rates of SVR observed in post-marketing "real world" cohorts of patients treated outside of clinical trials with ledipasvir/sofosbuvir regimens where the SVR rate has been >90 percent across multiple diverse patient cohorts [29, 30].

References

1. Liang TJ, Ghany MG. Current and future therapies for hepatitis C virus infection. N Engl J Med 2013;368:1907-17.
2. Afdhal N, Zeuzem S, Kwo P, et al. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. N Engl J Med 2014;370:1889-98.
3. Afdhal N, Reddy KR, Nelson DR, et al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. N Engl J Med 2014;370:1483-93.
4. Kowdley KV, Gordon SC, Reddy KR, et al. Ledipasvir and sofosbuvir for 8 or 12 weeks for chronic HCV without cirrhosis. N Engl J Med 2014;370:1879-88.
5. Lawitz E, Poordad FF, Pang PS, et al. Sofosbuvir and ledipasvir fixed-dose combination with and without ribavirin in treatment-naive and previously treated patients with genotype 1 hepatitis C virus infection (LONESTAR): an open-label, randomised, phase 2 trial. Lancet 2014;383:515-23.
6. Lawitz EJ, Gruener D, Hill JM, et al. A phase 1, randomized, placebo-controlled, 3-day, dose-ranging study of GS-5885, an NS5A inhibitor, in patients with genotype 1 hepatitis C. J Hepatol 2012;57:24-31.
7. Rong L, Dahari H, Ribeiro RM, et al. Rapid emergence of protease inhibitor resistance in hepatitis C virus. Sci Transl Med 2010;2:30ra32.
8. Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology 2010;138:447-62.
9. Wong KA, Worth A, Martin R, et al. Characterization of Hepatitis C virus resistance from a multiple-dose clinical trial of the novel NS5A inhibitor GS-5885. Antimicrob Agents Chemother 2013;57:6333-40.
10. G Cheng BP, A Corsa, M Yu, M Nash, YJ Lee, Y Xu, T Kirschberg, Y Tian, J Taylor, J Link, W Delaney IV. Antiviral Activity and Resistance Profile of the Novel HCV NS5A Inhibitor GS-5885 In EASL 47th Annual Meeting, Barcelona, Spain, April 18th - 22nd 2012, 2012.
11. Lam AM, Espiritu C, Bansal S, et al. Genotype and subtype profiling of PSI-7977 as a nucleotide inhibitor of hepatitis C virus. Antimicrob Agents Chemother 2012;56:3359-68.
12. Dutartre H, Bussetta C, Boretto J, et al. General catalytic deficiency of hepatitis C virus RNA polymerase with an S282T mutation and mutually exclusive resistance towards 2'-modified nucleotide analogues. Antimicrob Agents Chemother 2006;50:4161-9.
13. Le Pogam S, Jiang WR, Leveque V, et al. In vitro selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance. Virology 2006;351:349-59.
14. Tong X, Le Pogam S, Li L, et al. In vivo emergence of a novel mutant L159F/L320F in the NS5B polymerase confers low-level resistance to the HCV polymerase inhibitors mericitabine and sofosbuvir. J Infect Dis 2014;209:668-75.
15. Svarovskaia ES, Dvory-Sobol H, Parkin N, et al. Infrequent development of resistance in genotype 1-6 hepatitis C virus-infected subjects treated with sofosbuvir in phase 2 and 3 clinical trials. Clin Infect Dis 2014;59:1666-74.

16. Svarovskaia ES, Gane E, Dvory-Sobol H, et al. L159F and V321A Sofosbuvir-Associated Hepatitis C Virus NS5B Substitutions. J Infect Dis 2015.
17. P. Kwo NG, R. Nahass, D. Bernstein, S. Rojter, E. Schiff, M. Davis, P.J. Ruane, Z. Younes, R. Kalmeijer, M. Peeters, O. Lenz, B. Fevery, G. De La Rosa, J. Scott,, R. Sinha JW. A phase 3, randomised, open-label study to evaluate the efficacy and safety of 8 and 12 weeks of simeprevir (SMV) plus sofosbuvir (SOF) in treatment-naive and -experienced patients with chronic HCV genotype 1 infection without cirrhosis: OPTIMIST-1 In 50th Annual Meeting of the European Association for the Study of the Liver Vienna Austria, April 22-26, 2015.
18. E. Lawitz GM, E. DeJesus, E. Yoshida, F. Felizarta, R. Ghalib, E. Godofsky, R. Herring, G. Poleynard, A. Sheikh, H. Tobias, M. Kugelmas, R. Kalmeijer, M. Peeters, O. Lenz, B. Fevery, G. De La Rosa, J. Scott, R. Sinha, J. Witek. A phase 3, open-label, single-arm study to evaluate the efficacy and safety of 12 weeks simeprevir (SMV) plus sofosbuvir (SOF) in treatment-naive or -experienced patients with chronic HCV genotype 1 infection and cirrhosis: OPTIMIST-2 In 50th Annual Meeting of the European Association for the Study of the Liver, Vienna Austria, April 22-26, 2015.
19. Manns M, Pol S, Jacobson IM, et al. All-oral daclatasvir plus asunaprevir for hepatitis C virus genotype 1b: a multinational, phase 3, multicohort study. Lancet 2014;384:1597-605.
20. Sulkowski MS, Gardiner DF, Rodriguez-Torres M, et al. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. N Engl J Med 2014;370:211-21.
21. Gane EJ, Stedman CA, Hyland RH, et al. Efficacy of nucleotide polymerase inhibitor sofosbuvir plus the NS5A inhibitor ledipasvir or the NS5B non-nucleoside inhibitor GS-9669 against HCV genotype 1 infection. Gastroenterology 2014;146:736-743 e1.
22. HCV Phenotype Working Group HDDAG. Clinically relevant HCV drug resistance mutations figure and tables (Updated). Ann Forum Collab HIV Res 2012;14:1-10.
23. Shih I-h, Vliegen I, Peng B, et al. Mechanistic characterization of GS-9190 (tegobuvir), a novel non-nucleoside inhibitor of hepatitis C virus NS5B polymerase. Antimicrobial Agents and Chemotherapy 2011;55:4196-4203.
24. Lohmann V, Korner F, Koch J, et al. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.
25. Bartels DJ, Sullivan JC, Zhang EZ, et al. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J Virol 2013;87:1544-53.
26. Suzuki F, Sezaki H, Akuta N, et al. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J Clin Virol 2012;54:352-4.
27. Bourliere M, Bronowicki JP, de Ledinghen V, et al. Ledipasvir-sofosbuvir with or without ribavirin to treat patients with HCV genotype 1 infection and cirrhosis non-responsive to previous protease-inhibitor therapy: a randomised, double-blind, phase 2 trial (SIRIUS). Lancet Infect Dis 2015;15:397-404.
28. E. Lawitz SF, J.C. Yang, P.S. Pang, Y. Zhu, E. Svarovskaia, J.G. McHutchison, D. Wyles, P. Pockros. Retreatment of patients who failed 8 or 12 weeks of ledipasvir/sofosbuvir-based regiments with ledipasvir/sofosbuvir for 24 weeks, In 50th Annual Meeting of

the European Association for the Study of the Liver Vienna, Austria April 22-26, 2015.

29. Terrault N, Zeuzem S, Di Bisceglie AM, Lim JK, Pockros PJ, Frazier LM, Kuo A, Lok AS, Shiffman ML, Ben Ari Z, Stewart T, Sulkowski MS, Fried MW, and Nelson DR for the HCV-TARGET Study Group. Treatment Outcomes With 8, 12 and 24 Week Regimens of Ledipasvir/Sofosbuvir for the Treatment of Hepatitis C Infection: Analysis of a Multicenter Prospective, Observational Study. Annual Meeting of the American Association for theStudy of Liver Diseases (AASLD), Boston, MA, Nov 13-17, 2015. Abstract 94
30. Afdhal NH, Bacon B, Dieterich D, Flamm SL, Kowdley KV, Lee Y, Younossi ZM, Tsai N, Younoss Z. Failure with All-oral DAA Regimens: Real-world experience from the TRIO Network. American Association for the Study of Liver Diseases (AASLD), Boston, MA, Nov 13-17, 2015. Abstract LB-17.

Figure Legends

Figure 1. Prevalence of NS5A RASs and Treatment Response. Patient baseline sequences generated by population and deep sequencing were pooled for treatment-naïve 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 percent cutoff for deep sequencing and population sequencing with a substitution detection of ~15 percent). (b) SVR12 rates in patients with or without baseline NS5A RASs (using a 15 percent cutoff for deep sequencing and population sequencing with a substitution detection of ~15 percent). LDV, ledipasvir. SOF, sofosbuvir. RBV, ribavirin. GT, genotype.

Figure 2. SVR12 by Level of NS5A RASs in those Treated with Ledipasvir/Sofosbuvir. Patient baseline sequences generated by population and deep sequencing were pooled (using 1 percent cutoff for deep sequencing and population sequencing with a substitution detection of ~15 percent). (a) Treatment-naïve. 3/5 failures who had baseline NS5A RASs with >100 fold resistance to ledipasvir had baseline viral load >6 x10^6 IU/mL. (b) Treatment-experienced. (c) SVR12 for patients with NS5A RASs with >100-fold-resistance to ledipasvir in treatment-naïve 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 due to documented noncompliance during the dosing period. LDV, ledipasvir. SOF, sofosbuvir. RBV, ribavirin.

Figure 3. Prevalence of NS5A RASs by Deep Sequencing Cutoff and Geographical Region. Substitution analyses were conducted on deep sequencing data (population sequences were not included). (a) Prevalence of NS5A RAS by 1 percent, 5 percent, 10 percent, 15 percent and 20

percent deep sequencing cutoff in patients treated with ledipasvir/sofosbuvir (n=1040). (b) Prevalence of specific NS5A RASs in patients with genotype 1a infection by deep sequencing cutoffs. (c) Prevalence of specific NS5A RASs in patients with genotype 1b infection by deep sequencing cutoffs. (d) Prevalence of NS5A RASs in patients with genotype 1a infection in US and EU. (e) Prevalence of NS5A RASs in patients with genotype 1b infection in US and EU. 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 cutoff (1 percent and 15 percent) in patients treated with ledipasvir/sofosbuvir. (b) Relapse rate in patients with 1, 2 or ≥3 NS5A RASs. The prevalence of 1 RAS, 2 RASs and ≥3 RASs is 12.6 percent, 2.3 percent and 0.9 percent, 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. VF, virologic failures. GT, genotype.

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**	**100-1000-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	100-1000-Fold
	L31M (25.45%)	TN	1a	100-1000-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	100-1000-Fold
	Q30H (>99%)	TE	1a	100-1000-Fold
	M28T (1.03%) Q30R (>99%) L31M (>99%)	TE	1a	100-1000-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	100-1000-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	100-1000-Fold
	Y93H (1.20%)	TE	1a	>1000-Fold
ledipasvir/ sofosbuvir+ ribavirin 24 weeks[a]	K24R (1.06%) Q30R (2.61%)	TE	1a	100-1000-Fold

GT, genotype; RAS, resistance-associated substitution; SRV12, sustained virologic response 12 weeks after treatment. TN, treatment naïve. TE, treatment experienced.

a Patient experienced breakthrough due to documented noncompliance during the dosing period

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

Patients with Baseline NS5B NI RASs			
NS5B RASs	**Genotype**	**No. of Patients with RASs (%)**	**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[b] (%)**	**SVR12 for Patients with PI RASs (%)**	**No. of Patients with Q80 Variants (%)**	**SVR12 for Patients with Q80 Variants (%)**
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%)

GT, genotype; NI, nucleotide inhibitor; RAS, resistance-associated substitution; SVR12, sustained virologic response 12 weeks after treatment.

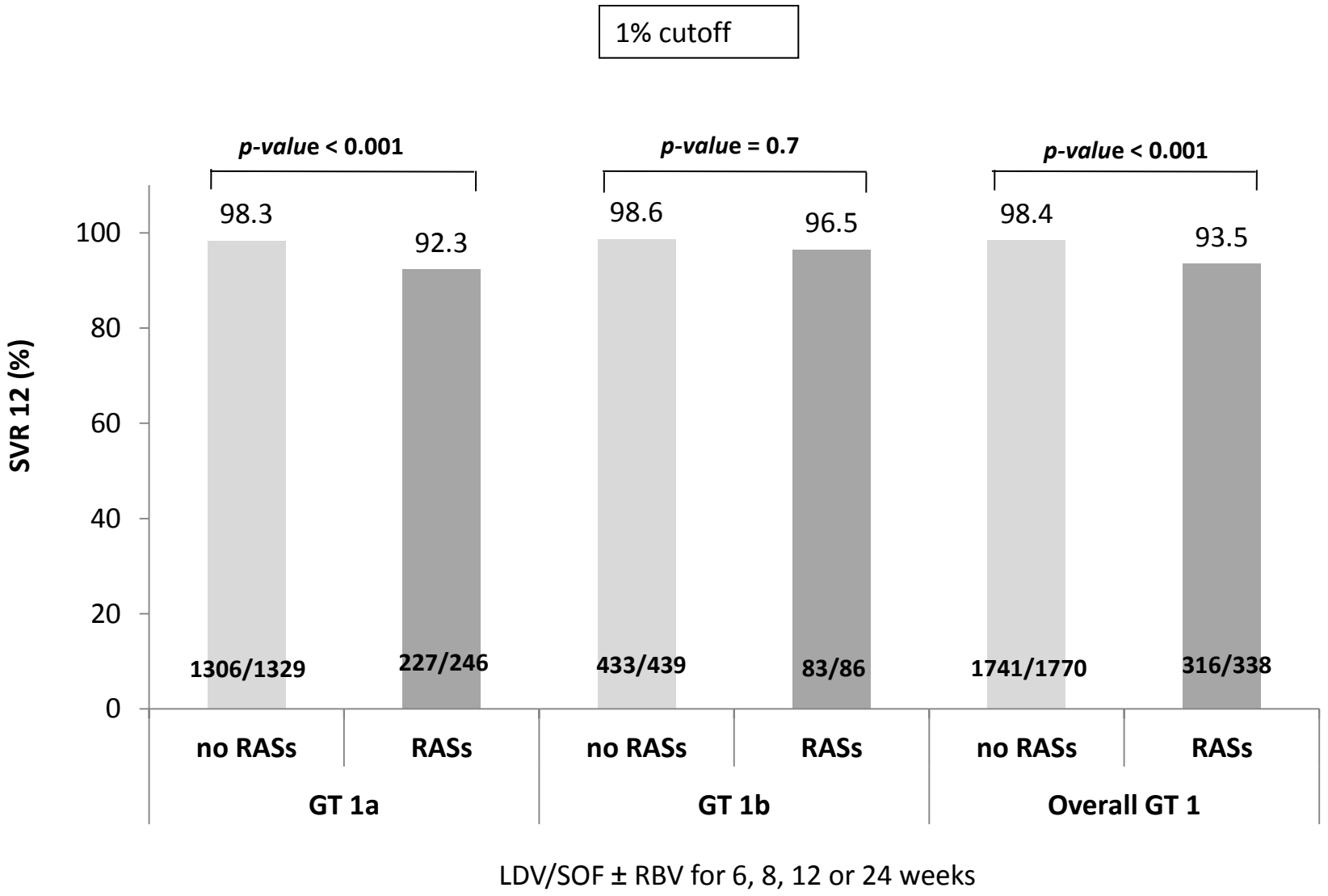
Variant analyses were conducted at 1% cutoff

[a] Substitutions observed at RAS sites.

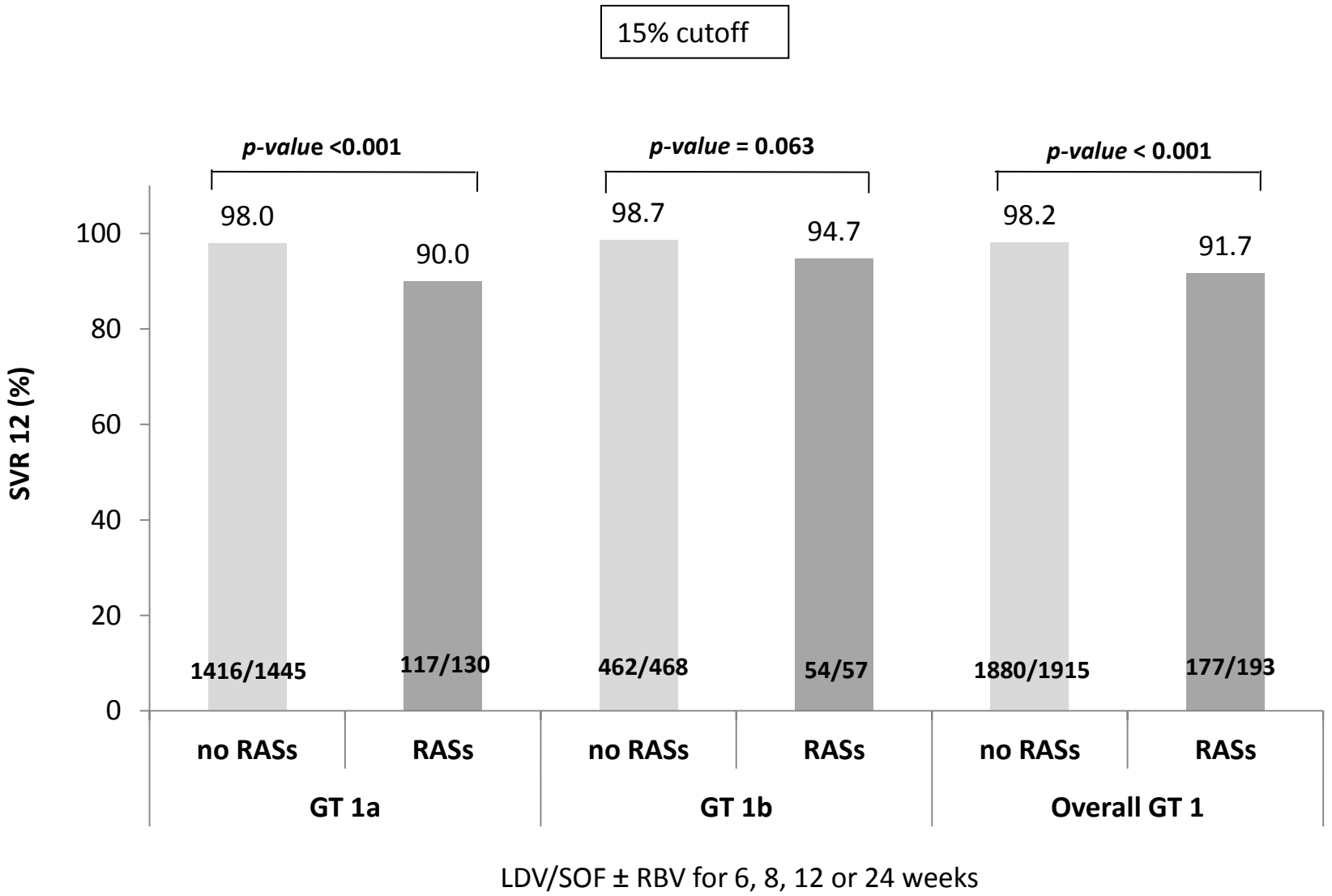
[b] RASs associated with resistance to protease inhibitors observed at baseline at positions V36, T54, V55, R155, A156, D168, I/V170, and M175L of the NS3 protease gene.

[c] Of the 110 patients with Q80 substitutions, 57 had a Q80 substitution and another NS3 RAS.

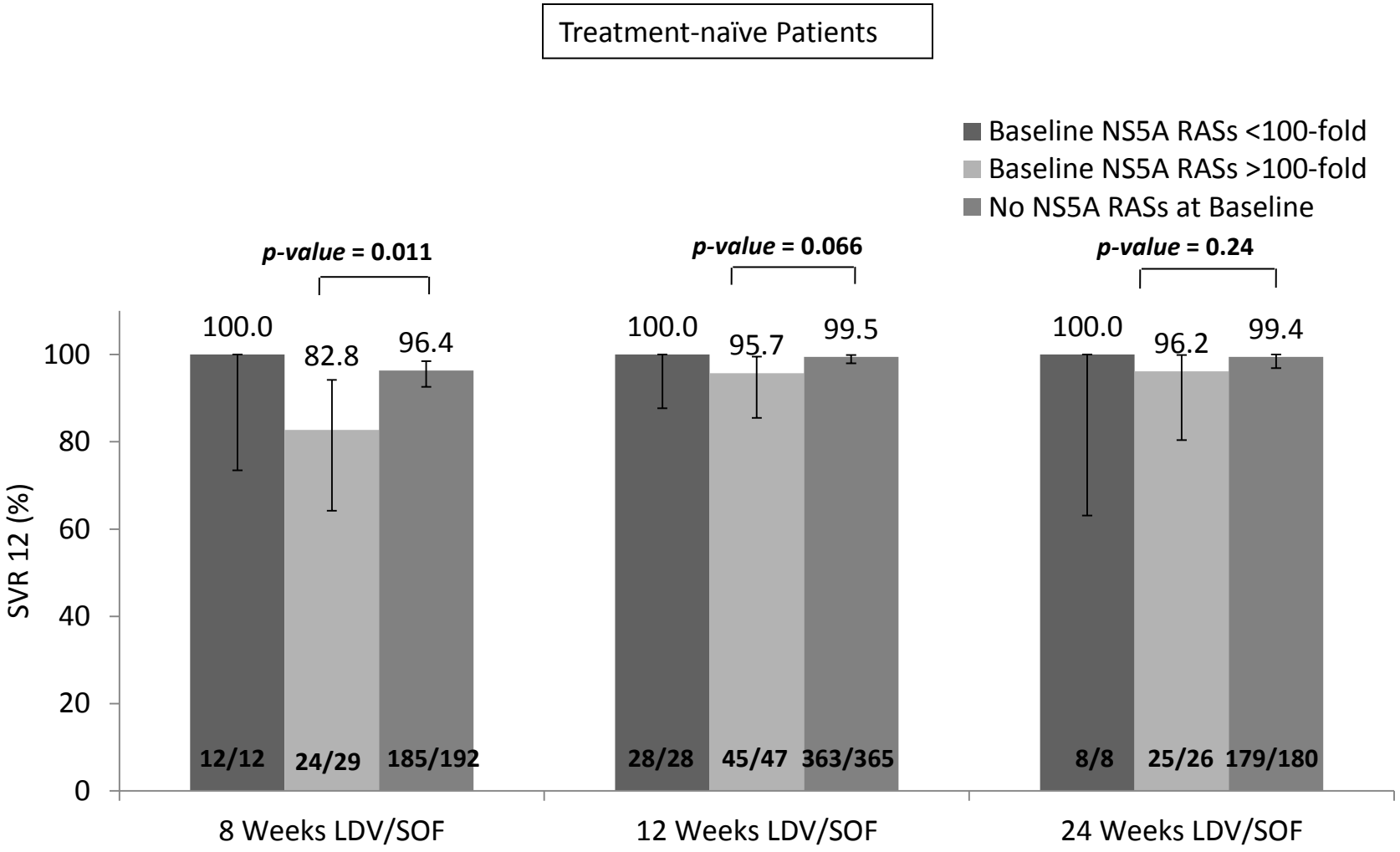
[d] Of the 93 patients with Q80 substitutions, 8 had a Q80 substitution and another NS3 RAS

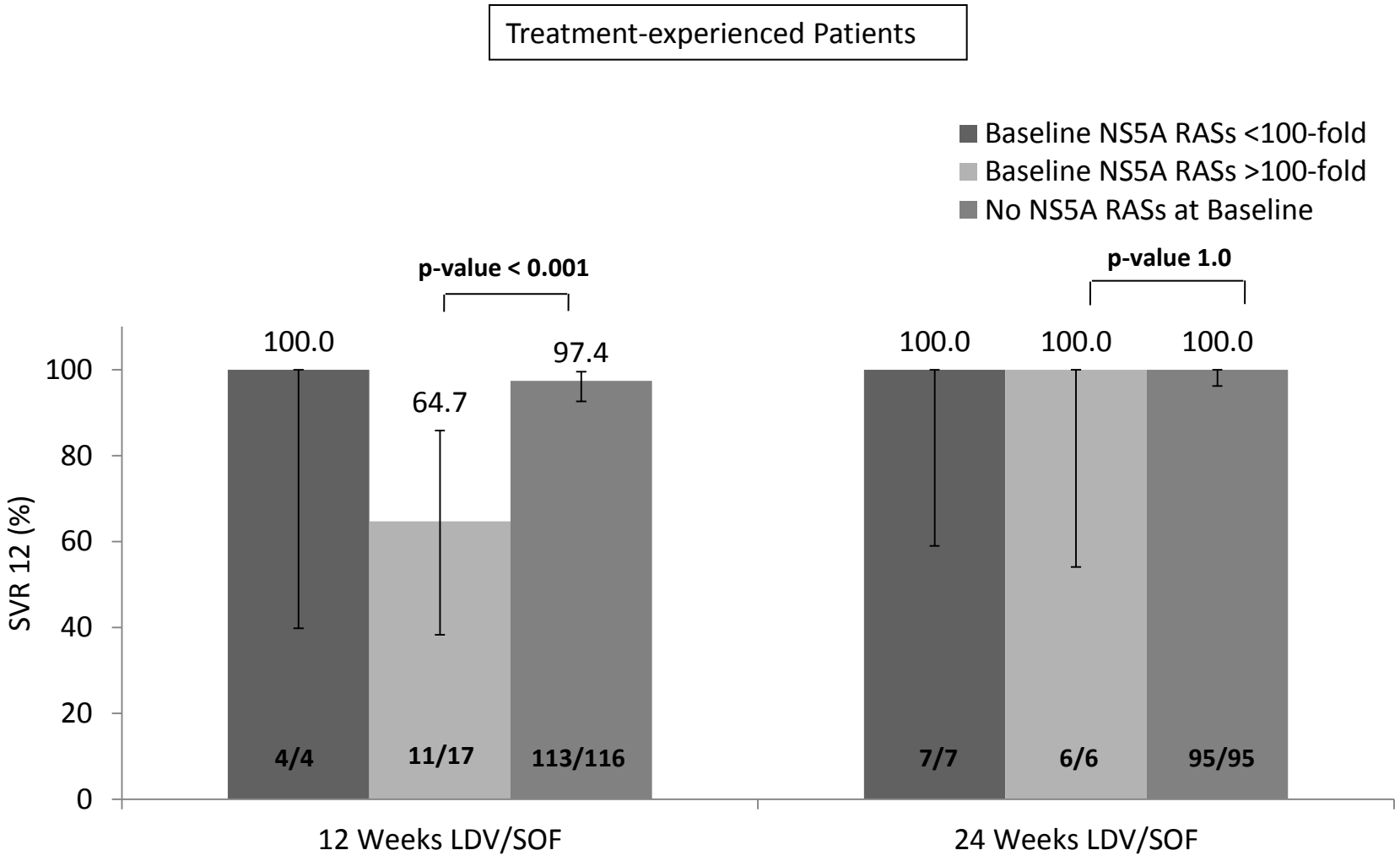


LDV/SOF ± RBV for 6, 8, 12 or 24 weeks

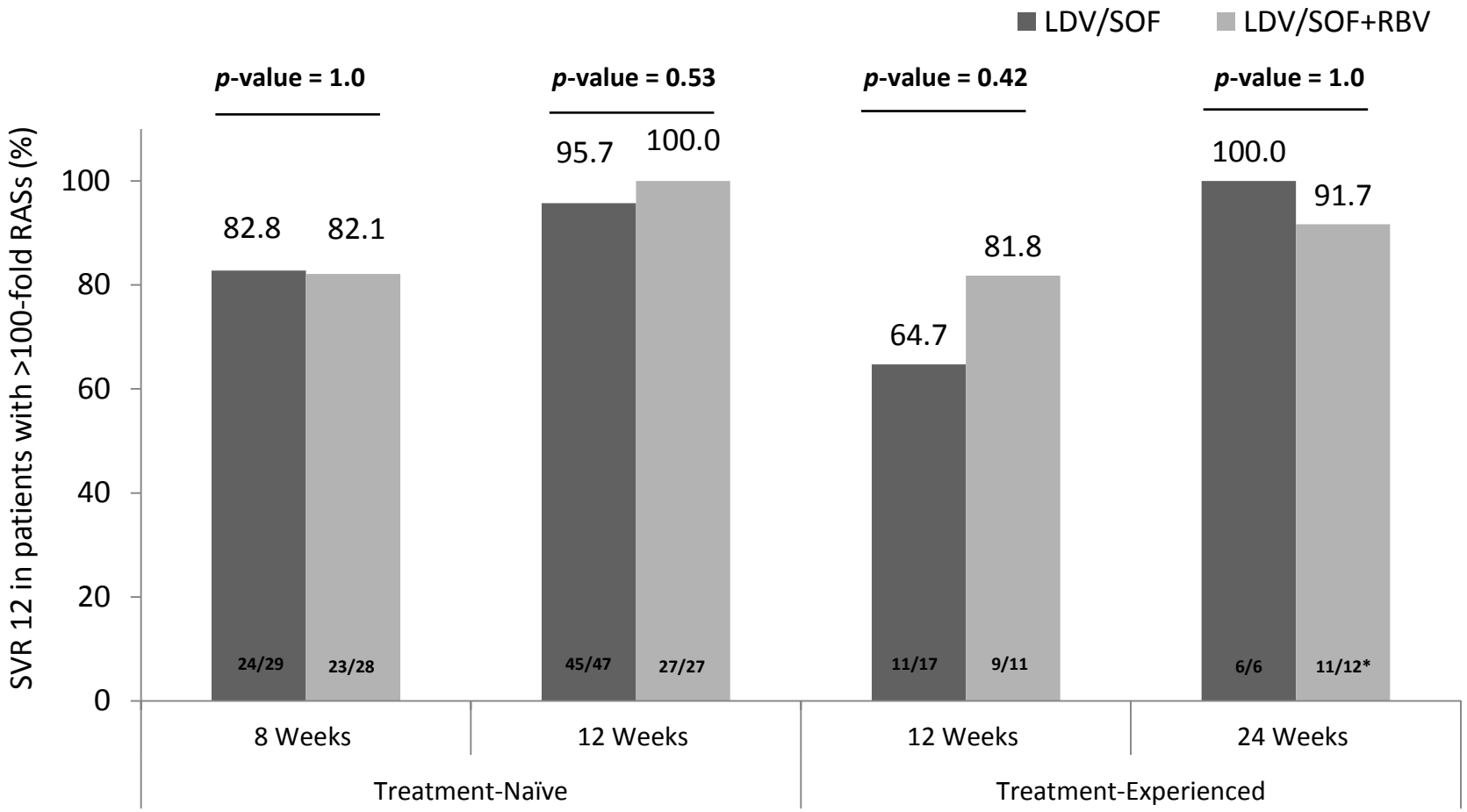


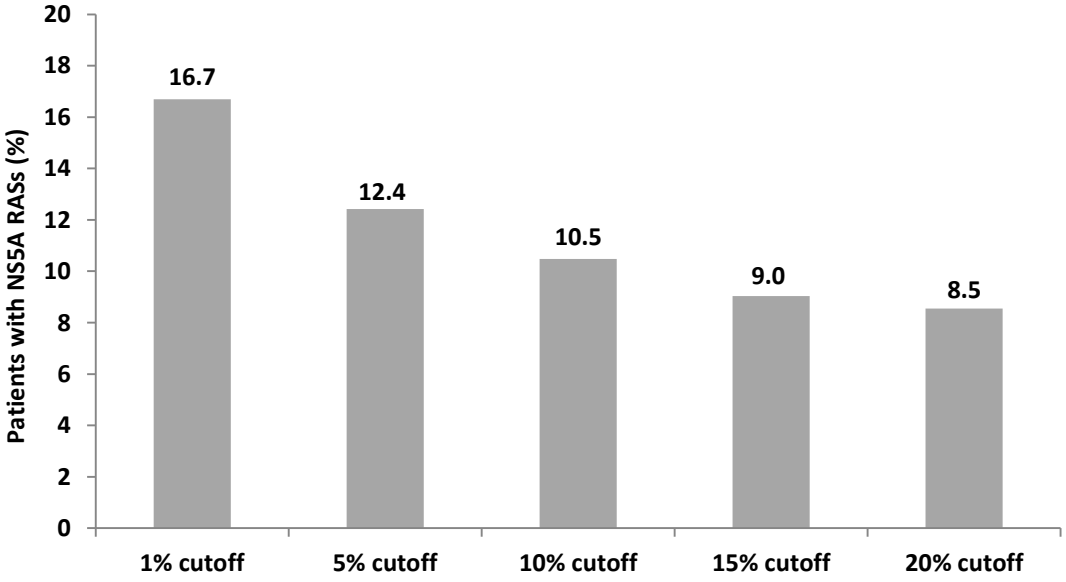
LDV/SOF ± RBV for 6, 8, 12 or 24 weeks

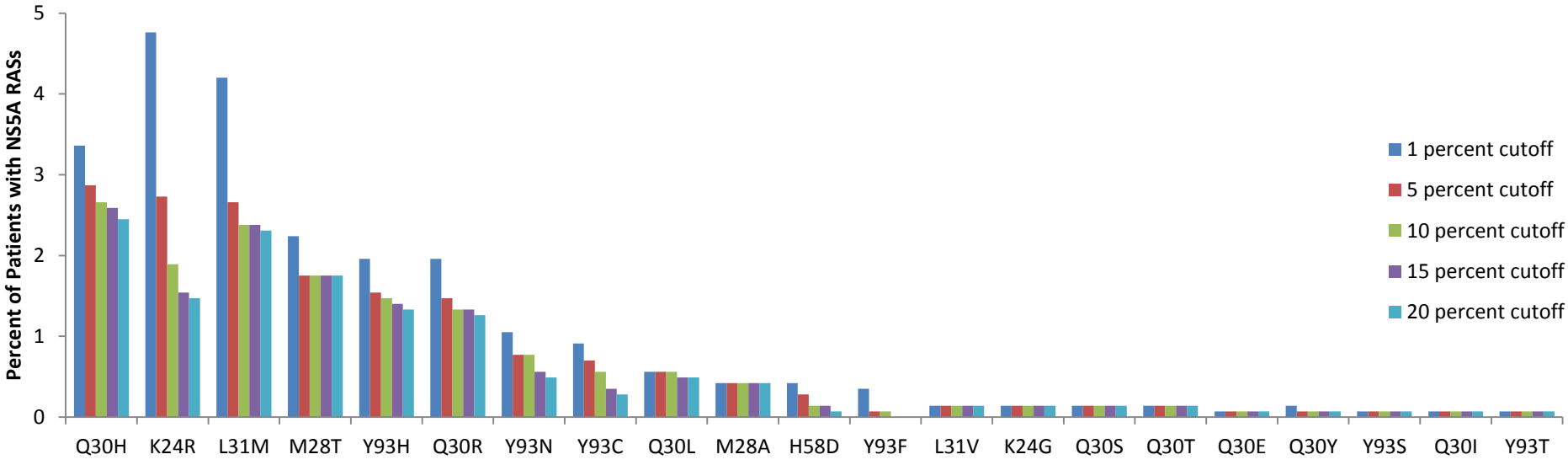

Treatment-naïve Patients
Baseline NS5A RASs <100-fold
Baseline NS5A RASs >100-fold
No NS5A RASs at Baseline
p-value = 0.011
p-value = 0.066
p-value = 0.24
SVR 12 (%)
100
80
60
40
20
0
100.0
82.8
96.4
100.0
95.7
99.5
100.0
96.2
99.4
12/12
24/29
185/192
28/28
45/47
363/365
8/8
25/26
179/180
8 Weeks LDV/SOF
12 Weeks LDV/SOF
24 Weeks LDV/SOF

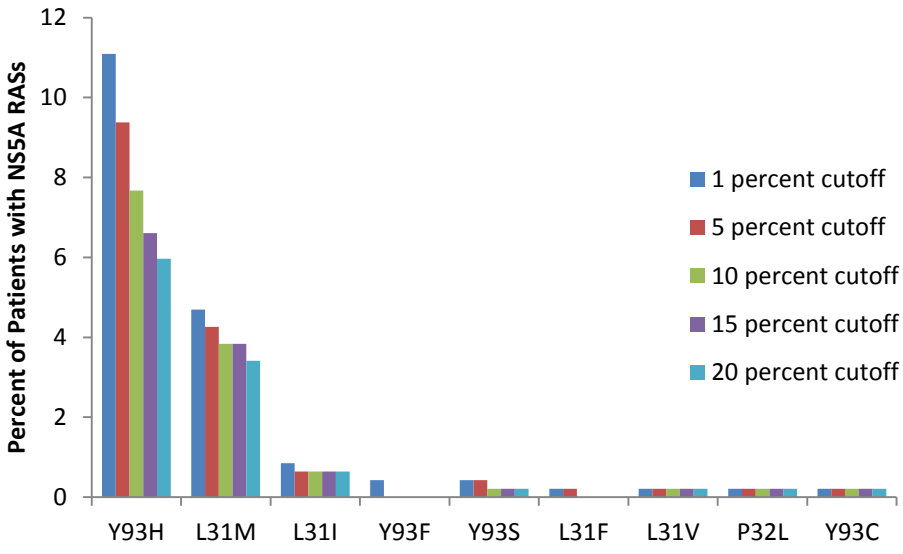

Treatment-experienced Patients
Baseline NS5A RASs <100-fold
Baseline NS5A RASs >100-fold
No NS5A RASs at Baseline
p-value < 0.001
p-value 1.0
SVR 12 (%)
100
80
60
40
20
0
100.0
64.7
97.4
100.0
100.0
100.0
4/4
11/17
113/116
7/7
6/6
95/95
12 Weeks LDV/SOF
24 Weeks LDV/SOF

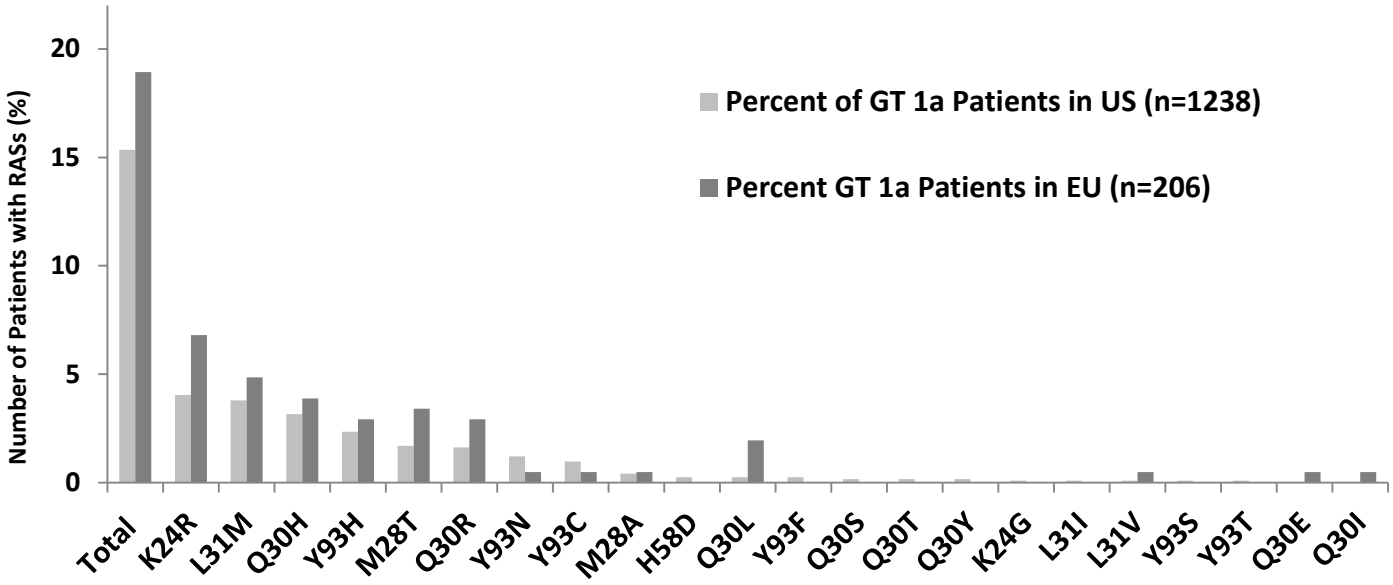
Patients with Highly Resistant NS5A RASs (LDV/SOF with and without RBV)

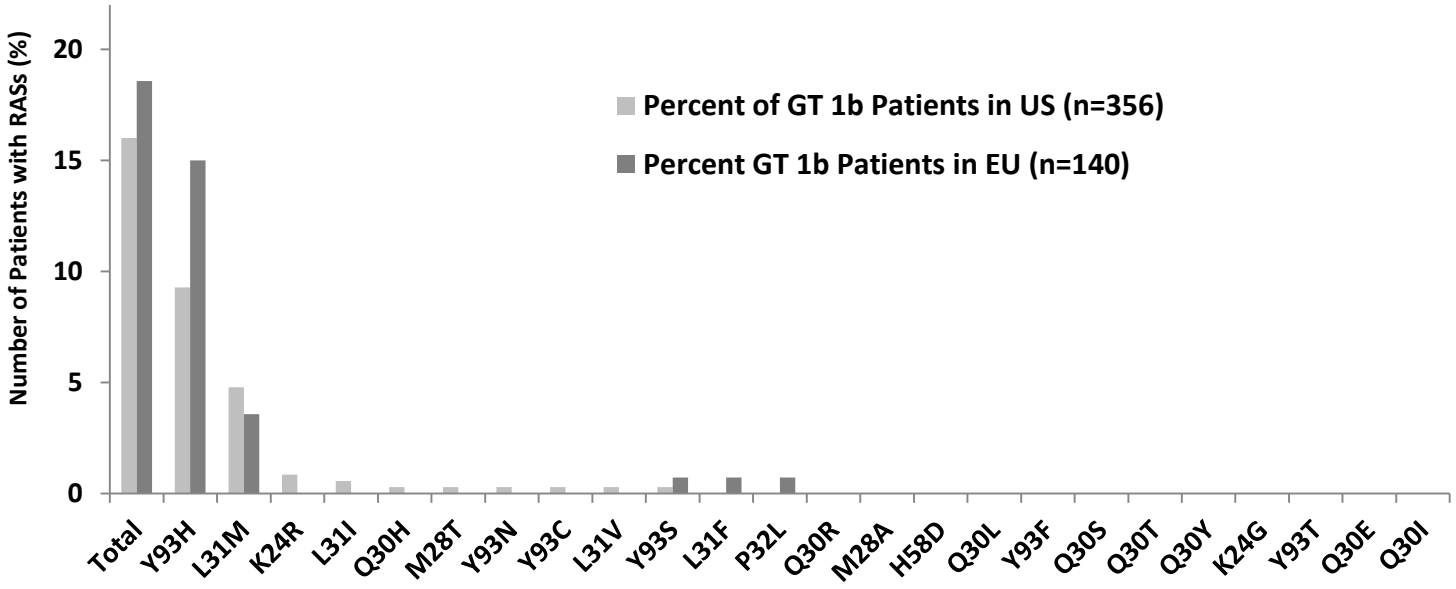


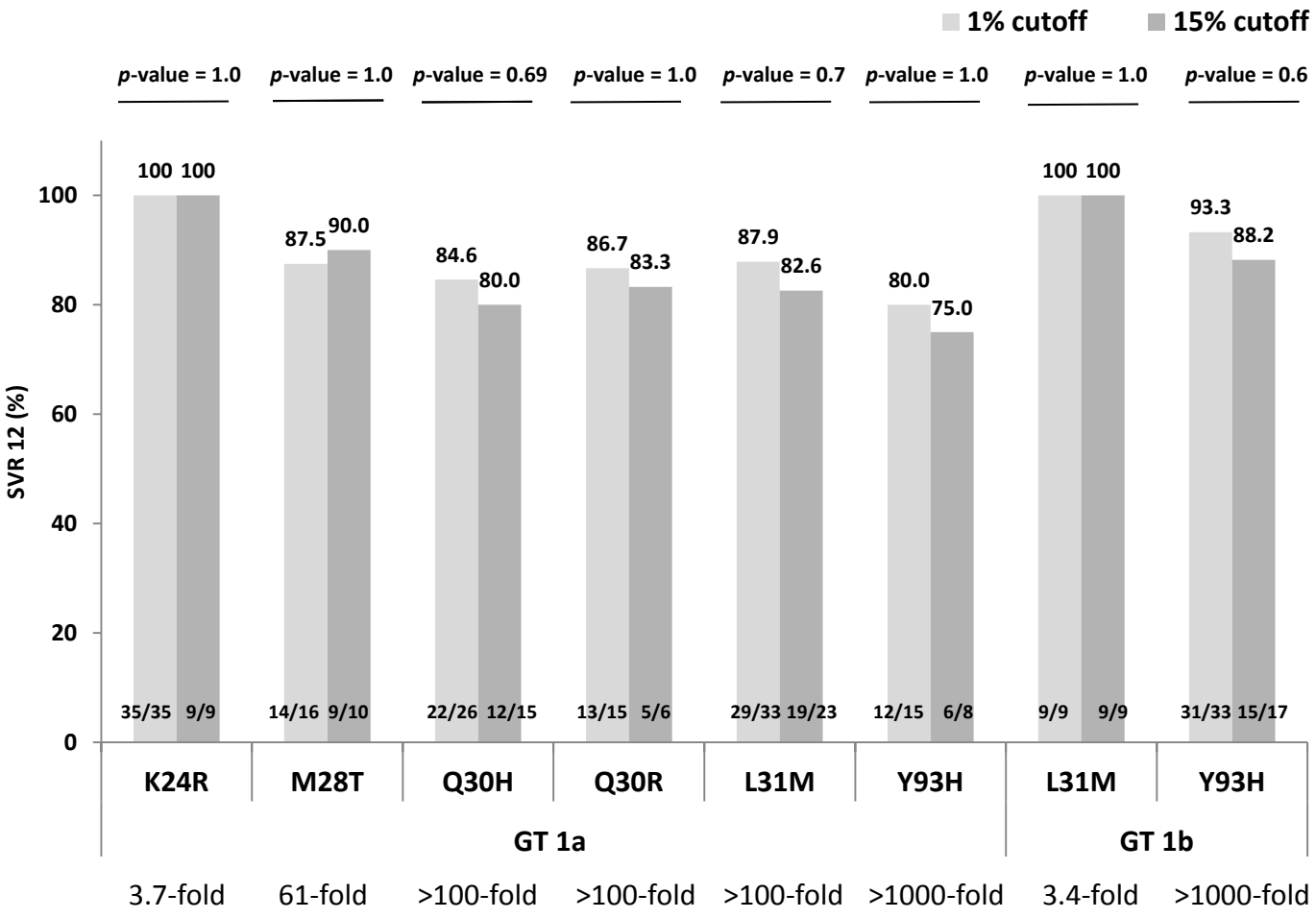

Patients with NS5A RASs (%)
20
18
16
14
12
10
8
6
4
2
0
16.7
12.4
10.5
9.0
8.5
1% cutoff
5% cutoff
10% cutoff
15% cutoff
20% cutoff

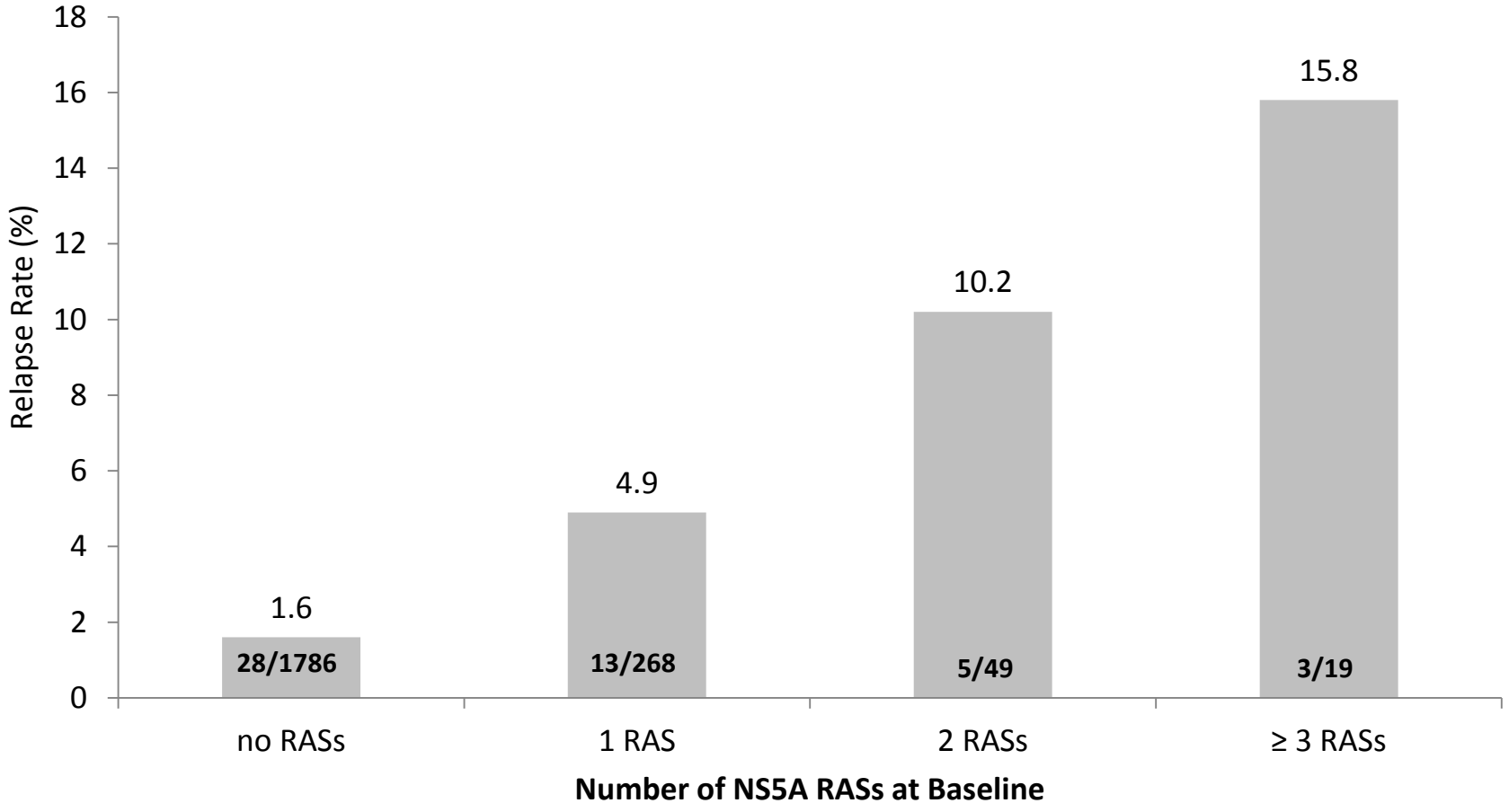

Percent of Patients with NS5A RASs
5
4
3
2
1
0
Q30H
K24R
L31M
M28T
Y93H
Q30R
Y93N
Y93C
Q30L
M28A
H58D
Y93F
L31V
K24G
Q30S
Q30T
Q30E
Q30Y
Y93S
Q30I
Y93T
1 percent cutoff
5 percent cutoff
10 percent cutoff
15 percent cutoff
20 percent cutoff


Percent of Patients with NS5A RASs
12
10
8
6
4
2
0
Y93H
L31M
L31I
Y93F
Y93S
L31F
L31V
P32L
Y93C
1 percent cutoff
5 percent cutoff
10 percent cutoff
15 percent cutoff
20 percent cutoff

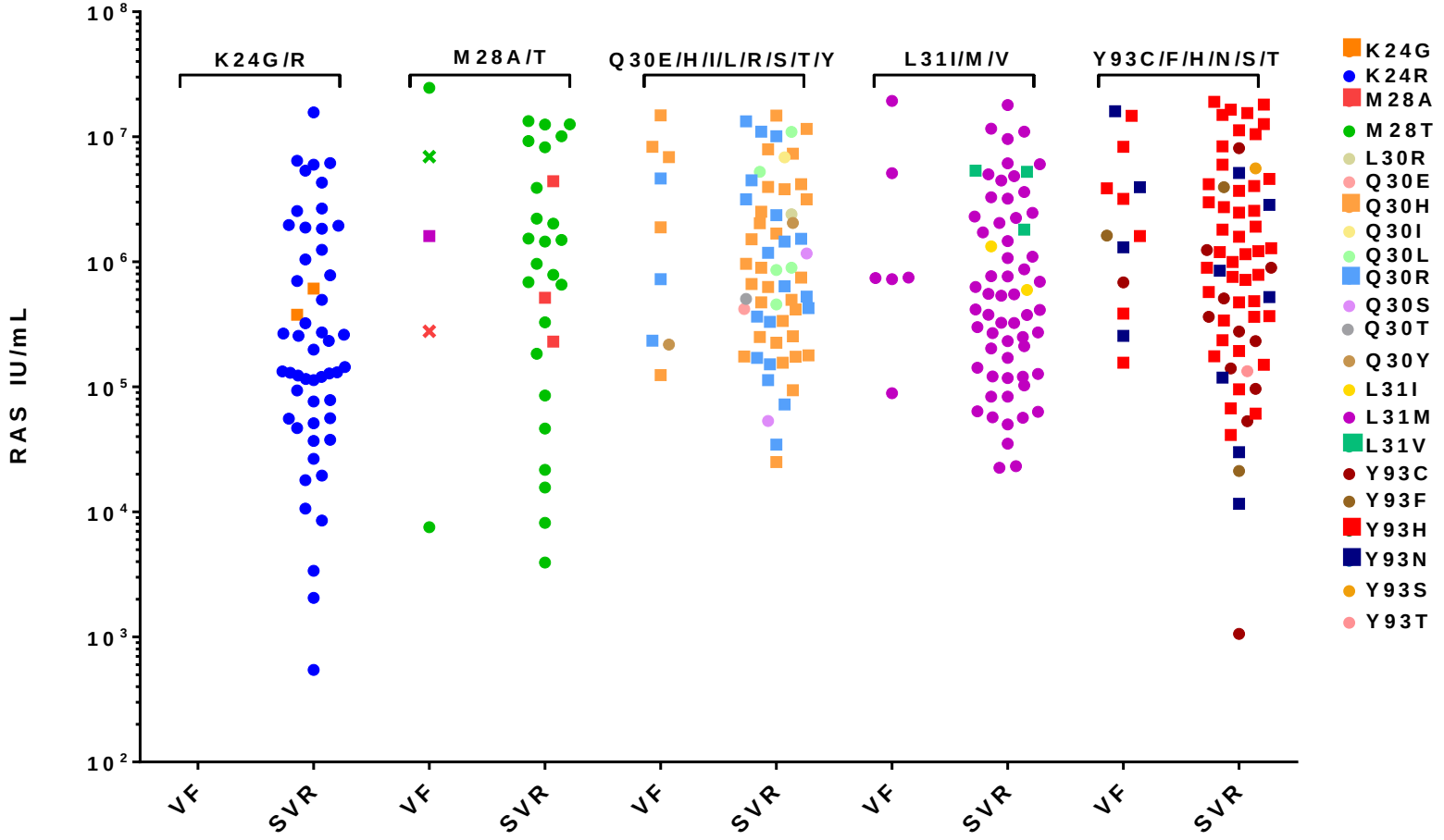

Number of Patients with RASs (%)
20
15
10
5
0
Percent of GT 1a Patients in US (n=1238)
Percent GT 1a Patients in EU (n=206)
Total
K24R
L31M
Q30H
Y93H
M28T
Q30R
Y93N
Y93C
M28A
H58D
Q30L
Y93F
Q30S
Q30T
Q30Y
K24G
L31I
L31V
Y93S
Y93T
Q30E
Q30I


Number of Patients with RASs (%)
20
15
10
5
0
Percent of GT 1b Patients in US (n=356)
Percent GT 1b Patients in 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

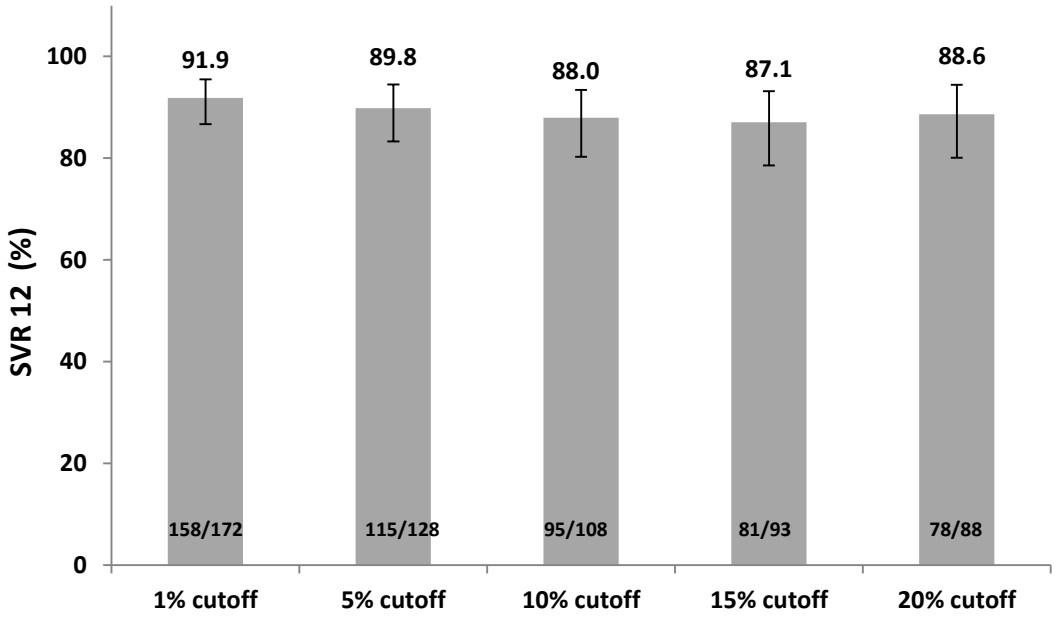

1% cutoff
15% cutoff
p-value = 1.0
p-value = 1.0
p-value = 0.69
p-value = 1.0
p-value = 0.7
p-value = 1.0
p-value = 1.0
p-value = 0.6
SVR 12 (%)
100
80
60
40
20
0
100 100
87.5 90.0
84.6 80.0
86.7 83.3
87.9 82.6
80.0 75.0
100 100
93.3 88.2
35/35 9/9
14/16 9/10
22/26 12/15
13/15 5/6
29/33 19/23
12/15 6/8
9/9 9/9
31/33 15/17
K24R
M28T
Q30H
Q30R
L31M
Y93H
L31M
Y93H
GT 1a
GT 1b
3.7-fold
61-fold
>100-fold
>100-fold
>100-fold
>1000-fold
3.4-fold
>1000-fold



Prevalence:	**84.2%**	**12.6%**	**2.3%**	**0.9%**


RAS IU/mL
10 8
10 7
10 6
10 5
10 4
10 3
10 2
K24G/R
M28A/T
Q30E/H/I/L/R/S/T/Y
L31I/M/V
Y93C/F/H/N/S/T
VF
SVR
VF
SVR
VF
SVR
VF
SVR
VF
SVR
K24G
K24R
M28A
M28T
L30R
Q30E
Q30H
Q30I
Q30L
Q30R
Q30S
Q30T
Q30Y
L31I
L31M
L31V
Y93C
Y93F
Y93H
Y93N
Y93S
Y93T

Supplementary Figure 1. SVR12 for Patients Treated with Ledipasvir/Sofosbuvir with NS5A RASs by Deep Sequencing Cutoffs. Substitution analyses were conducted on deep sequencing data (population sequences were not included).


SVR 12 (%)
100
80
60
40
20
0
91.9
89.8
88.0
87.1
88.6
158/172
115/128
95/108
81/93
78/88
1% cutoff
5% cutoff
10% cutoff
15% cutoff
20% cutoff