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NS5A resistance-associated substitutions in patients with genotype 1 hepatitis C virus: Prevalence and effect on treatment outcome

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Background & Aims: The efficacy of NS5A inhibitors for the treatment of patients chronically infected with hepatitis C virus (HCV) can be affected by the presence of NS5A resistance-associated substitutions (RASs). We analyzed data from 35 phase I, II, and III studies in 22 countries to determine the pretreatment prevalence of various NS5A RASs, and their effect on outcomes of treatment with ledipasvir-sofosbuvir in patients with genotype 1 HCV.

Methods: NS5A gene deep sequencing analysis was performed on samples from 5397 patients in Gilead clinical trials. The effect of baseline RASs on sustained virologic response (SVR) rates was assessed in the 1765 patients treated with regimens containing ledipasvir-sofosbuvir.

Results: Using a 15% cut-off, pretreatment NS5A and ledipasvir-specific RASs were detected in 13% and 8% of genotype 1a patients, respectively, and in 18% and 16% of patients with genotype 1b. Among genotype 1a treatment-naïve patients, SVR rates were 91% (42/46) *vs.* 99% (539/546) for those with and without ledipasvir-specific RASs, respectively. Among treatment-experienced genotype 1a patients, SVR rates were 76% (22/29) *vs.* 97% (409/420) for those with and without ledipasvir-specific RASs, respectively. Among treatment-naïve genotype 1b patients, SVR rates were 99% for both those with and without ledipasvir-specific RASs (71/72 *vs.* 331/334), and among treatment-experienced genotype 1b patients, SVR rates were 89% (41/46) *vs.* 98% (267/272) for those with and without ledipasvir-specific RASs, respectively.

Conclusions: Pretreatment ledipasvir-specific RASs that were present in 8–16% of patients have an impact on treatment outcome in some patient groups, particularly treatment-experienced patients with genotype 1a HCV.

Lay summary: The efficacy of treatments using NS5A inhibitors for patients with chronic hepatitis C virus (HCV) infection can be affected by the presence of NS5A resistance-associated substitutions (RASs). We reviewed results from 35 clinical trials where patients with genotype 1 HCV infection received treatments that included ledipasvir-sofosbuvir to determine how prevalent NS5A RASs are in patients at baseline, and found that ledipasvir-specific RASs were present in 8–16% of patients prior to treatment and had a negative impact on treatment outcome in subset of patient groups, particularly treatment-experienced patients with genotype 1a HCV.

Introduction

Due to high rates of viral replication and an error prone hepatitis C virus (HCV) RNA polymerase, tremendous variability of HCV has been observed within infected patients (quasispecies). These single mutations that do not abolish viral replication, are thought to be pre-existing [1] and as a result, NS5A resistance-associated substitutions (RASs) are observed at baseline in patients infected with chronic HCV. Deep sequencing enables detection of HCV substitutions, point deletions, or insertions within the quasispecies down to a frequency of 1%. However, commercially available assays based on standard population HCV sequencing or not cross-validated next generation, also called deep sequencing, report variants with a frequency of $\geqslant$15% of the quasispecies.

The prevalence of baseline NS5A RASs has been reported to be 6% to 16% using population sequencing (cut-off 15–25%) or deep sequencing (cut-off 1%) [2–4]. Interestingly, the prevalence and type of baseline NS5A RASs may vary by geographic regions. For example, the prevalence of the NS5A M28V in genotype 1a-infected patients was shown to be higher in the United States than in Europe, 7% *vs.* 0%, respectively [5]. Furthermore, the prevalence of genotype 3 NS5A Y93H varied between 0% and

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17% in different geographic regions [6]. A comparison of baseline prevalence of RASs in Japanese and Western patients showed that the prevalence of Q80L and S122G in NS3, and L28M, R30Q and Y93H in NS5A was significantly higher in Japanese patients than the Western counterparts [7].

Many currently approved interferon (IFN)-free regimens for the treatment of chronic hepatitis C (HCV) include an inhibitor of HCV NS5A. To date, there are five NS5A inhibitors approved for treatment of chronic HCV infection; ledipasvir (LDV), daclatasvir, and velpatasvir (which are all administered with the NS5B inhibitor sofosbuvir [SOF]), and ombitasvir (in a fixed-dose combination with the protease inhibitor paritaprevir, the nonnucleoside NS5B polymerase inhibitor dasabuvir, and ritonavir, a potent inhibitor of CYP3A4 enzymes), and elbasvir (in a fixed-dose combination with the protease inhibitor grazoprevir) [8–12]. The presence of baseline NS5A RASs may impact the treatment outcome of some NS5A inhibitor containing HCV regimens due to the intrinsic qualities of the NS5A inhibitor, drug pharmacology, or effects of the other compounds within the treatment regimen. However, depending on how NS5A RASs are defined and included in resistance analysis, as well as what level of variant detection is utilized, different results may be obtained. To date, three definitions of NS5A variants that are associated with resistance have been used most commonly; polymorphisms at RAS positions (RAPs), class RASs, and drug-specific RASs. Polymorphisms at RAS positions are defined as any change from reference sequence for a specific genotype at positions associated with NS5A inhibitor resistance. NS5A class RASs are substitutions that have been shown to emerge on treatment or confer a significant reduction in susceptibility *in vitro* (e.g., >2.5-fold change in EC_{50}) to any approved or investigational NS5A inhibitor. Drug-specific RASs refer to substitutions that have been shown to emerge on the specific drug treatment or confer significantly reduced susceptibility *in vitro* to the specific NS5A inhibitor. In addition, drug-specific RASs can be categorized into groups with different levels of reduced susceptibility to the drug.

To enable comparisons of resistance analyses between clinical trials, standardization of RAS definitions and sensitivity cut-offs are needed. In several studies, population sequences were used for resistance analysis (cut-off for variant detection 15–25%) and NS5A polymorphisms at RAS positions were defined as RAPs. In these studies, the presence of baseline NS5A polymorphisms at RAS positions had shown no significant impact on treatment outcome [5,13]. Further study is needed to understand the role of RASs present at frequencies below 15% and whether substitutions without an *in vitro* susceptibility change to the NS5A inhibitor may dilute a clinical signal by RASs that do confer reduced susceptibility to a specific NS5A inhibitor.

Here, we characterize the prevalence of baseline NS5A RASs in 5397 NS5A inhibitor-naïve HCV patients infected with genotype 1a or 1b according to geographic regions. Moreover, we assessed the effect of baseline NS5A RASs, defined as NS5A RAPs, NS5A class RASs or LDV-specific RASs using 1% and 15% sensitivity of substitution detection cut-offs, on treatment outcome among 1765 patients treated with currently recommended regimens containing LDV-SOF. A previous analysis using a portion of the same dataset has recently been published [14]. That analysis concerned the prevalence and effect on treatment of NS3, NS5A, and NS5B RASs, and included data on patients who had been treated with regimens/durations that have not been incorporated into label recommendations or treatment guidelines. The current study covers only NS5A RASs and includes data only from patients who received guideline-recommended regimens.

Materials and methods

Sequencing analysis

Deep sequencing of baseline plasma samples was performed in 5397 patients from 22 countries across the HCV Gilead clinical development program from 2010 to 2015. The list of clinical trials and identification numbers are included in the supplementary materials (Supplementary Table 1). The HCV NS5A coding regions were amplified by DDL Diagnostic Laboratory (Rijswijk, Netherlands) using proprietary amplification primers and standard reverse transcription polymerase chain reaction (RT-PCR) technology, if a plasma sample was available and baseline HCV RNA was >1000 IU/ml. Deep sequencing using MiSeq platform (Illumina, Inc., San Diego, CA) was performed by WuXi AppTec (Shanghai, China) or DDL Diagnostic Laboratory (Rijswijk, Netherlands). Deep sequencing data was split into one file per sample using only 100% matched barcodes to bin the reads. Sequence analysis was performed using internally developed software in a stepwise fashion. Briefly, raw reads from the FASTQ files were trimmed and filtered based on quality scores and read length. Trimming was carried out on reads when quality score decreased below 15, and reads shorter than 50 nucleotides were removed. Deep sequencing data was aligned using MOSAIK v1.1.0017. All aligned reads were then translated in-frame and changes from a reference sequence were determined. Assay sensitivity and assay background cut-offs were evaluated based on plasmid and RNA controls. There are no standardized HCV deep sequencing assays available as commercialized kits, therefore cross-validation of deep sequencing data from DDL and WuXi was performed on a subset of control samples.

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.

Definition of NS5A polymorphisms at RAS positions and resistance-associated substitutions

NS5A RAPs were defined as any change from genotype 1a or 1b reference strains (1a-H77 or 1b-Con1) at NS5A positions associated with NS5A drug resistance. NS5A class RASs were summarized by the HCV Drug Development Advisory Group group [15], and/or recently observed in clinical trials with LDV, velpatasvir, daclatasvir, pibrentasvir, and elbasvir [16–27], specifically variants at NS5A positions 24, 28, 30, 31, 32, 38, 58, 92, 93 that confer >2.5-fold reduced susceptibility to any NS5A inhibitor. LDV-specific RASs were classified as variants at NS5A positions 24, 28, 30, 31, 32, 38, 58, 92, 93 that confer >2.5–100 or >100-fold reduced susceptibility to LDV *in vitro* or were selected in clinical trials in patients treated with LDV-containing regimens [2,26,27] (Table 1).

Assessment of sustained virologic response in patients with and without pretreatment NS5A inhibitor RASs

SVR12 rates were assessed only in the 1765 patients who were treated with currently recommended regimens containing LDV-SOF (according to AASLD/IDSA and EASL guidelines) in 15 phase II and phase III Gilead-sponsored clinical trials (Supplementary Table 2). Only patients who were not previously exposed to NS5A inhibitors were included in these analyses. Patients were excluded from these analyses if they did not achieve SVR due to non-virologic failure (e.g., lost to follow up). The results were analyzed according to the 1% and 15% detection cut-offs of NS5A RAPs, class RASs or LDV-specific RASs.

Results

Patient baseline characteristics

Demographic and baseline clinical characteristics of the 5393 NS5A inhibitor-naïve patients included in the NS5A baseline prevalence RAS analysis are provided in Table 2. The majority

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Table 1. List of NS5A class RASs and ledipasvir RASs.

Genotype	Reference AA NS5A position	NS5A class RASs	Ledipasvir RASs	
		Substitutions that confer >2.5-fold change in EC_{50} to any NS5A inhibitor	Substitutions that confer >2.5–100-fold change in EC_{50} to ledipasvir (FC)	Substitutions that confer >100-fold change in EC_{50} to ledipasvir (FC)
1a	K24	G/N/R	G (43), N (28), R (4)	–
	M28	A/G/T/V	T (61)	A/G (>1000)
	Q30	C/E/G/H/I/K/L/R/S/T/Y	L (4), T (4)	E/G/H/K (>1000), R (632)
	L31	F/I/M/V	F (60)	I (370), M (554), V (683)
	P32	L	–	L (348)
	S38	F	F (54)	–
	H58	D/L	–	D (>1000)
	A92	K/T	T (15)	K (>1000)
	Y93	C/F/H/L/N/R/S/T/W	F (7)	C/H/N/S (>1000)
1b	Q24	-	-	–
	(L28)	M		–
	R30	-	-	–
	L31	F/I/M/V	F (8), I (29), M (3), V (43)	–
	P32	L	L (8)	–
	S38	-	-	–
	P58	D	–	D (238)
	A92	K	–	K (>1000)
	Y93	C/H/N/S	C (5)	H (>1000), N (110), S (142)

FC, fold change.

of patients were treatment-naïve (56%) and male (64%), with HCV genotype 1a (65%) and non-CC interleukin (IL) 28B alleles (73%). Approximately one-third (32%) of patients had cirrhosis.

Prevalence and type of pretreatment RASs across geographic regions

Baseline prevalence of NS5A polymorphisms at RAS positions, NS5A class RASs, LDV RASs and the specific Y93H NS5A variant was evaluated in genotype 1a (n = 3501) and 1b (n = 1887) patients using 1% through 50% sensitivity cut-offs (Fig. 1). Higher prevalence of all categories for NS5A RASs was observed at 1% sensitivity cut-offs and sharply declined with reduction in sensitivity of variant detection to 15%. No significant changes in NS5A RASs prevalence was observed with further reductions in assay sensitivity from 15% to 50%.

The prevalence of NS5A polymorphisms at RAS positions was significantly higher as compared to NS5A class RASs in both genotype 1a and 1b across all sensitivity cut-offs. The prevalence of NS5A class RASs was about 5% higher than that of LDV RASs in genotype 1a. This difference was mostly represented by

Table 2. Patient demographics and baseline characteristics.

	N. America (n = 3437)	Europe (n = 972)	Oceania (n = 387)	Asia Pacific (n = 597)	Total (n = 5393)
Median age, yr (range)	56 (18–81)	54 (18–80)	56 (22–74)	57 (20–80)	56 (18–81)
Male, n (%)	2322 (68)	610 (63)	272 (70)	268 (45)	3472 (64)
Race, n (%)					
White	2571 (80)	945 (97)	329 (85)	27 (5)	4052 (75)
Black	579 (17)	14 (1)	0	0	593 (11)
Asian	44 (1)	10 (1)	24 (6)	570 (96)	648 (12)
Other	34 (1)	3 (<1)	14 (4)	0	51 (<1)
Median BMI, kg/m^2 (range)	27 (17–66)	25 (17–57)	27 (18–57)	24 (16–42)	27 (16–66)
Genotype, n (%)					
1a	2635 (77)	531 (55)	314 (81)	27 (5)	3507 (65)
1b	802 (23)	441 (45)	73 (19)	570 (95)	1886 (35)
Median HCV RNA, log_{10} IU/ml (range)	6.5 (1.4–8.0)	6.4 (3.2–8.0)	6.4 (1.9–7.7)	6.7 (3.7–7.6)	6.5 (1.4–8.0)
Prior HCV treatment, n (%)					
Treatment-naïve	1961 (57)	559 (58)	184 (48)	332 (56)	3036 (56)
Non-responder	756 (22)	217 (22)	105 (27)	86 (14)	1164 (22)
Relapse/breakthrough	659 (19)	180 (19)	97 (25)	144 (24)	1080 (20)
Other	61 (2)	16 (2)	1 (<1)	35 (6)	113 (2)
IL28B, n (%)*					
CC	790 (23)	215 (22)	130 (34)	324 (54)	1459 (27)
CT	1922 (56)	568 (59)	197 (51)	247 (41)	2934 (55)
TT	697 (20)	187 (19)	59 (15)	26 (4)	969 (18)
Cirrhosis	1002 (29)	410 (42)	184 (48)	127 (21)	1723 (32)
Median ALT (range), U/L	60 (9–578)	61 (7–420)	66 (12–494)	50 (11–619)	60 (7–619)

* *IL28B* genotype was determined by sequencing of the rs12979860 single-nucleotide polymorphism.

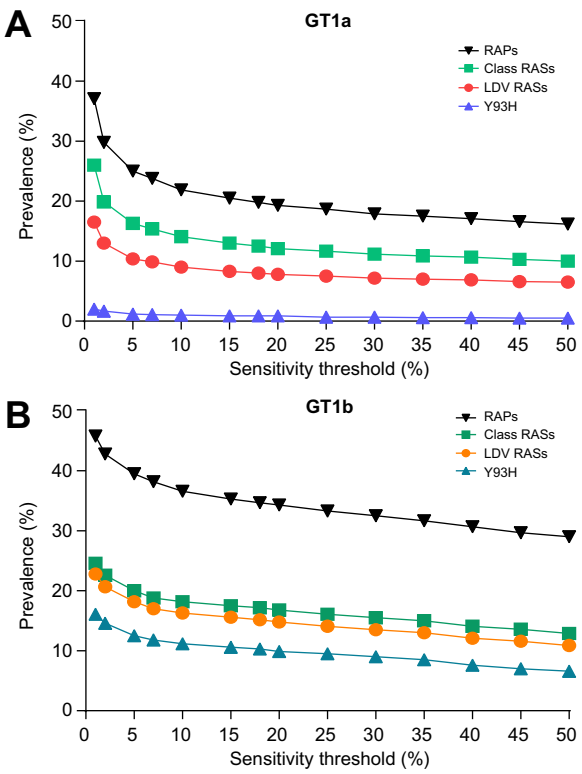

Fig. 1. Prevalence of RASs according to sensitivity threshold. The figures show the prevalence of polymorphisms at RAS positions (RAPs), NS5A class RASs, ledipasvir-specific RASs, and the Y93H RAS by sensitivity threshold. (A) Prevalence in patients with genotype 1a HCV. (B) Prevalence in patients with genotype 1b HCV.

prevalence of the M28V NS5A class RAS which is not an LDV RAS. There was little difference between NS5A class and LDV RASs in genotype 1b. Prevalence of Y93H was higher in genotype 1b as compared to genotype 1a across all assay cut-offs. Based on the observation of a sharp decline in prevalence from 1% to 15%, further analyses were performed with both 1% and 15% cut-offs.

Overall at the 15% assay cut-off, pretreatment NS5A class RASs were detected in 13.0% of genotype 1a patients (Table 3). The prevalence of NS5A class RASs overall in patients with genotype 1a HCV did not differ significantly across most of the geographic regions with the frequency ranging from 12.1% to 15.6%, but the prevalence of LDV RASs was significantly higher among patients in Oceania, than among those from other regions combined (12.7% *vs.* 7.9%, $p = 0.005$). The overall prevalence of baseline LDV RASs in genotype 1a patients was 8.3% with some numeric differences between different regions, the highest in Oceania (12.7%) and the lowest in Europe (7.7%). Specific RASs were detected at a similar frequency in genotype 1a patients across geographic regions, including K24R, M28V/T, Q30H/R, L31M and Y93H.

The overall prevalence of baseline NS5A class RASs was slightly higher (17.6%) in patients infected with genotype 1b than in those infected with genotype 1a (Table 3). The frequency of detection of NS5A class RASs ranged from 16.1% to 20.4% in genotype 1b patients with only minor numeric differences across geographic regions. The prevalence of baseline LDV RASs among genotype 1b patients was also similar across different regions (15.2–16.4%). Y93H was detected at a much higher frequency (10.6%) than other RASs, including L28M and L31I/M/V among genotype 1b patients, but differences in the prevalence of each RAS between regions were small. In both subtypes, the prevalence of multiple ($\geqslant 2$) RASs was low; ranging from 0 to 3.8% in genotype 1a, and less than 1.5% in genotype 1b.

Assessment of the effect of baseline RASs on treatment outcome with LDV-SOF by RAS categories and sensitivity cut-offs

To evaluate the effect of baseline RASs on treatment outcome, SVR12 rates were assessed in 1765 patients from 15 LDV-SOF clinical trials who were treated with currently recommended

Table 3. Prevalence of NS5A RASs in patients naïve to treatment with NS5A inhibitors by region (15% cut off).

Genotype	RAS	N. America	Europe	Oceania	Asia Pacific	Overall
1a	**N**	**2635**	**531**	**314**	**27**[†]	**3507**
	K24R	None	1.5%	1.6%	ND	1.1%
	M28T	None	1.1%	2.5%	ND	1.1%
	M28V	5.9%	4.7%	4.1%	ND	5.4%
	Q30H	1.8%	None	2.2%	ND	1.7%
	Q30R	None	1.7%	2.2%	ND	1.1%
	L31M	None	2.2%	4.1%	ND	2.3%
	Y93H	1.0%	None	None	ND	None
	Any LDV RASs	7.9%	7.7%	12.7%	ND	8.3%
	Any NS5A RASs	12.9%	12.1%	15.6%	ND	13.0%
1b	**N**	**802**	**441**	**73**	**570**	**1886**
	L28M	None	1.6%	None	5.4%	2.4%
	L31M	5.9%	4.8%	2.3%	2.1%	4.3%
	L31I	None	None	5.5%	None	None
	Y93H	9.4%	10.2%	9.6%	12.8%	10.6%
	Any LDV RASs	15.5%	15.2%	16.4%	16.0%	15.6%
	Any NS5A RASs	16.1%	16.8%	16.4%	15.6%	17.6%

N. America included: USA, Canada and Puerto Rico; Europe included: Austria, Belgium, Switzerland, Czech Republic, Germany, Spain, France, United Kingdom, Italy, Netherlands and Poland; Asia Pacific included: China, India, Japan, Korea, Russia and Taiwan; Oceania included: Australia and New Zealand. Only variants with prevalence >1% are listed. No LDV-specific RASs were observed at NS5A positions (26), 32, 38, 58, and 92 with prevalence >1%.

[†] The number of patients in the Asia Pacific region with genotype 1a HCV was too small to be the basis for prevalence estimates.

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regimens according to the 2015 AASLD/IDSA and EASL guidelines. The baseline characteristics of this population are given in Table 4. A systematic comparison of the effect on SVR12 rates was performed in genotype 1a and 1b treatment-naïve and treatment-experienced patients for NS5A RAPs, class RASs, and LDV RASs, and LDV RASs with >100-fold change, using a 15% sequencing assay cut-off (Fig. 2).

In treatment-naïve patients with genotype 1b HCV infection, the presence of baseline NS5A polymorphisms at RAS positions or NS5A class RASs did not impact the treatment outcome with LDV-SOF regimens with SVR12 rates of 98–99% in every group. The SVR12 rate in genotype 1a patients with baseline LDV RASs was 94% and 91% (1% and 15% cut-offs, respectively) compared to 99% in patients without LDV RASs (Table 5). The presence of baseline LDV RASs in treatment-naïve genotype 1b patients had no impact on SVR12 rates.

LDV RASs had the most notable impact on SVR12 rates in treatment-experienced patients (76–80% *vs.* 97–98% and 89–91% *vs.* 98% in genotype 1a and 1b, respectively). Even though similar results were obtained when 1% and 15% sensitivity assay cut-offs were used, SVR12 rates were slightly lower when 15% assay cut-off was used.

Taken together, the comparison of the different categories of NS5A RASs and assay cut-offs, LDV RASs detected with a 15% assay cut-off was identified as the most discriminating for LDV-SOF regimen baseline analyses and this cut-off was used to perform further subgroup evaluations.

Effect of baseline LDV RASs on treatment outcome by patient population

We calculated SVR12 rates by treatment history and cirrhosis status according to baseline LDV RASs using a 15% assay cut-off for HCV genotype 1a and genotype 1b (Fig. 3) infected patients. The SVR12 rate in treatment-naïve non-cirrhotic patients was not substantially impacted by the presence of LDV RASs at baseline (92% SVR). Numerically lower SVR12 rates (86%) were observed in treatment-naïve cirrhotic genotype 1a patients with baseline LDV RASs but the small number of patients (n = 7) limits the interpretability of this finding. Of genotype 1a patients, prior exposure to HCV treatment appeared to impact the SVR12 rates in both non-cirrhotic and cirrhotic groups (75% and 77% respectively) in the presence of baseline LDV RASs, but the number of patients in these groups was also small (<20). Among genotype 1b patients, the SVR12 rates remained >90% across the groups regardless of treatment history and presence of cirrhosis with or without baseline LDV RASs, except for the treatment-experienced non-cirrhotic group which showed an SVR rate of 87%, but it only included 23 patients. The number of patients with multiple RASs was small (N <30, <1%) for both LDV-specific and NS5A class RASs in genotype 1a and 1b. The overall SVR rates were 64% (9/14) in genotype 1a and 100% (2/2) in genotype 1b patients with multiple LDV RASs (Supplementary Table 3). Of those with multiple NS5A class RASs, the SVR rates were 74% (17/23) and 83% (5/6) among genotype 1a and 1b, respectively (Supplementary Table 3).

Among patients with cirrhosis, there were too few patients with baseline RASs to further assess the impact of treatment duration and/or the addition of ribavirin on treatment outcome (Supplementary Table 4).

Discussion

Current NS5A inhibitors show overlapping but distinct resistance profiles with RASs described at the NS5A amino acid positions 24, 28, 30, 32, 31, 38, 58, 92, and 93. There are advantages and disadvantages with each of the three main approaches to defining NS5A RASs. The advantage of using the NS5A RAPs definition is that it provides a uniform list of variants for all NS5A inhibitors. It does not require extensive phenotypic testing of all variants with several NS5A inhibitors and provides inclusive assessment

Table 4. Patient demographics and baseline characteristics.

	N. America (n = 1103)	Europe (n = 264)	Oceania (n = 67)	Asia Pacific (n = 331)	Total (n = 1765)
Mean age, yr (range)	53 (22–78)	55 (18–77)	55 (40–72)	57 (20–80)	54 (18–80)
Male, n (%)	795 (72)	165 (63)	50 (75)	140 (42)	1150 (65)
Race, n (%)					
White	817 (74)	258 (98)	50 (75)	0	1125 (64)
Black	251 (23)	5 (2)	0	0	256 (15)
Asian	15 (1)	1 (<1)	6 (9)	331 (100)	353 (20)
Other	20 (2)	0	11 (16)	0	31 (1)
Mean BMI, kg/m^2 (range)	28 (18–66)	25 (18–40)	29 (18–50)	24 (17–38)	27 (17–66)
Genotype, n (%)					
1a	829 (75)	139 (53)	51 (76)	17 (5)	1036 (59)
1b	271 (25)	124 (47)	16 (24)	313 (95)	724 (41)
1 (no confirmed subtype)	3 (<1)	1 (<1)	0	1 (<1)	5 (<1)
Mean HCV RNA, $\log_{10}$ IU/ml (range)	6.4 (1.4–7.8)	6.4 (3.7–7.5)	6.3 (4.9–7.7)	6.6 (3.7–7.6)	6.4 (1.4–7.8)
Treatment-naïve	682 (62)	135 (51)	28 (42)	178 (54)	1023 (58)
Treatment-experienced	421 (38)	129 (49)	39 (58)	153 (46)	742 (42)
IL28B, n (%)*					
CC	239 (22)	41 (16)	24 (36)	203 (61)	507 (29)
CT	626 (57)	166 (63)	27 (41)	119 (36)	938 (53)
TT	238 (22)	57 (22)	15 (23)	9 (3)	319 (18)
Cirrhosis	263 (24)	175 (66)	45 (67)	56 (17)	539 (31)
Mean ALT (range), U/L	75 (9–557)	82 (13–344)	100 (27–494)	66 (11–619)	75 (9–619)

* *IL28B* genotype was determined by sequencing of the rs12979860 single-nucleotide polymorphism.

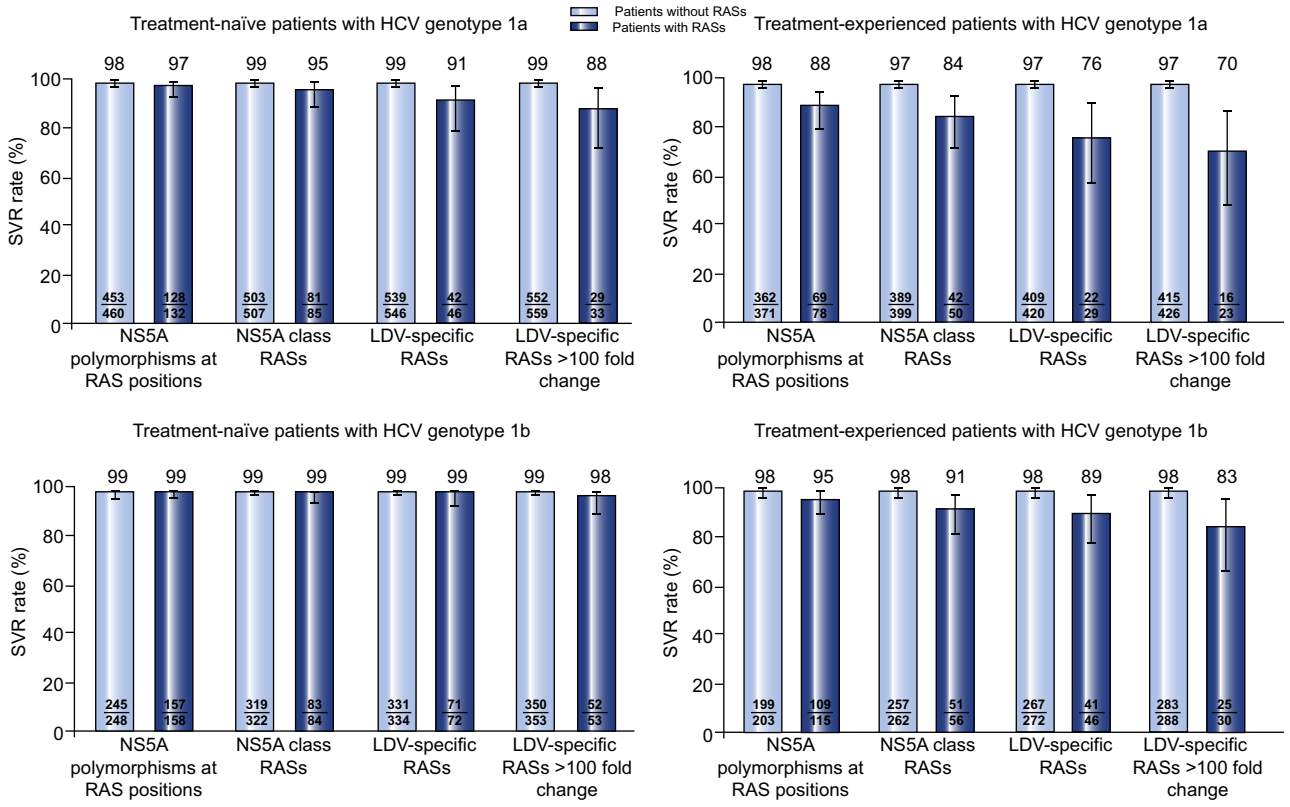

Fig. 2. SVR rates in patients with and without NS5A RASs. The rates of SVR12 by presence at baseline of NS5A polymorphisms at RAS positions (RAPs), NS5A class RASs, and LDV-specific RASs, and LDV-specific RASs that confer >100-fold change at a 15% sensitivity threshold.

Table 5. SVR12 rates in patients with and without LDV RASs using various sensitivity thresholds.

Genotype	Cut-off	Treatment-naïve		Treatment-experienced	
		With LDV RASs	No LDV RASs	With LDV RASs	No LDV RASs
1a	1%	94% (84/89)	99% (497/503)	80% (44/55)	98% (387/394)
	2%	93% (68/73)	99% (513/519)	78% (35/45)	98% (396/404)
	5%	92% (57/62)	99% (524/530)	77% (27/35)	98% (404/414)
	7%	92% (55/60)	99% (526/532)	77% (27/35)	98% (404/414)
	10%	90% (46/51)	99% (535/541)	76% (22/29)	97% (409/420)
	15%	91% (42/46)	99% (539/546)	76% (22/29)	97% (409/420)
	25%	93% (38/41)	99% (543/551)	77% (20/26)	97% (411/423)
	50%	94% (34/36)	98% (547/556)	76% (19/25)	97% (412/424)
1b	1%	99% (102/103)	99% (300/303)	91% (63/69)	98% (245/249)
	2%	99% (97/98)	99% (305/308)	90% (57/63)	98% (251/255)
	5%	99% (85/86)	99% (317/320)	88% (46/52)	98% (262/266)
	7%	99% (78/79)	99% (324/327)	88% (42/48)	99% (266/270)
	10%	99% (75/76)	99% (327/330)	87% (41/47)	99% (267/271)
	15%	99% (71/72)	99% (331/334)	89% (41/46)	98% (267/272)
	25%	98% (62/63)	99% (340/343)	88% (36/41)	98% (272/277)
	50%	98% (48/49)	99% (354/357)	85% (28/33)	98% (280/285)

of variants that developed in patients treated with NS5A inhibitors. However, for baseline analyses that investigate the role of pre-existing variants on treatment outcome, substitutions that are fully susceptible to a specific NS5A inhibitor dilute the investigated effect. To characterize NS5A class RASs, i.e., those that show reduced susceptibility to one or more NS5A inhibitors *in vitro*, standardized phenotypic testing is needed for each NS5A inhibitor. Even though the NS5A class RAS definition would exclude variants that are known to be sensitive to NS5A inhibitors and thus provide a more sensitive analysis of the effect of baseline RASs on SVR, some attenuation of the signal may still be observed due to different resistance profiles among the NS5A inhibitors. With further optimization of NS5A inhibitors to improve resistance profiles, the list of NS5A variants and positions that confer reduced susceptibility to the next generation drugs is shortening. Using drug-specific RASs is the most scientifically rigorous way to perform efficacy and baseline resistance analyses. However, extensive standardized phenotypic testing is needed to accurately define drug-specific RASs. Additionally, novel resistance substitutions that develop rarely *in vivo* may

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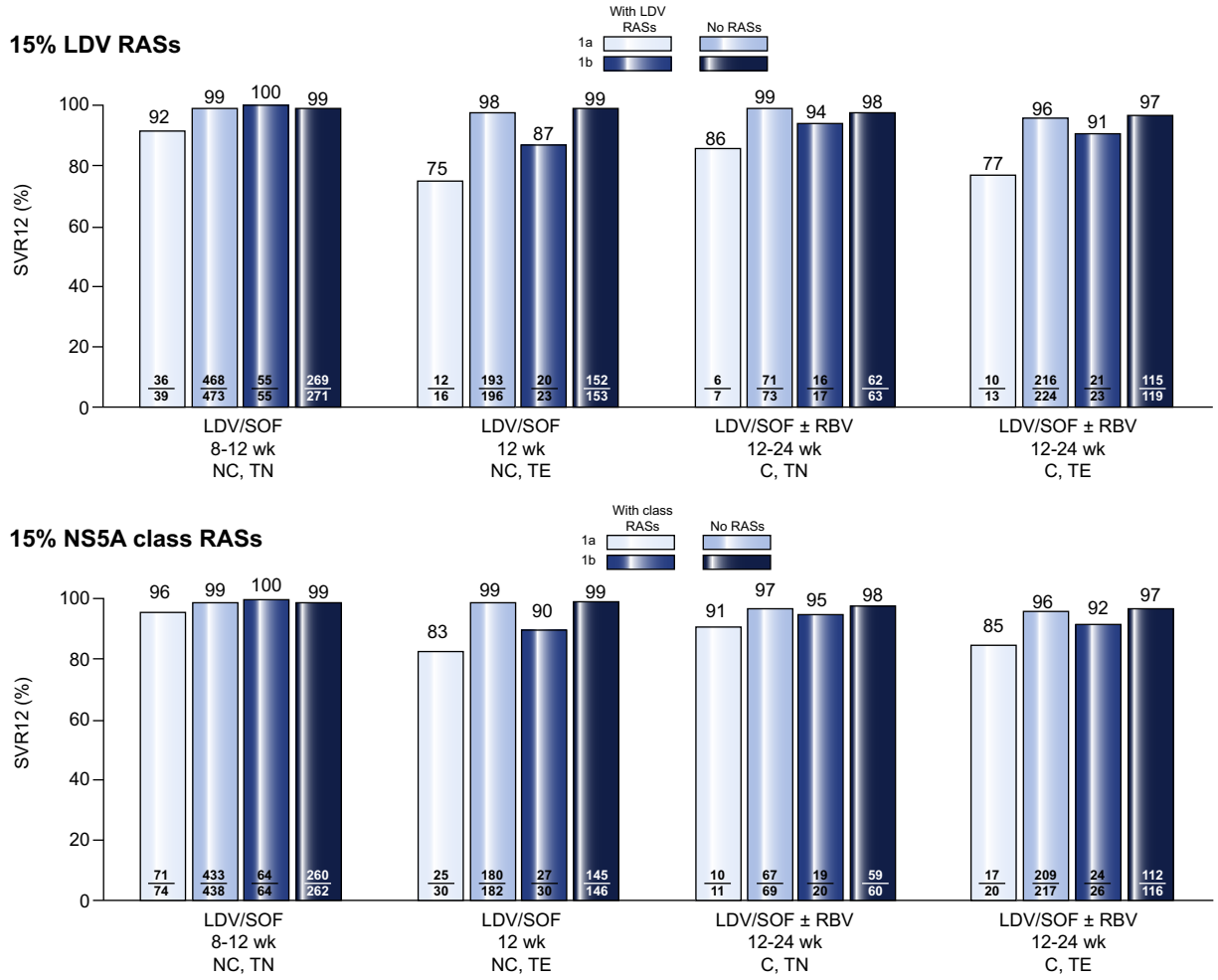

Fig. 3. SVR12 rates by treatment history and cirrhosis status. The rates of SVR12 by regimen, treatment history (naïve *vs.* previously treated), and cirrhosis status (present *vs.* absent). NC, non-cirrhotic; C, cirrhotic; TE, treatment-experienced; TN, treatment-naïve.

be missed during resistance monitoring and it may be difficult to compare results to those from other studies since drug-specific RASs will be different between various NS5A inhibitors. Another disadvantage of using drug-specific RASs is that this definition fails to capture relevant information regarding the response in patients with resistance to other NS5A inhibitors.

The results presented here show that analysis of LDV drug-specific RASs have more impact on LDV-SOF treatment outcomes overall as compared to the analysis of RAPs or class RASs, as would be predicted based on these RASs having demonstrated reductions in susceptibility to LDV. However, the presence of drug-specific RASs may affect SVR12 rates to a greater or lesser extent depending on the specific pharmacology of an inhibitor and the drug combination regimen being utilized for treatment. For example, previous analyses of LDV-SOF clinical trials have shown that only LDV-specific RASs contributing >100-fold reduction in susceptibility result in lower SVR rates with LDV-SOF regimens [2,14].

As multiple options for HCV treatment containing NS5A inhibitors have become available and more broadly applicable, understanding the prevalence of baseline NS5A RASs in specific regions has become more important. In this comprehensive analysis using >5000 patient samples from 21 countries in four continents, it is shown that the prevalence of both NS5A class and LDV RASs does not differ significantly across regions for both genotype 1a and 1b. Numerically lower prevalence of NS5A RASs is observed for genotype 1a in Asia Pacific, but there were small numbers of genotype 1a patients included from this region (n = 27) for epidemiological reasons. The prevalence of specific NS5A class and LDV RASs is also similar across regions for both genotypes 1a and 1b. For genotype 1b, the prevalence of Y93H was the highest in Asia Pacific whereas the prevalence of L31M/I/V was the lowest in this region. It must be noted, however, that large regions of the world – including much of Asia, and all of Africa, South America and the Caribbean – are not represented in this analysis.

The rates of SVR among patients without pretreatment LDV RASs at all detections thresholds were high regardless of subtype and treatment history, ranging from 97% to 99%. The greatest impact of LDV RASs on SVR was among treatment-experienced patients with genotype 1a HCV, who had an SVR rate of 76% (at the 15% cut-off). This difference was approximately the same at all detection thresholds. Among treatment-naïve patients with genotype 1b HCV, pretreatment LDV RASs appeared to have little

to no impact on SVR, with rates ranging from 98–99% for all detection thresholds. Treatment-naïve patients with genotype 1a HCV and treatment-experienced patients with genotype 1b HCV fell somewhere in between, with differences of 4% to 10% between those with and without LDV RASs.

The clinical interpretation of these findings remains challenging. The decision to perform pretreatment RAS testing may be made based on the magnitude of the effect of these RASs on treatment outcome. The effect of NS5A or LDV-specific RASs on treatment outcome was greatest in treatment-experienced patients and/or those with cirrhosis, groups that are at highest risk of disease progression. An argument in favor of pretreatment RAS testing could thus be made, with the decision to possibly extend treatment duration and/or add ribavirin for those with LDV-specific RASs. However, it should be noted that the number of patients within these subgroups was small ($\leqslant$23 patients) and these data may not be generalizable to the broader population.

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Conflict of interest

Stefan Zeuzem: Consultant: Abbvie, BMS, Gilead, Janssen, and Merck; Speaker: Abbvie, BMS, Gilead, Janssen, and Merck. Masashi Mizokami: Research: Asahi Kasei Pharma Corporation, Mitsubishi Tanabe Pharma Corp, G&G Science Co, SRL Technical Services; Speaker: Abbott, Astellas, Bristol-Myers Squibb, Chugai Pharmaceutical, Daiichi Sankyo, Dainippon Sumitomo, Eisai, G&G, GlaxoSmithKline, Minophagen, Merck Sharp & Dohme, Otsuka Pharmaceutical, Taisho Toyama Pharmaceutical, Takeda Pharmaceutical, The Chemo-Sero-Therapeutic Research Institute, Toray Industries, and Sysmex Corporation. Stephen Pianko: Personal Fees: Gilead Sciences, Brisol-Myers Squibb Alessandra Mangia: Consultant: BMS, Gilead Sciences, MSD; Advisory Board: BMS, Gilead Sciences, MSD; Research: Janssen, BMS. Wan-Long Chuang: Advisory Board: Gilead, Abbvie, BMS, Roche, PharmaEssentia; Speaker: Gilead, BMS, MSD, Roche. Ira Jacobson: Consultant: AbbVie, Achillion, Bristol-Myers Squibb, Gilead Sciences, Janssen, and Merck; Research: AbbVie, Bristol-Myers Squibb, Gilead Sciences, Janssen, Merck, and Tobira; Speaker: Bristol-Myers Squibb, Gilead Sciences, Janssen, and Merck. Gregory J. Dore: Consultant, Advisory Board and Research: AbbVie, BMS, Gilead, Merck. Mark Sulkowski: Advisory Board: Gilead, AbbVie, Janssen, Merck, Cocrystal, Trek; Research: Gilead, AbbVie, BMS, Janssen, Merck. Employees and stock holders of Gilead Sciences: Ross Martin, Evguenia Svarovskaia, Hadas Dvory-Sobol, Michael D. Miller, Brian Doehle, Charlotte Hedskog, Chohee Yun, Diana M. Brainard, Steven Knox, John G. McHutchison, Hongmei Mo. Nothing to declare: Kwang-Hyub Han.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2017.01.007.

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