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REVIEWS IN BASIC AND CLINICAL GASTROENTEROLOGY AND HEPATOLOGY

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Hepatitis C Virus Resistance to Direct-Acting Antiviral Drugs in Interferon-Free Regimens

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Treatment of hepatitis C virus (HCV) infection has progressed considerably with the approval of interferon-free, direct-acting antiviral (DAA)-based combination therapies. Although most treated patients achieve virological cure, HCV resistance to DAAs has an important role in the failure of interferon-free treatment regimens. The presence of viral variants resistant to NS5A inhibitors at baseline is associated with lower rates of virological cure in certain groups of patients, such as those with genotype 1a or 3 HCV, those with cirrhosis, and/or prior nonresponders to pegylated interferon–based regimens. DAA-resistant HCV is generally dominant at virological failure (most often relapse). Viruses resistant to NS3-4A protease inhibitors disappear from peripheral blood in a few weeks to months, whereas NS5A inhibitor–resistant viruses persist for years. Re-treatment options are available, but first-line treatment strategies should be optimized to efficiently prevent treatment failure due to HCV resistance.

Keywords: Resistance; Resistance-Associated Substitutions; Treatment Failure; Retreatment.

Treatment of hepatitis C virus (HCV) infection has progressed considerably since the approval in 2014 of direct-acting antivirals (DAAs) and the subsequent availability of interferon (IFN)-free, DAA-based combination therapies. Despite the high rates of virological cure achieved with these treatments, the infection is not eliminated from a substantial number of patients (1%–15%, depending on the patient group and regimen).[1,2] Factors that influence the ability of infected patients to be cured include the patients' metabolism of the DAA agents, their genetic background (eg, polymorphisms in the *IL28B* gene), whether they have extensive fibrosis or cirrhosis, their adherence to therapy, and resistance of the HCV to DAAs, which is an important factor in the failure of IFN-free regimens.

We review the principles of HCV resistance to DAAs, the role of HCV resistance in IFN-free treatment virological failures, the dynamics of resistant viruses after treatment failure, ways to prevent failure due to resistance, re-treatment options, and the utility of HCV resistance testing at different time points of therapy.

Principles of HCV Resistance to DAAs

Definition of Viral Resistance and Resistance-Associated Substitutions

HCV has a quasispecies distribution. Patients are infected by complex mixtures of genetically distinct but closely related viral populations of different sizes. Their respective proportions depend on their replication capacities in their environment (defined as fitness). HCV populations coexist in equilibrium at any time point, but any change in the environment tips the equilibrium and alters the quasispecies distribution.[3]

The viral populations that constitute the quasispecies differ by amino acid polymorphisms that emerge by mutation during replication and are subsequently selected based on their effects on viral fitness. Natural polymorphisms that lie in a viral protein region important for the antiviral effect of a DAA may confer reduced susceptibility to the DAA or DAA class. Such polymorphisms can be present in major, highly fit viral populations. However, they are more often present in minor viral populations because they generally reduce fitness compared with wild-type viruses (ie, viruses without these polymorphisms).

When a DAA is administered, positive selection of viral variants with reduced susceptibility to this drug defines viral resistance. Complete inhibition of DAA-sensitive wild-type viruses opens the replication space, allowing variants with reduced susceptibility to rapidly outgrow them. Additional, so-called compensatory or secondary amino acid substitutions, or fitness-associated substitutions—either naturally present or acquired by mutation during replication of the resistant virus on drug administration—may increase the fitness of resistant variants, leading to their rapid outgrowth on treatment (breakthrough) or after treatment (relapse) and influencing their posttreatment persistence.

Abbreviations used in this paper: **DAA, direct-acting antiviral; EC_{50}, 50% effective concentration; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; RAS, resistance-associated substitution; RdRp, RNA-dependent RNA polymerase; SVR, sustained virological response.**

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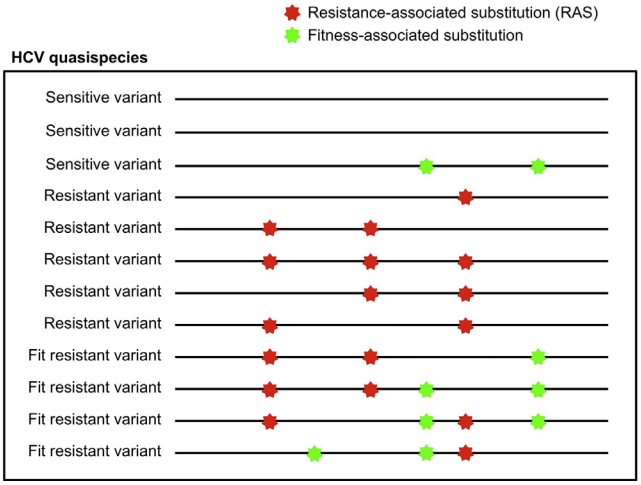

Figure 1. Definitions in HCV resistance. Viral variants are individual full-length viruses that constitute the HCV quasispecies in a patient. They are organized as viral populations, made of identical variants that are different from the variants in other populations. The sequence of sensitive variant genomes does not contain amino acids that confer reduced susceptibility to the antiviral action of an HCV DAA. Compared with the sequence of sensitive variants, the sequence of resistant variants contains one or several RASs, which are single amino acid changes that reduce susceptibility to a DAA or a class of DAAs. The sequence of resistant variants sometimes also contains one or several fitness-associated substitution(s), which are single amino acid changes that do not alter DAA susceptibility but increase the fitness of the resistant variants, giving them a replication advantage over other resistant variants. Fitness-associated substitution(s) can also be present in the sequence of sensitive variants, improving their replication capacity or having no effect in the absence of the RAS(s). The populations of viral variants coexist within each patient's viral quasispecies, under the pressure of Darwinian selection forces. In the absence of DAA treatment, sensitive viruses are generally (but not always) the fittest. When DAAs are administered, resistant variants (variants carrying RASs) are selected. Their outgrowth depends on their fitness in the presence of the drug more than on the level of resistance conferred by the RASs. When treatment is stopped, the outcome of competition between the variants also depends on their respective fitness.

The term "resistance-associated variant" is often used to indifferently describe the amino acid substitutions that reduce the susceptibility of a virus to a drug or drug class or, alternatively, the viral variants with reduced susceptibility that carry these substitutions. This term is inaccurate and should no longer be used. Instead, the amino acid substitutions that confer resistance must be called "resistance-associated substitutions" (RASs), and the viral variants that carry these RASs (and thereby have reduced susceptibility to the DAA) must be called "resistant variants" (see Figure 1). This terminology will be used throughout this review article.

Factors That Affect HCV Resistance to DAAs

Resistance of HCV to DAAs is determined by 3 major factors.[4] One is the genetic barrier to resistance, related to the number and type of nucleotide substitutions required for emergence of RASs during replication and to the number and type of RASs required for a viral variant to acquire full resistance to the drug. The genetic barrier to resistance varies with drug class, specific drug, and HCV genotype or subtype. It determines the likelihood that resistant viruses are generated during replication. Resistance is also determined by the fitness of resistant virus populations, which is independent of the level of resistance conferred by the RASs (the most resistant variants are not necessarily the fittest and vice versa). Fitness determines the likelihood that generated resistant viruses persist in minor or major populations. Resistance is finally determined by level of drug exposure compared with the drug's 50% and 90% inhibitory concentrations in vitro. In vivo exposure affects the ability of a drug to inhibit replication of resistant variants.

Characteristics of HCV Resistance to DAAs

In patients receiving antiviral treatment, HCV kinetics are typically biphasic.[5] The first-phase HCV RNA decline is rapid and results from the direct inhibitory effect of the drug(s) on viral replication. This phase depends on drug potency, exposure, and virus susceptibility. The second, slower-phase HCV RNA decline results from the progressive loss of HCV from cells due to degradation of nonreplicating viral RNAs by effectors of the innate immune system. This second phase is influenced by drug potency, genetic features of the host, and the severity of liver disease and can be accelerated by ribavirin through unclear mechanisms.[6]

In patients treated with a combination of DAAs, the absolute amount of each viral population present in the quasispecies at baseline evolves following individual kinetics that depend on the starting amount of the viral population, its susceptibility to the antiviral action of the drugs, and its fitness in the presence of the drugs (see Figure 2). Sensitive viral populations are rapidly eliminated following a typical biphasic decline if treatment duration is sufficient. In contrast, resistant variants, which are only partly or not at all inhibited, slowly decrease, remain at the same level, or expand. Some are still present in the liver when treatment is stopped, even though they were undetectable in peripheral blood during treatment. After treatment is withdrawn, these resistant variants start replicating again, eventually acquire mutations that increase their fitness, and propagate in the liver, ultimately causing virological relapse (Figure 2).

At treatment failure (breakthrough or relapse), if adherence and treatment duration have been appropriate, most if not all of the viral variants in the quasispecies are resistant to one or several of the drugs administered. After treatment, some variants (such as those resistant to NS3-4A protease inhibitors) disappear within a few weeks to months. They are replaced by wild-type, DAA-sensitive virus. The wild-type virus either persists in the liver—if therapy is not long enough to clear it—and outgrows them after treatment withdrawal, or it is generated by spontaneous mutation of resistant viruses (reversion to wild-type) if the original wild-type virus is cleared during therapy.

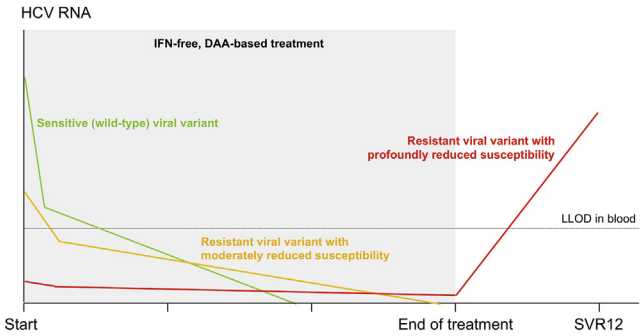

Figure 2. Schematic representation of the individual kinetics of different viral variant populations present in the quasispecies of an HCV-infected patient treated with an IFN-free, DAA-based regimen. In this example, the patient is infected at baseline with highly fit sensitive (wild-type) viral variants (*green*), less fit resistant variants with moderately reduced susceptibility to the DAA (*yellow*), and unfit resistant variants with profoundly reduced susceptibility to the DAA (*red*). Treatment administration efficiently blocks the replication of sensitive variants, inducing their biphasic decay and rapid clearance. The modest antiviral effect on resistant variants with moderately reduced susceptibility also induces their biphasic decay, although with a much slower second-phase slope, ultimately leading to their elimination because treatment duration is sufficient. In contrast, antiviral treatment has virtually no effect on the resistant variants with profoundly reduced susceptibility that keep replicating at the same low level during the full course of therapy. When treatment is stopped, their fitness acquisition through mutation causes the relapse with RAS-carrying resistant variants. If treatment were shorter, both resistant variants with moderately and profoundly reduced susceptibility would still replicate in the liver at withdrawal. The variants would then compete, causing relapse with the fittest RAS-carrying resistant variants. If treatment was much shorter, sensitive variants would still be present in the liver at withdrawal, causing relapse with wild-type variants. These HCV variant kinetics can be influenced by pharmacologic parameters that promote drug exposure and by host factors that modify the second-phase decay slope. LLOD, lower limit of detection; SVR12, SVR 12 weeks after treatment.

Other variants (such as those resistant to NS5A inhibitors) persist for years after treatment failure, either because they are naturally more fit or because they are unable to revert to wild-type virus for genetic reasons.

Resistance to Available DAAs

In 2016, patients with chronic hepatitis C are treated with a combination of 1 to 3 DAAs from 4 classes, with or without ribavirin.[1,2] Nucleotide analogue inhibitors of the RNA-dependent RNA polymerase (RdRp) or NS5B protein have a high barrier to resistance because the variants they select have modestly reduced susceptibility to these drugs and low fitness. Thus, breakthrough or relapse is the exception when these drugs are given as monotherapy. On the other hand, NS5A inhibitors, NS3-4A protease inhibitors, and non-nucleoside inhibitors of the RdRp have low barriers to resistance. When given as monotherapies, they rapidly select fit resistant variants. Second-generation NS3-4A and NS5A inhibitors have increased barriers to resistance. They are substantially more active against many but not all variants resistant to first-generation compounds. Table 1 shows the HCV DAAs approved, soon to be approved, or in late clinical developmental stages in early 2016.

Table 2 lists substitutions that reduce the susceptibility of different HCV genotypes and subtypes to currently available DAAs (>2-fold increase in the 50% effective concentration [EC_{50}] of the drug compared with wild-type virus in the replicon system in vitro) and those conferring high-level resistance. High-level resistance is also conferred in vitro by multiple substitutions at positions Q30, L31, and/or Y93 in the NS5A protein and at positions Y56, Q80, R155, and/or D168 in the NS3 protease.[7]

Prevalence of RASs at Treatment Baseline

Many studies have reported the proportion of detectable RASs at treatment baseline in various patient populations. These reports are generally based on population sequencing (or direct sequencing) via the Sanger method, which is available in most virology laboratories.[8] The sensitivity of population sequencing for the detection of viral populations in a quasispecies mixture is approximately 10% to 25%. Because population sequencing is not available in a standardized commercial format, in-house methods are used. As a result, studies based on population sequencing can hardly be compared, because their results strongly depend on the performance of the method used.

More sensitive techniques based on deep sequencing (or pyrosequencing) are available in some laboratories.[8] They detect viral populations that represent down to approximately 1% of the quasispecies. Their accuracy in predicting virological response depends on the cutoff used for analysis. Resistant variants present in low proportions (1%–15%) at baseline do not appear to significantly influence the response. A 15% cutoff, in the order of magnitude of population sequencing, better predicts treatment failure due to the selection of resistant viruses. Thus, there is consensus that the 15% cutoff should be used in all clinical trials and real-life studies and in clinical practice to report the presence of resistant variants assessed by deep sequencing and allow for comparison of results generated with different assays, including those based on population and deep sequencing.

Information about the prevalence of RASs at baseline is heterogeneous, partly biased by the technique used (lack of standardization, dependency on primers, device, reaction conditions, and so on), and often incomplete due to the choice to study some RASs but not others. Indeed, some studies have reported the prevalence of all RASs at positions possibly associated with resistance to a drug class, likely diluting the effect of clinically meaningful substitutions, whereas others have reported the prevalence of RASs proven to confer in vitro resistance (above a threshold that may vary from one study to another) to all drugs in the DAA class or to a specific DAA from the class. In addition, most studies do not report linkage (ie, whether the observed

Table 1. DAAs Approved for Treatment of HCV Infection or in Development (Beginning of 2016)

Class	Generation/wave		Compound	Manufacturer	Current status or phase of clinical development
Nucleotide analogues	First generation		Sofosbuvir	Gilead Sciences	Approved
			MK-3682	Merck	Phase 2
			AL-335	Janssen	Phase 2
NS5A inhibitors	First generation	First wave	Daclatasvir	Bristol-Myers Squibb	Approved
			Ledipasvir	Gilead Sciences	Approved
			Ombitasvir	AbbVie	Approved
		Second wave	Elbasvir	Merck	Approved (United States, European Union in 2016)
			Velpatasvir	Gilead Sciences	Phase 3 (Approval in 2016)
			Odalasvir	Janssen	Phase 2
			Ravidasvir	Presidio	Phase 2
	Second generation		ABT-530	AbbVie	Phase 3
			MK-8408	Merck	Phase 2
NS3-4A protease inhibitors	First generation	First wave	Telaprevir	Janssen, Mitsubishi	Approved
			Boceprevir	Merck	Approved
		Second wave	Simeprevir	Janssen	Approved
			Paritaprevir/r	AbbVie	Approved
			Asunaprevir	Bristol-Myers Squibb	Approved (Asia, Middle East)
			Vaniprevir	Merck	Approved (Japan)
	Second generation		Grazoprevir	Merck	Approved (United States, European Union in 2016)
			ABT-493	AbbVie	Phase 3
			GS-9857	Gilead Sciences	Phase 3
Nonnucleoside inhibitors of HCV RdRp	Palm-1 inhibitors		Dasabuvir	AbbVie	Approved

NOTE. Ledipasvir/sofosbuvir, ombitasvir/paritaprevir/ritonavir, grazoprevir/elbasvir, velpatasvir/sofosbuvir, ABT-530/ABT-493, and possibly other compounds are or will be available as single-pill, fixed-dose combinations.
/r, ritonavir-boosted.

RASs are on the same or on different viral variants). Finally, most of the information has been generated with HCV genotype 1, whereas few data are available for other genotypes.

Nucleotide Analogues

Substitutions in NS5B that reduce susceptibility to nucleotide analogues are rarely detected at baseline. In deep sequencing analyses with a cutoff of 1%, S282T was not found in any of 8598 patients included in phase 2 or 3 clinical trials of regimens that included sofosbuvir.[9] In a study in 1645 patients infected with HCV genotypes 1 to 6, population sequencing found L159F in 11 cases (0.7%), associated with C316N (a fitness-associated substitution when combined with L159F) in 9 cases. V321A was never detected at baseline.[10] Deep sequencing with a cutoff of 1% was performed in 3081 patients included in phase 2 and 3 studies of regimens that included sofosbuvir (1525 with genotype 1a, 439 with genotype 1b, 410 with genotype 2, 706 with genotype 3, and 1 with genotype 4). L159F was found at baseline in 33 of these patients (1.1%; 32 patients with HCV genotype 1b and 1 patient with genotype 1a); 31 of them (93.9%) also had C316N. V321V was not detected in any of these patients by deep sequencing.[11] Data using a 15% cutoff were not provided in this study.

NS5A Inhibitors

NS5A RASs are often detected at baseline in DAA-naïve patients. Deep sequencing of baseline samples was performed in 5397 patients included in phase 2 and 3 trials.[12] Using the 15% clinically relevant cutoff in patients infected with genotype 1a, at least 1 RAS was found at baseline in 13% of cases in North America, 14% in Europe, 7% in Asia-Pacific, and 16% in Oceania. Based on the 1% cutoff value, there were preexisting RASs in 26% (686/2638), 25% (130/517), 15% (4/27), and 27% (89/328) of patients with HCV genotype 1a in these regions, respectively. For patients with HCV genotype 1b, the 15% cutoff showed RASs at baseline in 16%, 17%, 20%, and 19% of patients in these regions, respectively. Using the 1% cutoff, baseline RASs were detected in 23% (184/802), 25% (105/416), 26% (150/570), and 26% (26/99) of patients in these regions, respectively.[12]

When only substitutions conferring a >100-fold increase in ledipasvir EC_{50} in vitro were considered with a 1% cutoff, Q30H/R was present in 5.0% (174/3483), L31M in 4.0% (140/3483), and Y93H in 2.0% (69/3483), and multiple substitutions were detected in 5.0% (174/3483) of patients with genotype 1a infection. In patients with genotype 1b infection, L31M/I/V was present in 7.5% (142/1887) and Y93H in 16.1% (304/1887), and multiple substitutions were detected in 1.4% (27/1887) of cases.[12] By

Table 2. List of Known RASs (Amino Acid Substitutions Reported to Reduce Susceptibility of Different HCV Genotypes or Subtypes to DAAs)

	Genotype or subtype							RASs that confer high-level resistance in the replicon model (genotype)
Amino acid	1a	1b	2	3	4	5	6	
Nucleotide analogues (sofosbuvir)								
159	L159F	L159F	L159F	L159F				
282	S282T/R	S282T	S282T					
320	L320F							
321				V321A				
NS5A inhibitors (first-generation drugs, including first wave and second wave)								
24	K24G/N/R		T24A				Q24H	
26	K26E							
28	M28A/G/T/S/V	L28M/T	L/F28M/V/S	M28T	L28V	L28I		M28A/G/T (1a) L28T (1b) F28S (2a) L28F (2b)
29		P29S						
30	Q30C/D/E/G/H/I/L/K/R/S/T/Y	R30G/H/P/Q	L30H/S	A30K	L30H			Q30H/G/R/E/K (1a)
31	L31I/F/M/V	L31I/F/M/V	L31M/V	L31M/V		L31V	L31V	L31M/V (all)
32	P32L/S	P32L/S					P32L/S	P32L/S (1a)
38	S38F							
58	H58D/L/R	P58D/S					T58A/N/S	H58D (1a) P58D (1b)
62		E62D						
92	A92K/T	A92K						
93	Y93C/F/H/L/N/R/S/T/W	Y93C/H/N/S	Y93H	Y93H	Y93H/R			Y93C/H/N/S (1a) Y93H (1b)
NS3-4A protease inhibitors (first-generation drugs, including first wave and second wave)								
36	V36A/C/G/L/M	V36A/C/G/L/M						
41	Q41R	Q41R						
43	F43L	F43I/S/V						
54	T54A/S	T54A/C/G/S						
55	V55A/I	V55A						
56	Y56H	Y56H/L	Y56H					
80	Q80H/K/L/R	Q80H/K/L/R						
122	S122R	S122R						
155	R155G/I/K/M/S/T/W	R155C/G/I/K/Q/M/S/T/W						
156[a]	A156S/T/V	A156G/F/S/T/V						A156T/V
158	V158I	V158I						
168[a]	D168A/C/E/G/H/K/N/T/V/Y	D168A/C/E/F/G/H/K/N/T/V/Y						D168H/T/K/V/Y
170	I/V170F/T/V	I/V170A/L/T						
175		M175L						
Nonnucleoside RdRp palm-1 inhibitors (dasabuvir)								
314	L314H							L314H
316	C316Y	C316H/N/Y/W						C316H/Y (1)
368		S368T						S368T (1b)
411		N411S						
414	M414T/V	M414I/T/V						
445		C445F/Y						
446	E446K/Q							
448	Y448C/H	Y448C/H						Y448C/H (1)
451	C451R							
553	A553T	A553V						A553T (1a) A553V (1b)
554	G554S	G554S						G554S (1)
555	Y555H							
556	S556G/R	S556G						S556R (1a)

Table 2. Continued

Amino acid	Genotype or subtype 1a	1b	2	3	4	5	6	RASs that confer high-level resistance in the replicon model (genotype)
557	G557R							
558	G558R	G558R						
559	D559G/N	D559G/N						D559G (1)
561	Y561H/N							

NOTE. This table was adapted from 2 review articles and data reported by drug manufacturers.[7,10–13,15] Reduced susceptibility is defined based on >2-fold increase in EC_{50} compared with wild-type HCV in the replicon system in vitro. The level of resistance conferred by a given RAS to different compounds from the same class may differ. The empty boxes indicate that no data are available for the corresponding genotype or subtype at the given amino acid position.
[a]Substitutions at positions 156 and 168 also confer resistance to the second-generation NS3-4A protease inhibitor grazoprevir.

extrapolation, an approximately 1.5-fold lower prevalence would be expected with a 15% cutoff, which is an estimation in keeping with previous studies based on population sequencing.[13]

NS3-4A Protease Inhibitors

NS3 protease RASs can also be detected at baseline. Their baseline prevalence was assessed by population sequencing in 2007 patients with genotype 1 infection.[14] The only frequent substitution was Q80K, found in 13.6% (273/2007) of cases. Q80K was mostly present in patients with genotype 1a infection (29.5% [269/911] vs 0.5% [5/1096] in genotype 1b), with geographical differences: 48.1% (185/385) in North America, 19.4% (73/377) in Europe, and 9.1% (2/22) in South America. Other NS3 protease substitutions were more rarely found at baseline: Q80G in 0.05%, Q80L in 1.9%, Q80N in 0.05%, Q80R in 0.6%, R155K in 0.3%, D168E in 0.4%, and the combination of Q80K and D168E in 0.05%.[14] The highly resistant but poorly fit A156T substitution was never found at baseline. These numbers are in keeping with older studies that were based on population sequencing.[13] In a small-scale study using deep sequencing with a cutoff of 1% in patients infected with HCV genotype 1a, the prevalence values were 3.4% (2/59) for V36A/L, 8.5% (5/59) for T54S, 8.5% (5/59) for V55A/I, 13.6% (8/59) for Q80K/L, 3.4% (2/59) for R155K, and 1.7% (1/59) for D168E.[15]

Non-nucleoside RdRp Palm-1 Inhibitors

Substitutions in NS5B that confer resistance to the RdRp palm-1 site inhibitor dasabuvir are rarely detected at baseline. Population sequencing found the preexisting dasabuvir RASs C316Y in 0.2% to 1.2%, M414T in 0.5%, Y448H in 0.2%, A553I/T/V in 6.0%, S556G in 0.6% to 3.1%, and S556N/R in 0.6% to 1.2% of patients with HCV genotype 1a infection. In patients with HCV genotype 1b infection, C316N was present in 10.9% to 35.6%, C316H in 1.9% to 2.1%, M414T in 0.4%, Y448H in 1.3%, and S556G in 7.0% to 16.0% of cases.[13] In an analysis of 332 patients infected with genotype 1a, dasabuvir RASs were detected at baseline in 1.5% of cases by deep sequencing, with a cutoff of 15% (5.9% with a 1% cutoff). In 151 patients infected with genotype 1b, dasabuvir RASs were detected in 29% of cases at baseline at a 15% cutoff.[16]

Effects of Baseline RASs on Virological Outcomes of IFN-Free Treatment

Resistance data have been generated from phase 2 and 3 studies of different IFN-free combinations. Thus far, partial data presentation, multiple subgroup analyses, and heterogeneity of the sequencing methods and reporting make their interpretation difficult. The data indicate that RASs that confer medium- to high-level resistance without profoundly altering fitness present in large proportions (>15% of the quasispecies) at baseline affect the virological outcome of DAA-based combination therapies. These effects vary with the combination regimen used.

Sofosbuvir/Ledipasvir

The presence of NS5A RASs at baseline affects chances for a sustained virological response (SVR) (see Table 3) to the fixed-dose combination of sofosbuvir and ledipasvir in a single pill (sofosbuvir/ledipasvir) in certain groups of patients. Effects of different baseline RASs on induction of an SVR in patients with HCV genotype 1 infection, with or without cirrhosis, have been described in 3 heterogeneous, partly overlapping post-hoc analyses of pooled data from phase 2 and 3 studies of sofosbuvir/ledipasvir, with or without ribavirin.[12,17,18] The presence of sofosbuvir or NS3-4A protease RASs at baseline did not affect patients' responses to sofosbuvir/ledipasvir.

The presence of NS5A RASs at baseline had no effect on SVR of treatment-naïve patients with or without cirrhosis treated for 12 or 24 weeks with or without ribavirin.[12,17] In contrast, NS5A RASs that conferred a high level of resistance to ledipasvir (>100-fold increase in EC_{50}) were associated

Table 3. Proportions of Patients With HCV Genotype 1 Infection Who Achieve SVR to Sofosbuvir/Ledipasvir According to the Presence of Baseline NS5A RASs

Pooled data analysis of the ION-1, ION-2, and ION-3 Phase 3 and ELECTRON Phase 2 trials, based on a combination of population sequencing and deep sequencing with a detection cutoff of 1%[17]

Cirrhosis status	Prior treatment	Treatment regimen	Rate of SVR		
			Patients with NS5A RASs at baseline (>100-fold increase in EC_{50})	Patients with NS5A RASs at baseline (<100-fold increase in EC_{50})	Patients without NS5A RASs at baseline
No split by cirrhosis	Treatment naïve	Sofosbuvir/ledipasvir +/− ribavirin for 8 wk	83% (24/29)	100% (12/12)	95% (184/193)
		Sofosbuvir/ledipasvir +/− ribavirin for 12 wk	96% (44/46)	100% (27/27)	97% (362/373)
		Sofosbuvir/ledipasvir +/− ribavirin for 24 wk	92% (24/25)	100% (8/8)	96% (174/183)
	Treatment experienced	Sofosbuvir/ledipasvir +/− ribavirin for 12 wk	65% (11/17)	100% (5/5)	95% (110/116)
		Sofosbuvir/ledipasvir +/− ribavirin for 24 wk	100% (6/6)	100% (7/7)	99% (95/96)

Pooled data analysis of 513 patients with compensated (Child–Pugh A) cirrhosis, based on deep sequencing with a detection cutoff of 1%[18]

Cirrhosis status	Prior treatment	Treatment regimen	Rate of SVR	
			Patients with NS5A RASs at baseline	Patients without NS5A RASs at baseline
Cirrhosis	No split by prior treatment	Sofosbuvir/ledipasvir for 12 wk	88% (23/26)	95% (86/91)
		Sofosbuvir/ledipasvir + ribavirin for 12 wk	94% (32/34)	97% (164/169)
		Sofosbuvir/ledipasvir for 24 wk	85% (17/20)	100% (113/113)
		Sofosbuvir/ledipasvir + ribavirin for 24 wk	100% (14/14)	100% (44/44)

Pooled data analysis of 1566 patients who received the current guideline-recommended sofosbuvir/ledipasvir regimens, based on deep sequencing with a detection cutoff of 1%[12]

Cirrhosis status	Prior treatment	Treatment regimen	Rate of SVR	
			Patients with NS5A RASs at baseline	Patients without NS5A RASs at baseline
No cirrhosis	Treatment naïve <6 million HCV RNA IU/mL	Sofosbuvir/ledipasvir for 8 wk	94% (30/32)	99% (107/108)
	Treatment naïve	Sofosbuvir/ledipasvir for 12 wk	99% (187/189)	99% (504/509)
	Treatment experienced	Sofosbuvir/ledipasvir for 12 wk	90% (79/88)	99% (298/300)
Cirrhosis	Treatment naïve	Sofosbuvir/ledipasvir for 12 wk	96% (26/27)	96% (65/68)
		Sofosbuvir/ledipasvir + ribavirin for 12 wk	100% (10/10)	100% (27/27)
		Sofosbuvir/ledipasvir for 24 wk	89% (8/9)	100% (19/19)
	Treatment experienced	Sofosbuvir/ledipasvir + ribavirin for 12 wk	89% (59/66)	96% (206/214)
		Sofosbuvir/ledipasvir for 24 wk	87% (13/15)	100% (84/84)

NOTE. Patients were included in phase 2 or 3 trials and received sofosbuvir/ledipasvir-based regimens. There is overlap between the 3 post-hoc pooled data analyses. NS5A RASs were assessed at baseline by population sequencing or deep sequencing with a detection cutoff of 1%.[12,17,18]

Table 4. Proportions of Patients With HCV Genotype 1a Infection Who Achieve SVR to Ombitasvir/Paritaprevir/Ritonavir and Dasabuvir Plus Ribavirin According to the Presence of Baseline RASs

Treatment regimen	RAS region	Type of RAS at baseline	Rate of SVR	
			Patients with RASs at baseline (>15% cutoff)	Patients without RASs at baseline
Ombitasvir/paritaprevir/ritonavir and dasabuvir + ribavirin (12 wk in patients without cirrhosis and 24 wk in patients with cirrhosis [Child–Pugh A])	NS5A	NS5A RASs	96% (48/50)	97% (271/280)
		Ombitasvir-specific RASs	95% (36/38)	97% (283/292)
	NS3 protease	Paritaprevir-specific RASs	100% (4/4)	97% (315/326)
		Q80K	96% (102/106)	97% (217/224)
	NS5B	Dasabuvir-specific RASs	80% (4/5)	97% (302/313)

NOTE. Patients received the combination of ombitasvir/paritaprevir/ritonavir and dasabuvir in phase 3 trials. Only patients treated with guideline-recommended regimens were included in the analysis, including 214 treatment-experienced patients with HCV genotype 1a infection without cirrhosis treated with ribavirin for 12 weeks and 118 treatment-naïve and -experienced patients with HCV genotype 1a infection and cirrhosis (Child–Pugh A) treated with ribavirin for 24 weeks. RASs were identified by deep sequencing, with a detection cutoff of 15%.[16]

with a lower rate of SVR in treatment-experienced patients with or without cirrhosis treated for 12 weeks without ribavirin (see Table 3). This effect was significant when RASs were present in large proportions (>15% of the baseline quasispecies) in patients infected with genotype 1a but not in those infected with genotype 1b.[12,18] Interestingly, all patients with genotype 1a infection who experienced a relapse had detectable pretreatment NS5A substitutions that reduced susceptibility to ledipasvir >1000-fold in vitro (H58D, Y93H/N/F, or multiple substitutions). The rate of SVR in patients with HCV genotype 1a infection with these RASs was only 72%.[12] The addition of ribavirin prevented the effect of preexisting NS5A RASs on SVR; 88% (23/26) of patients with cirrhosis treated for 12 weeks without ribavirin achieved an SVR compared with 94% (32/34) of patients who received ribavirin; these values were 85% (17/20) versus 100% (14/14) in patients treated for 24 weeks without and with ribavirin, respectively.[18]

Together, these data indicate that NS5A RASs that confer high to very high resistance to NS5A inhibitors, when they are present in substantial proportions at baseline (detectable by population sequecing or >15% by deep sequencing), affect SVR to sofosbuvir/ledipasvir in patients infected with genotype 1a, especially those with cirrhosis and/or patients who failed to respond to prior pegylated IFN–based treatment. Ribavirin appears to reduce the effects of preexisting NS5A RASs. Limited information is available for HCV genotypes 4, 5, and 6, which are also indications for sofosbuvir/ledipasvir.

Ombitasvir/Paritaprevir/Ritonavir Plus Dasabuvir

In a phase 2 study, 86% (19/22) of patients infected with HCV genotype 1a with RASs at baseline achieved an SVR, compared with 92% (185/201) of patients without baseline RASs. NS3 protease or NS5B RASs had no effect on SVR. All patients infected with HCV genotype 1b had an SVR.[19]

Researchers recently reported the effects of baseline RASs in genotype 1 HCV on SVR in patients with or without cirrhosis who were treated with guideline-recommended regimens of the one-pill combination of ombitasvir, paritaprevir, and ritonavir (ombitasvir/paritaprevir/ritonavir) plus dasabuvir, with or without ribavirin, for 12 or 24 weeks in 4 phase 3 trials.[16] All patients infected with genotype 1b who were treated without ribavirin achieved SVR. Table 4 shows SVR in patients with genotype 1a infection according to the presence of RASs in NS3 protease, NS5A, or NS5B (dasabuvir) at baseline. The presence at baseline of NS5A-class RASs, ombitasvir-specific RASs, paritaprevir-specific RASs, the Q80K substitution in the NS3 protease, or dasabuvir-specific RASs in more than 15% of the quasispecies variants, based on deep sequencing analysis, had no effect on SVR (Table 4). No data are available on ombitasvir/paritaprevir/ritonavir without dasabuvir in patients infected with HCV genotype 4.

Sofosbuvir Plus Daclatasvir

Researchers performed an integrated analysis of baseline NS5A RASs from phase 2 and 3 trials in 228 patients without cirrhosis infected with HCV genotype 1, including treatment-naïve and -experienced and human immunodeficiency virus (HIV)-positive and -negative patients, as well as patients who received liver transplants, treated with sofosbuvir plus daclatasvir for 12 weeks with or without ribavirin. All patients with NS5A RASs at baseline achieved SVR, whereas 2 patients without RASs did not.[20] Data generated with sofosbuvir/ledipasvir on a much larger, more homogeneous panel of patients with genotype 1 infection can probably be extrapolated to this equivalent combination. These data indicate that NS5A substitutions that confer a high to very high level of resistance affect SVR of patients infected with genotype 1a infection and cirrhosis, and/or patients who failed to respond to prior pegylated IFN–based treatment, and that the addition of ribavirin prevents this effect.

In a phase 3 study of patients infected with HCV genotype 3 treated with sofosbuvir plus daclatasvir for 12 weeks without ribavirin, SVR was achieved by high proportions of patients without cirrhosis (97% [73/75] and 94% [32/34] of treatment-naïve and -experienced patients, respectively)

but in lower proportions of patients with cirrhosis (58% [11/19] and 69% [9/13], respectively).[21] Among the 14 patients with preexisting NS5A RASs at position A30, 9 of 9 without cirrhosis but only 1 of 5 with cirrhosis achieved SVR; 2 of the 4 patients with cirrhosis who did not achieve SVR also had Y93H at baseline. Among the 13 patients with Y93H at baseline, 6 of 9 without cirrhosis but only 1 of 4 with cirrhosis achieved SVR.[21] These data indicate that the presence of NS5A RASs at baseline is associated with reduced rates of SVR in undertreated (too short duration, no ribavirin) patients with cirrhosis and genotype 3 infection. Data from patients receiving an optimal treatment regimen are awaited.

Sofosbuvir Plus Simeprevir

In a phase 2 study of sofosbuvir plus simeprevir in patients with genotype 1 infection, the presence of Q80K, detected by population sequencing at baseline, had a modest effect on SVR: 95% (35/37) of patients with genotype 1b infection, 88% (51/58) of patients with genotype 1a with Q80K, and 94% (68/72) of patients with genotype 1a without Q80K achieved SVR.[22]

In a phase 3 study of noncirrhotic patients with HCV genotype 1 infection who were treatment-naïve or had been treated with pegylated IFN–based regimens and received 12 weeks of therapy with sofosbuvir and simeprevir without ribavirin, SVR was achieved by 97% (38/39) of patients with genotype 1b infection, 96% (44/46) of those with genotype 1a with Q80K, and 97% (68/70) of those with genotype 1a without Q80K.[23] In treatment-naïve and -experienced patients with compensated cirrhosis (Child–Pugh A) treated for 12 weeks with sofosbuvir and simeprevir without ribavirin in another phase 3 study, SVR was achieved by 84% (26/31) of patients with genotype 1b infection, 74% (25/34) of those with genotype 1a with Q80K, and 92% (35/38) of those with genotype 1a without Q80K. Most treatment failures occurred in treatment-experienced patients.[24] No NS5B substitutions were found to affect SVR in either study.

Together, these data associate the presence of NS3 protease RAS Q80K with a reduced rate of SVR in patients with HCV genotype 1a infection and cirrhosis, especially if they failed to respond to pegylated IFN–based treatment. No data are available from patients with genotype 4 infection, the other indication of this combination.

Asunaprevir Plus Daclatasvir

The combination of asunaprevir and daclatasvir is not approved in the United States or Europe, but it has been widely used in Japan and other Asian and Middle Eastern countries in patients infected with HCV genotype 1b (this combination is not recommended for patients with genotype 1a). In a pooled analysis of 6 trials, 979 patients with genotype 1b infection treated with asunaprevir and daclatasvir for 24 weeks were studied by population sequencing.[25] When substitutions at positions 31 or 93 were absent, L28M and R30Q did not influence SVR (74% [29/39] vs 86% [813/940] and 79% [66/84] vs 87% [776/895] in patients with or without RASs at baseline, respectively). In contrast, major effects on SVR came from L31F/I/M/V (42% [18/43] vs 88% [824/936] with or without these RASs, respectively) and Y93H (37% [38/103] vs 92% [804/876] with or without this RAS, respectively).[25] RASs in the NS3 protease did not affect SVR.[26] These results indicate that patients with HCV genotype 1b with preexisting NS5A RASs at positions 31 or 93 should not be treated with the combination of asunaprevir and daclatasvir.

Grazoprevir/Elbasvir

The combination of the second-generation NS3-4A protease inhibitor grazoprevir and the NS5A inhibitor elbasvir into a single pill (grazoprevir/elbasvir) was approved in the United States in January 2016 and will be approved in the European Union in the second quarter of 2016. In a phase 3 trial of treatment-naïve patients with and without cirrhosis infected with HCV genotype 1 and treated for 12 weeks, the presence at baseline of NS3 protease RASs by population sequencing did not affect SVR. The presence of NS5A RASs did not affect SVR in patients with genotype 1b infection (94% [17/18] vs 100% [112/112] in patients with and without RASs, respectively). In contrast, in patients with HCV genotype 1a with NS5A RASs at baseline, 58% (11/19) achieved an SVR, compared with 99% (133/135) of those without RASs.[27]

Population sequencing data from genotype 1a and 1b–infected treatment-naïve and -experienced patients with or without cirrhosis, with or without HIV coinfection, from various phase 2 and 3 trials were pooled[28] (see Table 5). NS3 protease RASs had no effect on SVR, regardless of the treatment group and regimen received. The presence of NS5A RASs at baseline had no effect on SVR to 12 weeks of grazoprevir/elbasvir without ribavirin in treatment-naïve patients or those with prior relapse infected with genotype 1b. In patients infected with genotype 1a treated for 12 weeks without ribavirin, the presence of NS5A RASs was associated with a 12% reduction of the rate of SVR. Elbasvir-specific RASs were found in only 5% of patients, but the rate of SVR decreased to 58% when they were present at baseline versus 98% in patients not harboring them. In patients infected with genotype 1a or 1b who did not respond to prior treatment with pegylated IFN and ribavirin, the rates of SVR were substantially lower in the presence of NS5A RASs in those treated for 12 weeks without ribavirin, but all patients treated for 16 or 18 weeks with ribavirin achieved SVR (Table 5). The greatest effects occurred with substitutions at positions 30, 31, and 93, whereas substitutions at positions 24, 28, 58, and 92 had no effect.[28]

Together, these findings indicate that NS5A RASs have a substantial effect on the response to 12 weeks of treatment with grazoprevir/elbasvir without ribavirin in all patients with genotype 1a infection and in patients with genotype 1b infection who did not respond to pegylated IFN and ribavirin. This effect disappears if ribavirin is added and treatment is prolonged to 16 or 18 weeks.

Table 5. Proportions of Patients With HCV Genotype 1 Infection Who Achieve SVR to Grazoprevir/Elbasvir According to the Presence of Baseline NS5A RASs

Subtype	Prior treatment	Treatment regimen	Rate of SVR			
			Patients with any NS5A RASs	Patients without NS5A RASs	Patients with elbasvir RASs	Patients without elbasvir RASs
Subtype 1a	Treatment naïve and relapsers	Grazoprevir/elbasvir for 12 wk	86% (74/86)	98% (345/352)	58% (14/24)	98% (405/414)
	Nonresponders	Grazoprevir/elbasvir for 12 wk	64% (9/14)	96% (52/54)	29% (2/7)	97% (59/61)
		Grazoprevir/elbasvir + ribavirin for 16 or 18 wk	100% (8/8)	100% (44/44)	100% (1/1)	100% (51/51)
Subtype 1b	Treatment naïve and relapsers	Grazoprevir/elbasvir for 12 wk	99% (80/81)	99% (183/184)	98% (44/45)	100% (219/220)
	Nonresponders	Grazoprevir/elbasvir for 12 wk	83% (10/12)	100% (22/22)	67% (4/6)	100% (28/28)
		Grazoprevir/elbasvir + ribavirin for 16 or 18 wk	100% (16/16)	100% (22/22)	100% (12/12)	100% (26/26)

NOTE. Substitutions include M28A/G/T, Q30D/E/G/H/K/L/R, L31F/M/V, H58D, and Y93C/H/N/S in patients infected with HCV subtypes 1a or 1b, with or without cirrhosis, with or without HIV coinfection, included in phase 2 or 3 trials of grazoprevir/elbasvir, with or without ribavirin, administered for 12, 16, or 18 weeks. NS5A resistance was assessed at baseline by population sequencing.[28]

Sofosbuvir/Velpatasvir

The combination of sofosbuvir and velpatasvir in one pill (sofosbuvir/velpatasvir) is likely to be approved in the United States and Europe in 2016. Baseline resistance was assessed by deep sequencing, with a cutoff of 1%, in 3 phase 3 trials that included treatment-naïve and -experienced patients with or without compensated cirrhosis infected with HCV genotypes 1 to 6.[29,30] The presence of NS5A RASs at baseline had no effect on SVR in patients infected with genotypes 1a, 1b, 2, 4, 5, or 6 in whom only 2 virological failures occurred, both in patients with preexisting RASs in NS5A.[29,30] In contrast, in patients infected with genotype 3 HCV, 88% (38/43) of those with NS5A RASs at baseline (present in 16% of the population) achieved an SVR, compared with 97% (225/231) of patients without RASs. In particular, only 84% (21/25) of patients with Y93H at baseline achieved SVR.[30]

In another phase 3 trial, patients infected with genotypes 1 to 6 with decompensated cirrhosis (Child–Pugh B) received 12 weeks of treatment with sofosbuvir/velpatasvir with or without ribavirin or 24 weeks of treatment with sofosbuvir/velpatasvir without ribavirin.[31] In patients with genotype 1 infection, the rates of SVR with and without baseline NS5A RASs were 80% versus 96% in patients treated for 12 weeks without ribavirin, 100% versus 98% in those treated for 12 weeks with ribavirin, and 90% versus 98% in those treated for 24 weeks without ribavirin, respectively. In patients infected with genotype 3 with decompensated cirrhosis, SVR was achieved by 50% (7/14) and 50% (6/12) of patients treated without ribavirin for 12 or 24 weeks, respectively, and 85% (11/13) of patients treated for 12 weeks with ribavirin.[31] Analysis of the role of preexisting NS5A RASs was not possible in patients with genotype 3 infection because of the small numbers.

Together, these studies showed that baseline NS5A RASs do not affect the results of therapy with sofosbuvir/velpatasvir in patients without cirrhosis or patients with compensated cirrhosis, with the notable exception of those infected with genotype 3. NS5A RASs also appear to affect virological outcomes of patients with decompensated cirrhosis (Child–Pugh B). The addition of ribavirin reduces the effects of preexisting NS5A RASs to a greater extent than prolongation of therapy to 24 weeks without ribavirin.

Selection of Resistant Variants in Patients Who Do Not Achieve SVR

In adherent patients, virological breakthroughs are exceptional, whereas most treatment failures are relapses. At virological failure, a large proportion, if not all, of the quasispecies variants are resistant to at least one of the DAAs administered. However, if treatment has been too short to clear wild-type, DAA-sensitive virus, this virus may be dominant (Figure 2). RASs found at virological failure in patients receiving current or soon-to-be-approved regimens included in clinical trials are shown in Table 6.

Sofosbuvir/Ledipasvir

In an integrated analysis of phase 2 and 3 trials of sofosbuvir/ledipasvir, virological failure occurred in 2.4% (51/2144) of cases.[17] At virological failure, NS5A RASs were present in 74% (38/51) of patients, including 71% (30/42) with genotype 1a infection and 89% (8/9) with genotype 1b infection (Table 6). Some patients harbored multiple NS5A RASs. Three patients, all with genotype 1a, also had sofosbuvir RASs at treatment failure.[17] In another study of 12 weeks of treatment with sofosbuvir/ledipasvir in treatment-naïve or -experienced patients with or without compensated cirrhosis, 3 of 44 patients with genotype 4 infection and 2 of 41 patients with genotype 5 infection did not achieve SVR.

Table 6. RASs Present at Virological Failure in Patients Without SVR

IFN-free, DAA-based regimen	Trial(s)	Genotype or subtype	No. of patients with virological failure analyzed	RASs present at virological failure		
				NS3 protease RASs	NS5A RASs	NS5B RASs
Sofosbuvir/ledipasvir	Integrated analysis of phase 2 and 3 trials[17]	1a	42	NA	M28A/T, K24R, Q30E/H/K/L/R/Y, L31M/P, S38F, Y93C/H/N	S282T, L320I/V, L159F, V321A
		1b	9	NA	L31I/M/V, Y93H	
	HCV genotype 4 and 5 trial[32]	4	1	NA	S93C	S282T
		5	1	NA		S282T
Ombitasvir/paritaprevir/ritonavir plus dasabuvir	Integrated analysis of phase 2 and 3 trials[34]	1a	67	R155K, D168A/F/H/I/L/N/T/V/Y	M28A/T/V, Q30E/K/R	C316Y, M414I/T, S556G/R
		1b	7	D168A/F/H/I/L/N/T/V/Y	Y93H	C316Y, M414I/T, S556G/R
Ombitasvir/paritaprevir/ritonavir	PEARL-1[35]	4	3	Y56H, D168V	L28S/V, M31I/M, T58P/S	NA
Sofosbuvir plus daclatasvir	ALLY-2[37]	1a	10	NA	Q30E/Q/R, Y93N	
		1b	1	NA		
		2	1	NA	L31M	
		3	1	NA	A30S	
	ALLY-3[21]	3	16	NA	L31I, Y93H	
	ALLY-3+[38]	3	4	NA	Y93H	
Sofosbuvir plus simeprevir	COSMOS[22]	1a	6	R155K, D168E, I170T	NA	
	OPTIMIST-1[23]	1	31	R155K, D168E, I170T	NA	
	OPTIMIST-2[24]	1	16	R155K, D168E, I170T, N174G	NA	
Asunaprevir plus daclatasvir	International trial[39]	1b	101	F43S, Y56H/L, Q80K/R, S122D/G/I/N/T, R155G/K, D168A/E/F/H/T/V/Y	L28M/T, P29S/X, R30G/H/P/Q/R, L31F/I/M/V, P32F/L/X, P58S, Q62D, Y93H/N	NA
	Japanese trial[40]	1b	34	V36G, T54S, N77S, V78A, Q80L, S122G, R155Q, D168A/E/T/V/Y	L28M, R30H/Q, L31I/M/V, Q54H/Y, P58S, Y93H/N	NA
Grazoprevir/elbasvir	C-WORTHY[41]	1	25	V36M, Y56H, V170I, A156G/T/V, D168A/N/V/Y	M28G/T, Q30H/R/Y, L31M/V, H58D, Y93H/N	NA
	C-SALVAGE[42]	1a	2	V36L, Q80K, R155K/T, A156A/T, D168N	M28T, Q30H/R, H58D, Y93H	NA
		1b	1	T54S, A156T	L31M, Y93H	NA
	C-EDGE TN[27]	1a	10	V36M, Y56H, Q80K, S122G, D168A/V	M28A/G/V, Q30H/L/R, L31M/V, Y93H/N,	NA
		1b	1	T54S, V170I	L31F, Y93H	NA
		6	2	V36I, Y56H/Y, L80K/Q, S122T, I132L, D168E/Y, I170V	F28L, L31M	NA
	C-EDGE COINFECTION[43]	1a	4	Q80K, D168A	Q30K/R/Q, L31M, Y93S	NA
		4	1		L28S	NA
	C-SALT[44]	1a	2	S122G, D168A	M28T, Q30R, Y93N	NA
	C-SWIFT[45]	3	2	Q168R	Y93H	NA

Table 6. Continued

IFN-free, DAA-based regimen	Trial(s)	Genotype or subtype	No. of patients with virological failure analyzed	RASs present at virological failure		
				NS3 protease RASs	NS5A RASs	NS5B RASs
Sofosbuvir/velpatasvir	ASTRAL-1[29]	1a	1	NA	Y93N	
		1b	1	NA	Q30R, L31I/M/V, Y93H	
	ASTRAL-3[30]	3	10	NA	A30K, Y93H	
	ASTRAL-4[31]	1a	6	NA	Q30H/R, H58D, Y93H/N	L159F, S282T
		1b	3	NA	L31M/V, Y93H	L159F, S282T
		3	13	NA	M28T, Y93H	N142T, E237G, L302I

NOTE. Data from clinical trials of currently approved or soon-to-be-approved IFN-free, DAA-based regimens.
NA, not applicable.

The S93C RAS in NS5A was detected in 1 patient with genotype 4 infection, and S282T in NS5B was detected in 1 patient with genotype 4 infection and 1 patient with genotype 5 infection at virological failure.[32] NS5B S282T and L159F were more frequently selected when patients previously exposed to sofosbuvir/ledipasvir for 8 or 12 weeks were re-treated with the same regimen for 24 weeks (3 and 2 of 12 virological failures, respectively, in 41 re-treated patients).[33]

Ombitasvir/Paritaprevir/Ritonavir With or Without Dasabuvir

In a pooled analysis of HCV genotype 1 resistance in 2510 participants in phase 2 and 3 trials, 2.9% (74/2510) of patients did not achieve an SVR (67 with genotype 1a and 7 with genotype 1b infection). If only guideline-recommended regimens were considered, 1.8% (19/1083) of the patients did not achieve an SVR.[34] At virological failure, 78% (52/67) of patients with genotype 1a infection had a substitution at any NS3 protease RAS position, 72% (48/67) at any NS5A RAS position, and 58% (39/67) at any NS5B RAS position. In patients infected with genotype 1b, the numbers were 57% (4/7), 29% (2/7), and 29% (2/7), respectively (Table 6). The presence of RASs in 2 or 3 different regions at failure was common with this regimen.[34] In another trial, 3 of 135 (2.2%) patients infected with genotype 4 treated for 12 weeks with ombitasvir/paritaprevir/ritonavir, without ribavirin, did not have an SVR. NS3 protease and/or NS5A RASs were present at virological failure (Table 6).[35]

Sofosbuvir Plus Daclatasvir

In a phase 3 trial in patients with different genotypes, the 13 patients who did not achieve an SVR, including 10 with advanced cirrhosis (8 with subtype 1a, 1 with genotype 2, and 1 with genotype 3) and 3 patients who received liver transplants (1 with HCV subtype 1a, 1 with subtype 1b, and 1 with genotype 3), had NS5A RASs at treatment failure.[36] In HIV-coinfected patients, 13 patients had viral breakthrough ($n = 1$) or relapse ($n = 12$). Five of them had NS5A RASs at virological failure (Table 6). The remaining 8 patients, all of whom had received only 8 weeks of treatment, had a dominant wild-type HCV at relapse.[37] In 2 phase 3 trials of patients with genotype 3 infection, Y93H was the dominant species at relapse in 19 of the 20 patients with posttreatment relapse (Table 6).[21,38]

Sofosbuvir Plus Simeprevir

In a phase 2 trial, 5 of the 6 patients who experienced virological relapse had dominant NS3 protease RASs at the time of failure (Table 6). None of them selected sofosbuvir-resistant RASs.[22] In a phase 3 trial of patients without cirrhosis, 17% (27/155) and 3% (4/154) of patients experienced a relapse in the 8- and 12-week treatment groups, respectively. Only 2 patients (1 of 25 in the 8-week arm and 1 of 3 in the 12-week arm) had dominant simeprevir RASs at failure (Table 6).[23] Among patients with compensated cirrhosis treated for 12 weeks without ribavirin, 16% (16/

103) did not have an SVR, including 3 with viral breakthrough and 13 with relapse. Among 14 patients for whom sequencing data were available, 79% (11/14) had RASs in the NS3 protease at the time of failure. No sofosbuvir RASs were detected (Table 6).[24]

Asunaprevir Plus Daclatasvir

In a pooled analysis of phase 2 and 3 studies, the rate of virological failure was 14% (63/437) in patients without cirrhosis and 16% (32/206) in patients with cirrhosis. Two-thirds of patients experienced on-treatment breakthrough or nonvirological response at the end of treatment, whereas one-third experienced a relapse.[26] The most frequent resistant variants selected at treatment failure had RASs at position D168 of the NS3 protease and at position Y93 of the NS5A protein; each was present in approximately 75% of cases in the international trial (Table 6).[39] Of the 34 patients with virological failure in a phase 3 trial in Japan, 29 had both daclatasvir RASs (predominantly L31M/V and Y93H) and asunaprevir RASs (predominantly D168 RASs) at failure (Table 6).[40]

Grazoprevir/Elbasvir

In a phase 2 study, virological failure occurred in 5% (26/514) of patients with HCV genotype 1 infection (including 32% with cirrhosis) treated with grazoprevir/elbasvir. Fifty-eight percent (14/24 tested) and 56% (14/25 tested) had dominant NS3 protease and NS5A RASs at virological failure, respectively (Table 6).[41] Relapse occurred in 4% (3/79) of treatment-experienced patients (2 with genotype 1a and 1 with genotype 1b), all previously exposed to telaprevir or boceprevir, in another trial. All of them harbored dominant NS3 protease and NS5A RASs at virological failure (Table 6).[42] In a phase 3 study of treatment-naïve patients with genotype 1, 4, or 6 infections, with or without cirrhosis, virological failure occurred in 4% (13/316) of cases, including 10 infected with genotype 1a, 1 with genotype 1b, and 2 with genotype 6.[27] Three of these cases had RASs only in the NS3 protease region, whereas the remaining 10 had RASs in the NS3 and NS5A regions.[27]

Relapse occurred in 2.3% (5/218) of HIV-coinfected patients, including 4 with subtype 1a and 1 with subtype 4 (Table 6).[43] Both patients from the decompensated (Child–Pugh B) cirrhosis study who experienced a relapse during treatment with grazoprevir/elbasvir harbored RASs in the 2 target regions (Table 6).[44] Finally, in a phase 2 trial assessing short durations (4, 6, or 8 weeks) of treatment with grazoprevir/elbasvir and sofosbuvir, NS3 protease and NS5A RASs were detected in 1 of 30 and 9 of 30 patients with genotype 1 infection, respectively, who experienced a relapse. One patient with genotype 3 infection of 2 patients who experienced a relapse had NS3 protease and NS5A RASs at virological failure.[45]

Sofosbuvir/Velpatasvir

In phase 3 trials of sofosbuvir/velpatasvir in patients with no or compensated cirrhosis, 2 patients of 624 (0.3%) infected with genotypes 1a, 1b, 4, 5, or 6 experienced a relapse (1 patient with genotype 1a and 1 patient with genotype 1b), with NS5A substitutions.[29] In contrast, 4% (11/277) of patients with genotype 3 infection did not have an SVR. One was reinfected. The remaining 10 patients harbored dominant NS5A RASs at relapse.[30] In patients with decompensated cirrhosis (Child–Pugh B), 2 patients had virological breakthroughs and 20 experienced a relapse. Three had wild-type virus at virological failure. The remaining 19 patients all had RASs in NS5A, whereas RASs in NS5B were found in 5 cases together with NS5A RASs (Table 6).[31]

Evolution of Resistant Variants After Treatment Failure

Variants Resistant to Sofosbuvir

Because sofosbuvir-resistant variants are poorly fit, they rapidly disappear after treatment withdrawal in the rare patients in whom they are selected. Their transient selection does not affect sofosbuvir-based re-treatment.[9,11]

Variants Resistant to NS5A Inhibitors

Fifty-eight patients treated with ledipasvir (without sofosbuvir) who did not have an SVR and had dominant NS5A inhibitor–resistant variants at treatment failure were followed up for 96 weeks after treatment. The proportions of patients with dominant NS5A-resistant variants at follow-up weeks 12, 24, 48, and 96, based on deep sequencing analysis (cutoff of 1%), were 98% (42/43), 100% (45/45), 95% (52/55), and 86% (50/58), respectively. These findings indicate that these variants persist for many months and probably many years.[46] These results were confirmed in 67 patients with genotype 1a infection treated with ombitasvir/paritaprevir/ritonavir and dasabuvir who did not have an SVR using population sequencing analysis; 97% (68/70) and 96% (49/51) of these patients still had dominant NS5A resistant variants at weeks 24 and 48 after treatment, respectively.[34] In patients treated with grazoprevir/elbasvir, NS5A RASs were still present at follow-up week 24, based on population sequencing, in the same proportions as at virological failure.[41,42]

Together, these results indicate that variants bearing NS5A RASs selected by IFN-free therapies are long lasting, present as dominant species for several years (maybe forever), and likely to affect the results of re-treatment.

Variants Resistant to NS3-4A Protease Inhibitors

In contrast to NS5A RASs, NS3 protease RASs progressively disappear after treatment has been withdrawn. Among patients treated with telaprevir in combination with pegylated IFN and ribavirin in phase 3 trials, 77% (299/388) of those who did not achieve an SVR harbored resistant variants at virological failure. Kaplan–Meier estimates of loss of telaprevir-resistant variants over time, as assessed by population sequencing, estimated the median time for reversal to dominant wild-type virus to be 10.6 months for genotype 1a

and 0.9 months for genotype 1b. All patients had lost detectable resistant variants by 17 and 13 months after treatment, respectively.[47] In phase 2 and 3 trials of boceprevir, pegylated IFN, and ribavirin, 69% (314/452) of patients who did not have an SVR had dominant resistant variants at the time of treatment failure. Three years after treatment, 73% of the patients (228/314) had only wild-type HCV, based on population sequencing analysis. The median time for return to wild-type was approximately 14 months.[48] In 197 patients given simeprevir in combination with pegylated IFN and ribavirin, 91% (n = 180) who did not have an SVR had resistant variants present as the dominant species at the time of treatment failure. Kaplan–Meier analysis using the results of population sequencing showed a median time to loss of resistant variants of 9 months after treatment for genotype 1a and 6 months for genotype 1b.[14]

This was confirmed in patients receiving IFN-free regimens. In 67 patients with genotype 1a infection who did not have an SVR to the combination of ombitasvir/paritaprevir/ritonavir and dasabuvir, 46% (31/67) and 9% (5/57) still had detectable NS3 protease-resistant variants at 24 and 48 weeks after treatment, respectively.[34] In patients treated with grazoprevir/elbasvir, variants bearing A156G/T/V RASs disappeared within 30 to 120 days posttreatment, whereas D168 RASs needed more time to be replaced by wild-type virus.[41,42]

Together, these results indicate that variants with RASs in the NS3 protease selected by IFN-free therapies are no longer the major populations within a few months after treatment withdrawal. It is not clear whether treating physicians should wait until resistant variants are undetectable before re-treatment if the regimen is to include an NS3-4A protease inhibitor. Also, it is not clear whether RASs that were selected and then disappeared affect re-treatment with NS3-4A protease inhibitor–containing regimens.

Variants Resistant to Dasabuvir

In 67 patients with genotype 1a infection who did not have an SVR to ombitasvir/paritaprevir/ritonavir and dasabuvir, 75% (33/44) and 57% (20/35) had detectable dasabuvir-resistant variants 24 and 48 weeks after treatment, associated with RASs in NS5A.[34] Little is known about their effects on re-treatment with dasabuvir-containing regimens.

Re-treatment Strategies After Failure of IFN-Free Treatment

In patients who did not have an SVR after 8 or 12 weeks of treatment with sofosbuvir/ledipasvir without ribavirin, re-treatment with the same regimen for 24 weeks was disappointing; only 71% of patients (29/41) had an SVR. All failures occurred in patients with NS5A RASs at re-treatment baseline.[33]

Re-treatment strategies based on alternative combinations have been tested in small-scale studies. Fifteen patients who failed to respond to daclatasvir-based regimens were re-treated with sofosbuvir and simeprevir without ribavirin for 12 weeks; 87% (13/15) of them achieved SVR, including 8 of 10 with genotype 1a, 3 of 3 with genotype 1b, and 2 of 2 with genotype 4 infections.[49] In another study, 22 patients with failure to respond to DAA therapy, including 16 who were exposed to an NS5A inhibitor, were re-treated with sofosbuvir, ombitasvir/paritaprevir/ritonavir, and dasabuvir, with or without ribavirin. The proportions of patients with an SVR were 92% (13/14) after 12 weeks with ribavirin in patients with genotype 1a without cirrhosis, 100% (6/6) after 24 weeks with ribavirin in patients with genotype 1a with cirrhosis, and 100% (2/2) after 12 weeks without ribavirin in patients with genotype 1b.[50] Twenty-three patients with genotype 1 infection who did not have an SVR after a first course of 4, 6, or 8 weeks of sofosbuvir plus grazoprevir/elbasvir were re-treated for 12 weeks with the same combination. All of them achieved SVR.[51] We re-treated 11 patients with stage F3 or F4 fibrosis who did not have an SVR to various IFN-free regimens with a combination of sofosbuvir, daclatasvir, simeprevir, and ribavirin for 24 weeks; 2 patients had to discontinue therapy due to severe side effects, 6 patients achieved an SVR, 3 patients experienced a relapse, and SVR data are pending for the remaining 2 patients (Hézode et al, unpublished data, April 2016).

Together, these results indicate that re-treatment strategies can lead to an SVR in large proportions of patients who did not have an SVR to previous DAA regimens, including those patients with RASs at the time of re-treatment. The combination of sofosbuvir with 1 to 3 other DAAs should be considered. The addition of ribavirin and/or prolongation of treatment duration to 24 weeks are recommended in patients with factors of poor response. Careful monitoring is recommended, because the safety of these multiple combinations is unknown.

Resistance Testing in Clinical Practice Versus Treatment Optimization to Prevent Treatment Failure

Tools to Test for HCV Resistance in Clinical Practice

Tools that can be used to test for resistance of HCV to different drugs have been recently reviewed.[52] In contrast to HIV resistance tests, no standardized assays are available as purchasable kits. Resistance testing therefore relies on in-house techniques based on population or deep sequencing. A limited number of reference laboratories have made such tests available in the United States and Europe, and the performance of these assays has not been externally validated. In-house assays are also available in some local laboratories, but their performance and reliability vary. Our experience is that drug resistance testing for HCV is not trivial. It requires polymerase chain reaction amplification of large fragments in 3 genes encoding the NS3 protease, NS5A protein, and NS5B protein. This can be problematic even in experienced hands, especially for HCV genotypes other than 1 and 4. It is a challenge to interpret results because the sensitivity of population sequencing varies among laboratories, whereas deep sequencing cutoffs are not standardized. Not all RASs are clinically meaningful. Their effects depend on their proportion in the viral quasispecies and the

presence of other RASs in the same or other regions. Overall, the number of possible resistance patterns at treatment baseline is almost infinite, and their positive and negative predictive values on SVR are unknown.

Resistance Testing Before First-Line Therapy

There are many conditions for broad use of HCV resistance tests in clinical practice. First, a standardized assay should be available as a purchasable kit, externally validated for its performance, and easy to use routinely in any virology laboratory with experience in molecular biology. Whatever the technology used, the assay should reliably report the presence of RASs with a validated and repeatable sensitivity of 15%, equivalent to population sequencing. Second, interpretation and reporting of HCV resistance data should be homogenized and standardized through recommendations by an international organization. Thirdly, clinically relevant RASs should be clearly identified, and only these should be reported and used for treatment decisions. Finally, guidelines should be provided by international societies for treatment decisions based on results of drug resistance tests. The guidelines should be based on data from clinical trials and real-life studies that reported strong predictive values of the different RAS profiles.

Because none of these conditions have yet been met, systematic testing for HCV resistance before treatment should not be recommended. Systematic testing would seriously limit access to care and lead to erroneous decisions for a number of cases. Instead, treatment can be optimized for groups of patients known to have specific RASs in NS5A that reduce response to therapy. In patients with cirrhosis, guidelines already recommend adding ribavirin to 12 weeks of treatment with sofosbuvir/ledipasvir, sofosbuvir plus daclatasvir, or sofosbuvir plus simeprevir or to prolong therapy to 24 weeks to reduce the rate of failure.[53,54] Resistance data from the integrated analysis of phase 2 and 3 studies with sofosbuvir/ledipasvir[12] indicate that the same measures would reduce treatment failure in patients without cirrhosis who are prior nonresponders to pegylated IFN–based regimens.

On the other hand, the guideline-recommended use of ribavirin with 12 weeks of treatment with ombitasvir/paritaprevir/ritonavir, with or without dasabuvir, in patients with genotype 1a or 4 infection, as well as prolongation of treatment to 24 weeks in patients with genotype 1a infection and cirrhosis (especially those who did not respond to pegylated IFN–based treatment), reduces the rate of failure of this regimen. With grazoprevir/elbasvir, patients who do not respond to treatment with pegylated IFN and ribavirin (partial and null responders) can be treated for 16 weeks with ribavirin without a pretreatment resistance test, making this regimen more widely accessible.[28] Finally, patients with HCV genotype 3 infection (particularly those with advanced liver disease) need reinforcement of therapy with sofosbuvir/velpatasvir.[30,31] These simple strategies can be used to optimize conditions for achievement of an SVR, without the need for resistance testing. This will be made easier by the rapid diminution of these treatment groups in areas in which IFN-free treatments are easily accessible.

Physicians who have easy access to reliable resistance tests and easy communication with the virologists who perform the tests can use these results to guide their decisions. Although clear recommendations cannot be made, detection (by population sequencing or deep sequencing with a cutoff of 15%) of high-level resistance RASs in NS5A (see Table 2) indicates that treatment should be reinforced with ribavirin and/or that therapy should be extended. HCV resistance testing at baseline (for RASs in NS5A at positions 28, 30, 31, or 93) is recommended only in the US label for treatment-naïve and pegylated IFN/ribavirin-experienced patients infected with HCV genotype 1a before treatment with grazoprevir/elbasvir.[55]

Resistance Testing at Treatment Failure (Breakthrough or Relapse)

Patients who experience a relapse after an IFN-free treatment regimen have large proportions of drug-resistant viruses, so resistance testing at the time of relapse does not provide any useful information. Resistance tests should therefore not be performed for these patients.

Testing for Resistance Before Re-treating Patients Who Failed to Respond to DAA-Based Therapy

When the decision is made to re-treat DAA-exposed patients, resistance tests are not absolutely necessary but can guide re-treatment decisions. The decision should be made in a referral center that has access to reliable resistance tests by a multidisciplinary team that includes the virologists who perform the tests. Several re-treatment options exist, based on the combination of sofosbuvir with 1, 2, or 3 other DAAs, with or without ribavirin for 12 to 24 weeks. Too few patients have been included in re-treatment studies for us to address all of the possible situations and make strong recommendations, but careful analysis of patients' resistance profiles at the time of re-treatment can help in selection of an option that will provide the best chance for a cure. This is particularly important for patients with advanced fibrosis or cirrhosis (compensated or decompensated) and patients who have received liver transplants who need rapid cures for their infection.

Conclusions

HCV resistance to DAAs can keep patients from achieving SVR to IFN-free regimens. The presence of more than 15% of HCV variants with resistance to NS5A inhibitors in the patient's quasispecies population at baseline affects the chances for an SVR, especially in specific groups of patients, such as those with genotype 1a or 3 infection and cirrhosis and/or prior nonresponders to pegylated IFN–based treatment. At virological failure, drug-resistant viruses dominate. Viruses that are resistant to NS3-4A protease inhibitors disappear from peripheral blood within a few weeks to months, whereas NS5A inhibitor–resistant viruses persist for years, potentially

impairing the results of re-treatment. Re-treatment options are available based on the combination of multiple DAAs with or without ribavirin. Treatment strategies should be optimized according to current international recommendations to efficiently prevent treatment failure due to HCV resistance without the need for pretreatment resistance testing.

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