



Review

Update on hepatitis C virus resistance to direct-acting antiviral agents[☆]Eva Poveda^{a,*}, David L. Wyles^b, Álvaro Mena^a, José D. Pedreira^a, Ángeles Castro-Iglesias^a, Edward Cachay^b^a Grupo de Virología Clínica, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complejo Hospitalario Universitario de A Coruña (CHUAC), Sergas, Spain^b Department of Medicine, Owen Clinic and Division of Infectious Diseases, UC San Diego, USA

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ABSTRACT

Resistance to direct-acting antiviral (DAA) agents against hepatitis C virus (HCV) infection is driven by the selection of mutations at different positions in the NS3 protease, NS5B polymerase and NS5A proteins. With the exception of NS5B nucleos(t)ide inhibitors, most DAAs possess a low genetic barrier to resistance, with significant cross-resistance between compounds belonging to the same family. However, a specific mutation profile is associated with each agent or drug class and varies depending on the genotype/subtype (e.g., genotype 1b showed higher rates of sustained virological response (SVR) and a higher genetic barrier for resistance than genotype 1a). Moreover, some resistance mutations exist as natural polymorphisms in certain genotypes/subtypes at frequencies that require baseline drug resistance testing before recommending certain antivirals. For example, the polymorphism Q80K is frequently found among genotype 1a (19–48%) and is associated with resistance to simeprevir. Similarly, L31M and Y93H, key resistance mutations to NS5A inhibitors, are frequently found (6–12%) among NS5A genotype 1 sequences. In particular, the presence of these polymorphisms may be of relevance in poorly interferon-responsive patients (i.e., null responders and non-CC IL28B) under DAA-based therapies in combination with pegylated interferon- α plus ribavirin. The relevance of pre-existing resistance mutations for responses to interferon-free DAA therapies is unclear for most regimens and requires further study.

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1. Introduction

According to the 2013 World Health Organization report, about 150 million people are chronically infected with hepatitis C virus (HCV) worldwide and more than 350,000 people die every year due to HCV-related complications (WHO, 2013). Until recently, the only therapeutic option was the combination of pegylated interferon-plus ribavirin (pegIFN-RBV), also known as dual therapy. Both drugs are indirect antiviral agents, because they do not target a specific HCV protein or nucleic acid. More importantly, dual therapy is characterized by both limited efficacy and poor tolerability (European Association for the Study of the Liver, 2011; McHutchison et al., 2009).

Advances in our knowledge of the molecular biology of the HCV replication life cycle have led to the discovery of several molecules that specifically block various viral proteins (Pawlotsky et al., 2007; Soriano et al., 2011). These compounds are globally called direct-acting antiviral (DAA) agents and target different viral non-structural proteins, including the NS3/4A protease, the NS5B polymerase, and the NS5A protein (Table 1).

The first DAAs approved for the treatment of chronic HCV infection were the protease inhibitors (PI) telaprevir and boceprevir. The addition of PI to interferon and ribavirin therapy (triple therapy) significantly improved the efficacy of treatment (Poordad et al., 2011; Bacon et al., 2011; Jacobson et al., 2011; Zeuzem et al., 2011). However, these specific triple-therapy regimens were more complex, with an increased pill burden (e.g., boceprevir-based regimens require four pills every 8 h for a year in addition to pegIFN α -RBV), complex viral monitoring and stopping rules, and the need for frequent clinical monitoring due to worsened side-effect profiles with the potential for severe adverse events.

Two safer and more effective compounds have been recently approved for HCV treatment: the protease inhibitor simeprevir and the nucleotide polymerase inhibitor sofosbuvir. The current quest of HCV therapy development is to find the most effective, tolerable and affordable DAA combination with the least pill burden and highest viral resistance threshold that can cure people infected with HCV in the shortest period of time.

HCV interferon-dependent therapies rely on host factors such as IL28B polymorphism, liver fibrosis stage, and prior pegIFN α -RBV history to predict treatment response (Pawlotsky, 2013; Asselah and Marcelin, 2013; Poveda et al., 2012). While these factors may also affect responses to IFN-free DAA therapies; their impact diminishes as the potency of DAA regimens increases. One key component of the potency of DAA regimens is the resistance barrier for compounds in the regimen and the overall regimen itself. HCV is an RNA virus with an error-prone RNA polymerase, for which some analogies to HIV reverse-transcriptase can be drawn. Drug resistance frequently emerges in HIV patients treated with antiretrovirals and therefore limits the efficacy of these therapies. Given the

known high virion production of HCV (100-fold higher than that of HIV) and error rate of the RNA polymerase (10–4 substitutions per base per year, approximately 10-fold higher than that of HIV reverse transcriptase), the potential for the existence of baseline resistant polymorphism and/or the short-term resistance development following PI exposure is greater than HIV (Neumann et al., 1998; Martell et al., 1992). However, one key difference is the presence of an extremely long-lived viral reservoir in the case of HIV; such a reservoir does not exist for HCV (Soriano et al., 2008).

As we are just entering the era of IFN-free DAA therapy for hepatitis C, many key questions regarding these therapies and resistance remain. What is the clinical significance of baseline DAA resistance polymorphisms? If significant, do these baseline resistance polymorphisms impact different classes of DAAs equally? Is PI cross-resistance a consideration for patients who failed prior HCV PI regimens? If so, for how long, and does resistance testing play any role in determining this? In this review, we present the DAAs according to their mechanism of action, discuss important clinical differences among licensed PI, review the relevant drug resistance profile for each class, according to available *in vitro* and *in vivo* data, and address clinical implications, where appropriate. Finally, we discuss the utility of performing baseline resistance testing to detect baseline DAA polymorphism and discuss specific situations where its use could be clinically meaningful.

2. Main HCV resistance patterns and mutations for DAA agents

Resistance to DAAs is driven by the selection of mutations at different positions in the NS3 protease, NS5B polymerase and NS5A protein (Sarrazin and Zeuzem, 2010; Kieffer et al., 2010; Vermehren and Sarrazin, 2012; Poveda and Soriano, 2012). Each compound or drug family displays a specific mutation profile that may be influenced by the genotype/subtype. Furthermore, each class of DAAs is characterized by a difference in the genetic barrier to resistance; though this general characterization differs for individual agents in the class. Cross-resistance between compounds in the same inhibitor class is of most concern for NS3 protease and NS5A inhibitors.

2.1. HCV protease inhibitors

The HCV protease is a heterodimer located within the endoplasmic reticulum of infected hepatocytes, which results from the combination of viral NS3 with NS4A proteins. The latter acts as a cofactor of the protease. The catalytic site of the HCV NS3–4A serine protease is flanked by the amino-acid triad serine–histidine–aspartate. However, the catalytic site is located in a shallow substrate-binding groove with strong solvent features that does not facilitate tight binding of inhibitors (Sarrazin and Zeuzem, 2010). Therefore, current available PIs depend on few interactions with the enzyme for

Table 1

Direct acting antiviral (DAA) agents approved or in more advanced stages of clinical development.

DAA family			
NS5B nucleos(t)ide analogues inhibitors	NS5B non-nucleoside analogues inhibitors	NS3/4A inhibitors	NS5A inhibitors
Sofosbuvir (Gilead) □	Deleobuvir (NNI-site 1 inhibitor; Boehringer Ingelheim)	Telaprevir (Janssen)*	Daclatasvir (Phase III; Bristol-Myers Squibb)
Mericitabine (Roche, Phase II)	ABT-333 (NNI-site 3 inhibitor; Phase III; AbbVie)	Boceprevir (MSD)*	Ledipasvir (Phase III; Gilead)
IDX-184 (Idenix)	VX-222 (NNI-site 2 inhibitor; Phase II; Vertex)	Simeprevir (Janssen)†	ABT-267 (Phase III; AbbVie)
VX-135 (Phase II)	ABT-072 (NNI-site 3 inhibitor; Phase II; AbbVie)	Faldaprevir (Phase III; Boehringer Ingelheim)	MK-8742 (Phase II; Merck)
GS-6620 (Gilead)	Setrobuvir (NNI-site 3 inhibitor; Phase II; Roche)	Asunaprevir (Phase III; Bristol-Myers Squibb)	Samatasvir (Phase II; Idenix)
INX-189 (Bristol-Myers Squibb)	GS-9669 (NNI-site 2 inhibitor; Phase II; Gilead)	Vaniprevir (Phase III; Merck)	ACH-3102 (Phase II; Achillion)
	Tegobuvir (NNI-site 5 inhibitor; Gilead)	Danoprevir (Phase III; Roche)	
	Filibuvir (NNI-site 2 inhibitor; Pfizer)	Sovaprevir (Phase II; Achillion)	
		ABT-450/r (Phase III; AbbVie)	
		GS-9451 (Phase II; Gilead)	
		MK-5172 (Phase II; Merck)	

The dark background identifies those compounds for which developments has been stopped.

* Approved by the FDA and EMA in 2011.

† Approved by the FDA in November 2013.

ψ Approved by the FDA and EMA in December 2013 and January 2014, respectively.

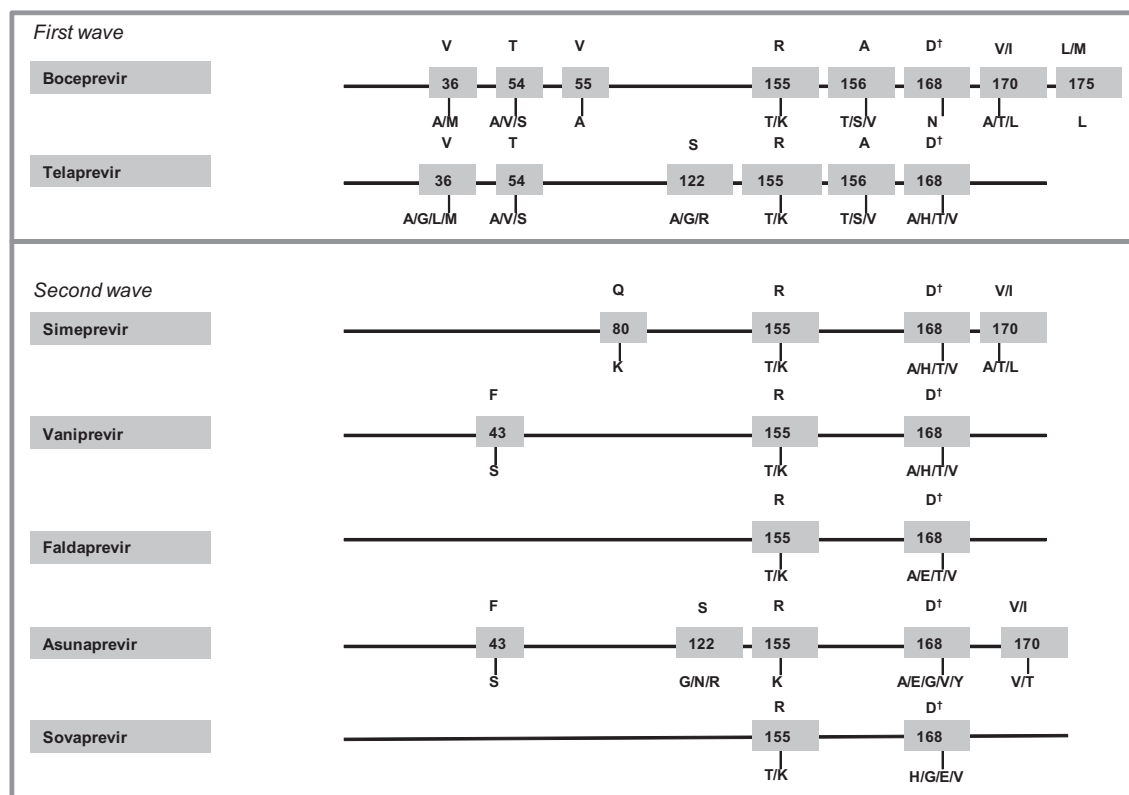


Fig. 1. Main resistance mutations associated with first and second wave of first generation of protease inhibitors. *Q80K is a natural polymorphism found in 19–48% of HCV genotype 1a and is associated with loss of susceptibility to simeprevir. †D168Q is found in almost all HCV genotype 3 conferring natural resistance to most protease inhibitors. Adapted from Poveda and García (2013).

Table 2

Main characteristics of the genotype activity and resistance of DAA classes.

	Genotype activity	Cross-resistance	Key resistance mutations
NS3 protease inhibitors	First PI generation: genotypes 1 (1b > 1a) Second PI generation: across all but genotype 3 (D168Q) Pawlotsky (2013)	High	First PI generation WHO (2013), European Association for the Study of the Liver (2011), McHutchison et al. (2009), Pawlotsky et al. (2007), Soriano et al. (2011), Poordad et al. (2011), Bacon et al. (2011), Jacobson et al. (2011) and Zeuzem et al. (2011): G1a: R155K, V36M G1b: V36M, T54A/S, A156T Second wave and second PI generation Pawlotsky (2013), Asselah and Marcelin (2013) and Neumann et al. (1998): F43S, Q80K, R155K, D168A/E/H/T/V Sofosbuvir* Martell et al. (1992): G1a: S282T+(I434M) G1b: S282T G2a: S282T+(T179A, M289L, I293L, M434T, and H479P) Mericitabine* Soriano et al. (2008) and Soriano et al. (2011): S282T+(K81R,S84S/P, I239L, A300F/L/C, A421V, and Y586C) NNI-site 1 Kieffer et al. (2010): A421V, P495L/S, V499A NNI-site 2 Vermehren and Sarrazin (2012) and WHO (2013): L419S, R422K, M423I/L/T NNI-site 3 Kieffer et al. (2012): C316Y/NS368T, Y448C/H, S556G NNI-site 5 Barnard et al. (2012): C316Y/N, Y448C/H G1a Ogert et al. (2013) and Osinusi et al. (2013): M28T, Q30E/R, L31F/M/V, Y93C/H/N G1b Ogert et al. (2013) and Osinusi et al. (2013): L31F/M/V, Y93C/H/N
NS5 nucleos(t)ide analogs inhibitors	Across all genotypes Sofosbuvir displays less antiviral activity againsts genotypes 3 and requires 24 weeks of sofosbuvir + RBV therapy Kieffer et al. (2012) and Kuntzen et al. (2008)	High	
NS5B non-nucleoside analogs inhibitors	Genotypes 1 (1b > 1a)	Low Overlapping resistance profile for NNI-site 3 and NNI-site 5 inhibitors (C316Y/N and Y448H)	
NS5A inhibitors	Across all genotypes (1b > 1a)	High	

* *In vitro* data. Mutations included in brackets are compensatory mutations improving the replication capacity of S282T variants. References: 1. Sarrazin and Zeuzem (2010); 2. Kieffer et al. (2010); 3. Vermehren and Sarrazin (2012); 4. Poveda et al. (2012); 5. Kieffer et al. (2012); 6. Barnard et al. (2012); 7. Ogert et al. (2013); 8. Halfon and Locarnini (2011); 9. Poveda and García (2013); 10. De Nicola and Aghemo (2014); 11. Summa et al. (2012); 12. Manns et al. (2013); 13. Lenz et al. (2013a); 14. Lam et al. (2012); 15. Pawlotsky et al. (2012); 16. Ali et al. (2008); 17. Larrey et al. (2013); 18. Troke et al. (2012); 19. Lawitz et al. (2012); 20. Lawitz et al. (2010); 21. Zeuzem et al. (2012); 22. Fridell et al. (2011); 23. Gao (2013).

tight binding, and this explain why a few critical mutations may confer significant loss of inhibitory activity (Kieffer et al., 2012; Barnard et al., 2012; Ogert et al., 2013; Halfon and Locarnini, 2011).

In April 2011, telaprevir and boceprevir were the first generation of HCV PIs approved for the treatment of genotype 1-infected patients in combination with pegIFN-RBV. Both drugs are orally bioavailable, linear ketoamide inhibitors which bind covalently but reversibly to the protease catalytic site (Kieffer et al., 2012; Barnard et al., 2012; Ogert et al., 2013; Halfon and Locarnini, 2011).

Several mutations in different positions at the NS3 protease have been associated with loss in susceptibility to PIs (Fig. 1). Resistance to first-generation PIs is characterized by selection of mutations at positions 36, 54, 55, 155, 156, and 170 and the resistance mutation profile is influenced by genotype subtypes (Sarrazin and Zeuzem, 2010; Kieffer et al., 2010, 2012; Vermehren and Sarrazin, 2012; Poveda and Soriano, 2012; Barnard et al., 2012; Ogert et al., 2013; Halfon and Locarnini, 2011; Poveda and García, 2013). Patients infected with HCV genotype subtype 1a mainly select mutations at positions 36 and 155. However, individuals with HCV subtype 1b select changes at codons 54, 55, 156 and 170. This difference in resistance pathways has been explained by the number of nucleotide changes needed at position 155 by genotype 1a and 1b. For example, subtype 1b needs two nucleotide changes at position 155 to produce resistance (R155K: CGG to AAG) whereas only one is needed for subtype 1a (R155K: AGG to AAG). This difference in nucleotide changes required combined with the higher relative fitness of the R155 variants compared to those selected in 1b, such as A156, explains the clinical observation that patients infected with genotype subtype 1b have a greater barrier to resistance than those with subtype 1a (Sarrazin and Zeuzem, 2010; Kieffer et al., 2010, 2012; Vermehren and Sarrazin, 2012; Poveda and Soriano, 2012; Barnard et al., 2012; Ogert et al., 2013; Halfon and Locarnini, 2011; Poveda and García, 2013).

In December 2013, simeprevir was approved by the FDA for the treatment of patients infected with genotype 1 and represents the second wave of the first generation of PIs. The second wave of PIs

includes agents with improved potency and dosing but with resistance profiles that are similar to telaprevir and boceprevir (Lenz et al., 2013a). Both first and second-wave of first generation of PIs are characterized by a low genetic barrier for resistance and broad cross-resistance between compounds (Table 2) (Poveda and García, 2013). It is proposed that the term “second-generation” PIs be used for agents that have an improved resistance profile, such as several that are currently in development (e.g., ABT-450 and MK-5172) (De Nicola and Aghemo, 2014).

2.1.1. Which advantages enhance second-wave and second-generation of protease inhibitors?

First, in contrast to telaprevir or boceprevir, second-wave protease inhibitors represent a significant improvement for dose administration, being administered once daily and are generally better tolerated (Summa et al., 2012). Second, while the first generation PIs are most active against genotype 1, the second wave of protease inhibitors generation are active against all genotypes with the exception of genotype 3, due to the presence of the natural polymorphism D168Q that confers resistance to available PIs (Lenz et al., 2013a). Third, although broad cross-resistance exists between PIs mainly due to the selection of mutations at positions 155 and 156 (the first wave of the first generation) and 168 (second wave or second generation), resistance to the first wave of PIs (telaprevir and boceprevir) does not completely overlap with the second wave or second generation, such as simeprevir, ABT-450, faldaprevir or asunaprevir (Fig. 1) (Kieffer et al., 2010, 2012; Vermehren and Sarrazin, 2012; Poveda and Soriano, 2012; Barnard et al., 2012; Ogert et al., 2013; Halfon and Locarnini, 2011; Poveda and García, 2013; Lenz et al., 2013a; De Nicola and Aghemo, 2014).

MK-5172 is also a second generation PIs that is administered as a once a day pill that seems to be very potent with a broader HCV genotype coverage. *In vitro*, MK-5172 is very potent and retains activity against HCV viruses that harbor resistance mutations to other HCV PIs, such as V36A/M, T54A/S, R155K/Q/T, A156S, V36M+R155K or T54S+R155K. Moreover, MK-5172 is expected to

Table 3

Prevalence of key polymorphisms at NS3/4A, NS5B polymerase and NS5A protein sequences associated with resistance to DAA agents.

Drug family	Mutation	Fold-change in EC50	1a	1b	2	3	4	DAA agents potentially affected by specific polymorphisms
NS3/4A protease inhibitors	Q80K	10.9	19–48%	0	0	0	0	Simeprevir WHO (2013) Asunaprevir European Association for the Study of the Liver (2011) Sovaprevir WHO (2013) Second PI generation McHutchison et al. (2009)
	D168Q	>700	0	0	0	99.2%	0	
NS5B non-nucleoside analogs inhibitors	C316N	>30*		13.3%				Setrobuvir Pawlotsky et al. (2007) (NNI-site 3 inhibitors) ABT-072 Pawlotsky et al. (2007) (NNI-site 3 inhibitors) ABT-333 Pawlotsky et al. (2007) (NNI-site 3 inhibitors)
	L419V	<4				13%		Filibuvir Soriano et al. (2011) (NNI-site 2 inhibitors) VX-222 Poordad et al. (2011) (NNI-site 2 inhibitors) GS-9669 Poordad et al. (2011) (NNI-site 2 inhibitors)
NS5A inhibitors	L31M	3–341		7%				Daclatasvir Bacon et al. (2011) Ledipasvir Jacobson et al. (2011)
	Y93H	5.4–24		6–12.5%				Daclatasvir Bacon et al. (2011) Ledipasvir Jacobson et al. (2011)

* In combination with mutations Y448H, D559G or Y555C. 1. Bae et al. (2010); 2. McPhee et al. (2012a,b); 3. Lenz et al. (2013); 4. Lawitz et al. (2010); 5. Troke et al. (2012); 6. Lawitz et al. (2012); 7. Fridell et al. (2011); 8. Gao (2013).

be broadly active against multiple HCV genotypes (Summa et al., 2012). Recently, results from a phase 2 clinical trial showed promising results with MK-5172-based therapy with sustained viral response (SVR) rates of 89–100% in HCV genotype 1 patients (Manns et al., 2013a).

2.1.2. What is the impact of the natural polymorphism Q80K on the SVR?

The prevalence of natural polymorphisms associated with resistance to HCV PIs has been evaluated in treatment-naïve patients. Using population-based sequencing, <1% of subjects harbored mutations at codons 36, 155, 156 or 168; whereas changes at residues 54 or 55 were seen in 3–7% of patients. However, the polymorphism Q80K is frequently found (19–48%) among NS3 protease sequences from genotypes 1a (Table 3) (Kieffer et al., 2012; Lenz et al., 2013a; Bartels et al., 2008; Kuntzen et al., 2008; Trimoulet et al., 2011; Treviño et al., 2011).

In vitro studies have shown that Q80K reduces susceptibility to simeprevir but not to other second wave PIs such as sofosbuvir, asunaprevir or faldaprevir. Having a Q80K reduces 10-fold susceptibility to simeprevir (Bae et al., 2010) and confers ≤5-fold reduction in the replicon susceptibility to sofosbuvir and minimally increases the EC50 to asunaprevir (3-fold increase) (McPhee et al., 2012a).

In vivo studies support the *in vitro* observations. The Phase II ASPIRE trial evaluated the impact of Q80K polymorphism on the virologic response to simeprevir (Lenz et al., 2013c). Patients infected with HCV genotype 1a that harbored the Q80K polymorphism had a significant decrease in the SVR to simeprevir (100 mg dose) compared to patients without Q80K (22% vs. 70%, respectively). However, an increase in the simeprevir doses to 150 mg was enough to achieve similar rates of SVR irrespective of Q80K status (Q80K 61% vs. Q80Q 66%).

The overall rates of SVR were lower for genotype 1a than in 1b (63% vs. 80%, respectively), similar to that observed for other PIs. The presence of Q80K did not affect sofosbuvir SVR rates in genotype 1 patients after a 28-day administration with pegIFN-RBV (Fabrycki et al., 2012). No significant effect of Q80K on the SVR at week 12 was recognized during the phase II clinical trial evaluating the efficacy of faldaprevir in combination with pegIFN-RBV (75% if wild type vs. 82% if Q80K was present) (Berger et al., 2013).

The Phase III clinical trials QUEST-1 and QUEST-2 assessing the safety and efficacy of simeprevir in combination with pegIFN-RBV in patients infected with genotype 1 refined our understanding of

the impact of Q80K on SVR rates to simeprevir. Compared with those patients infected with HCV genotype 1a without Q80K, lower SVR rates were observed among patients with the baseline Q80K treated with simeprevir (58% vs. 84%, respectively) (Jacobson et al., 2013).

Recent studies have defined at least two clades (clade 1 and clade 2) for subtype 1a, which display different geographic distribution and prevalence of the Q80K polymorphisms. Clade 1 is more frequently observed in NS3 sequences coming from the Americas and is associated with a high prevalence of Q80K (48.9%). Conversely, clade 2 is more frequent in sequences coming from Europe and has a lower frequency of Q80K (De Luca et al., 2013; Pickett et al., 2011). These findings have recently been corroborated with data from simeprevir Phase IIB/III studies (PILLAR, ASPIRE, QUEST-1, QUEST-2 and PROMISE) which showed a higher prevalence of Q80K among genotype 1a protease sequences from North America, compared with those from Europe (48.1% vs. 19.4%, respectively) (Lenz et al., 2013b).

The package insert and the most recent American HCV Guidelines for the treatment of chronic hepatitis C (AAS, 2014) recommend performing baseline resistance testing for Q80K in genotype 1a patients with consideration of an alternative to simeprevir if this mutation is present.

2.2. HCV nucleos(t)ide analog polymerase inhibitors

This family of compounds blocks HCV RNA synthesis by inhibitory competition with the physiologic nucleotide triphosphates for binding to the catalytic site of the enzyme (Ranjith-Kumar et al., 2006). Nucleos(t)ides are also called chain terminators because following incorporation the subsequent nucleotide triphosphate cannot be added to the RNA strand. Nucleos(t)ide analogs display a uniquely high barrier to resistance and possess antiviral activity across all genotypes (Table 2). *In vitro* studies demonstrated that drug resistance mutations to this class are selected within or near the polymerase catalytic site. Thus, the enzymatic activity appears to be impaired to such a large degree that viral replication is seriously compromised. The S282T is the *in vitro* signature resistance mutation to this class. However, the S282T mutation has rarely been detected in patients who failed treatment with nucleos(t)ide polymerase inhibitors in clinical trials. Several nucleotide analogs have shown very promising results and sofosbuvir is the first DAA in this family to gain regulatory approval (Gerber et al., 2013; Pockros, 2013; Soriano et al., 2012).

2.2.1. Is there any influence of HCV genotype/subtype on the resistance profile to nucleoside analogs?

In contrast to NS3 protease-, NS5B non-nucleoside- and NS5A-inhibitors where resistance mutations are subtype-dependent, little is known about NS5B nucleos(t)ide analogs genotype and subtype-dependent resistance mutations.

2.2.2. *In vitro* resistance data for sofosbuvir

The antiviral activity of sofosbuvir against different HCV genotypes and subtypes was tested using *in vitro* replicon systems. For example, sofosbuvir was active against genotype 1a, 1b, and 2a (strain JFH-1) replicons and chimeric replicons containing genotype 2a (strain J6), 2b, and 3a NS5B polymerase. The S282T mutation was the most common one selected among all genotypes, but it only conferred resistance to sofosbuvir in genotypes 1a and 1b (7.8 and 13-fold change, respectively). Genotypes 2a replicons harboring the S282T mutation experienced only a modest reduction in susceptibility to sofosbuvir (2-fold change) (Lam et al., 2012).

Sequence analyses also revealed differences in the resistance profile to nucleoside analogs among genotypes/subtypes. For genotype 1b, S282T was the only change selected after sofosbuvir exposure while for genotype 1a an additional mutation I434M was observed in combination with S282T. In the case of genotype 2a replicons, at least five additional mutations (T179A, M289L, I293L, M434T, and H479P) were selected prior to and after the emergence of S282T. Specifically, S282T together with mutations from both the finger (T179A) and palm (M289L and I293L) domains was essential to conferring resistance to sofosbuvir, while changes at the surface of the thumb domain (M434T and H479T) act as compensatory mutations improving the fitness of S282T variants (Lam et al., 2012).

Other *in vitro* studies evaluated the impact of the S282T mutation on the replication capacity of different genotypes/subtypes in the presence of sofosbuvir. Fitness assays demonstrated that the genotype 1a S282T replicon was the least fit compared with the wild type (3%) followed by genotype 1b S282T (12%), while genotype 2a S282T replicon was the most fit (30%) (Gerber et al., 2013).

2.2.3. *In vivo* data related to sofosbuvir resistance

The S282T mutation has been found infrequently in patients failing sofosbuvir-based regimens. First, in a patient infected with HCV genotype 2b who had detectable HCV RNA (virologic relapse) after 12 weeks of sofosbuvir monotherapy (Gene et al., 2013), second, in a HCV genotype 1 patient who relapsed in the SPARE trial after treatment with sofosbuvir/ribavirin for 24 weeks (Osinusi et al., 2013) and finally in another patient infected with HCV genotype 1 who had a virologic relapse after 8 weeks of treatment with sofosbuvir and the NS5A inhibitor ledipasvir (Lawitz et al., 2014).

2.2.4. *In vitro* data related to mericitabine resistance

Mericitabine is a prodrug of a cytosine nucleoside analog. It is active against all genotypes (1–6) but it has been most extensively studied against genotype 1. The S282T mutation resulted in a moderate 3- to 6-fold reduction in susceptibility to mericitabine but significantly impacted replication capacity with a reduction to 15% compared to wild type replication levels. Similarly to what was seen in genotype 2a with sofosbuvir, S282T can be accompanied by the selection of other mutations (K81R, S84S/P, I239L, A300A/T, L320F/L/J, A421V, and Y586C) that appear to enhance replication capacity (Pawlotsky et al., 2012; Ali et al., 2008).

2.2.5. *In vitro* data related to mericitabine resistance

To date, there is no evidence of selection of S282T in studies of patients exposed to mericitabine in combination with pegIFN α -RBV (JUMP-C trial). However, during the INFORM-1 trial which evaluated the safety and efficacy of mericitabine in combination

with the protease inhibitor danoprevir plus ribavirin, one genotype 1a patient who experienced a viral breakthrough had dual resistance to mericitabine (S282T) and danoprevir (R155K) (Gane et al., 2012). Recently, a double mutant L159F/L320F with impaired replication capacity was identified in one genotype 1b-infected patient failing mericitabine plus pegIFN α -RBV in the PROPEL and JUMP-C trials (Tong et al., 2014).

Mutations associated with resistance to nucleos(t)ide analogs have not been recognized in NS5B polymerase sequences derived from drug-naïve infected patients. Moreover, in patients with HIV/HCV coinfection, the use of HIV nucleos(t)ide analog inhibitors does not increase the rate of primary resistance mutations at the HCV NS5B polymerase, reflecting the large divergence between HIV and HCV polymerases (Plaza et al., 2011).

2.2.6. Is the antiviral activity of nucleos(t)ide analogs inhibitors influenced by genotype/subtype?

Unlike many other DAA, HCV nucleos(t)ide analog inhibitors display pangenotypic antiviral activity. This feature, together with its high resistance barrier and lack of primary resistance makes these drugs very attractive as key components of future DAA interferon-free regimens. However, initial data from sofosbuvir clinical trials (FISSION, POSITRON and FUSSION trials) have demonstrated lower frequencies of SVR against genotype 3 compared to genotype 2. In fact, the rate of SVR for patients infected with genotypes 3 under sofosbuvir/ribavirin therapy varied between 30% for interferon-treatment experienced patients (FUSION trial) to 56% for interferon-naïve patients (FISSION) compared with SVR rates of 86% and 97% in genotypes 2, respectively (Table 2) (Asselah, 2013). Subsequent data from two new studies (VALENCE and LONESTAR-2) have shown increased cure rates for HCV genotype 3 patients under sofosbuvir/ribavirin therapy by increasing treatment duration from 12 to 24 weeks, or by the addition of pegIFN α (85% and 83%, respectively) (Zeuzem et al., 2013; Lawitz et al., 2013b).

Regarding subtypes 1a and 1b, no significant differences were observed in SVR rates to sofosbuvir. The NEUTRINO trial evaluated the efficacy of sofosbuvir in combination with pegIFN α -RBV in naïve patients. This study reported rates of SVR of 92% and 82% for HCV genotypes 1a and 1b, respectively, similarly to that found in recent data from the ATOMIC and NEUTRINO trials (Foster et al., 2014). For genotype 2 interferon-naïve or treatment-experienced patients, sofosbuvir-ribavirin provided excellent results showing rates of SVR of 97% and 86%, respectively. Among genotypes 4 and 5/6 the SVR rates were up to 96%; however, these results must be taken with caution, since only 35 patients infected with these genotypes were included in the trial (Lawitz et al., 2013a).

The efficacy of mericitabine in combination with pegIFN α -RBV in the JUMP-C trial was similar between genotypes 1a and 1b. However, during the INFORM-1 study, which evaluated the interferon-free regimen of mericitabine + danoprevir + ribavirin a significant difference was observed in rates of SVR between genotypes 1a and 1b (26% vs. 71%, respectively) (Gane et al., 2012). In this case, different SVR between 1a and 1b are probably due to lower rates of response to danoprevir for genotypes 1a compared with 1b. Finally, of the five patients receiving mericitabine in the JUMP-C trial, three (60%) achieved SVR (Pockros et al., 2013).

2.3. HCV non-nucleoside polymerase inhibitors

Non-nucleoside polymerase inhibitors (NNI) are non-competitive blockers of HCV-RNA synthesis that interact with the HCV polymerase at allosteric sites outside the catalytic site and prevent conformational changes in the polymerase that are critical to its function. At least five different allosteric binding sites have been identified as targets for non-nucleoside inhibitors (NNI-1, NNI-2,

NNI-3, NNI-4, and NNI-5) as determined by their unique resistance patterns (Pauwels et al., 2007). These sites are located at the thumb (sites 1 and 2) and palm (sites 3, 4 and 5) of a right-hand modeled polymerase enzyme (Bressanelli et al., 2002; De Francesco and Carfi, 2007).

Overall, non-nucleoside inhibitors display the lowest genetic barrier to resistance among DAA. Limited to no cross-resistance exists between NNI belonging to distinct classes, as they interact with different sites of the polymerase. However, some overlapping resistance profiles have been observed at the palm sites. Specifically, the mutations C316Y/N and Y448H associated with resistance to both NNI-3 and NNI-4 site inhibitors, respectively, which reduces the activity of site 3 and 5 inhibitors (Table 2) (Sarrazin and Zeuzem, 2010; Vermehren and Sarrazin, 2012; Poveda and Soriano, 2012). The following sections review changes conferring resistance to each class, which have been well characterized:

2.3.1. Resistance to NNI-site 1 inhibitors

Resistance mutations to deleobuvir are selected at residues 495, 496 and 499 of the NS5B polymerase in genotype 1b patients. The most predominant variants detected at failure were P495L or P495S, which decrease susceptibility to deleobuvir 123–130-fold and 91-fold, respectively. V499A, a polymorphism detected in the majority (>96%) of genotypes 1a, 2, 3 and 4, confers 5.6-fold increase in the EC50 to deleobuvir in genotype 1b. Mutation A421V caused 5.8-fold increase resistance to deleobuvir in genotype 1a (Larrey et al., 2013). The clinical development of deleobuvir has been recently halted due to its lower efficacy against genotype 1a.

2.3.2. Resistance to NNI-site 2 inhibitors

Filibuvir binds to the thumb 2 domain of the NS5B polymerase. Mutations at residue M423I/L/T represent the predominant pathway to filibuvir resistance displaying high-level resistance and reduced replicative capacity relative to the wild-type (Trope et al., 2012). For VX-222, another NNI-site 2 inhibitor in phase II development; breakthrough was associated with the selection of mutations at codons L419, R422, and M423. In the case of GS-9669, a NNI-site 2 inhibitor in early clinical development, substitution at codons L419, R422, and A486 were commonly selected in genotype 1a and 1b following 3 days of GS-9669 monotherapy (Lawitz et al., 2012).

2.3.3. Resistance to NNI-site 3 inhibitors

Setrobuvir is under Phase II investigation. *In vitro* studies have associated the selection of mutations at positions M414, G554, and D559 with setrobuvir resistance. ABT-072 and ABT-333 are also NNI-site 3 inhibitors currently under evaluation in combination with other DAA (i.e., ABT-450, a protease inhibitor) and RBV in IFN-free regimens. *In vitro* studies have revealed mutations to these inhibitors at the following positions: C316, S368, M414, Y448, and S556 (Lawitz et al., 2010).

2.3.4. Resistance to NNI-site 5 inhibitors

Finally, tegobuvir binds to the β -hairpin in the thumb domain of NS5B, representing the first-in-class site 5 inhibitor. The most commonly detected mutation in patients failing tegobuvir-based therapy in combination with pegIFN α -RBV was Y448H. Tegobuvir was evaluated in double, triple and quadruple regimens with the protease inhibitor GS-9256, with or without RBV and/or pegIFN α (Zeuzem et al., 2012). Finally, because tegobuvir was associated with pancytopenia when administered with GS-9256 plus peg-IFN-ribavirin, its development has been halted (Gerber et al., 2013).

The presence of natural polymorphisms associated with reduced susceptibility to some of these agents is very common and differs among distinct HCV genotypes/subtypes. For example the mutation V499A is found in most (>96%) NS5B sequences among genotypes 1a, 2, 3, and 4. Of note, V499A has only been associated with resistance among genotypes 1b (5.6-fold change). Interestingly, naturally occurring polymorphisms potentially causing resistance to NNI were not found among genotype 1b, except mutation C316Y/N that causes resistance to NNI-3 and can be identified in ~13% of NS5B polymerase sequences belonging to genotype 1b (Table 3) (Sarrazin and Zeuzem, 2010; Treviño et al., 2011; Ali et al., 2008).

2.4. NS5A inhibitors

The NS5A protein plays a crucial role in regulating HCV replication and host-cell interactions as an essential component of the viral replication complex. It has a three domain organization: domain I (amino acids 1–213) located at the N-terminal region, domain II (amino acids 250–342) and domain III (amino acids 356–447) at the C-terminal region. A new family of antivirals against HCV was recently found to inhibit the NS5A replication complex by unclear mechanisms involving interaction with NS5A (Tellinghuisen et al., 2004).

Daclatasvir is the first compound in this class, a small-molecule inhibitor that targets NS5A domain I. It has demonstrated potent antiviral activity against HCV replicons from different genotypes. However, higher rates of virological responses to daclatasvir have been observed among genotype 1b patients, compared to genotype 1a. These findings might be explained by a higher barrier to resistance for daclatasvir in genotype 1b than 1a (Table 2) (Sulkowski et al., 2014). Indeed, *in vitro* studies have highlighted that the loss of susceptibility in genotype 1a by the selection of resistance mutations ranged from 233 to 3350-fold, whereas mutations at similar positions only resulted in a 3–28-fold loss in susceptibility to genotype 1b (Gao et al., 2010; Fridell et al., 2010).

Overall, resistance substitutions identified in the *in vitro* replication system correlate well with those observed in the clinic. Several amino acid changes at the N terminus of NS5A domain I at positions M28, Q30, L31 and Y93 for HCV genotype 1a and L31 and Y93 for genotype 1b have been associated with daclatasvir resistance. Daclatasvir is characterized by a low genetic barrier to resistance, especially for HCV genotype 1a in which the selection of a single mutation is enough to lose susceptibility to daclatasvir. Diminished replication capacity has been associated with resistance substitutions at residue Y93 (Fridell et al., 2011; Gao, 2013).

Second-wave NS5A inhibitors (i.e., ledipasvir (GS-5885), BMS-766, ACH-3102, MK-8742, and IDX-719) displaying improved potency *in vitro* against resistant variants selected by daclatasvir are in development. As an example, the mutation Q30E frequently selected in genotype 1a confers high level resistance to daclatasvir (~7500-fold), but only confers 5–50-fold resistance to BMS-766, ledipasvir and MK-8742. However, a broad cross-resistance between NS5A inhibitors is expected by the selection of mutations at codons 31 and/or 93 causing a loss in susceptibility to the majority of these compounds (Gao, 2013).

2.4.1. Resistance to daclatasvir

Natural polymorphisms in NS5A at positions that may influence susceptibility to daclatasvir are less frequently observed in genotypes 1a and 3, with the exception of the polymorphism Q30A found in almost all NS5A sequences from genotype 3 (Plaza et al., 2012). However, Q30A is not a relevant change for resistance to NS5A inhibitors. Mutation L31M is frequently observed among NS5A sequences from genotype 2 (range 50–85%), one of the key mutations to this family among genotypes 1a and 1b. However,

the impact of this mutation has recently been assessed during a clinical trial evaluating the antiviral activity of daclatasvir against genotypes 2 and 3. This study demonstrated that the presence of L31M does not predict failure in genotype 2 patients receiving short-term treatment (12 weeks) with daclatasvir in combination with pegIFN α -RBV (Dore et al., 2013).

Conversely, the presence of natural polymorphism associated with resistance to NS5A inhibitors is very common among genotypes 1b and genotype 4 NS5A sequences (McCormick et al., 2013). For example, mutation Q30R, associated with resistance to NS5A inhibitors in genotypes 1a represents the wild-type (~90%) in NS5A genotypes 1b sequences. More interestingly, the prevalence of the two key mutations associated with resistance to NS5A inhibitors, L31M and/or Y93H, ranged from 6% to 12% among NS5A genotype 1b sequences (Table 3) (McCormick et al., 2013; McPhee et al., 2012b). The impact of baseline mutations associated with daclatasvir resistance was assessed in the COMMAND-1 study which evaluated the efficacy of daclatasvir plus pegIFN α -RBV in genotype 1 treatment-naïve patients. For genotype 1a patients, from 32.7% of failures, 10% had pre-existing variants associated with resistance to NS5A inhibitors (M28T, Q30R/H, L31M/V/I, Y93H/N/C/S/T). At failure, predominant emergent NS5A resistance mutations were at codons 30, 31 and/or 93. Among genotypes 1b, from 14.5% of failures, 20% had baseline mutations associated with resistance to daclatasvir. Of note, in this study the prevalence of Y93H was 10% among genotypes 1b. The most prevalent resistance mutations selected at the time of failure were Y93H, Q30H-Y93H, L31V-Y93H, L31I-Y93H, L31M-Y93H (McPhee et al., 2012b).

Subsequently, the impact of baseline polymorphisms associated with loss of susceptibility to NS5A inhibitors was evaluated in a Phase III trial of daclatasvir combined with asunaprevir. This study highlighted that the presence of mutations at amino acids L31 and Y93 may reduce the barrier to resistance and influence virologic outcome for those patients who carry these polymorphisms at baseline (McPhee et al., 2013). Very recent data from the HALLMARK-dual study suggested that even in genotype 1b patients, the rate of SVR in patients with baseline resistance associated polymorphisms to asunaprevir/daclatasvir was only 40% compared to over 80% with no baseline resistance (Manns et al., 2013b). More recently, the results of safety and efficacy for the combination daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV-infected patients have been published. In this study, although the prevalence of baseline polymorphisms associated with daclatasvir resistance was around 8% for genotype 1 NS5A sequences, all but one patient achieved SVR (Sulkowski et al., 2014).

In addition, recent data have pointed out that due to massive protein–protein interactions in the HCV replication complex the emergence of changes at NS3, NS4B and NS5B proteins might modulate the susceptibility to NS5A inhibitors (Yang et al., 2013).

2.4.2. Resistance to ledipasvir

Ledipasvir is a new NS5A inhibitor with demonstrated antiviral activity against HCV genotypes 1a and 1b. Very high rates of sustained virological response (93–100%) have been reported from Phase II and Phase III clinical trials in combination with sofosbuvir among untreated and previously treated patients (LONESTAR and ION-1 & ION-2 trials, respectively) (Lawitz et al., 2014; Afdhal et al., 2014a,b). Although virological failure is rare using this DAA combination, baseline polymorphisms associated with loss of susceptibility to NS5A inhibitors have been found in half of the patients who relapsed (Afdhal et al., 2014a,b). Cross-resistance is expected between daclatasvir and ledipasvir, mainly due to the presence of mutations at positions L31 and Y93 (Gao, 2013).

3. Clinical implications of DAA resistance: Lessons from clinical trials

Although the antiviral potency of the majority of DAA is extraordinary, the ability of HCV to rapidly evolve in the setting of drug pressure and the presence of baseline natural polymorphisms associated with resistance to DAA must be considered as possible threats to the success of these new therapies. In addition, with the exception of nucleos(t)ide NS5B inhibitors, and second generation of protease inhibitors (i.e., ABT-450 and MK-5172), most DAA agents are characterized by a low genetic barrier to the development of resistance. This is the reason most current DAA-based therapies under evaluation must be co-administered with either pegIFN α and ribavirin or different compounds belonging to different DAA classes. Recent data from clinical trials evaluating the efficacy of DAA have yielded new data that merit particular attention.

3.1. The importance of HCV genotype/subtype

Clinical trials evaluating the efficacy of DAA have shown higher rates of SVR among genotype 1b than genotypes 1a. Uniformly, genotypes 1b displayed a higher barrier to resistance than genotypes 1a for protease inhibitors, non-nucleosides inhibitors and NS5A inhibitors (Sarrazin and Zeuzem, 2010; Kieffer et al., 2010). These findings have highlighted the need for a correct subtype identification in genotypes 1 for an optimized clinical management of patients (European Association for the Study of the Liver, 2011).

In vitro studies have also demonstrated the lack of efficacy of the majority of protease inhibitors against genotypes 3 (with the exception of MK-5172), mainly due to the presence of the natural polymorphism D168Q among HCV genotype 3 protease sequences (Lenz et al., 2013a). Data from sofosbuvir clinical trials demonstrated lower efficacy against genotypes 3 showing SVR rates which ranged from 30% to 56%, compared with rates of 86% and 97% for genotype 2. However, these sub-optimal cure rates have been partially offset by increasing treatment duration from 12 to 24 weeks or by the addition of pegIFN α (Asselah, 2013). Interestingly, this difference in efficacy for genotype 3 is not clearly related to a different antiviral potency or barrier to resistance for sofosbuvir.

3.2. IL28B genotype

Single nucleotide polymorphisms (SNP) in the IL28B gene were identified in 2009 as strong predictors of treatment response to pegIFN α -RBV in HCV-infected patients. The SNP most strongly linked to SVR was rs12979860. Individuals carrying one (CT) or two copies (TT) of the T allele had a higher probability of failure compared with individuals carrying genotype CC (Ge et al., 2009). More recently, data obtained from different clinical trials evaluating the efficacy of several DAA compounds in combination with pegIFN α -RBV also point out the role of the IL28B polymorphism in the virologic response to these new therapies. In fact, in patients receiving a DAA-based therapy in combination with pegIFN α -RBV, virologic failure is more frequent in genotype 1a non-CC IL28B patients.

In this context, it has been observed that the presence of baseline polymorphisms and/or resistance-associated variants might be clinically relevant in IFN non-responder patients, genotypes 1a and non-CC IL28 patients (Barnard et al., 2012; Dore et al., 2013; McPhee et al., 2012b, 2013). However, as new clinical guidelines are moving rapidly away from interferon-based combinations (AAS, 2014), the clinical utility of IL-28 testing is likely to be limited, particularly with more potent, all-DAA regimens.

3.3. Baseline natural polymorphisms

The impact of natural polymorphisms at positions involved in DAA resistance may be negligible in the context of combination therapies when other compounds of the regimen retain full activity. Using DAA-based therapies in combination with pegIFN α -RBV the presence of baseline polymorphisms or resistance associated variants might negatively influence the virologic response in poorly interferon-responsive patients (i.e., genotypes 1a and non-CC IL-28B) (Barnard et al., 2012; McPhee et al., 2012b, 2013). It is noteworthy the high prevalence among patients infected with genotype 1a (19–48%) of the polymorphism Q80K and its negative impact on virological response to simeprevir/pegIFN α -RBV. For these reasons, baseline resistance testing for Q80K is strongly recommended for genotype 1a, and alternative treatments to simeprevir should be considered if this mutation is present.

In theory, for interferon-free regimens the presence of baseline polymorphisms and/or resistance associated variants may have a clinically significant impact. The low genetic barrier to resistance for many DAAs, with the exception of nucleos(t)ide analogs, might facilitate the on-therapy emergence of resistance variants in patients harboring baseline polymorphisms and/or resistance mutations. Indeed, baseline polymorphisms associated with resistance to NS5A inhibitors has a non-negligible prevalence (10–15%) and their presence has been associated with lower rates of virologic response in some daclatasvir-based regimens (Dore et al., 2013; McPhee et al., 2012b, 2013).

3.4. Resistance variants for re-treatment strategies

In the case of telaprevir- and boceprevir-experienced patients, the selection of resistance mutations in the HCV protease might be relevant, if re-treatment with another protease inhibitors (e.g., simeprevir, faldaprevir) is considered. It has been demonstrated that after telaprevir or boceprevir discontinuation, resistance mutations tend to disappear after a median follow-up of 30 months in most patients (>85%) (Sherman et al., 2011). However, there are limited data evaluating re-treatment strategies with protease inhibitors. The C219 study examined the efficacy of a re-treatment strategy with telaprevir/pegIFN α -RBV in 9 patients who had received 14 days of telaprevir monotherapy a mean of 5.7 years prior to re-exposure. In this case, 5/9 had SVR with telaprevir/pegIFN α -RBV (Sarrazin et al., 2013). The OPERA study evaluated the efficacy of re-treatment with simeprevir/pegIFN α -RBV in 5 patients who had been exposed to a short course of simeprevir monotherapy 1.5 years prior. While 3 patients achieved SVR, in the other 2, persistence of low-level resistant variants may have contributed to the observed virologic failure (Lenz et al., 2012). Therefore, due to the potential risk of persistence of protease-resistant variants at low frequencies, re-treatment strategies with low-resistance barrier protease inhibitors in combination with pegylated interferon and ribavirin would not be advisable (AAS, 2014).

Conversely, very recent data from the LONESTAR trial demonstrated the success of a retreatment strategy with sofosbuvir/ledipasvir/RBV in one patient who previously had failed 8 weeks of sofosbuvir/ledipasvir. This favorable response was seen despite the presence of mutations associated with resistance to both NS5A inhibitors (Q30L, L31M, and Y93H) and the S282T mutation associated with sofosbuvir resistance at the onset of re-treatment (Lawitz et al., 2014). Whether this result is generalizable is not known and requires confirmation from ongoing studies.

4. Current role of HCV drug resistance in clinical practice

In summary, there are many relevant clinical questions which remain unanswered, given limited data and the large number of

DAAs soon to be approved for clinical use. For example, can the combination of sofosbuvir and simeprevir be used successfully in patients harboring the Q80K polymorphism? The phase 2 COSMOS study suggests this is the case; however the numbers are too small for certainty. Perhaps resistance in the interferon-free DAA era may not possess the clinical relevance initially expected; largely a result of several promising, highly potent combination therapies (Lawitz et al., 2014; Sulkowski et al., 2014). Undoubtedly, the relevance of pre-existing resistance mutations for responses to interferon-free DAA therapies needs to be better studied. In the meantime, in certain situations resistance testing might help to select the most optimized treatment option.

4.1. Treatment-naïve patients and re-treatment after pegIFN α -RBV treatment failure

In these patient populations, baseline resistance testing for Q80K among genotype 1a is recommended, and alternative treatments to simeprevir should be considered if this mutation is present (AAS, 2014). Although there is a link between baseline resistance variants to NS5A inhibitors and treatment failure, in the context of very potent DAA combinations (e.g., sofosbuvir/daclatasvir) failures seem not to be associated with the presence of pre-existing resistance variants.

4.2. Re-treatment after failure of conventional telaprevir or boceprevir/pegIFN α -RBV

Since cross-resistance exists between first- and second-generation protease inhibitors, and due to the potential risk of persistence of protease resistance variants at low frequencies, re-treatment strategies with protease inhibitors would not be advisable.

Transparency declarations

None to declare.

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