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REVIEW

Hepatitis C virus resistance to the new direct-acting antivirals

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Abstract

Introduction: The treatment of hepatitis C virus (HCV) infection has dramatically improved in recent years with the widespread use of interferon-free combination regimens. Despite the high sustained virological response (SVR) rates (over 90%) obtained with direct-acting antivirals (DAAs), drug resistance has emerged as a potential challenge. The high replication rate of HCV and the low fidelity of its RNA polymerase result in a high degree of genetic variability in the HCV population, which ultimately explains the rapid selection of drug resistance associated variants (RAVs).

Areas covered: Results from clinical trials and real-world experience have both provided important information on the rate and clinical significance of RAVs. They can be present in treatment-naive patients as natural polymorphisms although more frequently they are selected upon treatment failure. In patients engaged in high-risk behaviors, RAVs can be transmitted.

Expert opinion: Although DAA failures generally occur in less than 10% of treated chronic hepatitis C patients, selection of drug resistance is the rule in most cases. HCV re-treatment options are available, but first-line therapeutic strategies should be optimized to efficiently prevent DAA failure due to baseline HCV resistance. Considerable progress is being made and next-generation DAAs are coming with pangenotypic activity and higher resistance barrier.

Keywords: drug resistance, HCV, resistance testing, simeprevir, sofosbuvir, NS5A inhibitors

Article highlights box

- The rapid development of DAAs has replaced interferon-based regimens as HCV therapy. However, the effectiveness of DAAs may be compromised by drug resistance.

- NS3/4A inhibitors and NS5A inhibitors display low barrier to resistance and broad cross-resistance to compounds within the same drug family. However, combination DAA therapy generally allows to overcome antiviral resistance.

- Sofosbuvir exhibits a high barrier to resistance, being mutation S282T rarely been recognized in vivo.

- RAVs causing resistance to NS5A inhibitors tend to compromise SVR in patients with advanced cirrhosis, infection with genotype 1a and 3 and/or prior interferon failure.

- The elevated costs of all-oral DAA therapies may push tailoring therapy; DAA resistance testing may be useful to help to identify the most convenient (cost-effective) treatment and retreatment option for each patient.

- Viral gene sequencing may recognize RAVs, with rates depending on HCV geno/subtype and sensitivity of methods used.

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver disease which can progress to cirrhosis and hepatocellular carcinoma (HCC) and it is the most common indication for liver transplantation in Europe and the United States [1]. According to WHO reports, approximately 120 million people worldwide are infected with HCV, with an estimated global prevalence of 2-3% [2].

Until 2011, the combination of pegylated-interferon (pegIFN) and ribavirin (RBV) was the standard treatment for HCV infection, leading to sustained virologic response (SVR) rates below 40% in HCV genotypes (GT) 1 and 4. Besides its limited effectiveness, interferon-based therapy was associated with a long treatment duration and with frequent and severe adverse effects, especially in cirrhotic patients.

The advent of direct acting antivirals (DAAs) has revolutionized therapeutic options for patients with HCV. New oral interferon-free therapies provides cure rates above 90% in most patients, regardless geno/subtype, prior IFN-experience, and fibrosis stage [3,4]. However, the effectiveness of new DAAs may be compromised by the rapid development of resistance-associated variants (RAVs) [5].

Currently available DAAs are classified into four categories on the basis of their molecular target in the viral lifecycle and mechanism of action: NS3/4A protease inhibitors, NS5A inhibitors, nucleotide analogue inhibitors of NS5B RdRp and non-nucleoside inhibitors of RdRp. The high specificity of DAAs against their viral targets makes them sensitive to small changes in the viral sequence, resulting in emergence of antiviral resistance which plays a key role in IFN-free treatment failure. Given the large HCV genetic variability, the outcome of DAA-based therapies may be altered by the selection of mutations at different positions in the NS3 protease, NS5B

polymerase and NS5A protein which affect viral susceptibility to the administered compounds. Each drug or class of DAAs is characterized by a specific resistance profile that influences the genetic barrier to resistance and differ between viral geno/subtypes [6] as shown in **Table 1**. Currently, different interferon-free combination therapies with DAAs are approved (**Table 2**) and should provide additive or synergistic antiviral potency and prevent the emergence of DAAs resistance [7].

A sequence diversity analysis at drug resistance-associated aminoacid positions is important to evaluate the risk of naturally occurring resistance-related variants present at baseline or the risk for development of drug resistance variants under drug selective pressure [5]. The identification of treatment-emergent resistant variants as well as the impact of preexisting baseline mutations on treatment outcome in patients failing treatment with DAA therapy is essential to predict the rate of cure with distinct DAA combinations and to assess treatment and retreatment options.

2. HCV variability and emergence of Resistance Associated Variants (RAVs)

The combination of a high HCV replication rate, the low fidelity of HCV polymerase and the selective pressures exerted by the host immune system has driven the evolution of HCV towards the development of a global diversity. Phylogenetic and sequence analysis of entire viral genomes splits HCV into seven major distinct genotypes and more than 60 subtypes [8].

HCV has a high rate of turnover with 10^{10} to 10^{12} virions produced per day in an infected patient. The RdRp of HCV has a poor fidelity because it lacks an exonucleolytic proofreading activity. Therefore, HCV replication is error-prone with an error rate of 10^{-3} to 10^{-5} mutations per nucleotide per genomic replication cycle. Consequently, the virus population in a chronic HCV infection exists as a group of genetically distinct but closely related variants, termed “quasispecies” [9]. While the

majority of those variants are cleared by the host immune system or are unable to replicate as a result of mutations that confer loss of function to essential HCV encoded proteins, some variants remain replication competent. Thus, within an HCV-infected individual, this heterogeneous pool of genetic variants consists of a dominant (or "wild-type") HCV strains that replicates to high efficiency, within a background of less fit HCV variants present at lower frequencies [10]. Moreover, some of these minor viral variants can carry aminoacid substitutions which determine conformation changes of a drug-target binding site, and are therefore less susceptible to the drug's inhibitory activity, subsequently leading to a virological breakthrough during treatment or a relapse after treatment cessation [11]. Although these drug resistant variants represent only minor percentages of the total virus population (frequencies <1%), they can be selected and become the predominant viral species during drug exposure, as shown in **Figure 1**.

It is therefore not surprising that RAVs naturally occur in HCV-infected treatment-naive patients [12]. The frequency of baseline RAVs is extremely variable and depends on many factors, such as the replicative fitness of the natural variant, the characteristic of the drug administered, the drug-binding region in the HCV genome and the viral geno/subtype.

The likelihood that a drug will select for and allow outgrowth of viral variants carrying resistance-associated mutations within the quasispecies depends on several factors including (i) the drug's genetic barrier to resistance, (ii) the viral fitness of the resistant variant, and (iii) drug selective pressure [13].

(i) The genetic barrier to resistance refers to the number and type of nucleotide changes needed to result in aminoacid substitutions required to acquire resistance to the antiviral drug and is different between genotypes and also varies on the HCV subtype level. When a single aminoacid substitution is sufficient to confer a high-level resistance, the drug is considered to have a low genetic barrier,

whereas a drug with a high genetic barrier requires multiple mutations within the HCV genome to generate a resistant variant.

(ii) The replication fitness of a resistant variant is defined as its ability to survive and replicate in a highly mutagenic environment. A selected resistant variant must be able to replicate efficiently in order to fill in the replication space left vacant by the susceptible "wild-type" virus during drug exposure. Therefore, a highly resistant but poorly fit variant may not emerge to become the dominant viral species under drug selection pressure and will be less clinically significant than a variant with a preserved replication fitness that can replicate efficiently in the presence of the drug.

(iii) The drug selective pressure is influenced by the drug potency, the level of drug exposure, defined as the drug concentration achieved in vivo relative to the IC_{50}-IC_{90}/EC_{50}-EC_{90} values of resistant variants, and the patient adherence to therapy.

3. Major HCV resistance patterns and mutations

3.1 Resistance to NS3/4A Protease Inhibitors

The NS3/4A protease inhibitors (PIs) bind to the catalytic site of the enzyme and block post-translational processing of the viral polyprotein at cleavage sites, preventing the release of functional proteins, necessary for the production of infectious viral particles.

First-generation HCV PIs telaprevir and boceprevir are no longer recommended, given their limited efficacy (restricted to GT1), troublesome toxicities and low genetic barrier to resistance and considerable cross-resistance. The second wave of first generation PIs includes simeprevir, asunaprevir, paritaprevir and vaniprevir. These drugs exhibit an improved safety profile, a higher genetic barrier to resistance and a better antiviral activity against multiple genotypes, except GT3.

A summary of NS3/4A protease inhibitors resistance associated mutations is given in **Table 3.**

3.1.1 Simeprevir

Simeprevir (SMV) was approved to be used with pegIFN/RBV or as part of all-oral regimens in patients with HCV GT1 or GT4 who are treatment-naive and prior treatment-failures.

Resistance to SMV was reported *in vitro* for several aminoacid changes at key position in NS3: 80,122,155 and 168 [14]. Results from QUEST-1 and QUEST-2 phase III trials assessing the efficacy and safety of the combination of SMV plus pegIFN/RBV in treatment-naive GT1 patients confirmed the replicon studies [15,16]. Most patients with treatment failure had emerging mutations in the HCV NS3 protease domain, which were mainly D168V in patients with GT1b or R155K alone or in combination with aminoacid substitutions at positions 80 or 168 in those with GT1a.

Pre-treatment natural resistance to SMV is rare among HCV GT1-infected patients [17]; however, Q80K variant is associated with a much higher natural prevalence in HCV subtype 1a isolates, leading to reduced susceptibility to SMV when combined with pegIFN/RBV [15,16], but not in association with the nucleos(t)ide NS5B polymerase inhibitor sofosbuvir (SOF) as pointed out in the COSMOS randomised study [18].

The OPTIMIST-1 and 2 studies showed that the effect of Q80K on clinical outcome to SMV plus SOF seems to be substantially attenuated or possibly eliminated. High SVR12 rates were achieved, including in subjects with baseline Q80K, showing the strength of combinatorial treatment [19,20].

No data is available for GT4, the other indication of this combination.

3.1.2 Asunaprevir

Asunaprevir (ASV) has been approved in Japan in combination with the NS5A inhibitor daclatasvir (DCV) in patients chronically infected with HCV GT1b.

In HCV replicon systems and in short-term ASV monotherapy studies [21], the most commonly NS3 substitutions identified were R155K and D168E, which conferred low- to moderate-level ASV resistance in GT1a, and D168V associated with high-level ASV resistance in GT1b.

The impact of pre-existing drug-resistant substitutions on clinical outcome of the combination treatment with DCV and ASV was studied in GT1b infected patients [22,23]. While pre-existing DCV-resistant variants at positions 31 or 93 might compromise the response to this regimen, no ASV-resistant variants were detected at baseline. However, treatment failure was associated with the emergence of both NS5A-L31/Y93 and NS3-D168 variants. While ASV-RAVs that emerged during therapy returned to wild-type, DCV-resistant variants tended to persist in the absence of the drug, suggesting a higher relative fitness of NS5A variants [24].

3.1.3 Paritaprevir

Paritaprevir is coadministered with the pharmacokinetic enhancer ritonavir (paritaprevir/r) and approved in combination with the non-nucleoside NS5B inhibitor dasabuvir and the NS5A inhibitor ombitasvir ± RBV for treatment of GT1-infected patients. This interferon-free regimen is referred to as 3-drug combination (3D).

Aminoacid variants conferring resistance to paritaprevir were detected in NS3 at positions 155 and 168 in GT1a and at positions 156 and 168 in GT1b, *in vitro* or following monotherapy, with the D168V variant conferring the highest level of resistance to paritaprevir in both subtypes [25].

These findings are consistent with resistance analyses conducted in the AVIATOR phase II trial in which the most prevalent NS3 treatment-emergent variant among patients with virologic failure were D168V and R155K [26].

The phase 3 SAPPHIRE-I and SAPPHIRE-II trials evaluating the safety and efficacy of the combination of paritaprevir/r-ombitasvir and dasabuvir with RBV in HCV GT1 infected patients naive or previously treated with pegIFN/RBV, respectively [27,28], showed that the patients who experienced virologic failure during treatment or relapse had at least one aminoacid variant that was known to confer resistance to one of the three DAA agents included in the regimen. The most frequently detected variants in patients with GT1a infection who did not achieve SVR were D168V in the protein NS3, M28T/V and Q30R in the protein NS5A, and S556G/R and M414T in the

protein NS5B. The GT1b-infected with virologic failure had Y56H and D168A/V in NS3, L31M and Y93H in NS5A, and C316N and S556G in NS5B.

No data are available for the 2D regimens of ombitasvir and paritaprevir/r (without dasabuvir) in patients infected with HCV GT4.

3.1.4 Vaniprevir

Vaniprevir is an investigational PI currently approved only in Japan, which exhibits potent antiviral activity in GT1-infected patients when added to pegIFN/RBV.

In vitro resistance selection experiments and sequence data from Phase I and II clinical studies have identified several NS3 variants at positions R155, A156 and D168 associated with decreased susceptibility to vaniprevir [29].

RAVs from patients failing to achieve SVR on vaniprevir-containing regimens from a trial of triple-combination therapy were R155K and D168T/V/Y in GT1a patients and D168H/T/V in GT1b-infected patients. Moreover, R155K variants were observed at baseline in 2 naive patients, who subsequently experienced virologic failure [30]. It is difficult to draw general conclusions from this observation, however, due to the limited data set.

3. 2. Resistance to NS5B Polymerase Inhibitors

The NS5B polymerase inhibitors which interfere with viral replication by binding to the NS5B RdRp can be divided into two distinct categories [31].

Nucleos(t)ide analogue inhibitors (NIs) mimic the natural substrates of the polymerase and are incorporated into the nascent RNA chain causing direct chain termination. This class of DAA shows a high potency, a pan-genotypic activity and a high genetic barrier to resistance because the active site of the HCV NS5B polymerase is strongly conserved among all HCV genotypes.

Non-nucleoside inhibitors (NNIs) usually bind to several discrete sites on the HCV polymerase, which results in conformational protein changes before the elongation complex is formed. A

limitation of this mechanism of action is that these allosteric binding sites are less conserved among genotypes compared to the active site. As a consequence, lower cross-genotypic activity and higher probability of resistance development is observed.

A summary of NS5B polymerase inhibitors resistance associated mutations is given in **Table 4.**

3.2.1 Sofosbuvir

Sofosbuvir (SOF) is the first nucleos(t)ide NS5B polymerase inhibitor approved for the treatment of HCV infection as part of IFN-based and IFN-free regimens.

Using HCV replicon systems, the S282T mutation was most commonly selected. The S282T known as signature NS5B mutation associated with resistance to SOF from *in vitro* studies, has been rarely detected at baseline in Phase II or III SOF-containing clinical trials.; this could be explained by the low replicative fitness of this variant [32].

However, in the ELECTRON trial, the S282T substitution was detected in a patient infected with HCV GT2 who suffered a virologic relapse after 12 weeks of SOF monotherapy [33].

This polymorphism was also found in two GT1-infected patients who relapsed after treatment with SOF/RBV for 24 weeks [34] and with SOF plus the NS5A inhibitor ledipasvir for 8 weeks [35], respectively.

In a pooled analysis of SOF phase III clinical trials for which drug resistance analyses were performed, low-frequency treatment-emergent NS5B substitutions including L159F and V321A were associated with virological failure in some SOF-treated subjects [36].

In a pooled analysis of SOF phase III clinical trials (FISSION, POSITRON, FUSION and NEUTRINO) for which drug resistance analyses were performed, low-frequency treatment-emergent NS5B substitutions including L159F and V321A emerged in several patients infected with HCV GT3 who experienced post-treatment relapse.

3.2.2 Dasabuvir

Dasabuvir (DSB) currently is the only non-nucleoside NS5B inhibitor binding to the palm I site approved as a component of the 3D combination.

A number of RAVs have been selected in HCV replicon or monotherapy studies at several aminoacid positions in the NS5B protein: S556G and C316Y in GT1a, while C316Y and M414T in GT1b. The C316Y variant in both subtypes conferred >900 fold resistance to DSB [37].

3.3 Resistance to NS5A Inhibitors

The NS5A inhibitors target the Domain I of NS5A protein and block phosphorylation of NS5A, which is important for viral replication assembly and release of HCV particles.

HCV NS5A inhibitors are likely to be a component of any multi-drug combination regimens with pan-genotypic activity potent enough to prevent the emergence of resistance mutations. Currently, available NS5A inhibitors are daclatasvir, ledipasvir and ombitasvir. Although NS5A inhibitors are quite potent and have a broad genotypic coverage (which is explained by a more conserved interaction site within the NS5A protein), they are also characterized by their relatively low viral barrier to resistance and long-time persistence of RAVs, as viral fitness seems not to be impaired [24]. All resistance mutations to this class of inhibitors were mapped to the N-terminal region of NS5A (domain I).

A summary of NS5A inhibitors resistance associated mutations is given in **Table 5.**

3.3.1 Daclatasvir

Daclatasvir (DCV) shows a very potent antiviral effect on several HCV genotypes.

NS5A mutations emerged in vivo and associated with failure of DCV mono- or combination-therapy are similar to those selected in the HCV replicon system or with the infectious clone [38].

The primary resistance conferring mutations observed in vivo for GT1a infected patients who did not achieve SVR were M28T, Q30E/H/R, L31M/V, P32L, H58D and Y93H/N and for GT1b the major resistance substitutions were L31M/V, P32L and Y93H/N.

NS5A residues 30, 31 and 93 were the major sites associated with resistance to DCV in most of the genotypes [39], suggesting that the location of the DCV binding site is conserved among diverse HCV strains.

Due to a relatively low genetic barrier, DCV was developed as part of an interferon-free dual therapy in combination with asunaprevir. Subsequently, the impact of baseline polymorphisms associated with loss of susceptibility to NS5A inhibitors was evaluated in an open-label phase III clinical trial of DCV plus asunaprevir in GT1-infected patients. This study highlighted that the presence of mutations at aminoacids L31 and Y93 may reduce the barrier to resistance and influence virologic outcome for those patients who carry these polymorphisms at baseline [40].

More recently, the results of safety and efficacy for the combination DCV plus sofosbuvir for previously treated or untreated chronic HCV GT1-infected patients have been published. In this study, although the prevalence of baseline polymorphisms associated with DVC resistance was around 8%, all but one patient achieved SVR [41].

3.3.2 Ledipasvir

Ledipasvir (LDV) was approved in combination with sofosbuvir against HCV genotypes 1a and 1b. The NS5A aminoacid substitutions Q30E/R, L31M and Y93C/H/N in GT1a and Y93H in GT1b, both in cell culture and in clinical trials, have been associated with high levels of reduced susceptibility to LDV [42,43].

In phase II and III clinical trials, the combination of LDV/SOF ± RBV resulted in high rates of SVR among untreated and previously treated patients with HCV GT1 infection, including those with compensated cirrhosis [44-46]. Although virological failure was rare using this DAA regimen,

NS5A-resistant variants have been found in half of the patients who relapsed both at baseline and at the time of relapse, without NS5B RAVs.

3.3.3 Ombitasvir

Ombitasvir is an HCV NS5A inhibitor with pan-genotypic efficacy, co-formulated as a single tablet with the PI paritaprevir/ritonavir and administered along with the NNI dasabuvir in the 3D combination regimen.

The *in vitro* profile of ombitasvir and the results in the 3-day monotherapy study identified variants conferring resistance at aminoacid position 28, 30, 31, 58 and 93 in the NS5A gene across genotypes 1 to 6; however, the resistance conferred by variants at these aminoacid positions to ombitasvir varied by genotype [47].

3.4. Next generation DAA therapies

As the availability of broad pan-genotypic DAAs remains scarce and the emergence of resistance remains challenging, there is still a search for more potent DAA combination therapies with increasing SVR rates and shorter treatment duration. The second-generation DAAs exhibit improved barrier to resistance as they aim to overcome restrictions in terms of resistance profile of the previous drug classes as well as concerning the coverage of distinct HCV geno- and subtypes. In the coming years, 2016/17, hopefully at least three other DAA-based combinations could be approved: grazoprevir in co-formulation with elbasvir, sofosbuvir with velpatasvir +/- voxilaprevir, ABT-493 plus ABT-530 combination therapy.

3.4.1. Grazoprevir/Elbasvir

Grazoprevir (GZR), a HCV protease inhibitor has been approved in the United States in 2016 in combination with the NS5A inhibitor elbasvir (EBR) either ± RBV for the treatment of chronic Hepatitis C Virus Genotype 1 or 4 infection. Due to their improved structure, GZR and EBR

showed, in vitro, increased potency against some common clinical NS3 and NS5A RAVs selected by previous first generation compounds.

Virologic findings in patients treated with GZR/EBR from phase II and III clinical studies were consistent with the preclinical observations. This therapy combines two DAA agents with distinct action mechanisms and non-overlapping resistance profiles to target HCV at multiple steps in the viral lifecycle, resulting in SVR12 rate up to 95%, even in difficult to treat patients such as cirrhotic, HIV co-infected, or those who previously failed antiviral therapy [48].

The C-SALVAGE study demonstrated that GZR/EBR combination plus RBV for 12 weeks provides a promising retreatment option for HCV-infected patients with genotype 1 with a history of failure on a triple regimen containing earlier-generation protease inhibitor [49].

SVR12 was attained in the 91% of the patients with prior virologic failure harboring virus with documented NS3 RAVs conferring decreased susceptibility to boceprevir, telaprevir, and/or simeprevir at baseline. This new regimen exerts a potent effect on HCV RNA replication and presents a high genetic barrier to resistance and not cross-resistance to the failed protease inhibitor.

The presence of NS3 RAVs at baseline did not significantly affect the efficacy of GZR/EBR ± RBV, although NS5A baseline RAVs had some effect on SVR12 . Results from the C-WORTHY trial suggests that pre-existing NS5A RAVs pose a bigger clinical problem than NS3/4A RAVs (SVR12 82% vs 92%) [50]. Although EBR shows a higher barrier of resistance, NS5A polymorphisms at the same positions as for first generation HCV NS5A inhibitors were observed in patients with treatment failure (M28, Q30, L31, Y93), especially in genotype 1a patients.

3.4.2. Sofosbuvir/velpatasvir +/- voxilaprevir

Promising pan-genotypic regimens in development are the co-formulation of sofosbuvir with the second generation NS5A inhibitor, velpatasvir (VEL) and SOF/VEL plus voxilaprevir (VOX), an experimental macrocyclic HCV NS3/4A protease inhibitor.

In ASTRAL phase III clinical trials, a fixed-dose combination of SOF/VEL for 12 weeks was highly effective in both treatment -naïve and -experienced patients, infected with genotypes 1 to 6, including those with compensated and decompensated cirrhosis and those who did not achieve SVR after prior treatment with other DAA regimens. At baseline, the presence of NS5A resistance-associated variants had no impact on SVR (99%) in patients infected with genotypes 1a, 1b, 2, 4, 5 and 6 in whom only two virologic failures occurred, both in patients with HCV genotype 1 infection. Those two patients who had a relapse, had NS5A-resistant variants at baseline (Q30R and L31M) and at the time of relapse (Y93H) [51].

Otherwise, among patients with HCV genotype 3, the rate of SVR was 88% in patients who had NS5A resistance-associated variants at baseline and 97% among those who did not, with the lowest rate (84%) observed among patients with the Y93H variant at baseline [52].

Data from a phase II clinical trial demonstrated that the combination SOF/VEL plus VOX for 8 weeks was effective, achieving SVR rates over 95% across different patient populations including previously difficult-to-treat patients with cirrhosis, genotype 3 HCV infection and previous nonresponse to treatment. In addition, VOX and VEL retain potent activity in the presence of most commonly detected NS3 and NS5A RAVs, respectively. No specific baseline NS3, NS5A, or NS5B RAV alone or in combination predicted virologic failure, even for those patients with prior treatment experience [53]. Baseline RAVs, including Y93H, the only NS5A substitution which confers high-level resistance to velpatasvir, did not appear to affect response to short durations of this treatment, confirming a very high barrier to resistance of this regimen and further suggesting its potential as a salvage regimen for DAA-experienced patient with longer treatment duration.

3.4.3. ABT-493/ABT-530

One of the exciting new combinations with several phase II clinical trials results being presented is the new second generation HCV protease inhibitor ABT-493 and the NS5A inhibitor ABT-530. In vitro, both compounds demonstrated potent pan-genotypic antiviral activity, with a high barrier to

resistance and maintained potent antiviral activity against key RAVs that often negatively affect the potency of other DAAs .

Moreover, the presence of baseline NS3 and NS5A RAVs did not appear to affect viral load declines during ABT-493 and ABT-530 monotherapy, respectively in treatment-naive adults with HCV genotype 1 infection, with or without compensated cirrhosis [54]. Taken together, these results suggest that the combination of these next-generation DAAs holds promise for more difficult-to-treat patients who harbor NS5A RAVs that are known to confer resistance to currently approved NS5A inhibitors. The combination of ABT-493 and ABT-530 has been advanced into phase II clinical studies in both treatment-naive and previously treated HCV patients with patients with genotype 1 to 6 infections, including patients with compensated cirrhosis, achieving encouraging SVR rates between 97-100%.

4. RAVs before and after treatment failure

4.1. Clinical significance of baseline RAVs

The error-prone nature of HCV polymerase determines that pre-treatment RAVs are likely to occur. Indeed, standard population sequencing and NGS technologies have described the natural existence of RAVs for all DAAs classes [55-57].

RAVs may be present in the inherent sequence of some genotypes and subtypes which could explain the reduced activity of certain DAAs to different HCV genotypes. For example, S556G which confers resistance to dasabuvir is present in 97-100% of HCV genotypes 2, 3, 4 and 5 isolates. The high frequency of this natural variant, together with RAVs at other positions within the NS5B polymerase (M289I/L, C316N), could explain the lack of antiviral activity of this DAA in non-HCV genotype 1 infected patients [57].

It is currently unclear which frequency of RAVs is clinically relevant for the prediction of virologic treatment failure as the impact of pre-treatment RAVs on therapy efficacy is also variable. Several clinical studies have revealed that in most cases, the pre-existence of a RAV is not always related to treatment failure, thus suggesting that many others factors are also implicated. First, the level of resistance of a certain RAV is not necessarily related to treatment failure in a DAA-based antiviral therapy. For example, Q80K -a low level RAV to NS3 PIs- significantly influences virologic treatment outcome in a triple therapy with SMV/pegIFN/RBV; whereas for the combination therapy of SMV/SOF the baseline presence of Q80K seems to be less relevant [18,58,59]. Secondly, in some cases, viral and host negative predictors of virologic treatment response together with baseline RAVs seem to be of clinical relevance. It has been reported that for the combination therapy of SOF/LED, response to antiviral therapy depends not only on the pre-existence of high level resistance NS5A variants (for example, Y93H with a baseline frequency of 3.8-14.1% in HCV GT1b patients), but also on other predictive factors, such as treatment duration and the stage of liver fibrosis [60]. Finally, the antiviral activity and the genetic barrier to resistance of the chosen DAA or combination of different drug classes influence therapy response. In fact, it has been reported that the pre-existence of RAVs seems to have a greater impact on treatment schemes that include DAAs with low barrier to resistance; whereas for regimens with DAAs with high antiviral activities and high genetic barrier to resistance the presence of baseline resistance leads only to a small reduction of SVR rates [5].

4.2. Persistence of RAVs

In HIV and HBV infection, resistant variants are archived for prolonged periods after virologic failure and reselected during retreatment with the same type of drug [61,62] based on their replication with stable DNA intermediates. In contrast, in the case of HCV, studies are contradictory. While in some reports no evidence for long-term persistence and re-selection of

isolates with RAVs during retreatment with the same drug was observed, in others indirect evidence pointed to the possibility of persistence and re-selection [63-65]

After stopping DAA treatment, the frequency of many RAVs with impaired replicative fitness within the HCV quasispecies rapidly decline to levels undetectable by population and clonal sequencing [66,67]. For example, immediately after treatment failure with telaprevir and boceprevir, 82% of patients exhibited RAVs, but persistent variants were detected one year later only in 18% of HCV GT1a infected patients [68]. In the case of patients with repeated protease inhibitor-based therapy, clonal and deep sequencing analysis revealed a continuous evolution of the NS3 genomic region with no clear evidence of persistence and reselection of RAVs but strong signs of independent de novo generation of resistance [65].

In contrast, RAVs to NS5A inhibitors are associated with high replicative fitness and frequently additional compensatory mutations. Indeed, in clinical studies it has been reported persistence of NS5A RAVs over 1-2 years after treatment failure in over 85% of patients [69-70]. After 24 weeks of retreatment with sofosbuvir plus ledipasvir, in patients with detectable NS5A RAVs at baseline the overall SVR rate was 60% which highlights the importance of persistent NS5A RAVs for the selection of effective retreatment options [35].

Regarding the long-time persistence of NS5B RAVs, preliminary data suggest that at least some RAVs (M414T, S556G) may tend to persist during long-term follow-up for at least one year after treatment failure. Interestingly, the persistence rate of NS5B RAVs which occur together with NS5A RAVs appears to be higher in comparison to isolated NS5B RAVs [71].

Although RAVs are almost always observed in patients with virologic breakthrough during treatment, in relapse patients the detection rate of RAVs varies between 53-91% depending on the

duration of treatment, the DAA class and regimen [72-76]. This is most likely explained by the low sensitivity of the DNA sequencing method used, potential rapid reversion to wild-type between end-of-treatment and the day of blood sampling for sequence analysis and a very low frequency of isolates containing RAVs within HCV quasispecies. Moreover, in patients with short duration of DAA-based antiviral therapies, wild-type virus may not be completely eradicated yet which also justifies relapse with a predominantly wild-type variant.

All oral DAA combination therapies have exhibited high efficacy in the majority of patients with chronic hepatitis C. However, given the large number of infected individuals around the world, treatment failure is still expected as a consequence -in most cases- of the combined presence of RAVs and negative predictive host or viral factors, reduced susceptibility to additional antiviral agents or suboptimal treatment duration. Therefore, the problem of persistence, transmission and re-selection of RAVs will be more relevant in the near future [77].

5. HCV drug resistance testing

HCV resistance testing is fundamental to understand the clinical impact of drug resistance, optimize treatment schemes, increase SVR rates and reduce treatment failure. In clinical research or in the clinical setting, the available tools could be used either to determine the individual variant pattern of a patient's quasispecies (genotypic analysis) or to characterize resistance substitutions (phenotypic analysis) in samples collected at baseline (pre-treatment), in case of virological breakthrough or relapse (virologic failure), and after treatment cessation (follow-up period).

The genotypic analysis is based on sequencing technologies which include population sequencing (also called direct sequencing) [78,79], clonal sequencing [80,81] and next-generation sequencing (NGS) [82-84]. The direct sequencing of the HCV genome only exhibits appropriate sensitivity to

determine those dominant HCV variants that are present in the sample's quasispecies with a frequency ≥20%; thus, it is a method useful for generating a consensus sequence. For many years, the only alternative method to population sequencing was genetic cloning followed by Sanger sequencing. Due to the fact that each clonal sequence represents a single variant present in the viral population, this technique shows high sensitivity to detect minor viral variants. However, the number of clones that can be analyzed is limited and the method is time-consuming and laborious. As a consequence, clonal sequencing is now being replaced by deep-sequencing technologies, as they allow reliable and fast detection of numerous viral variants with a frequency down to 0.5-1% [85].

The choice of one method over the other mainly depends on the aim of research and time point of sample collection. For example, pre-treatment samples are analyzed to detect the pre-existence of known or unknown resistance substitutions and/or provide a comparator for on- and post-treatment changes. Because of its high sensitivity, baseline resistant variants are more frequently revealed with NGS targeting short regions of a specific gene for quasispecies analysis [86,87]. On the other hand, at the time of virological breakthrough or relapse, it is important to identify aminoacid changes relative to baseline that confer resistance to the administered drugs. This type of analysis is best performed by NGS or clonal sequencing to describe quasispecies changes. Finally, in the follow-up period, population sequencing would useful if the resistant variant is present as a dominant viral population, but more sensitive techniques (i.e., clonal sequencing or NGS) are required to fully characterize the dynamics of RAV decay after treatment cessation.

Due to the error-prone activity of the HCV polymerase, all possible variants are continuously generated in the mixture of viral populations [88]. Therefore, all above-mentioned sequencing methods may miss some RAVs as a result of their low frequencies within HCV quasispecies below the detection limits of available assays. In addition, other methodological restrictions such as non-

amplification based on HCV RNA secondary structures and primer selection may increase the lack of detection of RAVs.

In some cases, the inability to explain virologic failure requires the selection of candidate resistance substitutions detected in treated patients to perform phenotypic assays. In order to ensure an accurate assessment of the level of reduced susceptibility conferred by the selected substitution and a correct evaluation of its viral fitness cost, it is recommended to carry out this analysis by using site-directed mutagenesis or viral sequence insertion on a chimeric replicon backbone with the same genotype as the original isolate. In addition, to take into account the quasispecies distribution of HCV populations and the fact that resistant substitutions are present in mixtures of viral variant populations, phenotypic assays are best performed on a mixture of isolated clones [89].

The usefulness of performing HCV resistance testing before starting a DAA treatment scheme is still under debate [90]. However, in some situations, resistance testing can be suitable in the clinical practice to decide which DAA is the best treatment option for a given patient.

In the case of treatment-naive patients and patients after failure with pegIFN/RBV treatment, DAA resistance testing is not justified as it has been reported that treatment success with these new drugs occurs in high rates independently of the pre-existence of RAVs. For regimens that include DAAs with high antiviral activities and high genetic barrier to resistance, the presence of pre-treatment RAVs is related to a small reduction of SVR rates. In these cases, it is important to take into consideration additional predictors of response such as the stage of liver fibrosis or baseline viral load.

However, a well-known exception is the Q80K substitution which confers resistance to NS3 protease inhibitors. Unlike other RAVs to this group of DAAs, Q80K exhibits no loss of replicative fitness in the majority of patients and a high probability of pre-existence in HCV GT1a. International guidelines recommend testing for the presence of Q80K in DAA-naive patients

infected with HCV subtype 1a who are being considered for treatment with SMV plus pegIFN/RBV in cirrhotic patients due to the relative high frequency of pre-existing Q80K variants in GT1a in South American (9%), European (18%) and North American populations (48%) [91]. It has been reported that SVR rates in treatment-naive HCV GT1a infected patients with and without Q80K were 58% and 84%, respectively. Therefore, SMV-based triple therapy is not recommended in patients with detectable Q80K substitution at baseline.

In regions with economic limitations, pre-treatment resistance testing may be cost effective in order to avoid virologic failure and the need of retreatment due to the high costs of these new drugs. In addition, HCV resistance testing may also be useful to select optimal treatment schemes in patients with shortened treatment duration, in those with liver cirrhosis, or in patients experiencing virological breakthrough or post-treatment relapse [92], particularly when their treatment comprises NS5A inhibitors. Indeed, in contrast to NS3 protease variants, NS5A RAVs can remain detectable several years after treatment withdrawal in 85% of patients [93], as viral fitness seem not to be impaired. The AASLD/IDSA guidelines recommend testing for RAVs that confer decreased susceptibility to NS3 protease and to NS5A inhibitors, for retreatment of cirrhotic patients or other patients who require retreatment urgently when these patients have history of failure to NS5A inhibitor-containing regimen [94].

Currently, there is no available recommendation for patients who failed to all oral DAA regimens. A proposed approach is longer retreatment with the same class of drugs although lower SVR rates are expected. Due to the high probability of the presence of multiple RAVs as well as the accumulation of negative predictive factors in these patients, monitoring resistance for the persistence of RAVs may be useful to determine the most appropriate and effective DAAs for second-line therapy, thus reinforcing the need of resistance testing in the context of virological failure in the clinical setting. Up to now, it is suitable to wait for results of clinical studies or in case

of urgent need of retreatment, select a different class of DAAs and take data obtained from resistance analysis into thorough consideration [95].

6. Expert Opinion

The confluence of high viral replication turnover and the error-prone nature of the virus polymerase accounts for the large genetic variability displayed by HCV. Within each single infected person, the dynamic quasispecies nature of the viral population explains that mutations causing reduced susceptibility to antivirals are constantly been produced and that they would be selected under drug pressure. Combination antiviral therapy may overcome viral escape due to drug resistance in most instances and halting viral replication for time enough would lead to HCV elimination. This is in contrast with HIV or HBV, for which there is no stable cellular reservoir for the HCV genomic material, namely proviral DNA in HIV and cccDNA in HBV [96]. In the latest, antiviral treatment generally must be keep forever.

Viral gene sequencing may recognize drug resistance substitutions for almost all DAA, with rates depending on HCV geno/subtype [12] and sensitivity of methods used. The choice of the methods for RAV testing depends on the research subject and on the sensitivity expected from the sequencing technique, and on the financial supports of each clinical laboratory. As the investigation of RAVs has to be performed on several genomic regions, with the use of different classes of DAAs in combination, could therefore gain from a complete genome sequencing techniques [97]. In our opinion, the use of NGS in the clinical laboratory and thus the implementation of HCV whole-genome sequencing in clinical practice could help in identifying compensatory mutations located on the outside of the usually investigated regions, directly targeted by antiviral drugs, and recombinant or rare viral types. Besides expanding genomic region coverage, deep sequencing methods may provide unique information on the impact of minor quasispecies that otherwise would be missed

using crude population sequencing. The study of both the natural history of HCV infection and drug resistance could therefore benefit from the advantages of new molecular tools. However, the rapid and constant evolution of assays and their original high costs tend to slow down as NGS is steadily entering clinical practice.

Drug resistance in HCV has reached enough maturity to be considered a key factor in hepatitis C therapeutics. A reduced susceptibility to antiviral agents may be present in both drug-naive and treatment-experienced patients. The rate of natural polymorphisms at positions associated with drug resistance varies across HCV geno/subtypes and each antiviral agent [98]. In contrast, selection of drug resistance following treatment failure occurs in most instances, although long-term persistence is mainly a concern for NS5A inhibitors. Considerable progress is being made and next-generation DAAs are coming with activity against drug-resistant viruses to either NS5A or protease inhibitors. Moreover, these new agents are pangenotypic and exhibit higher resistance barrier [98].

The robustness of sofosbuvir against DAA resistance largely accounts for its pivotal inclusion within most current DAA regimen combinations, being taken along with NS5A inhibitors, protease inhibitors and/or non-nucleoside polymerase inhibitors. All of the latest exhibit low resistance barrier. Besides the well-characterized S282T mutation, two additional changes (C316N and L159F) have recently been shown to confer reduced susceptibility to sofosbuvir. Whereas codon 282 changes dramatically impair viral fitness, C316N and L159F do not. Accordingly, they are recognized as naturally occurring polymorphisms in never treated patients, especially in persons infected with HCV GT1b [99].

Newer DAA are being designed that display greater resistance barrier and could allow building soon sofosbuvir-free, alternative therapeutic options, following the path of 3D for HCV GT1b. As

example, phase 3 trials are ongoing with ABT-530 plus ABT-493, drugs that are pangenotypic NS5A and protease inhibitors, respectively [98].

Although DAA failures generally occur in less than 5-10% of treated chronic hepatitis C patients, selection of drug resistance is the rule in most cases. Of note, most treatment failures are relapses rather than viral breakthroughs on therapy. HCV re-treatment options are available, but first-line therapeutic strategies should be optimized to efficiently prevent DAA failure due to baseline HCV resistance [100]. For patients with cirrhosis or in whom previous treatment with any HCV NS5A inhibitors has failed and require retreatment urgently, testing for RAVs that confer decreased susceptibility to NS3 protease inhibitors (eg, Q80K) and to NS5A inhibitors should be performed using commercially available assays prior to selecting HCV treatment regimen. Given that baseline NS5A RAVs are one of the strongest pre-treatment predictors of treatment outcome with certain regimens, testing for these RAVs should be considered prior to use of ledipasvir/sofosbuvir. If ledipasvir associated RAVs are detected consideration should be given to adding RBV to the regimen and extending therapy to 24 weeks; otherwise treatment with simeprevir, sofosbuvir, and RBV for 24 weeks is recommended. For patients who have both NS3 and NS5A inhibitor RAVs detected, limited data suggest a retreatment approach based on sofosbuvir combined with either grazoprevir/ elbasvir may be efficacious.

Besides compromising therapeutic options, drug-resistant viruses may also be transmitted. This caveat is of particular concern for NS5A inhibitor resistance associated mutations that once selected may persist for years. Transmission of DAA-resistant viruses may occur from patients that have failed drugs within this family and are engaged in high-risk practices, i.e., needle sharing among injection drug users or promiscuous sex among men who have sex with men. As proof of concept, sexual transmission of protease inhibitor resistant HCV has already being reported [77]. However,

the major concern is for NS5A inhibitors, given that resistance mutations to these compounds generally persist for years and produce wide cross-resistance to most agents within this family.

Implementation of HCV drug resistance testing is challenged by the lack of commercial assays, difficult interpretation rules, and the rapid progress of the HCV armamentarium with next-generation DAA that would overcome the impaired response to current DAA driven by resistance-associated mutations, present either at baseline or following prior treatment failure. The usefulness of resistance testing in the clinical setting requires continued scrutiny as the use of different classes of DAAs for chronic HCV infection becomes increasingly widespread. Not all baseline and emergent RAVs will actually confer clinically significant drug resistance. HCV drug resistance testing prior to first-line therapy currently is not recommended [95]. Indeed, the SVR rates are very high both in patients without and with detectable amounts of pre-existing RAVs; therefore, the detection of RAVs will not influence the treatment decision. Resistance testing may be useful in patients experiencing virological breakthrough or post-treatment relapse, particularly when their treatment comprises NS5A inhibitors. So in the context of DAA failure, monitoring resistance for the persistence of RAVs will lead to better management of second-line therapy. Otherwise, the usefulness of performing HCV resistance testing before starting a DAA treatment scheme is still under debate. However, the elevated costs of all-oral DAA therapies may push tailoring therapy, and in some situations like in patients with advanced cirrhosis, also baseline resistance testing can be suitable in the clinical practice to decide which DAA is the best (cost-effective) treatment option for a given patient.

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Declaration of Interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Table 1. Direct acting antiviral (DAA) agents approved

CLASS OF DAAs				POTENCY	GENOTYPIC COVERAGE	BARRIER TO RESISTANCE	CROSS RESISTANCE
NS5B nucleos(ti)de analogue Inhibitors	First generation		SOFOSBUVIR	High	Pan-genotypic	High	High
NS5B non nucleoside Inhibitors	First generation		DASABUVIR	Low	Limited to genotypes 1 (1b >1a)	Low	Low
NS3-4A protease Inhibitors	First generation	1° wave	TELAPREVIR	Medium	Limited to GT1 (1b > 1a)	Low	High
			BOCEPREVIR	Medium	Limited to GT1 (1b > 1a)	Medium	High
		2° wave	SIMEPREVIR	High	Across all but genotype 3	Low	High
			PARITAPREVIR	High	Across all but genotype 3	Low	High
			ASUNAPREVIR	High	Limited to GT1 (1b > 1a)	Medium	High
			VANIPREVIR	High	Across all genotypes less effective for GT3	Intermediate	High
	Second generation		GRAZOPREVIR	Very High	GT1, GT4 and GT6	High	Low
			VOXILOPREVIR	Very High	Pan-genotypic	High	Low
			ABT-493	Very High	Pan-genotypic	Intermediate	High
NS5A Inhibitors	First generation		DACLATASVIR	Very High	Pan-genotypic	Low	High
			LEDIPASVIR	High	GT1, GT4 and GT5	Low	High
			OMBITASVIR	High	GT1 and GT4	Medium	High
	Second generation		ELBASVIR	Very High	GT1, GT4 and GT6	High	Low
			VELPATASVIR	Very High	Across all genotypes less effective for GT3	High	Low
			ABT-530	Very High	Pan-genotypic	High	Low

Table 2. The current recommended treatment options for each genotype for HCV-monoinfected or HCV/HIV coinfected patients with chronic hepatitis C without or with cirrhosis (EASL. Recommendations on treatment of hepatitis C 2015).

	GENOTYPE	
IFN-FREE REGIMENS	HCV-monoinfected or HCV/HIV coinfected patients with chronic hepatitis C without cirrhosis	HCV-monoinfected or HCV/HIV coinfected patients with chronic hepatitis C with compensated cirrhosis
Sofosbuvir + Ribavin (RBV)	2, 3	2
Sofosbuvir/Ledipasvir ± RBV	1,4,5,6 without RBV	1,4,5,6 with RBV or without RBV
Ombitasvir/Paritaprevir/Ritonavir + Dasabuvir ± RBV	1 with RBV (Gt1a) or without RBV (Gt1b)	1 with RBV
Sofosbuvir + Simeprevir ± RBV	1,4 without RBV	1, 4 with RBV or without RBV
Sofosbuvir + Dacalatsvir ± RBV	All without RBV	All with RBV or without RBV
Ombitasvir/Paritaprevir/Ritonavir ± RBV	4 with RBV	4 with RBV

Table 3. Main resistance mutations associated with HCV NS3/4A protease inhibitors in genotypes (GT) 1a and 1b.

NS3/4A Inhibitors	**HCV NS3 Protease Wild-type aminoacid, position and resistance-associated substitution(s)**										
	Genotype (GT)	**V36**	**T54**	**V55**	**Y56**	**Q80**	**S122**	**R155**	**A156**	**D168**	**I/V170**
Simeprevir	GT1a					Q80K/R	S122G/R	**R155K**		**D168/E/V**/A/H	I/V170T
	GT1b					**Q80R**/K/H	S112A/I/T	R155Q		**D168/E/V**/A/F/H/T	
Asunaprevir	GT1a	V36L/M			Y56H/L			**R155K**		**D168E**/A/T/V/Y	
	GT1b	V36G		V55A	Y56H/L	Q80R/K	S122D/G/I/N/T	R155G/Q		**D168E/V/Y**/F/G/H/T	I/V170A
Paritaprevir	GT1a	V36A/M		V55I	Y56H			R155K		**D168A/V/Y**/E/F/I/L/N/T	
	GT1b				**Y56H**					**D168V**/A/K/F/H/I/L	
Vaniprevir	GT1a							**R155K**		**D168T/V/Y**/A/F/H/N/S	
	GT1b									**D168H/T/V**/A/E/F	
Grazoprevier	GT1a	V36L/M			Y56H	Q80K	S122G	R155K/T	A156A/T	D168N/A/V	

	GT1b		T54S						A156T		V170I

*The amino acid substitutions most commonly observed in patients who did not achieve SVR are visualized in bold.

Table 4. Main resistance mutations associated with HCV NS5B Polymerase inhibitors in genotypes (GT) 1a and 1b.

NS5B Inhibitors	**HCV NS5B Polymerase inhibitors Wild-type aminoacid, position and resistance-associated substitution(s)**						
	Genotype (GT)	**L159**	**S282**	**V321A**	**C316**	**M414**	**S556**
Sofosbuvir	GT1a	L159F	S282T/R	V321A	C316N		
	GT1b	L159F	S282T		C316N		
Dasabuvir	GT1a				C316Y	**M414T**/I	S566G/R
	GT1b				C316Y	M414T/I	S566G/R

*The amino acid substitutions most commonly observed in patients who did not achieve SVR are visualized in bold.

Table 5. Main resistance mutations associated with HCV NS5A inhibitors in genotypes (GT) 1a and 1b.

NS5A Inhibitors	**HCV NS5A Wild-type aminoacid, position and resistance-associated substitution(s)**					
	Genotype (GT)	**M/L28**	**Q/R30**	**L31**	**H/P58**	**Y93**
Daclatasvir	GT1a	**M28T**/A/S/V	**Q30E/H/R**/D/G/K/T	**L31M**/I/V	**H58D**/R	**Y93H/N**/C
	GT1b	L28M/T	R30G/H/P/Q	**L31M/V**/F/I	P58S	**Y93H**/N
Ledipasvir	GT1a	M28T/A	**Q30E/H/R**/K/L/R/Y	**L31M**/P	H58D	**Y93C/H/N**/S
	GT1b			L31I/M/V		**Y93H**/C
Ombitasvir	GT1a	**M28T**/V/A	**Q30R**/K/E		H58D	Y93C/H/N/S
	GT1b					Y93H/C
Elbasvir	GT1a	M28T/G/A	Q30R/H/Y/L	L31M/V	H58D	Y93H/N
	GT1b			L31M/F		Y93H

*The amino acid substitutions most commonly observed in patients who did not achieve SVR are visualized in bold.

Figure 1. Emergence and selection of drug resistant variants.