

REVIEW

Causes of treatment failure for hepatitis C in the era of direct-acting antiviral therapy

Joaquín Cabezas, Susana Llerena, Ángela Puente, Emilio Fábrega and Javier Crespo

Digestive Health Service. Hospital Universitario Marqués de Valdecilla. IDIVAL. Facultad de Medicina. Universidad de Cantabria. Santander, Cantabria. Spain

ABSTRACT

Hepatitis C therapy in the era of the newer direct-acting antiviral agents has radically changed our treatment schemes by achieving very high rates of sustained virological response. However, treatment with direct antiviral agents fails in a subgroup of patients. This group of so-called difficult-to-treat individuals is the subject of this paper, which reviews the causes of virological failure, their clinical implications, and some final recommendations.

Key words: Hepatitis C. Direct-acting antiviral therapy. Resistance-associated variants. Treatment failure.

INTRODUCTION

Hepatitis C virus (HCV) was discovered in 1989 (1). Two decades later, the in-depth understanding of this virus's life cycle has led to impressive therapeutic innovation.

At present, antiviral therapy can cure the infection in most patients using short interferon (IFN)-free schemes with a minimum of side effects. Also, while multiple aspects remain investigational, we shall likely significantly reduce morbidity and mortality with both hepatic and extrahepatic, particularly cardiovascular and renal, causes. This revolution has been associated with some paradigm changes regarding this infection. First, its discovery itself, which used a till then unknown strategy searching for a viral infection that had been suspected but hidden from the view of researchers for years. Secondly, aiming at speeding up the development of novel direct-acting antiviral agents (DAAs), major regulatory agencies such as the FDA and EMA approved fast clinical trials by avoiding comparison with standard therapy. Thirdly, in many regions, particularly in Spain, intense societal mobilization, only simi-

lar to the early days of the HIV pandemics, has occurred. Scientific societies have had to change their strategy for consensus meetings, often born obsolete, in view of rapid drug innovation. Even from a financial standpoint, both the pharmaceutical industry and authorities have made a considerable effort to replace the standard practice of purchasing small volumes at a high price by a reverse approach. Finally, strategic plans have been developed to thoroughly address infection management in both the short and the long run (2).

All the above nurtures optimism. When witnessing the results obtained by the newer IFN-free therapies our eyes are irresistibly drawn to their huge effectiveness. This is in fact higher than 90% in virtually all clinical trials regardless of viral load, IL28B haplotype, genotype or sub-genotype, presence of cirrhosis, absence of response to a previous IFN-based regimen, coinfection with HIV, or presence of advanced kidney failure. When we examine sustained viral response (SVR) rates in the real world, not even results differ much from those obtained in clinical trials, with effectiveness rates oscillating between 82% and 93% (3). However, when we focus on the picture negative, patients failing to this therapy are nowhere near a residual population. It is estimated that, in the USA, nearly 150,000 people have received combined sofosbuvir and simeprevir. By simple cross-multiplication, if 7% to 17% of these 150,000 cannot be cured, between 10,500 and 27,000 patients will have failed to respond to therapy. If we extrapolate these figures to our country setting, results are scarcely reassuring; thanks to our Plan Estratégico Nacional for hepatitis C (2) we are in a position to treat over 50,000 within a short period of time, probably shorter than the 3 years initially foreseen. Using the same rule of three and assuming that real-world results in Spain will

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Correspondence: Susana Llerena. Department of Digestive Diseases. Hospital Universitario Marqués de Valdecilla. IDIVAL. Facultad de Medicina. Universidad de Cantabria. Avda. Los Castros, s/n. 39005 Santander, Cantabria. Spain
e-mail: sllerena@humv.es

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be similar to those obtained in the US, patients failing to IFN-free antiviral therapy will amount to a figure between 2,600 and 10,400 (Fig. 1).

The expressions “difficult-to-cure” and “difficult-to-treat” are used indistinctly in the literature, which renders challenging establishing a reason why some patients cannot be freed from infection (4-7). In this review we shall use the term “difficult-to-cure patient” to refer to failures related to virological characteristics, which we shall discuss in depth, and the term “difficult-to-treat patient” to failures associated with one of the following three reasons: a) poor adherence; b) early therapy discontinuation because of an unlikely side effect or a not so unlikely clinically relevant interaction; and c) loss to follow-up, in close relationship to poor adherence, which precludes the assessment of SVR endpoint attainment (Table I).

HOW SHOULD THE DIFFERENTIAL DIAGNOSIS OF IFN-FREE THERAPY FAILURE BE APPROACHED?

We usually categorize therapy failure according to the time of its development: within-treatment recurrence (virological rebound or breakthrough), post-treatment recurrence (relapsing infection), and primary absence of response. However, this long useful classification is not truly of value just now, when DAA therapy failure usually develops after treatment, with breakthroughs being exceptional. The virological causes of therapy failure (Table II) may be categorized as: a) genotyping errors; b) genetic recombination phenomena; c) treatment-resistant variants (whether pre-extant or acquired following initial exposure to DAAs); d) persistent infection, usually with the emergence of new predominant isolates; e) reinfection; and f) superinfection. We shall discuss these in greater detail.

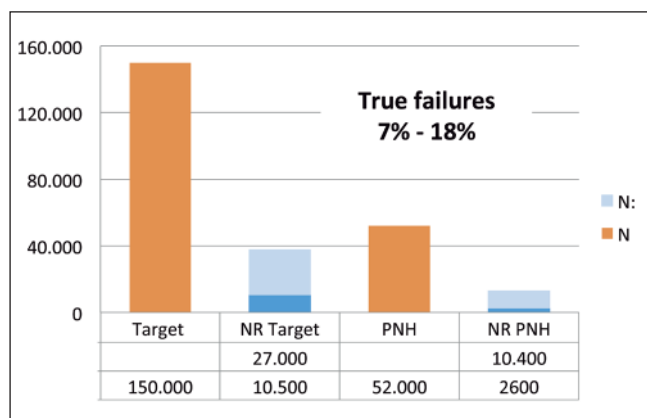


Fig. 1. Number of subjects that may fail to respond to IFN-free therapy (NR: Non-responders; PNH: Plan Estratégico Nacional frente a la Hepatitis C).

Table I. Difficult-to-cure patients

- Difficult-to-cure patients with IFN-based therapies:
 - Genotype 1
 - High viral load
 - Untoward IL28B polymorphism
 - Absence of response to prior therapy
 - Compensated cirrhosis
 - Coinfection with HIV
 - Advanced renal failure (grade 4 or 5)
- Difficult-to-cure patients at present (IFN-free therapy)
 - Genotype 3, particularly in previously treated or cirrhotic individuals
 - Compensated cirrhosis
 - DAA therapy failure

Table II. Virological causes of failure

- Genotyping error
- Treatment resistance associated variants (RAVs):
 - Present before treatment onset
 - Developed as a consequence of treatment
- Genetic recombination phenomena
- Persistent infection
- Reinfection
- Superinfection

Genotyping errors

HCV has high genetic heterogeneity (8). According to their homology degree, HCV isolates have been clustered together into seven genotypes showing up to 30% divergent nucleotide sequences. In turn, the various genotypes show notable differences in their sequence (up to 20%), which allows their classification into subtypes. Their geographical distribution is also different, with genotype 1 predominating in the US, Japan, and Europe, whereas genotype 4 is found in Egypt and genotype 3 is dominant in the Indian subcontinent. For years we have known that genotype conditions SVR, particularly for IFN-based regimens, even though the reason was never definitively elucidated. In the era of direct-acting antiviral therapy appropriate viral isolate genotyping remains crucial since most DAAs directed against HCV have variable sensitivities according to genotype and even subtype, which entails various SVR rates and resistance patterns (9).

HCV genotyping is a well-established technique involving the assessment of a highly preserved viral region where differences exist between genotypes and subtypes. Other genotyping techniques include: direct sequencing and subsequent phylogenetic analysis; PCR followed by hybridization using genotype- and subtype-specific probes; and serological techniques. Genotyping using serologic methods lacks sensitivity and specificity, which renders its use marginal and we shall not discuss it. Sequencing and subsequent phylogenetic analysis for the obtained sequence

is the gold standard technique; however, routine use is not recommended given the high genetic variability of the regions usually sequenced (NS5b, NS3), which renders results interpretation challenging. Therefore, in order to genotype a sample we usually use PCR to amplify a highly preserved region in the HCV genome, namely non-coding region 5' or 5'-UTR. Furthermore, commercial essays have been developed in the last few years that may perform genotyping depending on differences in region NS5b (10). However, despite advances in HCV genotyping, the inability to genotype or subtype a sample is not uncommon. Let us see some recent examples: a) in a sample of 1,052 patients, Benedet et al. (10) demonstrate that commercial assays cannot discriminate approximately 9-10% of cases; b) similarly, Josep Quer et al. (11), assuming the hypothesis that current genotyping techniques result in false identifications, analyze the value of deep sequencing and phylogenetic analysis for the correct identification of genotype, subtype, and the odd mixed infection. Following the analysis of 32 samples with undetermined genotype and 81 samples with genotype 1 and undetermined subtype, they observed that, while Lipa 2 improves genotype and subtype identification, deep sequencing is the most valuable method for appropriate genotyping, achieving satisfactory results in all cases; c) finally, Silberstein et al. (12) performed a phylogenetic analysis for 343 HIV-infected patients before initiating an IFN-free antiviral therapy regimen. In this subgroup of patients a classical genotyping approach was used, and region NS3 was sequenced for most subjects (NS5a for 9% and NS5b for 14% of samples). When more than a genomic region per patient was tested (n = 52), sequencing results were 100% consistent, which confirms specific genotype/subtype allocations. In contrast, consistency between commercial genotyping and sequencing was 91.8%. Furthermore, sequencing resulted in appropriate typing for all patients with indeterminate or mixed genotyping. Hence, nearly 8% of patients needed sequencing for proper genotype and/or subtype assignment. Highly similar results had been previously reported (13). Although not currently established, genotype characterization via sequencing is probably interesting for patients

with indeterminate genotypes or genotype 1 and impossible subtyping before prescribing a DAA-based regimen. On the other hand, a group in which viral genome sequencing will be essential is that of patients who failed to respond to a prior IFN-free regimen including two or more DAAs.

Genetic recombination processes

HCV is a flavivirus that replicates using RNA intermediates. Some *Flaviviridae* are known to infect cells with two different strains. When this happens crossover phenomena may take place between RNA intermediates that result in hybrids, recombinant viruses with both sets of characteristics. The potential for this genetic setup had been recognized for HCV, but was thought to be a rare occurrence of uncertain clinical impact (14). However, such claim might be partly untrue, as shown in a recent paper by Hedskog et al. (15) we had the opportunity to contribute to. This paper assesses divergences in genotyping using two systems: a test for region 5' NC (InnoLipa) and a sequencing technique for region 3' NC. Most of the over 2,000 samples analyzed were consistent but two patients had inconsistent results, with the testing of region 5' suggesting a genotype 2 and the sequencing of region 3', a genotype 1 (Fig. 2). Later the virus was thoroughly sequenced, which showed the presence of hybrid viruses with a recombination site in region NS2/NS3. Also, this virus had a significant clinical impact. For instance, our patient was categorized as genotype 2 and treated as such with a short regimen containing sofosbuvir and RBV. After an excellent response to therapy, viral load recurred. This recurrence developed when the virus behaved as genotype 1. In fact, all hybrid viruses behaved similarly; only 3 of 11 patients with a theoretical genotype 2 responded to the above regimen. Obviously, this genetic recombination process may account for some (probably few) failures to respond to cutting-edge antiviral therapy through choice of suboptimal regimens. Furthermore, while recombination is only described between genotypes 1 and 2, other recombination events cannot be excluded.

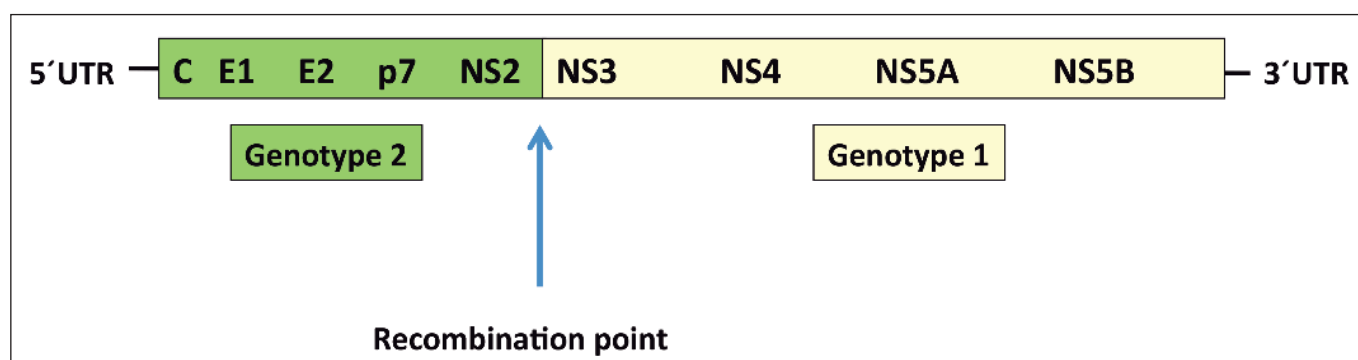


Fig. 2. Genetic recombination phenomenon between genotypes 1 and 2.

Resistance-associated variants (RAVs)

The huge amount of virions produced daily (10^{12}), high rate of mutation (10^{-4} a $\times 10^{-5}$ per nucleotide), and poor ability to repair RNA-dependent RNA polymerase errors explain the wide genetic diversity of HCV. As a result of such variability HCV exists as multiple closely-related genetic variants called quasispecies. This genetic variability accounts for the higher development of resistance-associated variants (RAVs) as compared to HBV or HIV (16,17).

RAV identification primarily depends on the method used (Fig. 3). Using population sequencing we may detect variants representing at least 10% of the viral pool; with more sensitive techniques, such as clonal sequencing, we may detect variants amounting to only 1% of the entire viral population; finally, when using extraordinarily sensitive techniques such as next generation sequencing (NGS) we may even test for populations representing less than 0.5-1.0% of the total sample. RAVs alone do not account for resistance to therapy. Indeed, resistance is associated with: a) the quantitative relevance of RAVs; b) antiviral regimen potency; c) genetic barrier to DAAs (number of mutations necessary for HCV to become resistant to

DAAs); and d) viral fitness. Also, though still lacking scientific support, RAV development is likely to increase as a result of suboptimal adherence to treatment.

A description of the most common RAVs (Fig. 4)

RAVs involving NS3 are seen with a relatively low frequency before treatment (< 3% in naïve and < 7% in experienced patients) (18). Those most commonly associated with failed therapy emerge as a consequence of treatment with telaprevir and boceprevir.

The most important one is maybe R155K/T, whose fitness may improve when in association with another RAV, namely V36M. RAVs most commonly associated with second-generation protease inhibitors again include R155K and D168A/V/E/T. Polymorphism Q80K, frequent in infection with genotype 1a, is associated with resistance to simeprevir (19). As may be seen in figure 4, multiple potential NS5A variants may induce resistance to NS5A complex inhibitors. For daclatasvir, L31V/M and Y93H/N are most common (20). Variants resistant to ombitasvir are identified at positions 28, 30, 58 and 93, virtually exclusively in infection with genotype 1a. Resistance patterns

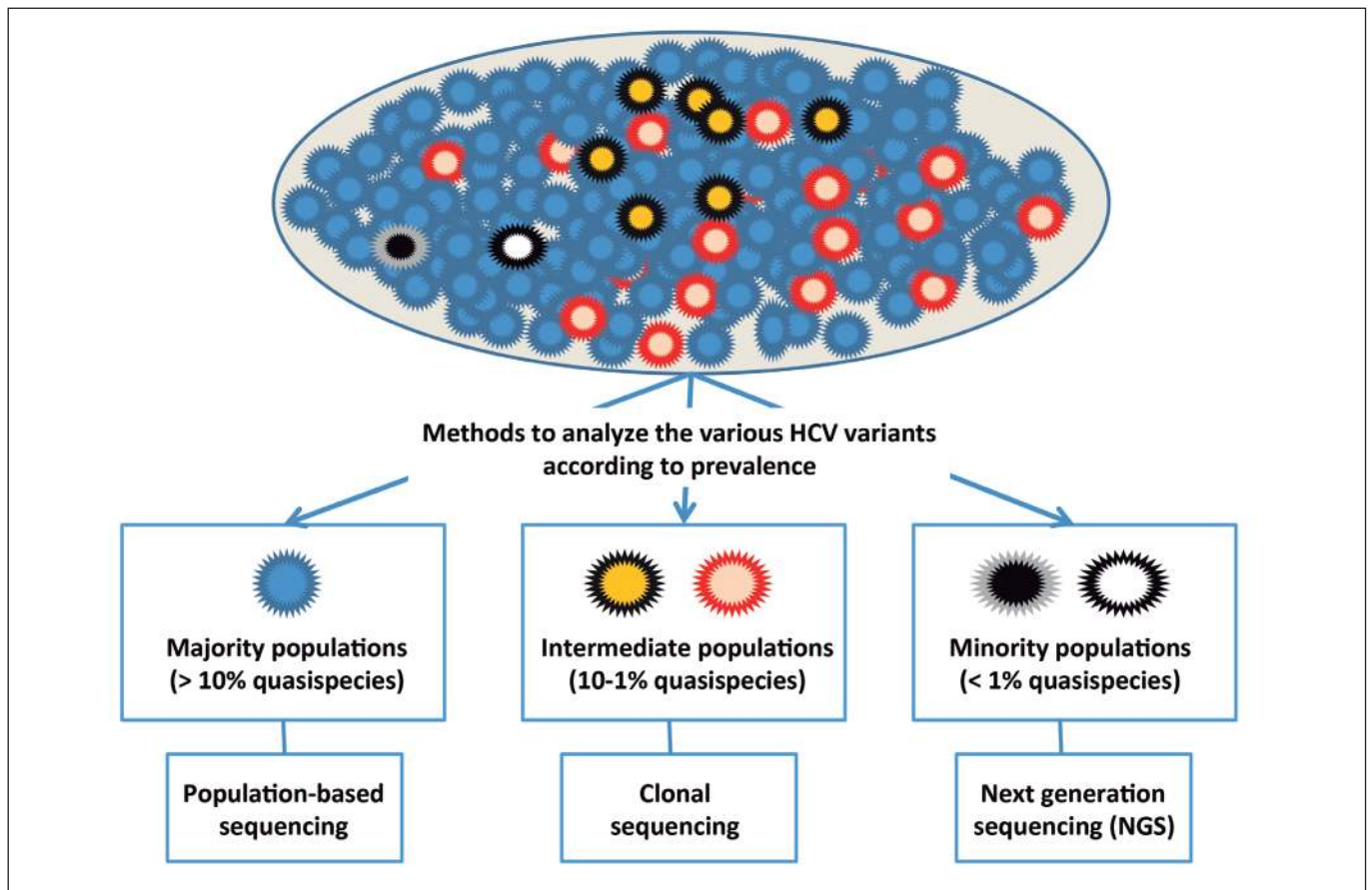


Fig. 3. Methods to analyze the various HCV variants according to prevalence.

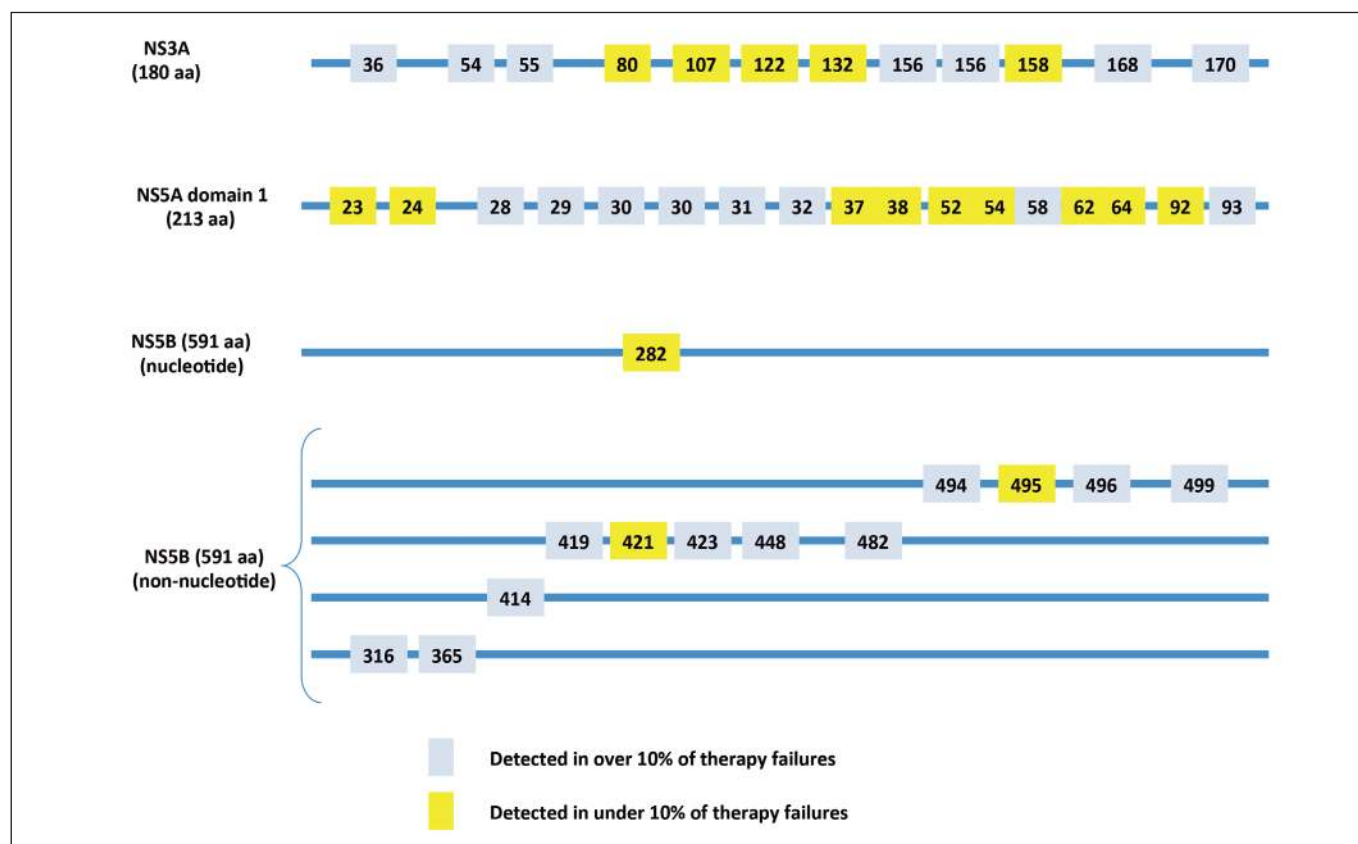


Fig. 4. Variants associated with resistance to direct antiviral agents.

regarding ledipasvir are similar. The NS5B S282T substitution is the only resistance mutation associated with reduced susceptibility to NS5B nucleotide inhibitors, with sofosbuvir being the paradigmatic molecule; it develops minimally and is unrelated to other NS5B RAVs (21). It was initially detected in the Electron study with sofosbuvir monotherapy in a patient infected with genotype 2 who relapsed at week 4 post-treatment (22). Thus far this RAV was only detected in 4 patients. NS5B RAVs conferring resistance to non-nucleoside NS5B inhibitors (such as dasabuvir or tegobuvir) are much more common than those involving nucleotide inhibitors, and are also associated with viral resistance and breakthrough. Overall, they are more common in genotype 1a than in genotype 1b (23), although variant C316N is more often seen in genotype 1b (24). RAV C316N/H/F has been identified at baseline in 6 patients with genotype 1b HCV who failed to respond to sofosbuvir, and in a patient with genotype 1a who later recurred; however, further studies are needed to appropriately establish the role of RAVs as a cause of resistance to sofosbuvir (25).

Clinical significance of RAVs

The information available on RAVs capable of conferring resistance to DAAs is increasingly extensive; how-

ever, many aspects are not fully understood at present (26-28). Based on incomplete data, and on statements that might well be qualified in the upcoming future, most relevant questions include:

- *Does the baseline presence of RAVs correlate with lower SVR?* No single answer to this question is available. As a rule, baseline RAVs do not decisively affect SVR probabilities, particularly NS3-related RAVs in minority populations (< 1%). The impact of NS5A-related RAVs is variable, with SVR being most commonly absent when other negative predictive factors, including cirrhosis, are present (29). Some authors have shown that RAV-conditioned NS5A inhibitor activity compromise is higher in genotype 1a as compared to genotype 1b (30). A potential impact on retreatment has also been demonstrated in two studies: in the first one all therapy failures occur in the group of patients with RAVs (31); in the second study, virtually all 22 patients not achieving SVR (out of 471 patients on grazoprevir and elbasvir) had RAVs, often present before treatment onset (32). However, not all studies are consistent, as shown by the fact that among 94 patients with baseline RAVs (from a series of 511, 18% with cirrhosis) receiving sofosbuvir and ledipasvir, SVR rates were similar for patients with and without baseline RAVs (33).

In contrast, the ION-3 study showed that in patients who had previously received treatment for 8 weeks and could not achieve SVR, retreatment for 24 weeks was associated with very low SVR rates (below 50%) when two or more NS5A-related RAV were present at baseline (34).

- *Do RAVs persist or tend to disappear?* Long-term persistence has been assessed in a very high number of patients and was recently reported at the last EASL meeting. Protease inhibitor-related RAVs disappear both following therapy with simeprevir (35) and regimens including paritaprevir (36) or grazoprevir (32). However, RAVs associated with NS5A inhibitors persist over time in patients receiving ledipasvir (31), ombitasvir (36) or elbasvir (32). Similarly, RAVs associated with non-nucleotide NS5B inhibitors such as dasabuvir also persist over time (36). Furthermore, such persistence may be long protracted as shown by Dvory-Sobol et al., who acknowledged the presence NS5A-related RAVs up to 96 weeks after treatment completion, which suggests fitness for these variants (31).
- *RAV-driven therapy?* A highly relevant aspect is whether therapy should be guided by the prior presence of RAVs. In 312 European patients RAVs were studied in regions NS3, NS5A and NS5B, potentially relevant for treatment with telaprevir, simeprevir, asunaprevir, daclatasvir, ledipasvir, ombitasvir, and dasabuvir. No SOF-resistant RAVs were identified at baseline. NS3-related RAVs were identified in 20.5% of cases; NS5A-related RAVs were seen in 11.5% (most commonly in genotype 1b versus 1a); and NS5B-related RAVs were found in 21.5 of subjects (also more commonly in genotype 1b). The authors conclude that the study of baseline RAVs may help in deciding the best therapy possible and in optimizing therapy costs (37). As of today, major scientific societies either do not approve or do not position themselves regarding the study of resistance before treatment onset (38-40) except when searching for polymorphism Q80K in patients with genotype 1a about to receive a regimen containing simeprevir.

Reinfection, superinfection and coinfection

Reinfection with HCV is common in some populations, particularly in male homosexuals co-infected with HIV (41). The incidence of acute hepatitis C has increased among HIV-infected males who have sex with other males (MSM) both in Europe and in Australia and the US (42,43). In their November 2014 meeting, the American Association for the Study of Liver Diseases (AASLD) reported an extensive study, later published in *Clinical Infectious Diseases* (44), with the primary goal of analyzing the clinical

benefits of SVR. It also analyzed reinfection risk in a population with over 8,000 subjects who achieved SVR following antiviral therapy, reporting that, over a period of 3 to 5 years, reinfection risk varied according to the population examined (0.9% in patients with no risk factors, 8.2% in parenteral drug users and/or inmates in custody, and up to 23% in co-infected patients, particularly MSMs). However, the aforementioned studies lack consistent virological studies. To establish a correct definition for the various infections extant in a given patient with failed antiviral therapy technology clinicians are as yet unfamiliar with is required: deep sequencing using NGS and phylogenetic analysis. With these we may distinguish several situations (Fig. 5):

- *Persistent infection:* This refers to infections where we may identify identical variants before and after treatment. The relative weight (or dominance) of each variant may change considerably, but their phylogenetic analysis demonstrates divergence below 10% (single phylogenetic origin). Relapse is probably nothing but a persistent infection that at some point became low-level and undetectable.
- *Reinfection or new infection with HCV:* This is defined as the presence of variants after treatment that bear no phylogenetic resemblance with baseline variants (divergence over 10%). Reinfection is obvious when genotype is changed.
- *Superinfection:* Variants are identified post-treatment with a clear phylogenetic relation with pre-treatment variants (persistent infection) besides variants unrelated to original isolates (reinfection).

Recently, the need to discuss the issue of virological failure using NGS was clearly demonstrated (45). Through the study of quasispecies dynamics using conventional, clonal, deep sequencing in patients with a high risk for reinfection (homosexuals co-infected with HCV and HIV), differentiation between persistent infection and reinfection was attempted. Of 99 treated subjects, 15 did not achieve SVR; these patients are the focus of the analysis. Both clinically and through conventional sequencing it was interpreted that 10 of these patients had reinfection. However, the use of NGS revealed that all 15 patients had evidence of persistent infection, although the variant had not been previously identified in 6. The authors concluded that, even in groups with a high risk for reinfection, persistent infection is more likely than reinfection, the latter being overestimated by conventional sequencing. This work provides many original aspects that should be borne in mind in the future: a) multiple viral strains can be identified before treatment, a fact that may result from concurrent infection or superinfection (46). On the other hand, the presence of new variants (undetected in baseline samples but with identical phylogenetic origin) may be accounted for by therapy-induced selection or the presence of different variants in different compartments, such as the central nervous system (47);

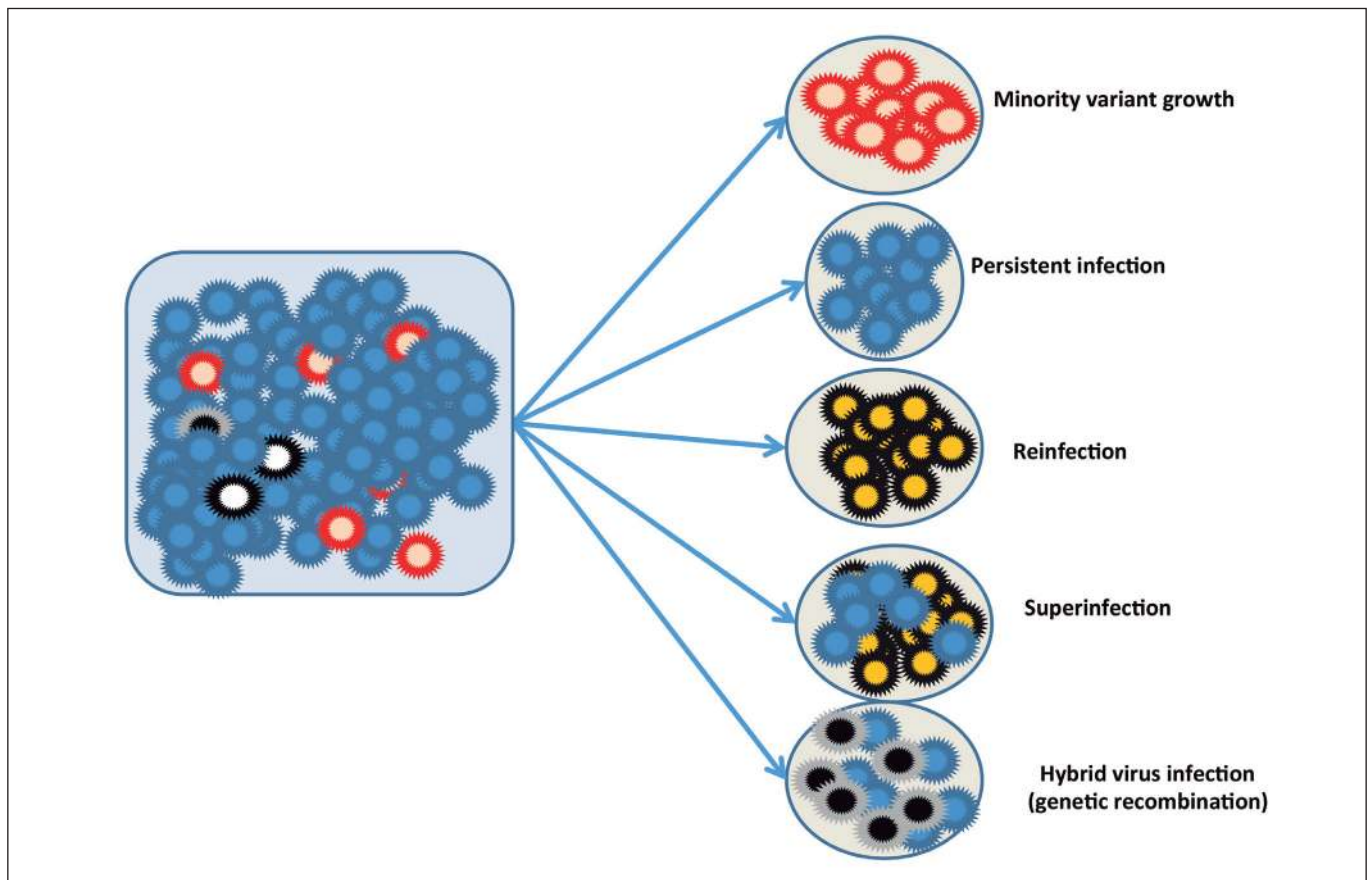


Fig. 5. Differentiation between persistent infection, reinfection and superinfection.

b) therapeutic implications: should the patient have persistent infection, the best approach is likely prolonged therapy, whereas for reinfection a combination of DAAs is to be adjusted according to the newer strain (48); c) finally, this study also questions the truthfulness of the high rate of reinfection reported, and posits that a major part of such presumed reinfections actually are persistent infections. This statement is also supported by some reports that show extremely low long-term relapse rates amongst the co-infected (49).

However, while the above study questions the presence of at least some infections, Sarrazin provides an interesting approach to this issue. He analyzes the consistency of SVR at 12 and 24 weeks in over 3,000 subjects included in phase-3 sofosbuvir trials, and reports consistency for all but 12 patients. In these 12 subjects, he examines viral sequences (complete for 10, of NS5B for 2). He presumes that, when a different genotype is found, reinfection has occurred; however, when said genotype is the same the decision between reinfection and persistent infection will depend on phylogenetic distance. His results show that both situations are possible (7 reinfections, 5 persistent infections) and cannot be told apart from a clinical standpoint (50).

Potential implications and some recommendations

Now that therapy with DAAs can cure HCV infection in most patients, we desirably should pay special attention to refractory patients. This is driven by an intrinsic difficulty in understanding the mechanism of therapy failure and, more importantly, the absence of clear guidelines to define patients failing to respond to IFN-free antiviral therapy. Having discussed therapy failure-associated causes, some reflections –not at all dogmatic– may be due here.

- Appropriately genotyping all patients before antiviral therapy onset is essential. Presently, genotyping is performed with conventional PCR. Considering the high prices of drugs and the potential risk of establishing an inadequate therapy regimen, particularly for patients with advanced disease, as well as the relatively low cost of sequencing, we believe that baseline sequencing will be increasingly common for ever more patients in the near future. Before implementing this new technology cost-effectiveness studies should be undertaken to support it. Furthermore, this sequencing would allow not only adequately typing all patients but also knowing the potential presence of RAVs, thus favoring custom therapy.

– As clearly discussed in the last EASL guidelines, update scientific evidence is not enough to recommend any specific regimen following a failed IFN-free antiviral therapy including two or more DAAs. However, as treatment has to be urgent on occasion, a practical approach does seem reasonable for the present issue:

- First, we must appropriately categorize our patient: cirrhosis status, liver function, decompensation risk, portal hypertension level, potential inclusion in a liver transplant wait list, and liver graft reinfection. Indeed, we must assess retreatment urgency. Also, we should consider potential reinfection or superinfection, and ask the patient accordingly.
- Secondly, we should carefully assess the characteristics of the prescribed antiviral regimen, including type of drugs, RBV status, potential adherence issues, time of therapy failure, etc.
- And thirdly, we must carefully examine virological characteristics: viral load, genotype, and subtype. As previously seen, reinfection development, genotyping or subtyping errors, presence of treatment-resistant variants are not exceptional, and more rarely genetic recombination may occur. In this respect, while no guidelines explicitly recommend it, we think complete HCV sequencing is desirable for all patients failing to a last generation regimen in order to exclude or confirm some of the potential virological failure causes we just discussed in the present review. Also, since sequencing technology is not affordable for all sites, homologation of reference institutions to this end seems crucial. In any case, what is actually affordable for all sites is storage of a baseline sample at -70 °C to allow for in-depth virological testing when possible.

Bearing the above considerations in mind, some highly generic recommendations, which will presumably change in a short time, may be offered:

- Since sofosbuvir has a high genetic barrier to resistance and RAVs are exceptional, most patients failing to antiviral therapy with DAAs should receive a regimen including sofosbuvir. This regimen should be IFN-free, and if possible a DAA from a previously unused class should be selected.
- Treatment should likely include RBV, but the addition of this compound or prolonged therapy are unknown to be effective. Whether ribavirin may reduce treatment resistance remains unclear.
- Importantly, we should recall that subjects failing to regimens containing an NS5A inhibitor likely harbor RAVs with cross-resistance for any NS5A inhibitor, and that these variants do not tend to disappear (51-53).
- Patients without an urgent need for treatment may wait until the emergence of additional data and/or alternative therapy options validated in relevant clinical trials.
- Requesting from trial sponsors a road map, a pre-defined strategy for actions to be taken in case of antiviral therapy failure may also be appropriate, and said actions should be adjusted according to the cause of failure.

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