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FULL PAPER

# Resistance Associated Mutations in HCV Patients Failing DAA Treatment

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SUMMARY

Currently, treatment of chronic hepatitis C is based on a combination of direct-acting antiviral agents (DAAs) which achieve HCV clearance in more than 95% of patients. Despite this high rate of cure, treatment failures can occur in about 3-5% of treated patients. Resistance associated substitutions (RAS) are commonly detected after virological failure, although their role in real-life DAA failures is still debated.

This study aimed to evaluate in real-life DAA-failing patients the prevalence of clinically relevant RASs for the different DAA classes and to identify possible predictors.

Fifty consecutive HCV-infected patients who experienced a virological failure to a DAA-containing regimen were included in the study.

Direct sequencing of HCV regions involved in DAA resistance (NS3, NS5A and NS5B) was performed with Sanger-based homemade protocols.

The presence of mutations in the NS3 and NS5A regions was statistically associated with regimens containing protease inhibitors ($p<0.0032$) and NS5A inhibitors ($p<0.0006$), respectively.

On the contrary, for the NS5B region, the known mutations associated with the NS5B RNA polymerase inhibitors were detected in treated HCV patients, although there was no statistical significance ($p>0.5$).

A significant correlation was found between the presence of RASs and advanced fibrosis/cirrhosis, but not with age, sex and viral load.

Our study demonstrates a high frequency of RASs in patients with DAA failure, thus highlighting the usefulness of genotypic tests in this setting. The identification of RASs may guide the choice of the most appropriate drugs for HCV re-treatment.

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## INTRODUCTION

Recently, treatment of chronic hepatitis C has been significantly improved by oral direct-acting antiviral agents (DAAs) that act directly on the hepatitis C virus (HCV) at various points in the viral life cycle, achieving a sustained virological response in more than 95% of cases (WHO 2016; Ji *et al.,* 2019). Despite this high rate of HCV cure, treatment failures can occur in about 3-5% of treated patients (Perpinan *et al.,* 2018). Many factors contribute to DAA treatment failure, including patient adherence, suboptimal regimens or drug resistance. In particular, resistance associated substitutions (RASs) are commonly detected at the failure of DAA regimens (Sorbo *et al.,* 2018; Di Maio *et al.,* 2018). HCV has high genetic heterogeneity due to the lack of proofreading mechanisms of the HCV-RNA-dependent RNA-polymerase (NS5B) and high viral replication activity. During HCV replications a large number of viral variants are produced (viral quasispecies) that may include variants with reduced susceptibility to DAAs (Lontok *et al.,* 2015; Paolucci *et al.,* 2012; Paolucci *et al.,* 2017; AASLD-IDSA., 2019; WHO 2019; Pawlotsky *et al.,* 2009). If complete suppression of viral replication is not obtained during DAAs treatment, preexisting strains with reduced susceptibility to the drugs administered can be selected, leading to a virological breakthrough or relapse after therapy discontinuation (Pawlotsky *et al.,* 2011).

Moreover, in patients who failed DAA regimens, sev-

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eral hosts and viral factors have been associated with failure, including advanced cirrhosis, HCV genotype 3 and 1a, baseline high viral load, HIV co-infection and an unfavorable polymorphism in the region of the gene IL28 beta (Osinusi *et al.,* 2012; Elsheredy *et al.,* 2020). Therefore, several efforts are still devoted to better understanding of the relevance of RASs in DAA-failing patients and to defining the usefulness of genotypic testing prior to retreatment to guide the choice of the best second-line therapy. The presence of RASs for HCV is mostly evaluated by analysis of the genomic nucleotide sequence; to date, Sanger sequencing and Next generation sequencing are used to detect RAS in NS3, NS5A and NS5B (Bellocchi *et al.,* 2019; Caputo *et al.,* 2020). Both of these methods have been demonstrated to be equivalent (Bellocchi *et al.,* 2019; Guinoiseau *et al.,* 2017). However, errors can occur during Next generation sequencing generated data analysis (Howison *et al.,* 2019); therefore, Sanger sequencing is still considered the gold standard for the detection of HCV mutations (Caputo *et al.,* 2020).

The aim of this study was to evaluate in real-life DAA-failing patients the prevalence of clinically relevant RASs in all three genes and to identify RASs predictors.

## MATERIALS AND METHODS

### *Patients*

Fifty consecutive patients referred to the Infectious Diseases Unit of Foggia between January 2016 and July 2018 were enrolled in the study.

The inclusion criteria were:

1) chronic HCV infection,
2) DAA treatment,
3) DAA failure by experiencing a breakthrough or a relapse.

Relapse and breakthrough are defined based on the virological response to treatment:

– *Virological relapse:* HCV RNA decreases and remains below the limit of detection during treatment but becomes detectable after discontinuation of treatment.
– *Virological breakthrough:* HCV RNA rebounds and becomes detectable before treatment is completed (Dieterich *et al.,* 2009).

Serum samples obtained from patients with virological breakthrough or relapse were stored at -20°C until testing.

Based on the progressive availability of new DAA and according to national and international guidelines (AISF, EASL), patients were treated with the following DAA regimens: Sofosbuvir (SOF) + Ribavirina (RBV); Simeprevir (SMV) + SOF+/-RBV; 3D (Ombitasvir (OBV), + Paritaprevir (PTV) + Dasabuvir (DSV), SOF + Ledipasvir (LDV); SOF + Velpatasvir (VEL); Elbasvir (EBR) + Grazoprevir (GZR); Glecaprevir (GLV) + Pibrentasvir (PBV) and Interferon (IFN)+ RBV + SMV.

All specimens were tested for HCV-RNA levels by a commercially available method (ABBOTT GmbH & Co KG Max Plank-Ring 2 65205 Wiesbaden Germany); detection limit was 12 IU//ml.

### *Amplification and sequences of NS3, NS5A and NS5B*

HCV RNA was extracted from 140 µl of sera using the Qiamp viral RNA minikit following the manufacturer's instructions (Qiagen GmbH, Qiagen Strasse 1, 40724 Hilden, Germany). Synthesis and amplification of cDNA was then carried out in a single step using the commercial Superscript III One-step RT-PCR system with Platinum Taq (Invitrogen by Life Technologies, 5791 Van Allen Way Carlsbad, CA, 92008 USA) and primers for RT-PCR for each HCV genotype/subtype were designed from known sequences based on the NS3, NS5A and NS5B, as reported elsewhere (Di Maio *et al.,* 2017). If necessary, nested PCR was also performed with specific HCV genotype/subtype primers (Di Maio *et al.,* 2017). Finally, NS3, NS5A and NS5B amplified products were purified and sequenced by an automated DNA sequencing analyzer (ABI-3130) in sense and antisense orientation using the Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA). Wild-type amino acids were defined according to reference sequences from Geno2Pheno HCV tool.

The basic local alignment search tool (BLAST) and phylogenetic analysis were used to identify HCV genotypes.

First, the nucleotide sequences from NS3, NS5A and NS5B regions were analyzed by HCV BLAST in the Los Alamos HCV sequence database (http://hcv.lanl.gov/content/index). Then, the NS3, NS5A and NS5B sequences were aligned using the Clustal W algorithm integrated into the BioEdit software. The sequences of HCV strains were then aligned with a reference panel of sequences representative of each subtype (Genebank Accession numbers: HCV-G1a M62321; HCV-G1b D90208; HCV-G2a D10988; HCV-G2c D50409 and HCV-G3a D17763), the same proposed by Geno2pheno HCV tools (Di Maio VC *et al.,* 2017). The sensitivity for detection of RASs using Sanger sequencing is approximately 10-20% (Sarrazin *et al.,* 2016).

### *Statistical analysis*

A Chi-Square test and multivariate analysis of variance (ANOVA) were used to investigate the significance relationship between HCV Genotypes and presence of RASs, and among presence of RASs and liver fibrosis, sex, viral load and age. A p value less than 0.05 was considered significant.

## RESULTS

The clinical and virological characteristics of the 50 patients who failed different DAA regimens are reported in *Table 1*.

Forty-two patients were males and eight were females, age ranged from 38 to 84 years (mean age =57 years), 13/50 (26%) had cirrhosis, and 22/50 (44%) were drug addicts. Regarding HCV genotype (GT), 27 patients were infected by GT1 (10 GT1a and 17

**Table 1** - *Clinical and virological characteristics of 50 HCV infected patients with DAA failure.*

| *Patient* | *Age* | *Drugabuse* | *HCVGT* | *Fibrosis stage* | *Sex* | *Viral Load (IU/ml)* | *DAA failure* |
|---|---|---|---|---|---|---|---|
| 1 | 56 | Yes | 3a | F2 | M | 1000000 | Relapse |
| 2 | 50 | Yes | 3a | F2 | M | 34245 | Relapse |
| 3 | 44 | Yes | 3a | F2 | M | 750000 | Relapse |
| 4 | 49 | No | lb | F4 | F | 1132000 | Breakthrough |
| 5 | 47 | No | lb | F4 | M | 723000 | Breakthrough |
| 6 | 50 | No | lb | F3 | M | 1010000 | Breakthrough |
| 7 | 75 | No | 2c | F2 | M | 31844 | Relapse |
| 8 | 68 | No | lb | F2 | M | 500000 | Breakthrough |
| 9 | 50 | No | 3a | F3 | M | 195000 | Breakthrough |
| 10 | 63 | No | 3a | F3 | M | 299514 | Breakthrough |
| 11 | 48 | No | 3a | F2 | M | 1608000 | Relapse |
| 12 | 67 | No | lb | F4 | M | 888000 | Breakthrough |
| 13 | 48 | Yes | la | F3 | M | 7598000 | Breakthrough |
| 14 | 61 | Yes | la | F4 | M | 730000 | Breakthrough |
| 15 | 59 | No | 2c | F3 | M | 6026000 | Relapse |
| 16 | 46 | Yes | 3a | F3/4 | M | 4342 | Breakthrough |
| 17 | 47 | Yes | 3a | F3 | M | 2468000 | Breakthrough |
| 18 | 56 | Yes | 3a | F3 | M | 112500 | Breakthrough |
| 19 | 66 | No | 2c | F3 | M | 4093000 | Relapse |
| 20 | 38 | No | la | F2 | M | 16000 | Breakthrough |
| 21 | 65 | No | lb | F4 | M | 1253000 | Breakthrough |
| 22 | 50 | No | la | F3 | F | 7000000 | Breakthrough |
| 23 | 49 | Yes | la | F3 | M | 1300000 | Relapse |
| 24 | 75 | No | lb | F4 | M | 257000 | Breakthrough |
| 25 | 73 | No | lb | F4 | M | 820000 | Breakthrough |
| 26 | 43 | Yes | la | F4 | M | 4160 | Breakthrough |
| 27 | 84 | No | lb | F3 | M | 113800 | Breakthrough |
| 28 | 57 | No | lb | Fl/2 | F | 2050 | Relapse |
| 29 | 71 | No | lb | F4 | M | 1217000 | Relapse |
| 30 | 50 | Yes | la | F3/F4 | M | 2596000 | Breakthrough |
| 31 | 44 | Yes | lb | F2 | M | 965000 | Breakthrough |
| 32 | 51 | Yes | 3a | F3 | M | 2170000 | Breakthrough |
| 33 | 48 | No | la | F2 | M | 694000 | Relapse |
| 34 | 57 | No | lb | F4 | F | 1376000 | Breakthrough |
| 35 | 72 | Yes | lb | F3 | M | 12800 | Breakthrough |
| 36 | 50 | No | lb | F2 | M | 19100 | Relapse |
| 37 | 55 | Yes | 3a | F3 | F | 450000 | Breakthrough |
| 38 | 52 | Yes | 3a | F3 | M | 2194000 | Breakthrough |
| 39 | 45 | Yes | 3a | F4 | M | 11450 | Relapse |
| 40 | 77 | No | lb | F3 | F | 12840 | Breakthrough |
| 41 | 50 | Yes | la | Fl/2 | M | 1553000 | Breakthrough |
| 42 | 67 | No | 2c | Fl/2 | F | 414000 | Breakthrough |
| 43 | 49 | Yes | 3a | F3 | M | 350000 | Relapse |
| 44 | 41 | Yes | 3a | F3 | M | 824000 | Breakthrough |
| 45 | 52 | No | la | F2 | F | 34700 | Relapse |
| 46 | 72 | No | lb | F4 | M | 380000 | Breakthrough |
| 47 | 61 | No | 3a | F4 | M | 700000 | Breakthrough |
| 48 | 58 | Yes | 3a | F2 | M | 220000 | Relapse |
| 49 | 47 | No | 3a | F4 | M | 2300000 | Breakthrough |
| 50 | 65 | Yes | 2a | FI | M | 4100000 | Relapse |

GT1b), 5 patients by GT2 and the remaining 18 patients by GT3a. The HCV-RNA levels were higher than 800,000 IU/ml in 23/50 patients (46%). Distribution of RAS according to DAA regimen is shown in *Figure 1*. Overall, 33 patients showed a breakthrough and 17 were relapsers. According to DAA failure (breakthrough vs relapse), 28/33 patients with breakthrough (85%) and only 2/17 relapsers showed the presence of RAS (*Table 2*). None of the relapsing patients in whom resistance testing was performed after at least 18 months from DAA discontinuation presented mutations associated with drug resistance. In 20/50 failed patients, sequence analysis showed no RAS in any of the three regions.

### *Sequencing of NS3 region*

NS3 sequences analysis showed the presence of RAS only in GT1 failed patients (*Table 2*). Overall, the Q80K mutation was detected in 5/27 (18%) of GT1 patients treated with protease inhibitors (SMV, PTV and GZR). As expected, the presence of mutations in the NS3 region was statistically associated with regimens containing protease inhibitors ($p<0.0032$).
In addition, a significant correlation was found between the presence of Q80K and GT1a ($p<0.001$). Interestingly, one GT1b patient showed in the NS3 region the presence of multiple mutations (V36L+S122N+V170I) associated with DAA resistance (*Table 2*). The remaining sequences showed the presence of a wild type genotype.

### *Sequencing of NS5A region*

The presence of mutations associated with drug resistance in the NS5A region was found in 26/50 (52%) of patients who failed DAA therapy. The presence of known mutations associated with resistance to NS5A inhibitors was found to be statistically significant in patients treated with regimens containing NS5A inhibitors ($p<0.0006$). Among GT1a infected patients, 5/10 (50%) presented NS5A RASs conferring resistance (4 OMV, 1 GZR), while among GT1b infected patients, 13/17 (77%) had mutations associated with resistance (8 LDV, 2 OMV, 3 EBR). In GT3a infected individuals, the presence of amino acid mutations in the NS5A region was observed in 8/18 (44%) of patients (4 DCV, 2 OMV, 2 VEL). No amino acid mutations were found in the NS5A region of GT2 infected patients.
The most commonly detected NS5A RAS was Y93H/C, found in 15/27 (55%) of GT1 infected patients, and in all eight GT3a infected patients (*Table 2*). In 12 GT1b patients, Y93H was found in combination with other RASs in the NS5A region, such as L31I/M/V found in 11 patients and L28M detected in one patient (*Table 2*). Mutation at position 31 was significantly associated with GT1b ($p<0.004$), whereas the presence of Y93H/C was observed in all HCV patients who failed DAA treatments, regardless of the viral genotype (*Table 2*).

### *Sequencing of NS5B region*

The NS5B region showed drug-related mutations in only 5/50 (10%) of patients. In particular, S556G was observed only in 3 GT1a and 1 GT1b patients, while one patient infected with GT1b showed the presence of S556G combined with C451N (*Table 2*). Although mutations associated to NS5B inhibitors were found more frequently in patients treated with these agents, the difference was not statistically significant ($p>0.5$). The remaining sequences from the NS5B region from HCV-GT2 and/or HCV-GT3 patients were wild type.

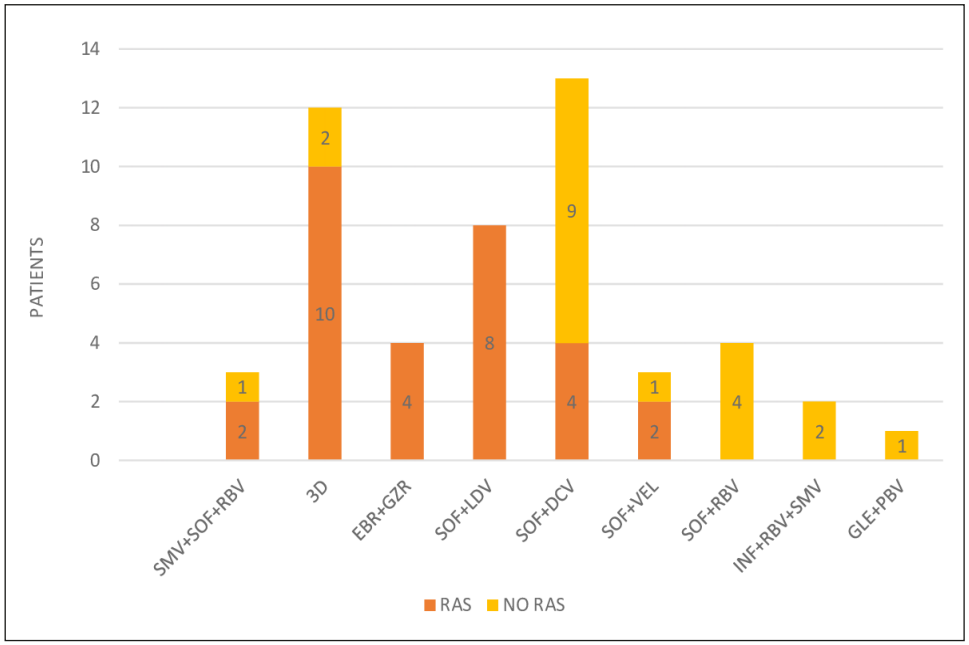


**Figure 1** - *Distribution of RAS according to DAA regimen in 50 HCV patients with DAA failure.*

Legend: SMV, Simeprevir; SOF, Sofosbuvir; RBV, Ribavirina; 3D: OBV(Ombitasvir) + PTV (Paritaprevir) + RTV(Ritonavir) + DSV (Dasabuvir); EBR, Elbasvir; GZR, Grazoprevir; LDV, Ledipasvir; DCV, Daclatasvir; VEL, Velpatasvir; INF(Interferon); GLE, Glecaprevir; PIB, Pibrentasvir.

**Table 2** - *Analysis of NS3, NSSA and NSSB RASs detected in 30/50 patients who failed DAA treatment.*

| *Patient ID* | *HCV GT* | *DAA regimen* | *Failure RASs* | | |
|---|---|---|---|---|---|
| | | | NS3 | NSSA | NSSB |
| PT13 | la | SMV+SOF+RBV | Q80K | | |
| PT14 | la | OBV+PTV+RTV+DSV | Q80K | Y93C | S556G |
| PT20 | la | OBV+PTV+RTV+DSV | | Q30R | |
| PT26 | la | SMV+SOF+RBV | Q80K | | |
| PT30 | la | OBV+PTV+DSV | Q80K | Q30L+Y93H | S556G |
| PT33 | la | EBR+GZR+RBV | Q80K | Q30H+L3lM | |
| PT41 | la | OBV+PTV+DSV | | M28T | |
| PT45 | la | OBV+PTV+DSV | | | S556G |
| PT4 | lb | SOF+LDV | | L31V+Y93H | |
| PT5 | lb | SOF+LDV | | L28M+Y93H | |
| PT6 | lb | OBV+PTV+DSV | | | C451N+S556G |
| PT8 | lb | SOF+LDV | | L31M+Y93H | |
| PT12 | lb | SOF+LDV | | L31I+Y93H | |
| PT21 | lb | OBV+PTV+DSV | V36L+S122N+V170I | L31V+Y93H | S556G |
| PT24 | lb | SOF+LDV | | L31I+Y93H | |
| PT25 | lb | SOF+LDV | | L31M+Y93H | |
| PT27 | lb | SOF+LDV | | L31V+Y93H | |
| PT31 | lb | OBV+PTV+DSV | | L31I+Y93H | |
| PT34 | lb | EBR+GZR | | L31M+P58S+Y93H | |
| PT35 | lb | EBR+GZR | | L31M+P58S+Y93H | |
| PT40 | lb | SOF+LDV | | Y93H | |
| PT46 | lb | EBR+GZR | | L31M+Y93H | |
| PT9 | 3a | OBV+PTV+DSV | | Y93H | |
| PT10 | 3a | OBV+PTV+DSV | | Y93H | |
| PT16 | 3a | DCV | | Y93H | |
| PT17 | 3a | DCV+SOF | | Y93H | |
| PT32 | 3a | DCV+SOF | | Y93H | |
| PT37 | 3a | DCV+SOF | | Y93H | |
| PT38 | 3a | SOF+VEL | | Y93H | |
| PT47 | 3a | SOF+VEL | | Y93H | |

### *Resistance associated mutations do not affect fitness and correlate with liver fibrosis*

No correlation was observed between the presence of RASs and HCV RNA levels, age, sex, and drug abuse (p:>0.5). On the contrary, a significant correlation was found between mutations associated with drug resistance and advanced fibrosis/cirrhosis assessed by transient elastography (p<0.008).

## DISCUSSION

Currently, DAA-based regimens achieve HCV cure in most chronic hepatitis C patients. Nevertheless, virological failure can occur in <5% of patients, usually in association with development of RASs. In DAA-failing patients, the presence of RASs may compromise the efficacy of second-line therapy and is therefore an important issue for successful re-treatment (Lontok *et al.,* 2015).

Recently, resistance testing for all three regions (NS3, NS5A and NS5B) at virological failure after a DAA-based regimen was proposed by a panel of Italian experts to better guide retreatment decisions (Di Maio *et al.,* 2017).

In this real-life study, we performed the sequence analysis of the NS3, NS5A and NS5B regions in 50 HCV patients who failed different DAA regimens. In line with other studies (Di Maio *et al.,* 2018; Sorbo *et al.,* 2018), we found the presence of clinically relevant RASs in 30/50 tested patients.

Twenty-six patients showed at least one mutation in one of the three regions involved in DAA failure, one patient exhibited the presence of RAS in both NS3 and NS5A regions, while three patients showed the presence of RAS in all three regions.

A strong correlation was detected between the presence of RASs and the therapeutic regimen. In fact, 6/16 (38%) and 26/28 (93%) of patients treated, respectively, with regimens containing NS3 or NS5A inhibitors had RASs associated with therapeutic failure.

On the contrary, RASs associated to NS5B RNA polymerase inhibitors were found more frequently in patients treated with these agents, but the difference was not significant. This result is due to the high genetic barrier of sofosbuvir compared with dasabuvir. In fact, we found RASs associated with resistance to

the NS5B inhibitors only in patients treated with dasabuvir (5/10 patients).

In line with published data (Parlati *et al.,* 2018; Kileng *et al.,* 2018; Dietz *et al.,* 2018; Martinez *et al.,* 2017), we observed a significant correlation between the presence of RASs and viral genotype. In particular, the Q80K mutation in the NS3 region was observed only in patients with GT1a, compared to other genotypes; while in the NS5A region, the presence of mutations at position 31 was associated with GT1b. The presence of the Y93H mutation in the NS5A region, independent of viral genotype, significantly correlated with a therapeutic failure to all second and third generation NS5A inhibitors. In our study, RASs were detected in patients with a virological breakthrough and only in two relapsed patients, who were tested soon after relapse. On the contrary, the presence of RAS was reported in none of the relapsing patients, in whom the resistance testing was performed at least 18 months after therapy discontinuation and prior to re-treatment. This is not surprising, since it is known that HCV may revert to wild type after treatment is stopped, although RASs persist as minor variants in the viral quasispecies.

According to a previous study by Paolucci and colleagues, the presence of mutations associated with drug resistance was significantly correlated to advanced fibrosis/or cirrhosis (Paolucci *et al.,* 2017).

Moreover, we did not find any mutations associated with DAAs resistance in HCV-GT2 failed patients, as already reported in other studies (Kliemann *et al.,* 2016; Mizokami *et al.,* 2016; Schnell *et al.,* 2018).

There are two important limitations in our study:

a) the lack of RASs analysis in pre-treatment samples that were unfortunately not available. In the absence of baseline resistance testing, the presence of natural RAS cannot be ruled out;
b) the long time (18 months or more) elapsed from DAA discontinuation to the date of sampling for the genetic test in relapsed patients; during this time some RASs, such as NS3-RAS, may disappear, whereas NS5A-RAS, which are very fit, tend to persist longer. However, even with these limits, our results highlight the frequent presence of RASs in patients with failure to DAA regimens, especially in cirrhotic patients and in those treated with a regimen containing an NS5A inhibitor.

In conclusion, our data suggest that in patients failing DAA treatment, genetic testing is useful for detecting the presence and type of mutations associated to resistance, which in turn may aid in the choice of the optimal second line regimen.

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