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ORIGINAL ARTICLE

Baseline resistance associated substitutions in HCV genotype 1 infected cohort treated with Simeprevir, Daclatasvir and Sofosbuvir in Brazil

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KEYWORDS
HCV;
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DAA treatment;
Antiviral resistance

Summary
Background: The World Health Organization estimates that 1% of the world population (71 million) is infected with hepatitis C virus (HCV). In 2015, three direct-acting antivirals (DAAs), simeprevir (SMV), sofosbuvir (SOF) and daclatasvir (DCV) were included in the Brazilian protocol for the treatment of chronic hepatitis C. Despite the fact that the use of these drugs is associated with higher treatment response rates and with lower incidence of side effects, studies have shown the association between the presence of viral resistance mutations and the failure of pharmacological treatment.

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Aim: This way, this study aimed to evaluate the safety and effectiveness of treatment for HCV genotypes 1a and 1b infected patients with these DAAs, also analyzing the occurrence and prevalence of baseline resistance associated substitutions (RAS), observing the impact of these mutations into the treatment success.
Methods: Clinical data were collected from all the 262 HCV infected patients included for comparative analysis, while serum samples collected from 144 of these individuals, before treatment, were submitted to molecular biology approaches for mutation analysis into NS3, NS5A and NS5B regions.
Results: Regarding the treatment regimens, 49.6% of the patients received SOF + DCV ± ribavirin and 50.4% used SOF + SMV ± ribavirin. The sustained virological response at 12 weeks post-treatment (SVR12) rate was 92.7% (93.9% for SOF plus DCV and 91.7% for SOF plus SMV). No clinical or laboratorial factor was statistically associated with SVR. The most common adverse reactions were haematological events, nausea/vomiting, headache and asthenia. Out of 144 blood samples, 70 (48.6%) had detected RAS, 34.8% treated with SOF + DCV ± ribavirin and 61.3% SOF + SMV ± ribavirin. The resistance mutations against SMV were detected into NS3: substitutions G122S (28%), I170V (22.7%), Y56F (17.3%) and V132I (14.7%). The mutations against DCV R30Q (9.1%), P58H (6.1%) and Q62E (6.1%) were observed into NS5A, and for SOF the mutations A421V (10.6%), L159F (6.4%) and C316N (6.4%) were present inside NS5B viral protein. Four patients did not reach SVR, three of them presented viruses carrying RAS (1 treated with SOF + DCV and 2 with SOF + SMV). Some of these mutations, like R30Q (present in relapsing samples) and L159F, are well known by their influence on antiviral resistance, while others, like C316N, have a compensatory effect on viral fitness, maintaining these baseline RAS.
Conclusion: The use of treatment regimens composed of SOF and DCV or SOF and SMV showed a high SVR rate, despite of a high rate of RAS, and a good tolerability profile in patients with HCV genotype 1. However, the high occurrence of baseline RAS observed in this casuistic is still a concern and studies like this show the necessity to understand how they are maintained in the population and to direct more efficiently the use of DAAs.
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Introduction

Chronic infection of hepatitis C virus (HCV) affects about 71 million people worldwide, causing approximately 400,000 deaths every year due to complications like cirrhosis, liver failure and hepatocellular carcinoma [1]. In Brazil, the number of infected people is around 1.5 million, being the major cause of death among all viral hepatitis [2].

Until 2010, the pharmacological therapy for chronic hepatitis C (CHC) was based in the use of interferon what was associated with low effectiveness rates and significant side effects [3]. Advances on the virology field provided a better understanding on HCV life cycle and viral proteins function, allowing the development of specific drugs that could directly target these viral components, the direct acting antivirals (DAAs), with improved therapy success (response rates over 90%) and lower incidence of side effects [4–7].

The main objective of CHC treatment is to cure the infection or to achieve sustained virological response (SVR). SVR is defined as the non-detection of HCV RNA in the blood 12 (SVR12) or 24 weeks (SVR24) after the end of treatment [8]. The Brazilian guidelines has recommended the use of Sofosbuvir (SOF - NS5B polymerase inhibitor) plus Simeprevir (SMV - NS3/4A protease inhibitor) or Daclatasvir (DCV - NS5A inhibitor) for the treatment of HCV genotype 1 since 2015. These therapeutic regimens can also be associated with ribavirin (RBV) in some cases [2,9,10].

HCV is known to replicate in a high rate and this fact, associated with the lack of proofreading activity of the viral RNA polymerase NS5B, provides a high mutation rate that confers to HCV a high genetic variability. This variability can be observed inside the same host, where HCV can circulate as a pool of genetic related variants, with small differences among them, called quasispecies [11–13]. This characteristic, associated with the selective pressure exerted by the DAAs during treatment, can work as an important tool for viral resistance acquisition, selecting mutations that can lead to changes in the amino acid chain, modifying the viral protein and, consequently, preventing the interaction and inhibition by the DAAs. In some cases, these resistant variants can occur naturally, before the treatment with the antivirals [14–16].

Despite all the issues involving treatment and resistance acquisition and considering the incorporation of new antiviral treatments for CHC in Brazil, there are scarcity of studies that evaluate the success and safety of DAAs usage in this country. This way, this study aimed to evaluate the safety and effectiveness of treatment for HCV genotypes 1a and 1b infected patients with these DAAs, also analyzing the occurrence and prevalence of baseline resistance associated substitutions (RAS), observing the impact of these mutations into the treatment success.

Materials and methods

Study setting and ethical aspects

A prospective cohort study was performed at the University Hospital, Ribeirão Preto School of Medicine, University of

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São Paulo, Brazil (HCFMRP-USP), a tertiary teaching institution linked to the Brazilian Public Health System. All the molecular experiments and analysis were performed at Genomic Studies Laboratory of the São Paulo State University (UNESP). This study was approved by the Research Ethics Committee of HCFMRP-USP with the approval number 1.398.168.

Patients

Patients older than 18 years chronically infected with HCV genotype 1 who started treatment from December 2015 to June 2017 were included. Following the Brazilian protocol for HCV treatment at the time the study was conducted, patients with advanced fibrosis (F3 or F4) or diagnosis of F2 for more than 3 years were included. In addition, patients with mild/moderate fibrosis could be treated when they had cryoglobulinemia, porphyria cutanea tarda, chronic kidney disease, post-liver transplant setting or non-hepatic solid organ transplant [17].

Patients with decompensated cirrhosis and/or prior non-responders to first generation of protease inhibitors (PI) based treatment received SOF (400 mg daily) plus DCV (60 mg daily) with or without RBV for 24 weeks. The remaining genotype 1 patients were treated with SOF plus DCV or SMV (150 mg daily) with or without RBV for 12 weeks [2]. Patients coinfected with Human Immunodeficiency Virus and/or hepatitis B virus were excluded. All the individuals included signed the Informed Consent Form.

Clinical data collection

Clinical and laboratory data were collected on three moments (before, during and after treatment) through access to the hospital computerized system. Regarding characterization of study population in the pretreatment period, the following information were collected: sociodemographic data (age, sex, race), anthropometric data (weight and height), besides clinical information concerning hypertension, diabetes mellitus, obesity, liver fibrosis staging, hepatic encephalopathy, ascites, esophageal varices, prior CHC treatment history (treatment-naïve patients × treatment-experienced patients), prior CHC treatment response (relapsers × non-responders), HCV subgenotype, RASs, length treatment, use of RBV. The results of the following laboratory tests performed before treatment were recorded: alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total serum bilirubin (TB), international normalized ratio (INR), creatinine and HCV viral load. Cirrhosis staging was performed according to Child-Turcotte-Pugh score and Model for End-Stage Liver Disease (MELD) score was also calculated.

The liver fibrosis stage was determined by biopsy (METAVIR score) or through non-invasive exams, point shear wave elastrography (pSWE) or transient elastography, considering the most recent result when two exams were available. Patients with esophageal varices, as cites and/or splenomegaly were considered as F4. The Body Mass Index (BMI) over 30 Kg/m^2 were considered obese [18].

During treatment, the safety of the therapy was analyzed by the incidence of adverse events. Anemia, neutropenia and thrombocytopenia where considered using the respective thresholds: serum hemoglobin levels < 13.9 g/dL (men) and < 12 g/dL (women), neutrophils < 1700/mm^3 and platelets < 130,000/mm^3.

To verify the therapy response, the HCV viral load was determined at the end of treatment and 12 weeks after the end. The viral load quantification was carried out by real-time polymerase chain reaction-based method, with limit of detection < 12 UI/mL.

Investigation of mutations prevalence

To evaluate the presence and prevalence of resistance associated mutations in the viral genome, serum samples from the patients included in the study were collected before the beginning of the DAAs treatment and viral targets were determined according to the treatment regimen for each individual. For those treated with SMV and SOF, NS3 and NS5B viral proteins were analyzed, while for those treated with DCV and SOF NS5A and NS5B were investigated.

RNA extraction and cDNA synthesis

The viral RNA was extracted from the serum samples using a TRIzol extraction protocol. In 1.5 ml tubes, for each sample, 250 μl of serum and 750 μl of TRIzol (Thermo Fisher Scientific) were added and homogenized, followed by 5 min incubation at room temperature. After that, the RNA separation, precipitation and elution were performed according to manufacturer's protocol. For the cDNA synthesis, the High Capacity cDNA Archive Kit (Life Technologies) was used following the manufacturer's guidance protocol.

Viral targets amplification

The viral genomic regions, correspondent to the DAAs' targeted viral proteins NS3, NS5A and NS5B were amplified by Nested PCR using the Long PCR Enzyme Mix (Thermo Fisher Scientific) following the manufacturer's instructions, with focus on the regions where the resistance associated mutations occur. Due to the genetic variability between subtypes 1a and 1b, specific primers were designed for each subtype (Supplementary Table 1). Since the mutations in NS5B are distributed within the entire protein, it was necessary to amplify the whole NS5B genomic region. For this, we performed two different Nested-PCR reactions, dividing NS5B into two different amplicons (NS5B-I and NS5B-II), corresponding to each half of the whole viral gene.

Sequencing analysis for resistance associated mutations

Once amplified, the PCR products were submitted to the sequencing reaction using the BigDye Terminator v3.1 (Life Technologies) and the internal primers of the Nested-PCR reaction. The sequences were obtained using the ABI 3130 XL Genetic Analyzer (Life Technologies) and then were aligned by the Clustal W method using the BioEdit 7.1.11 software [19,20], where resistance associated mutations were detected according to the comparison with reference

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Table 1 Clinical and demographical characteristic of studied population according to the treatment scheme.

Variables	SOF + DCV ± RBV (*n* = 130)	SOF + SMV ± RBV (*n* = 132)	All treated (*n* = 262)
Age (years), mean ± SD	56.7 ± 9.3	56.6 ± 10.8	55.0 ± 10.0
Male sex, *n* (%)	83 (63.8)	69 (52.3)	152 (58.0)
Obesity, *n* (%)			
BMI < 30.0 Kg/m^2	65 (50.0)	46 (34.8)	111 (42.4)
BMI ≥ 30.0 Kg/m^2	30 (23.1)	26 (19.7)	56 (21.4)
Missing data	35 (26.9)	60 (45.5)	95 (36.3)
Diabetes mellitus, *n* (%)	41 (31.5)	43 (32.6)	84 (32.1)
Hypertension, *n* (%)	72 (55.4)	61 (46.2)	133 (50.8)
AST[a] (≥ 3 ULN), *n* (%)	18 (13.8)	23 (17.4)	41 (15.6)
ALT[b] (≥ 3 ULN), *n* (%)	20 (15.4)	28 (21.2)	48 (18.3)
Liver fibrosis stage, *n* (%)			
F0/F1/F2	20 (15.4)	33 (25.0)	53 (20.2)
F3	15 (11.5)	32 (24.2)	47 (17.9)
F4	95 (73.1)	67 (50.8)	162 (61.8)
Cirrhosis stage, *n* (%)			
Child-Pugh A	65 (68.4)	58 (86.6)	123 (75.9)
Child-Pugh B	26 (27.4)	3 (4.5)	29 (17.9)
Child-Pugh C	2 (2.1)	0 (0.0)	2 (1.2)
Missing data	2 (2.1)	6 (8.9)	8 (4.9)
MELD score, mean ± SD	10.1 ± 4.1	8.1 ± 2.4	9.1 ± 3.4
HCC, *n* (%)	10 (7.7)	7 (5.3)	17 (6.5)
Liver transplantation, *n* (%)	2 (1.5)	1 (0.8%)	3 (1.1)
Previous treatment history, *n* (%)			
Naïve patients	42 (32.3)	72 (54.5)	114 (43.5)
Experienced patients	88 (67.7)	60 (45.5)	148 (56.5)
Previous treatment response[c], *n* (%)			
Relapsers	35 (39.8)	24 (40.0)	59 (39.9)
Non-responders	53 (60.2)	34 (56.7)	87 (58.8)
Missing data	0 (0.0)	2 (3.3)	2 (1.4)
HCV subgenotype, *n* (%)			
1[a]	70 (53.8)	65 (49.2)	135 (51.5)
1b	51 (39.2)	53 (40.1)	104 (39.7)
Non-subgenotyped	9 (6.9)	14 (10.6)	23 (8.8)
HCV-RNA level (UI/mL)[d], *n* (%)			
< 800,000	72 (55.4)	64 (48.5)	136 (51.9)
≥ 800,000	58 (44.6)	68 (51.5)	126 (48.1)
RASs[e], *n* (%)	24 (34.8)	46 (61.3)	70 (48.6)
Treatment duration, *n* (%)			
12 weeks	63 (48.5)	132 (100.0)	195 (74.4)
24 weeks	67 (51.5)	0 (0.0)	67 (25.6)
Use of RBV, *n* (%)	109 (83.8)	62 (46.9)	171 (65.3)

ALT: alanine aminotransferase. AST: aspartate aminotransferase. BMI: body mass index. DCV: daclatasvir. HCC: hepatocellular carcinoma. HCV: hepatitis C vírus. MELD: Model for End-stage Liver Disease. RASs: Resistance-associated substitutions. RBV: ribavirin. SD: standard deviation. SMV: simeprevir. SOF: sofosbuvir. ULN: upper limit of normal.

[a] Number of patients that presented the ratio between the last AST value before treatment and the value of ULN ≥ 3.

[b] Number of patients that presented the ratio between the last ALT value before treatment and the value of ULN ≥ 3.

[c] For the percentage calculation, the total number of patients refers to the total of experimented patients (*n* = 88, *n* = 60, *n* = 148, respectively).

[d] Values refer to the last viral load performed before starting treatment.

[e] Percentages calculated in relation to the total of clinical samples which viral genome regions were amplified and analyzed (*n* = 69, *n* = 75, *n* = 144, respectively).

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sequences H77 for genotype 1a (GenBank Accession number AF011753.1) and HCV-K1-R2 for genotype 1b (GenBank accession number D50481.1) [19].

Statistical analysis

The quantitative variables were shown as mean and standard deviation (SD) or median and interquartile range when the distribution was not normal. The qualitative variables were categorized and the absolute and relative frequencies were described. The chi-square test and the Odds Ratio (OR) calculation were proposed to verify the association between SVR and the categorized variables, the association between drug adverse reactions described with the used therapeutic regimen and to investigate the relation between before treatment mutations and variables related to the patient. To proceed with the analysis, global SVR results were considered and they were performed by the intention to treat. For all the association analysis, a 5% of significance level (α) was established and the tests were performed using the Statistical Package for Social Sciences software (SPSS Inc., Version 17.1.0).

Results

Relation among clinical variables, treatment response and RASs presence

Two hundred and sixty two patients were included, with predominance of male (58%) and Caucasian/white (79.4%). The main clinical and demographical characteristics of the studied population, according to the treatment regimen, are described in Table 1. Considering all patients enrolled in this study, the overall SVR rate was 94.3% (Fig. 1).

Regarding the endpoints cited in Fig. 1, SVR rates may range according to treatment responses in patients with missing data for the worst-case and best-case scenarios about SVR status. Considering patients treated with DCV+SOF $\pm$ RBV, the study showed a SVR rate of 95.4% (worst-case scenario) that could achieve 98.5% (all patients with missing SVR data would have achieved SVR). In relation to the group treated with DCV + SMV $\pm$ RBV, our results indicated SVR rate of 93.2% (worst-case scenario) that could achieve 97.7% (best-case scenario). All relapsing patients (two who used DCV + SOF + RBV and three who used SMV + SOF + RBV) had liver cirrhosis. Of all the relapsing patients, three of them completed the prescribed therapy, being treated for 12 weeks, and the other two patients (one which used SMV and SOF and one which used DCV and SOF) had discontinued treatment due to adverse event. All patients that died during the treatment already presented decompensated cirrhosis before starting therapy. Concerning patients with hepatocellular carcinoma history before the beginning of treatment, 14 (82.4%) achieved SVR12. The other three had no treatment response data. All individuals that had liver transplantation history achieved SVR12. The SVR12 rates, according to selected clinical variables associated with therapy response and treatment regimen are described in Table 2.

Regarding the safety of the interferon-free therapies evaluated, we performed an association analysis between the occurrence of most common side effects and the different antiviral treatment regimens (Table 4).

When analyzing the possible association between SVR and baseline clinical data by the chi-square test, we did not identify a relation among all the analyzed variables and the SVR12 endpoint (Table 3). The same side effects listed on Table 4 were analyzed for their association with the use of RBV (regimens with RBV vs regimens without RBV). We detected evidences of the relation between RBV administration and incidence of anemia ($P = 0.001$), nausea/vomiting ($P = 0.014$), appetite loss ($P = 0.028$), asthenia/fatigue ($P = 0.001$) and skin reactions ($P = 0.015$).

Amplification efficiency

Since different genomic regions, from different subtypes, were amplified by Nested-PCR, different amplification efficiency was observed. For genotype 1a we had 100% of efficiency for all regions (NS3, NS5A and NS5B). Meanwhile, genotype 1b had different efficiency rates for each region, where amplification was efficient in 90.9% of NS3 samples and 84.6% of NS5A samples. For NS5B, due to the partitioned amplification, the observed efficiencies were 90.14% and 74.65% for first fragment (NS5B-I) and second fragment (NS5B-II), respectively.

Resistance associated substitutions prevalence in the population

Investigation of substitutions related to DAAs resistance acquisition was performed in samples from 144 patients included in this study. Seventy patients (48.6%) presented variants carrying mutations related to antiviral treatment failure. When comparing the data between the treatment groups, 24 patients (34.8%) who were treated with DCV + SOF presented variants previously associated with therapy evasion before starting treatment, while 46 patients (61.3%) of those receiving SMV + SOF were carrying resistant mutants (Table 5).

Seventy-six patients were infected with subtype 1a and 63 patients with subtype 1b. However, in five patients it was not possible to determine the subtype. Twenty five (32.9%) with genotype 1a presented viral variants carrying resistance associated substitutions. Mutations were present in NS3 region in 14.5% (11/25), 2.6% (2/25) on NS5A and 19.7% (15/25) on NS5B. Forty-one out 63 (65.1%) subtype 1b presented resistance mutations detected in viral variants, 26/63 (41.3%) presented NS3 substitutions, 3/63 (4.8%) in NS5A and 17/63 (27%) showed NS5B mutations related to therapy escape.

The most prevalent mutation was against SMV with the substitution G122S observed in 28% of the cases, followed by I170V (22.7%), Y56F (17.3%) and V132I (14.7%). For mutations against DCV, R30Q (9.1%) was the leading one and followed by P58H (6.1%) and Q62E (6.1%), while resistance mutations for SOF were headed by A421V (10.6%), the most recurrent one, L159F (6.4%) and C316N (6.4%).

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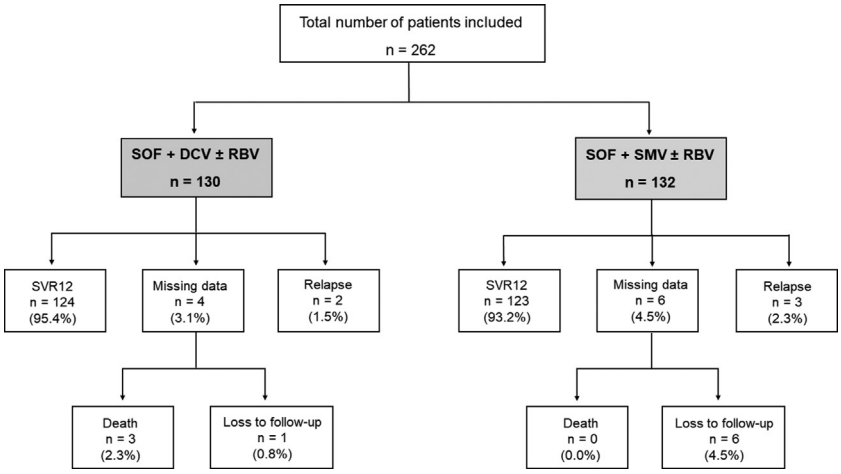

Figure 1 Clinical samples description and work flow. Treatment outcome for all included patients according to treatment scheme.

Relation between previous treatment, viral subtype and mutations occurrence

To evaluate the possible relation between viral subtype, previous treatment and occurrence of mutations, an association analysis was performed. According to the data, virus circulating in patients previously treated with first generation protease inhibitors were 20% more inclined to carry second generation DAAs RAS in NS3 than those who were not treated before (Table 6).

Regarding the relationship between viral subtype and the occurrence of mutations against SMV, DCV and SOF, HCV genotype 1b presented 20% more chances to carry RAS than genotype 1a, independent of previously treatment regimens (Table 6).

Discussion

Therapies based on DAAs usage are well known for their high SVR rate and low side effects occurrence, especially when compared to interferon based schemes, and here we describe a treatment effectiveness rate higher than 90%, corroborating to the observed in other genotype 1 studies with administration of SOF associated with DCV or SMV, with and without the addition of RBV [21—27]. The majority of the patients included had advanced liver fibrosis or even CHC complications as decompensated cirrhosis. Advanced liver fibrosis is considered the most important factor associated with treatment failure in the era of DAAs [26,28—30]. The characteristics of the population and the small number of non-responder/relapsers could explain the fact we did not find association between clinical and demographic variables and the response to treatment.

Regarding the therapy safety, some side effects were highly prevalent in both treatment groups, like nausea/vomiting, headache, asthenia/fatigue and skin reactions. The usage of RBV, according to our data, is possibly related with higher incidence of anemia, nausea/vomiting, appetite loss, asthenia/fatigue and skin reactions. These results consistently agree with data from literature and support the caution when prescribing RBV to patients with anemia or skin diseases history [25,26,30]. Two relapsing patients, from different therapy groups, did not complete the treatment due to adverse events. Both were treated with RBV and presented common side effects. The one treated with SOF + DCV +RBV presented asthenia, appetite loss and cough, while the other, treated with SOF + RMV + RBV presented asthenia and headache, with posterior anemia confirmation. Also, our data suggests that the combination SOF + DCV would be associated with nephrotoxicity and thrombocytopenia, but this result does not corroborate with published studies [26,27,31]. Additional studies evaluating the safety of DCV, SOF and SMV administration are necessary to confirm these results.

As observed in our results, for both subtypes, NS5A presented the low resistance mutation frequency when compared to other DAAs targets here investigated. This data differs from those published by Malta et al., where they found mutations inside NS5A in 14.6% of genotype 1a and 6% of genotype 1b infected patients. In our study, the frequency was 2.6% for 1a and 4.8% for 1b. The mutations that occurred in each case are also different, where for genotype 1a the substitutions Y93H and Q30H/R were present in only one sample (2.6%) while R30Q (15.15%) was the predominant in our genotype 1b samples. The most prevalent changes in their study were M28V and Q30H/R (14.6%) for genotype 1a and L31F/V and Y93H (6%) for 1b [32].

Our results regarding mutations inside NS5B are similar to another study from the literature, especially for genotype 1a. We observed the presence of resistance mutations in 19.7% of genotype 1a samples, close to the 20.1% described in literature. For genotype 1b, this number is a little more discrepant, 27% against 16.3% from the published work. The resistance associated mutations L159F, C316N and A421V were detected in both studies [33].

Since the aim of this study was to evaluate the frequency and prevalence of resistance associated mutations to the DAAs distributed by the Brazilian health system, it was important to understand how these mutations contribute to the antiviral escape. According to the literature, the most important NS3 mutations related to SMV failure are V36M, R155K/G/T, Q80K/R, S122R and D168A/E/H/V [34]. In our data, none of these mutations were identified. The most

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Table 2 SVR12 rates according to demographic and clinical variables.

Variables	SOF + DCV ± RBV (*n* = 130)		SOF + SMV ± RBV (*n* = 132)	
	n (%)	SVR12, *n* (%)	*n* (%)	SVR12, *n* (%)
Age (years)				
< 55 years	59 (45.4)	56 (94.9)	57 (43.2)	54 (94.7)
≥ 55 years	71 (54.6)	66 (93.0)	75 (56.8)	69 (92.0)
Sex				
Male	83 (63.8)	78 (94.0)	69 (52.3)	65 (94.2)
Female	47 (36.2)	46 (98.0)	63 (47.7)	58 (92.1)
AST[a]				
< 3x ULN	112 (86.2)	106 (94.6)	109 (82.6)	103 (94.5)
≥ 3x ULN	18 (13.8)	18 (100.0)	23 (17.4)	20 (87.0)
ALT[b]				
< 3x ULN	110 (84.6)	104 (94.5)	104 (78.8)	96 (92.3)
≥ 3x ULN	20 (15.4)	20 (100.0)	28 (21.2)	27 (96.4)
Liver fibrosis				
F0/F1/F2/F3	35 (26.9)	35 (100.0)	65 (49.2)	63 (96.9)
F4 or cirrhosis	95 (73.1)	89 (93.7)	67 (50.8)	60 (89.6)
Previous treatment history				
Naïve patients	42 (32.3)	41 (97.6)	72 (54.5)	67 (93.1)
Experienced patients	88 (67.7)	83 (94.3)	60 (45.5)	56 (93.3)
Previous treatment response[c]				
Relapsers	35 (39.8)	33 (94.3)	24 (40.0)	24 (100.0)
Non-responders	53 (60.2)	50 (94.3)	34 (56.7)	30 (88.2)
Missing data	0 (0.0)	–	2 (3.3)	2 (100.0)
HCV subgenotype				
1a	70 (53.8)	64 (91.4)	65 (49.2)	60 (92.3)
1b	51 (39.2)	51 (100.0)	53 (40.2)	51 (96.2)
Non-subgenotyped	9 (6.9)	9 (100.0)	14 (10.6)	12 (85.7)
HCV-RNA level (UI/mL)				
< 800,000	72 (55.4)	69 (95.8)	64 (48.5)	60 (93.8)
≥ 800,000	58 (44.6)	55 (94.8)	68 (51.5)	63 (92.6)
RASs[d]				
Yes	24 (34.8)	23 (95.8)	46 (61.3)	44 (95.7)
No	45 (65.2)	44 (97.8)	29 (38.7)	29 (100.0)
Treatment duration				
24 weeks	66 (50.8)	65 (98.5)	0 (0.0)	–
12 weeks	59 (45.4)	58 (98.3)	131 (99.2)	123 (93.9)
Others[e]	5 (3.8)	1 (20.0)	1 (0.8)	0 (0.0)
Use of RBV				
With RBV	109 (83.8)	104 (95.4)	60 (45.5)	55 (91.7)
Without RBV	21 (16.2)	20 (95.2)	72 (54.5)	68 (94.4)

ALT: alanine aminotransferase. AST: aspartate aminotransferase. DCV: daclatasvir. HCV: hepatitis C vírus. RASs: Resistance-associated substitutions. RBV: ribavirin. SMV: simeprevir. SOF: sofosbuvir. SVR12: undetectable HCV-RNA 12 weeks after treatment completion. ULN: upper limit of normal.

[a] Values referred to ratio between the last AST before treatment value and ULN.

[b] Values referred to ratio between the last ALT before treatment value and ULN.

[c] For the percentage calculation, the total numbers of patients are referred to the total of experimented patients (*n* = 88 and *n* = 60, respectively).

[d] Percentages calculated in relation to the total of clinical samples where the viral genome regions were amplified and analyzed after blood collection (*n* = 69, *n* = 75, respectively).

[e] Patients that were treated in a shorter time in relation to the prescribed therapy according to the occurrence of adverse event, death or follow-up loss.

prevalent was G122S, followed by I170V, Y56F and V132I. This substitution in the position 122 from a Glycine (G) to a Serine (S) in our samples probably has no impact on treatment failure, once the Serine amino acid is considered the wild type in the work of Sorbo et al., [34]. Since the occurrence of a substitution is a random event and its fixation depends on the evolutionary forces acting on the genome, virus from different geographic regions present differences in positions that are not subjected to negative selection conferring population characteristics according

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Table 3 Analysis of the association of demographic and clinical variables with SVR12.

Variable	OR (CI 95%)	*P*
Treatment regimen[a]	1.512 (0.248; 9.208)	0.654
Age	1.058 (0.968; 1.157)	0.215
Sex	0.917 (0.150; 5.584)	0.925
AST[b]	1.375 (0.150; 12.640)	0.778
ALT[c]	0.870 (0.099; 7.622)	0.900
Liver cirrhosis	0.254 (0.030; 2.145)	0.208
Previous treatment history	0.322 (0.035; 2.920)	0.314
Previous treatment response[d]	2.137 (0.217; 21.077)	0.515
HCV subgenotype	0.405 (0.042; 3.955)	0.437
HCV-RNA level (UI/mL)[e]	4.373 (0.482; 39.682)	0.190
RASs[f]	0.452 (0.040; 5.101)	0.607
Use of RBV	0.319 (0.038; 2.697)	0.294
Treatment duration[g]	0.943 (0.096; 9.226)	0.960

ALT: alanine aminotransferase. AST: aspartate aminotransferase. CI: confidence interval. DCV: daclatasvir. HCV: hepatitis C vírus. OR: odds ratio. RASs: resistance-associated substitutions. RBV: ribavirin. SVR12: undetectable HCV-RNA 12 weeks after treatment completion.

[a] DCV + SOF ± RBV vs SMV + SOF ± RBV.
[b] Ratio between the last value of before treatment AST exam and the upper limit of normality (< 3.0 x ≥ 3.0).
[c] Ratio between the last value of before treatment ALT exam and the upper limit of normality (< 3.0 x ≥ 3.0).
[d] Relapsing patients vs non-responder patients.
[e] < 800,000 x ≥ 800,000 UI/mL.
[f] Presence of at least one RAS.
[g] 12 weeks treatment vs 24 weeks treatment.

Table 4 Analysis of the association between the most common adverse events according to treatment regimens adjusted by the use of RBV.

AE	SOF + DCV (*n* = 130)	SOF + SMV (*n* = 132)	OR (CI 95%)	*P*
Anemia	83 (63.8%)	51 (38.6%)	1.708 (0.984; 2.965)	0.060
Neutropenia	25 (19.2%)	13 (9.8%)	1.963 (0.916; 4.206)	0.080
Thrombocytopaenia[a]	50 (38.5%)	26 (19.7%)	2.164 (1.199; 3.906)	0.010
Abdominal pain	9 (6.9%)	6 (4.5%)	0.967 (0.322; 2.903)	0.950
Nausea/vomiting	24 (18.5%)	14 (10.6%)	1.319 (0.622; 2.797)	0.470
Diarrhea	14 (10.8%)	4 (3.0%)	2.818 (0.858; 9.257)	0.090
Appetite loss	15 (11.5%)	8 (6.1%)	1.311 (0.515; 3.338)	0.570
Headache	33 (25.4%)	29 (21.9%)	1.011 (0.547; 1.870)	0.970
Dizziness	7 (5.4%)	5 (3.7%)	1.081 (0.313; 3.727)	0.900
Asthenia/fadigue	41 (31.5%)	32 (24.2%)	0.857 (0.472; 1.558)	0.610
Psychiatric disorders[b]	7 (5.4%)	2 (1.5%)	2.716 (0.517; 14.278)	0.240
Myalgia	10 (7.7%)	7 (5.3%)	1.273 (0.438; 3.702)	0.660
Skin reactions	10 (7.7%)	15 (11.4%)	0.430 (0.178; 1.04)	0.060
Nephrotoxicity[c]	20 (15.4%)	7 (5.3%)	4.374 (1.621; 11.803)	< 0.001
Dry cough	10 (7.7%)	3 (2.3%)	3.465 (0.851; 14.109)	0.080
Xerostomia/disgeusia	7 (5.4%)	1 (0.8%)	6.025 (0.674; 53.862)	0.110

AE: adverse events. CI: confidence interval. DCV: daclatasvir. OR: odds ratio. RBV: ribavirin. SMV: simeprevir. SOF: sofosbuvir.

[a] For those patients who already showed thrombocytopenia before treatment, any decrease into platelets count compared to before treatment values were considered.
[b] Depression and/or anxiety.
[c] Presented by changes in creatinine and in glomerular filtration rate in relation to pretreatment values.

to sampling location, not necessarily related to therapy outcome [35]. Probably the substitution for Arginine (R) instead of Serine (S) or Glycine (G) is the responsible for resistance acquisition. The mutation Q80K is one of the most described but presents low resistance levels against SMV. In our results, this mutation was observed in only two clinical samples, from responding patients, corroborating with the low prevalence described for Q80K in HCV genotype 1 clinical samples from Brazil [36].

The substitutions M28A/T, Q30E/H/K/R, L31I/M/V, H58D and Y93C/H/N are the most described associated with resistance to DCV [34]. We were able to identify mutations like

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Table 5 Prevalence of resistance associated mutations prevalence in genotype 1 (subtypes a and b) infected patients against Simeprevir, Daclatasvir and Sofosbuvir. Mutations are presented according to the antiviral regimen and viral proteins targeted by the DAAs (NS3, NS5A and NS5B).

	SOF + DCV ($n = 69$)		SOF + SMV ($n = 75$)	
	n	%	*n*	%
NS3 RAS				
T54I	—	—	1	1.3
T54S	—	—	2	2.7
V55A	—	—	2	2.7
V55I	—	—	2	2.7
Y56F	—	—	13	17.3
Q80K	—	—	2	2.7
Q80L	—	—	1	1.3
S122G	—	—	5	6.7
S122N[a]	—	—	2	2.7
G122S	—	—	21	28.0
G122T	—	—	2	2.7
G122N	—	—	2	2.7
V132I	—	—	11	14.7
V132V	—	—	1	1.3
I170V	—	—	17	22.7
NS5A RAS				
L28V	1	1.4	—	—
L28M	3	4.3	—	—
R30Q[a]	6	8.7	—	—
L31M	1	1.4	—	—
Q30L	1	1.4	—	—
H58P	1	1.4	—	—
P58H	4	5.8	—	—
P58S	1	1.4	—	—
P58T	1	1.4	—	—
Q62E	4	5.8	—	—
Y93H	2	2.9	—	—
NS5B RAS				
L159F	4	5.8	5	6.7
C316N	4	5.8	5	6.7
V321F	0	0.0	2	2.7
V321N	0	0.0	1	1.3
A421V[a]	5	7.2	10	13.3
Y448H	1	1.4	0	0.0
A553G	0	0.0	1	1.3
A553S	0	0.0	1	1.3
A553V	0	0.0	1	1.3
S556G	4	5.8	1	1.3
S556T	0	0.0	1	1.3

SOF: sofosbuvir; DCV: daclatasvir; SMV: simeprevir; RAS: resistance associated substitution.
[a] Mutations detected in viruses circulating into relapsing patients.

Table 6 Association analysis between previously treatment regimens, HCV viral subtype and presence of resistance associated mutations.

	OR	CI 95%	*P*
Previous TT with dual therapy	4.375	0.487; 39.265	0.219
Previous TT with PI[a]	0.973	0.059; 16.153	0.747
Viral subtype[b]	0.228	0.112; 0.466	0.001

OR: odds ratio; CI: confidence interval; TT: treatment; PI: protease inhibitor.
[a] For this analysis, only resistance-associated substitutions into NS3 were considered.
[b] 1a x 1b.

L31M, H58P instead of H58D and Y93H, corroborating to the literature. However, the most prevalent ones were R30Q, P58H and Q62E. R30Q is just the opposite way from mutations at position 30 showed in the study from Sorbo et al. [34]. However, according to previous studies, in genotype 1b infected patients, R30Q confers resistance to DCV, and was detected together with P58S, a modification that could be acting as a compensatory mutation, reestablishing the viral fitness that could be altered when R30Q is present and enabling, its persistence in the population [37,38]. Interestingly, our results showed that R30Q was present in one of the genotype 1b relapsing patients, and was present together with P58H. These data could indicate that R30Q had an impact on antiviral treatment failure for this patient and that substitutions on site 58, regardless of the inserted amino acid, are possibly important for the resistance associated mutation persistence.

Considering the NS5A mutation L31M, Pawlotsky showed that, when tested *in vitro*, this mutation conferred high resistance rates in subgenomic replicon systems for all HCV genotypes, while Zhou et al. confirmed the relation with drug resistance and also described a compensatory effect of this mutation on viral fitness loss associated with substitutions at position 30 [39,40]. Despite the already described high resistance rates conferred against many NS5A inhibitors, the mutation Y93H was detected in low frequency in our study, only two patients, and both of them presented SVR [38].

In the case of SOF, the literature cites the mutations S282T, L159F and V321A as the most common in NS5B RNA polymerase [34]. In our results, the observed mutations, in order of recurrence, were A421V, L159F, C316N and V321N. The substitution S282T is known to confer, *in vitro*, high resistance rates against SOF due to the fact that this position is situated near the catalytic site [41—45]. In our study we did not find this mutation, and according to the literature it is really difficult to detect it in non-treated clinical samples, a situation that can be explained by the fact that this mutation leads to low viral fitness [46].

The mutation L159F is described to be structurally near to amino acid S282 (4 angstroms) of NS5B. Once S282 is near to the catalytic site and has influence on antiviral escape, L159F could interact with it as well, contributing to therapy failure [47]. An *in vitro* study showed that L159F confers high resistance to SOF, but is also associated with viral fitness decay when detected alone. However, when this mutation is followed by C316N, the viral fitness is recovered [48]. In our study, L159F and C316N were detected only in patients infected with subtype 1b and always occurred together in the same clinical sample, reinforcing this compensatory relation between them. Our data also corroborates with

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other studies showing that this double mutation is highly frequent, especially in Brazilian samples (25% against 3.78%, 6.05% and 1.16% for North America, Europe and Asia, respectively) [33,49].

Many clinical aspects are important to determine the success or failure of the treatment, while other characteristics turn these associations more difficult to be done. We screened here personal and clinical aspects of the individuals included in the study willing to determine the relation among them and SVR. The majority of patients presented advanced fibrosis during treatment and, with the low number of relapsing or non-responding individuals, the association among some clinical aspects and SVR was not possible to be made. Despite these limitations, with our data we can observe that therapies based on the usage of SMV, DCV and SOF, for genotype 1, are more effective and safe than the previously treatments offered in Brazil, presenting high SVR rate and high tolerability by the patients.

Our main focus was the observation of mutations related to antiviral resistance and how they were circulating in the studied population prior to treatment. We detected a high occurrence of baseline RAS that are well known to confer high resistance rates, like R30Q in NS5A and L159F into NS5B. We also observed the high frequency of compensatory mutations that enable the maintenance of these RAS, like L31 M and C316N into NS5A and NS5B, respectively. The combination of more than one DAA during therapy, targeting more than one viral protein, showed to be an efficient way to avoid resistance provided by these baseline RAS. However, this simultaneous circulation could imply in an important impact on SVR rates in the future, especially due to the continuous use of these DAAs, acting as a selective pressure. More studies are necessary to evaluate the impact of baseline RAS in the failure of treatment and its consequences. This could also allow the improvement of antiviral treatment by choosing the more adequate therapy scheme.

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Disclosure of interest

The authors declare that they have no competing interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.dx.org/10.1016/j.clinre.2019.07.015.

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