

Evolution of Resistance-Associated Variants of All-Oral Direct-Acting Antiviral Therapy of Hepatitis C in a Clinical Setting

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Abstract

Because of the high variability of Hepatitis C virus (HCV), it might be important to characterize *in vivo* the evolution of resistance-associated mutations (RAVs) to direct-acting antivirals (DAAs) in different genotypes.

NS3-, NS5A- and NS5B-HCV substitutions were studied by next generation sequencing (NGS) on 74 HCV-infected patients who started a DAA regimen. RAVs with frequencies of 1% and 15% were analyzed.

Globally, 43, 15, 12 and 4 patients were infected with subtype 1a, 1b, genotype 4 and subtype 3a, respectively. The majority of patients (64.8%) had cirrhosis, 70.3% were HIV-coinfected and 14.9% were DAA-experienced. Overall baseline prevalence of RAVs was 74.3%, 52.2%, 45.9% and 36.8% to any NS3, NS5B and NS5A inhibitors available at that time, respectively, and dropped to 39.2%, 26.1%, 22.8% and 16.2%, respectively, when only mutations associated with the ongoing regimen were considered. The highest proportion of mutations was detected in subtype 1a (81.4%, $p=0.026$), particularly in NS3 region (76.9%, $p<0.001$). Among the 7 failing patients, 57.1% had a baseline sequence showing substitutions as majority species. At the time of viral relapse two patients accumulated further RAVs that were missing even as minority variants at baseline.

Although almost half of the patients showed natural substitutions at baseline, these substitutions did not induce resistance to DAAs. A limited role of NGS with a low cut-off was suggested by our study, as the detection of minor species seems not to predict the selection for resistant variants at the time of failure. The impact of pre-treatment RAVs on the achievement of sustained virologic response with DAA is limited.

Keywords DAA regimens; HCV genotypes; Resistance associated variants

Introduction

Hepatitis C virus (HCV) infects around 80 million people worldwide and remains the major cause of cirrhosis and hepatocellular carcinoma [1]. Cure of HCV, namely the achievement of sustained virologic response (SVR), is associated with reduced complications of chronic liver disease, liver-related morbidity and death [2,3]. Direct-acting antivirals (DAAs) have become the new standard of care of chronic hepatitis C showing an extremely high rate of SVR with a minimal incidence of adverse events. However, 1-15% of patients fail to eradicate HCV infection in real world cohorts [4]. It has been demonstrated that baseline variables associated with reduced SVR are liver cirrhosis, high baseline viral load, HCV genotype 3 infection, prior treatment failure. In addition, host parameters such as IFNL4, IP10 levels and gender may play a role in the response to treatment. The presence of natural resistance mutations in NS5A or NS3 genes may also affect SVR [5,6].

As a result of the high replication rate and the lack of mechanism of proofreading in the RNA-dependent RNA polymerase, substitutions frequently occur in HCV RNA, generating a very high number of viral species and potentially leading to the development of resistance-associated mutations (RAVs) [7,8].

Although these polymorphisms are usually present in minor viral populations as they are associated with a lower replicative fitness compared to the wild-type virus, under DAAs pressure a positive selection may occur generating viral resistant variants associated with compensatory mutations [9].

Most of the information on such substitutions has been generated for genotype 1, whereas data remain scarce for other genotypes [10,11]. Although specific baseline mutations (i.e. NS3 Q80, NS5A L31, NS5A Y93) have been associated with reduced antiviral activity *in vitro*, their clinical impact in the prediction of virologic failure is still under investigation [12,13]. The probability that a DAA will select for and allow outgrowth of RAVs in viral populations depends on DAAs genetic barrier to resistance, drug concentration, length of therapy and viral fitness of the mutated variants [14,15]. With the exception of the Q80 variants, NS3 and NS5B substitutions are associated with a replicative impairment that explains their rather low pre-treatment

prevalence, as well as a relatively rapid replacement by wild type virus after therapy interruption. On the contrary, NS5A inhibitor-resistant viruses have good replicative fitness and may persist for years, potentially impairing the results of retreatment, even with the newest pangenotypic drugs [16,17].

It remains to be determined in clinical studies whether baseline RAVs might influence the response to therapy and the frequency of their occurrence in viral population during therapy.

The development of the next generation sequencing (NGS) technologies has enabled to identify mutations occurring with 1% frequency or less possibly predicting their occurrence at failure [18].

The aim of this study was to characterize in a cohort of HCV-infected patients starting a DAA regimen the preexistence and the evolution of resistance-associated variants in specific genotypes using a NGS approach comparing two different cut-offs of 1% and 15% and to measure their frequency at baseline and at virologic failure in patients who did not achieve SVR.

Patients and Methods

Study population

This study involved 74 HCV-monoinfected and Human Immunodeficiency Virus (HIV)/HCV coinfecting patients, at least 18 years old, who started an all-oral DAA regimen and were enrolled in a prospective, observational study conducted at the Clinic of Infectious Diseases of 'L. Sacco' Hospital, Milan, Italy, from November 2014 to December 2015.

Patients received an all-oral DAA combination according to the current European Association for Study of Liver recommendations [19]. Fibrosis stage and cirrhosis were determined by transient elastography (Fibroscan) and the cut-off values used were: 7.1 kPa for $F \geq 2$, 9.5 kPa for $F \geq 3$, and 12.5 kPa for $F=4$ [20].

Sustained virologic response was defined as HCV RNA concentration lower than 12 IU/mL (referred as undetectable) 12 weeks after the end of treatment by the real-time HCV assay (Abbott RealTime HCV quantitative assay; Abbott Molecular Inc., Des Plaines, IL, USA).

Hepatitis C viral breakthrough and viral relapse were defined as HCV RNA level higher than 12 IU/mL after having reached HCV undetectability at any treatment point or once therapy is discontinued, respectively.

The study was approved by the Institutional Review Board of the 'L. Sacco' Hospital and was conducted in compliance with the Good Clinical Practice guidelines and the 1964 Declaration of Helsinki. Written informed consent was obtained from all participants. Self-reported adherence was registered at each monthly visit.

Sample processing and HCV amplification

Viral RNA was extracted from 200 μ l of patient plasma using NucleoMag 96 Virus (Macherey-Nagel, Düren, Germany) and automated KingFisher™ ml Magnetic Particle Processors (Thermo Scientific) according to the manufacturer's protocol.

Reverse transcription and polymerase chain reaction (PCR) were carried out using SuperScript III reverse transcriptase (Thermo Fisher

Scientific Inc, Waltham, MA) and GoTaq[®] DNA Polymerase (Promega).

The NS3, NS5A, and NS5B fragments were amplified using HCV genotype-specific primers (Table S1). Amino acids 1-270 of HCV NS3 protease, amino acids 1-261 of NS5A and amino acids 1-560 of NS5B were included. The nested PCR products were analyzed on 1.3% agarose gel stained with ethidium bromide.

All amplicons were purified using the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands) in accordance with the manufacturer's instructions and eluted into a final volume of 50 μ l of distilled water. NS3, NS5A and NS5B regions were analyzed according to current regimen for 46, 57 and 74 patients, respectively.

Next generation sequencing and data analysis

Library preparation for Illumina sequencing was done using a Nextera[®] XT DNA Sample Preparation and Index kit (Illumina, San Diego, CA, USA) according to the manufacturer's manual. The quantified library concentrations were determined using the Invitrogen Quant-iT Picogreen dsDNA assay. Resulting libraries were normalized and pooled for subsequent sequencing on an Illumina MiSeq platform using the 2 \times 150 cycle paired-end sequencing protocol.

The sequence reads were analyzed by an in-house pipeline using VirVarSeq [21]. Minority species with a frequency above 1% and viral variants upon 15% were taken into account in the analysis (mean coverage of 3,000 reads). Results were mapped and aligned to the reference-genomes 'Hepatitis C virus genotype 1, complete genome H77' (accession number: NC_004102.1).

Resistance mutations were defined according to resistance tables indicated by Chen et al. [22]. The HCV Database (<http://www.hcv.lanl.gov/content/index>) was used to compare genotype 4 sequences.

Mutations and prediction of phenotypic resistance was also analyzed using Geno2pheno tool 0.92 (<http://hcv.geno2pheno.org/index.php>).

Consensus sequence was generated by Geneious software (v. 9.1.5; <http://www.geneious.com>) [23]. The genotype assignment obtained with the VERSANT[®] HCV Genotype 2.0 Assay^{*} Line Probe Assay (LiPA) was confirmed by phylogenetic analysis using MEGA 7 program (<http://www.megasoftware.net/>).

Statistical methods

Categorical variables were summarized as frequencies (%); continuous variables were expressed as median (interquartile range, IQR). Comparisons between groups were performed using the χ^2 or the Fisher exact test. For all the analyses an α -error of 5% was considered. Analyses were performed using the SPSS software package (v.22.0, SPSS, Inc., Chicago, IL).

Results

Patient characteristics

Of the 74 study patients 70.3% were males (n=52) with a median age of 51 years (IQR: 27-77) and a median HCV RNA of 6.4 \log_{10} (IQR: 3.8-7.2 \log_{10}).

Gene	Genotype/Subtype	Name	Sequence (5'>3')
NS3	ST 1a	P1a0	TCGTCTTYTCCRRATGGAG
		P1a1	ACCTTRTAGCCCTGAGCYGC
		P1a2	CTCATCACGTGGGGGGCRGA
		P1a3	TTGGTGCTCTTRCCGCTGCC
	ST 1b	Lod-IF	TCGTCTTTTCTGACATGGAG
		Lod-IR	TTGTACCCTTGGGCTGCATA
		Lod-IIF	TCATCACCTGGGGGGCAGAC
		Lod-IIR	GTGCTCTTGCCGCTGCCAGT
	GT 4	NS3-4 G4F	TGGGCAATGARATCTTGTTCGG
		NS3-4 G4R	GCARCCTCCATCRGCCAGGAA
NS5A	ST 1a	1a NS5A-F0	GACATCTGGGACTGGATATGYGA
		1a NS5A-R0	GTCCAGGWRTARGACATYGAGCA
		1a-NS5A-SeqF	ARCTGTCYGCWCCATCTCTCAAGG
		1a-NS5A-SeqR	AAGGAGTCCARRATCACCAC
	ST 1b	1b-NS5A-F0	GAYGTTTGGGAYTGGATATGCAC
		1b-NS5A-R0	GTCCAYGWRTARGACATYGAGCA
		1b-NS5A-SeqF	ARCTGTCYGCWCCATCTCTCAAGG
		1b-NS5A-SeqR	AARGAGTCCARRATYACYAC
	ST 3a	G3-out F	AGTGGATCAAYGARGACT
		G3-out R	GGYAGTTTYTCTCCTCRGCAC
		G3-innF	TCAAYGARGACTAYCCAA
		G3-innR	CACTRACGGTGGACCAAGAGT
	GT 4	G4-out F	ATACTATCATCCCTSACTGT
		G4-out R	CCTTCACTSTGGAKGCTCG
		G4-innF	CTGTGACMTCCCTTCTCAGA
		G4-innR	CTCGTGCCTTAATCTCCTT
NS5B	GT 1	G1-outF	ACGGARGAYGTCGTSTGCTGCTC
		G1-outR	CGGTTGGGGAGSAGGTARATGCCTACCCCTRC
		S3-F	TATGATACCCGCTGCTTTGACTCCAC
		Eno4-R	ARTACCTRGTCATAGCCTCCGTGAA
	ST 3a	G3-outF	CACCGGTGCCTCCYCCYCGSAGRAAAAGRAC
		G3-outR	GACACGCTGTGATAAATGTCGTTCCCGCC
	GT 4	G4-outF	TCTTGGTCCACYGTYAGCGGATCGG
		G4-outR	CGAGCAGGCAGCARRAAGATGCCTA

Table S1: Primers used for PCR.

Thirty-six (48.6%) patients were HCV treatment naïve, 27 (36.5%) were pegIFN plus ribavirin (RBV) treatment-experienced and 11 (14.9%) telaprevir (TPV) or simeprevir (SMV)-experienced.

Based on phylogenetic assignment, 58.1% were infected with subtype 1a (n=43), 20.3% with subtype 1b (n=15), 5.4% with subtype 3a (n=4) and 16.2% carried genotype 4 (n=12). Nine patients carried subtype 4d and three subtype 4v, 4a and 4n, respectively.

The majority of patients (64.8%, n=49) had cirrhosis and 70.3% (n=52) were HIV-coinfected; half of them were both HIV/HCV-coinfected and cirrhotic.

Twenty-nine patients (39%) received paritaprevir+ombitasvir +dasabuvir (PTV+OMV+DSV), 18 (24%) daclatasvir+sofosbuvir (DCV+SOF), 17 (23%) simeprevir+sofosbuvir (SMV+SOF) and 10 (14%) ledipasvir+sofosbuvir (LDV+SOF) with or without RBV.

Overall prevalence of DAA RAVs with a cut-off of 1%

A total of 46, 57 and 74 sequences of NS3, NS5A and NS5B, respectively, were included in a baseline dataset. At baseline the prevalence of RAVs to any available DAA was 74.3%; the prevalence of RAVs associated with NS3 protease inhibitors resistance was 52.2%, followed by 45.9% associated with NS5B polymerase inhibitors and 36.8% with NS5A inhibitors resistance. The combination of multiple RAVs in both NS3 and NS5A regions was 31% (9/29). Nine (19.6%, 9/46) and eleven (19.3%, 11/57) patients showed RAVs for NS3+NS5B or NS5A+NS5B inhibitors, respectively. Five subjects (17.2%, 5/29) showed RAVs in all regions.

Considering only RAVs associated with the regimens that patients were on treatment with, their overall prevalence were 39.2% of which 26.1%, 22.8% and 16.2% associated with NS3, NS5A and NS5B inhibitors, respectively. The combination of multiple substitutions restricted to clinically relevant RAVs was 10.3% (3/29) for NS3+NS5A, 8.7% (4/46) for NS3+NS5B, 5.3% (3/57) for NS5A+NS5B and 6.9% (2/29) for NS3+NS5A+NS5B.

RAVs more frequently identified were S122G (14/24), I170V (6/24) and T54S (5/24) in NS3 region; M28V (5/21), K24R (4/21) and L31M (3/21) in NS5A region; S556G (17/34), C316N (5/34) and L159F (5/34) in NS5B region.

A different frequency of RAVs was observed among different genotypes. At least one substitution was found in all the 4 subjects carrying subtype 3a, in 35/43 and 11/15 of those with subtype 1a and 1b, respectively, and in 5/12 patients with genotype 4 infection.

The highest prevalence of RAVs in patients infected with subtype 1a was observed in the NS3 region (20/26), while 4/10 subjects carrying subtype 1b with available NS3 sequence and none of those infected with genotype 4 or subtype 3a presented RAVs in the NS3 region. The prevalence of RAVs in NS5A region was highest in patients infected by subtypes 1a (14/39), followed by patients with genotypes 1b and 4 (5/12 and 2/2, respectively). None of the subjects infected by subtype 3a carried substitutions in NS5A. A significantly higher proportion of RAVs in NS5B region was observed in subtype 1b (9/15) or 3a (4/4) infected individuals compared to those infected with subtype 1a (18/43) or genotype 4 (3/12) (Figure 1).

Among subtype 1a patients the substitutions more frequently identified were S122G (13/20), I170V (6/20), Q80K/R (6/20), R155K (4/20) in NS3 region; M28V (5/14), K24R (4/14), H58P (2/14) in NS5A region; S556G (5/18) in NS5B region.

The substitutions most frequently identified in subtype 1b patients were T54S (2/4) in NS3 region; Y93H and P58S (2/5) in NS5A region; S556G and C316N (5/9), L159F (3/9) in NS5B region. Of note, the substitution Q80K/R was not found in patients with subtype 1b.

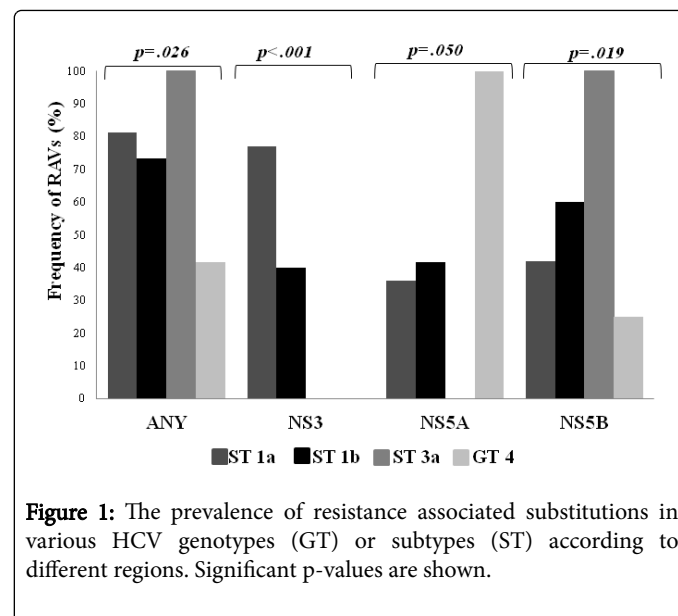


Figure 1: The prevalence of resistance associated substitutions in various HCV genotypes (GT) or subtypes (ST) according to different regions. Significant p-values are shown.

All patients harboring genotype 4 carried L31M in NS5A and S556G in NS5B region and every subject infected with subtype 3a showed the S556G substitution in the NS5B portion.

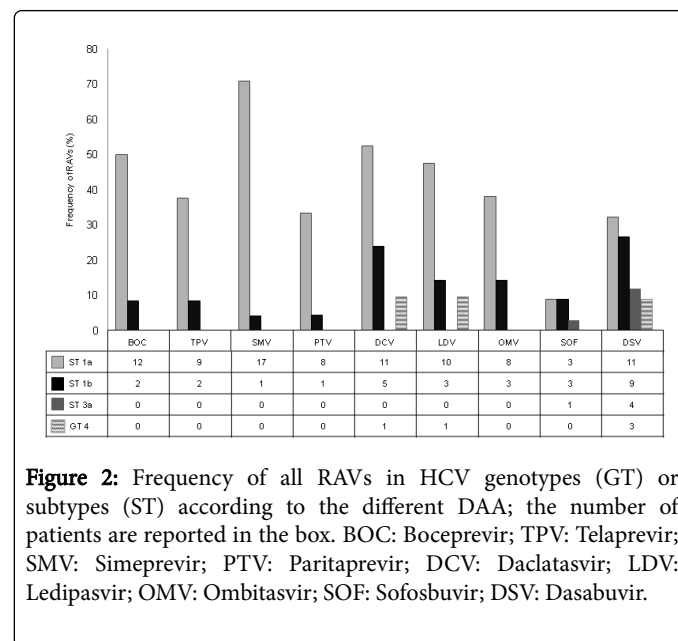


Figure 2: Frequency of all RAVs in HCV genotypes (GT) or subtypes (ST) according to the different DAA; the number of patients are reported in the box. BOC: Boceprevir; TPV: Telaprevir; SMV: Simeprevir; PTV: Paritaprevir; DCV: Daclatasvir; LDV: Ledipasvir; OMV: Ombitasvir; SOF: Sofosbuvir; DSV: Dasabuvir.

By stratifying RAVs according to the different DAA available at the time when the study was performed, substitutions for any available drug were identified in patients with subtype 1a and 1b, with a high prevalence of substitutions related to SMV (70.8%) in subtype 1a and DSV (26.5%) in subtype 1b. Subjects with subtype 3a infection showed RAVs for DSV (11.8%) and SOF (2.9%) while patients with genotype 4 harbored RAVs for DCV, LDV (9.5% each) and DSV (8.8%) (Figure 2).

No differences were observed in the overall distribution of RAVs between patients with or without cirrhosis ($p=.418$, $p=.142$, $p=.615$, $p=.083$ for any, NS3, NS5A and NS5B-associated, respectively); (Figure 3A). Similarly, the comparison of HCV and HIV-HCV-infected patients did not reveal significant differences in the frequency of RAVs ($p=.179$, $p=.686$, $p=.874$ and $p=.108$ for any, NS3, NS5A and NS5B, respectively) (Figure 3B).

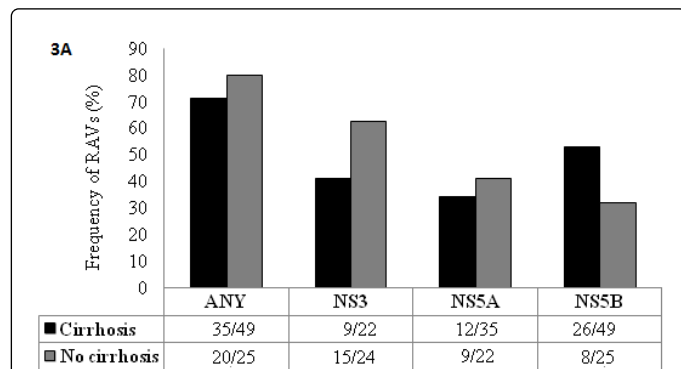


Figure 3A: Frequency of all RAVs by different regions in cirrhotic and non cirrhotic patients. Number of patients are reported in the box. Peg IFN- α : Pegylated Interferon- α ; RBV: Ribavirin; DAA: Direct Acting Antivirals. Statistically significant difference is indicated.

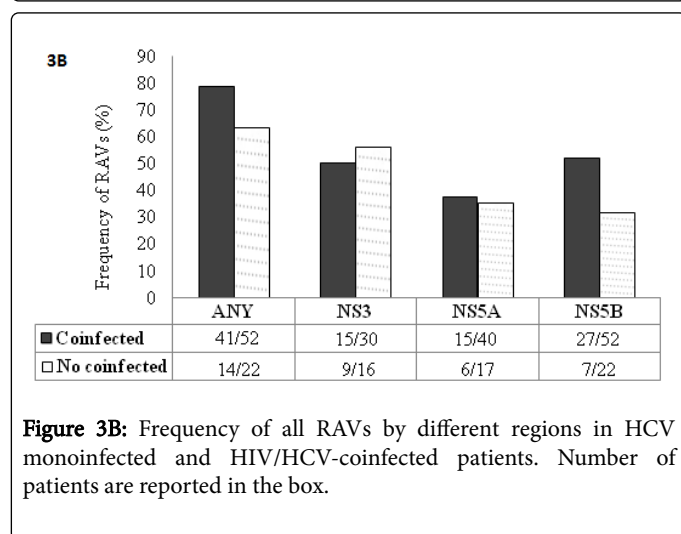


Figure 3B: Frequency of all RAVs by different regions in HCV monoinfected and HIV/HCV-coinfected patients. Number of patients are reported in the box.

Finally, no differences were detected in the overall distribution of RAVs between naïve patients and patients unsuccessfully treated with pegIFN and RBV or DAA (TPV or SMV) ($p=.325$, $p=.147$ and $p=.690$ for any, NS3 and NS5A, respectively). However, a significantly higher prevalence of RAVs was found in NS5B region of patients previously treated with DAA then those treated with pegIFN and RBV or naïve patients (81.8%, 40.7%, 38.9%, respectively; $p=.029$) (Figure 3C).

Prevalence of RAVs using a cut-off of 15%

Since treatment guidelines indicated as clinically significant only RAVs presents in more than 15% of the sequences generated, this cut-off was also considered.

At baseline, the prevalence of RAVs to any available DAA was 60.8%; the proportion of NS5B polymerase inhibitors RAVs was 40.5% followed by NS3 protease inhibitors (30.4%) and NS5A inhibitors RAVs (21.1%). Significant differences were observed in the prevalence of NS3 (52.2% vs. 40.5%, $p=.002$) and NS5A (36.8% vs. 21.1%, $p=.02$) RAVs using a cut-off of 1%.

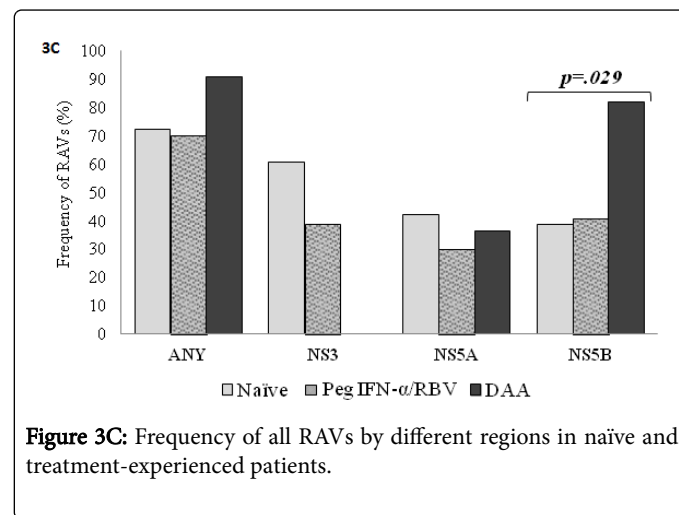


Figure 3C: Frequency of all RAVs by different regions in naïve and treatment-experienced patients.

Considering the overall prevalence of multiple RAVs combination, five patients showed RAVs in NS3+NS5A regions (5/29); three subjects showed RAVs for NS3+NS5B (3/46) or NS5A+NS5B (3/57) inhibitors. Only one subject (1/29) presented substitutions in all the regions. No significant differences were detected in prevalence of multiple RAVs considering all combinations with two different cut-offs.

Considering only RAVs associated with the ongoing regimens for each patient, the overall prevalence was 27% (20/74) of which 13%, 14% and 13.5% associated with NS3, NS5A and NS5B inhibitors, respectively. Only the prevalence of NS3 RAVs was significantly different between the two cut-offs considered (39.2% vs. 13%, $p=.03$).

The combination of multiple clinically relevant RAVs was 3.4% (1/29) for NS3+NS5A, 4.3% (2/46) for NS3+NS5B, 3.5% (2/57) for NS5A+NS5B and 3.4% (1/29) for NS3+NS5A+NS5B, with no difference between the two cut-offs.

By stratifying patients according to different genotypes, at least one substitution was observed in all 4 patients carrying subtype 3a, in 26/43 subjects with subtype 1a, in 10/15 with subtype 1b and in 5/12 of genotype 4 patients, with no difference between the two cut-offs. No different distribution of specific RAVs were observed through the different subtypes.

As already observed using a 1% cut-off, the highest prevalence of RAVs was in the NS3 portion (13/26) of subtype 1a ($p<.001$). In NS5A, the distribution of RAVs were 8/39, 2/12 and 2/2 patients with subtypes 1a, 1b and genotype 4, respectively ($p=.041$). A significant higher proportion of NS5B RAVs was observed in subtype 1b (9/15) than in subtype 1a (14/43) and genotype 4 patients (3/12) ($p=.007$). All subtype 3a patients showed RAVs in the NS5B regions.

Considering RAVs associated with the different DAA, patients with subtype 1a showed substitutions for all available drugs with a high prevalence of substitutions related to SMV (78.6%). Subjects with subtype 1b presented RAVs for all available drugs except for PTV and SMV; a high proportion was related to DCV (16.7%) and DSV (19.3%). Subtype 3a patients showed RAVs for DSV (12.9%) and SOF (3.2%)

and subtype 4 patients had substitutions for DCV (16.7%), LDV (16.7%) and DSV (9.7%).

By comparing patients with or without cirrhosis, no differences were observed in the overall distribution of RAVs for any, NS3, NS5A and NS5B portions.

Differently from the analyses conducted using a cut-off of 1%, a significant higher proportion of any (91.9% vs. 63.6%, p=.018) and NS5B (64.9% vs. 31.8%, p=.028) RAVs was observed in HIV co-infected than HCV mono-infected subjects.

Finally, no differences were observed in naïve or previously treated patients.

Analysis of HCV resistance-associated substitutions in failing patients

Table 1 shows the characteristics of the 7 subjects. (9.4%) who did not achieve SVR.

A higher proportion of RAVs was present in patients that were successfully treated compared with those who failed with both cut-offs (76.1% vs. 86%, p=.009 using 1% cut-off and 61.2% vs. 57.1%, p=.049 using 15% cut-off). One subject (#7) showed a viral breakthrough at week 12, 5 relapsed after the end of treatment and patient #5 prematurely interrupted therapy after 8 weeks.

Patients	Subtype	Therapy	Cirrhosis	HIV Coinfection	Time point	NS3		NS5A			NS5B		
						SMV	PTV	DCV	LDV	OMV	SOF	DSV	
#1*	1a	SMV+SOF+RBV	YES	YES	Baseline	Q80K						-	
					Failure	Q80K						-	
						R155K							
						R155K							
#2*	1a	PTV+OMV+DSV+RBV	NO	YES	Baseline		V36G			M28V		-	
							R155K						
					Failure		-		-		-		
#3*	4d	SMV+SOF+RBV	NO	YES	Baseline	-					-		
					Failure	-					-		
#4*	1b	PTV+OMV+DSV	YES	YES	Baseline		V36A			Y93H		E446Q	
											S556G		
											D559G		
					Failure		Y56H		Y93H		E446Q		
											S556G		
#5*	4d	SMV+SOF+RBV	YES	YES	Baseline	-					-		
					Failure	-					-		
#6*	4d	SMV+SOF+RBV	YES	NO	Baseline	-					-		
					Failure	-					-		
#7**	3a	DCV+SOF+RBV	YES	YES	Baseline			-			V321A		
					Failure			-			-		

*12 weeks of treatment, **24 weeks of treatment

Table 1: Evolution of NS3, NS5A, and NS5B Resistance-Associated Substitutions in seven cases, who did not achieved sustained virological response.

At baseline 4 subjects (patients #1, #2, #4 and #7) harbored RAVs. Two patients (#1 and #2) infected by subtype 1a showed the substitutions Q80K and V36G+R155K+M28V, respectively, while one patient (#4) with subtype 1b showed V36A+Y93H+E446Q+S556G+D559G. All these substitutions were detected as majority species of viral population (more than 90%). At the time of viral rebound, patient

#1 and #4 additionally accumulated R155K+D168E and Y56H+D168V, respectively. Interestingly, all the emerging mutations that were detected as majority species in more than 90% of strains at the virologic failure were not present as minority variants at baseline. Finally, patient #7 with subtype 3a showed at baseline the V321A

substitution as minority variant (12.5%), which was not present at time of failure.

Among subjects with subtype 4d, who failed SOF+SMV (#3, #5 and #6), none showed RAVs either at baseline or at virologic failure. In addition, by comparing these sequences with all the available sequences carrying this subtype in HCV Database, no variants differing from the natural polymorphisms associated with their subtype were observed.

Distribution of RAVs according to previous treatment experience

The analysis of the NS3 region in patients who had failed a previous TPV or SMV-containing regimen (n=11) showed mutations in 8 subjects (72.7%), with R155K being the most frequent substitution (7/8) (Table 2).

Interestingly, the available sequences from 6 patients performed before unsuccessful TPV or SMV-based treatments (ranging from 18 to 37 months before new DAA treatment, mean time: 25.8 months) were analyzed. Three patients showed RAVs before the first DAA treatment (37.5%) as majority species (Table 2). By comparing the mutations present before the first and the second DAA regimen, only substitutions Q80K and S122G were present at both time points.

Discussion

This study describes the prevalence of RAVs in a real life setting of patients who started a DAA regimen in an advanced stage of liver disease. At the time of this study, four NS3 protease inhibitors (BOC, TPV, SMV, PTV), three NS5A inhibitors (DCV, LDV, OMV), one non-nucleoside (DSV) and one nucleotide NS5B inhibitor (SOF) were available for the treatment of chronic hepatitis C in Italy.

Patient	Subtype	First DAA ^c regimen	Second DAA regimen	NS3 substitutions before first DAA treatment	NS3 substitutions before second DAA treatment
#58	1a	pegINF ^d +TPV+RBV ^e	LDV ^f +SOF ^g +RBV	NA ^h	T54S, R155K
#61	1a	pegINF+TPV+RBV	LDV+SOF+RBV	NA	R155K
#75	1a	pegINF+TPV+RBV	LDV+SOF+RBV	NA	-
#77	1a	pegINF+TPV+RBV	LDV+SOF+RBV	T54S, V55I, S122G	T54S, V55I, S122G, R155K
#84	1a	pegINF+TPV+RBV	SOF+DAC ⁱ	Q80K	T54S, Q80K, R155K
#92	1a	pegINF+TPV+RBV	SOF+DAC	-	R155K
#99	1a	pegINF+TPV+RBV	LDV+SOF+RBV	S122G	S122G, R155K
#111	1a	pegINF+TPV+RBV	LDV+SOF+RBV	-	V36M, R155K
#14	1b	pegINF+TPV+RBV	SOF+DAC+RBV	NA	Q41R, V55A, S122A, M175L
#56	1b	pegINF+TPV+RBV	LDV+SOF+RBV	NA	-
#79	4d	pegINF+SMV+RBV	LDV+SOF+RBV	-	-

^aTelaprevir; ^bSimeprevir; ^cdirect-acting antivirals; ^dPegylated interferon- α ; ^eRibavirin; ^fLedipasvir; ^gSofosbuvir; ^hNot available; ⁱDaclatasvir. Sequence analysis was performed using a cut-off of 15%.

Table 2: NS3 substitutions in patients previously failing TPV^a or SMV^b containing regimens.

The current report highlighted that the overall pre-treatment prevalence of DAA RAVs was high (more than 60%) in all the target regions using either 1% or 15% cut-off, being near 35% in NS3 and NS5B regions. This frequency is higher than that previously reported, particularly for the proportion of RAVs in NS5B region, that in this study was found similar to that observed in NS5A [5,9,22,24,25]. This discrepancy might in part depend on the different sensitivity of the sequencing method used; indeed, most of published studies used a direct sequencing with sensitivity around 20% rather than the NSG approach with a cut-off of 1%-15% used in the present investigation. However, we found a higher percentage of patients with baseline RAVs in NS5A and NS5B than what reported elsewhere even when a cut-off value of 1%-15% was used. [5,26] Despite the high prevalence in our case file of HIV-HCV co-infected patients (70.3%) who have been reported to harbor a high prevalence of HCV NS3 variants [27,28] and at least one RAV in up to 92.8% of genotype 1b in NS5B [29], we did not observe a significantly different rate of RAVs between HIV infected and uninfected subjects.

When only RAVs relevant for the ongoing regimen were considered, their frequency was similar to that previously described [22,27,30].

In accordance with other reports that used the two different cut-offs, RAVs occurred more frequently in subtype 1a than subtype 1b, particularly in the NS3 region where Q80K/R was detected in one third of cases. Interestingly, 33% of these subjects had been previously treated with TPV or SMV [22,27,25]. These data are in line with a recent work showing a higher genetic barrier to resistance for most protease inhibitors of HCV subtype 1b compared with subtype 1a [11].

In agreement with data on sequences from the European cohorts, NS5B RAVs were detected more frequently in subtype 1b (60%) compared to subtype 1a and genotype 4 (42%-32.5% and 25%, respectively) [31,25]. The substitution S556G, that confers intermediate resistance to DSV, appeared at a higher frequency in subtype 1b than in subtype 1a (56% vs. 28%). In addition, the C316N substitution, also known to confer intermediate resistance to DSV, was detected at a frequency of 56% in subtype 1b as naturally occurring variant. All

patients with subtype 3a showed only S556G substitution in NS5B, which is a natural variant in this genotype.

Regarding NS5A RAVs, although a recent paper [5] indicated as multiple mutations in this region are associated with a higher probability of viral relapse, we observed a limited number of NS5A mutations in patients with virologic failure.

In line with published data on the association between RAVs for different DAA and HCV genotype/subtype, this study indicated that subtype 1a and 1b presented RAVs especially for SMV and DSV, respectively, while RAVs for DCV, LDV and DSV were more represented in genotypes 3 and 4 [22,25].

In accordance with previous reports, no differences were observed in the distribution of RAVs according to cirrhosis or to a previous failure to pegINF+RBV therapy [32]. Unexpectedly, by the comparison of NS3 protease inhibitors-experienced patients with naïve or pegINF+RBV treated subjects, a higher frequency of NS5B RAVs was observed in the former group rather than the awaited higher proportion of RAS in the NS3 region. It could be of interest to investigate in a larger dataset whether a previous treatment with drugs directed against NS3 region might have exerted a selective pressure on the evolution of the NS5B region, as reported in HIV-1 infected patients who often show mutations in the gag or gp41 rather than in the protease region after a failure to protease inhibitor containing regimens [33].

Furthermore, the analysis of NS3 in patients retreated after an unsuccessful protease inhibitor-based regimen revealed the persistence of pre-selected mutations and the further addition of new RAVs. Indeed, although many NS3 RAVs are associated with a replicative impairment that leads to their rapid replacement by wild type virus after treatment interruption, we observed two substitutions, the Q80K and the S122G, carried by two patients with subtype 1a, that persisted for more than 30 months after treatment, longer than previously reported [34,35]. Indeed, Q80K mutation was not associated with loss of viral fitness [36], while only *in vitro* experiments would clarify the impact on HCV replicative capacity of S122G, known to confer resistance to SMV in subtype 1a [10]. In this study 9.4% of patients did not achieve SVR. From published data, the prevalence of RAVs in subjects who relapsed after a DAA regimen ranged from 53% to 91% [31,34]. Accordingly, 57.1% of our relapsing patients showed pre-existing resistance mutations. Interestingly, the presence at baseline of two major mutations, such as the Q80K and the Y93H, conferring intermediate to high level of resistance to all NS3 and NS5A inhibitors, respectively, and the emergence of additional mutations at failure could explain the virological failure in 2 of the 7 relapsers. The disappearance of baseline mutations at the time of failure in 2 other relapsing patients might be justified by the time-span between the treatment suspension and the sequence analysis (12 weeks) that could have allowed the emergence of wild type virus.

Finally, three patients harboring genotype 4 failed to SOF+SMV+RBV therapy despite the absence of any mutations.

Similarly to what recently observed in a large cohort of patients with genotype 4 treated with SMV plus pegIFN and RBV [37], a low frequency of RAVs in genotype 4 and none of them in NS3 was found in this study, confirming the high genetic barrier of genotype 4 to protease inhibitors [11].

Regarding the role of NGS with a cut-off of 1% for the detection of RAVs, its use seems to be of limited utility in predicting the selection of

substitutions under drug pressure. All the pretreatment RAVs were present as majority species and all the emerging mutations were not present at baseline not even as minority variant despite the very high sensibility of NGS approach. The exception is represented by the substitution V321A associated with a low decrease in susceptibility to SOF, which was present at baseline as minority variant and disappeared at virologic failure. It was suggested that V321A variant likely pre-exists treatment initiation at low abundance, due to its reduced replicative capacity; however the slightly reduced susceptibility to SOF allows its expansion over wild type during treatment, at the end of which V321A is rapidly replaced by wild-type that has a greater replication efficiency compared to mutant [26].

The limited number of patients analyzed represents the major limitation of the present study that could not investigate the prevalence of RAVs on genotypes 2, 5 and 6, absent in this cohort. Moreover, the study population was heterogeneous in terms of DAA regimens, HCV genotypes and HIV co-infection, reflecting the real life nature of our cohort analysis. However, the adherence to the EASL 2015 treatment guidelines should have ruled out the impact of the different regimens used on the clinical significance of pre-treatment RAVs testing. In conclusion, the impact of pre-treatment RAVs on the probability of SVR with DAA is limited and only NS5A testing possibly plays a role for treatment decisions. However, resistance testing should be useful to guide the choice of the new regimen in all DAA-failing patients, particularly in view of the long-term RAVs persistence after failure [38]. In conclusion, characterizing the RAVs scenario for each genotype is important to gain information on mutations involved in drug resistance in specific genotype and to study the impact of their persistence overtime.

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