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## No correspondence between resistance mutations in the HCV-NS3 protease at baseline and early telaprevir-based triple therapy



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### ABSTRACT

Direct-acting antiviral (DAA)-based therapy is the new standard treatment for chronic hepatitis C virus (HCV) infection. However, protease inhibitor (PI)-resistant viral variants have been often described. This study aimed to examine HCV-NS3 protease variants at baseline and at 4 weeks under triple therapy. To this end, we analyzed the presence of variants in HCV-NS3 protease region from peripheral blood samples of 16 patients infected with HCV-1 at baseline and at 4 weeks of combined therapy with telaprevir, pegylated interferon, and ribavirin, using next-generation sequencing. Several variants with synonymous and non-synonymous amino acid substitutions were detected at both time points. Variants detected at low frequency corresponded to 74% (HCV-1a) and 35% (HCV-1b) of non-synonymous substitutions. We found nine PI-resistance-associated variants (V36A, T54S, V55I, Q80K, Q80R, V107I, I132V, D168E, M175L) in HCV-NS3 of 10 patients. There was no correspondence of resistance-associated variant profile between baseline and at 4 weeks. Moreover, these resistance variants at baseline and short-term treatment are not good predictors of outcome under triple therapy. Our study also shows a large number of others minor and major non-synonymous variants in HCV-NS3 early in telaprevir-based therapy that can be important for further drug resistance association studies with newly developed PI agents.

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### 1. Introduction

Hepatitis C virus (HCV) infection, a worldwide public health problem, affects 135 million people, i.e., 3% of the world population [1]. Nearly 80% of infected individuals develop chronic disease, often associated with liver cirrhosis and hepatocellular carcinoma [1]. No vaccine has been developed yet for HCV. Conventional therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) is effective for only 40% of patients infected with the most prevalent genotype (HCV-1) [2,3]. Therefore, direct-acting antiviral (DAAs) agents, particularly protease inhibitors (PI), have emerged as a major advance in hepatitis management, with several DAAs currently under development [4].

Early emergence of viral resistance mutations has been, however, associated with PI monotherapy [5–8], with several of these mutations showing cross-resistance to multiple PI agents [9]. Still, PI resistance mutations are widespread and even present in naïve patients [10–15], indicating that monotherapy with PI may select for resistance mutations already present at baseline of treatment [6,16]. Consequently, previous studies have suggested that the presence of resistance mutations before treatment could be a reliable predictor of PI-based HCV therapy efficacy [12,17].

Even though routine baseline resistance mutation detection before PI therapy is still prescribed as a prognostic tool [18], the predictive value of these mutations at baseline remains controversial. In fact, recent research [19,20] has suggested that resistant variants that emerge during PI therapy might not be the same as those identified at baseline. Moreover, the presence of resistance mutations before treatment could not be associated with therapy outcome in samples of non-cirrhotic patients [7,21]. However, it is not yet possible to discriminate the influence of potentially confounding factors on these results, including previous

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**Table 1**

Profile of hepatitis C virus-infected patients at commencement of telaprevir, pegylated interferon, and ribavirin triple therapy.

No. of patients (men/women)	16 (8/8)
Age (years)	48.8 ± 13.4
Body weight (kg)	81.4 ± 13.5
Body mass index (kg/m <sup>2</sup> )	30.4 ± 6.8
Hemoglobin (g/dL)	14.8 ± 1.6
Platelet count (× 10 <sup>4</sup> /μL)	17.6 ± 6.5
Alanine aminotransferase (IU/L)	107.2 ± 53.8
HCV genotype (1a/1b)	8/8
HCV RNA level (IU/mL) [median (range)]	962,112 (9,106–13,629,444)
Previous response (naïve/null/relapse)	10/3/3
Liver fibrosis (F1–F2/F3–F4)	2/14
Cirrhosis (%)	31.3

treatment, host-dependent factors, HCV-genotype, and the specific antiviral agent used [21,22]. Also, it is not known whether these observations can be generalized to other populations of varying disease severity.

### 1.1. Objectives

Our study investigates and compares variants of HCV-NS3 protease—an enzyme with a key role in viral replication—in chronic PI-naïve patients infected with HCV genotype 1 (HCV-1) under triple therapy (telaprevir, PEG-IFN, and RBV) at baseline and after 4 weeks of treatment. To this end, we use high throughput sequencing technology to detect even low-frequency resistance mutations, which can be underestimated by conventional sequencing methodology.

## 2. Materials and methods

### 2.1. Study population

Sixteen patients infected with hepatitis C virus genotype 1 were recruited from August to December, 2013 at the Hepatology Service of the Hospital Universitário Clementino Fraga Filho (HUCCF), Rio de Janeiro (RJ), Brazil. Patients who were chronically mono-infected with HCV genotypes 1a or 1b were previously selected by their attendant clinician to enroll in a telaprevir-based triple therapy protocol (telaprevir (TVR) 750 mg, 3×/d + PEG-IFNα 1.5 μg/kg of body weight

once a week + RBV 1,000–1,500 mg/d, for 48 weeks). Triple therapy is recommended for mono-infection with HCV-1, advanced liver fibrosis or cirrhosis, compensated liver disease, and absence of previous protease inhibitor (PI) treatment. Written informed consent was obtained from every patient.

### 2.2. HCV RNA isolation, NS3 protease region amplification, and viral load determination

Serum from 16 patients was collected at baseline (PRE) and after 4 weeks (Week 4) of TVR/PEG-IFN/RBV treatment. HCV RNA was isolated, its NS3 protease region amplified and directly sequenced for HCV-genotype confirmation as previously described [15]. HCV genotypes were obtained by comparing their sequences with those at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Los Alamos HCV databases (<http://hcv.lanl.gov/content/index>).

HCV RNA levels were determined by real time PCR, using Abbott RealTime HCV assay (Abbott GmbH, Wiesbaden, Germany) (quantification range of 12 to 100 million IU/mL) [23].

### 2.3. Next-generation sequencing (NGS) and HCV-NS3 protease region analysis

High throughput sequencing was performed using Ion Torrent Personal Genome Machine (PGM)<sup>TM</sup> sequencer (Life Technologies, Carlsbad, CA). Ion Torrent adapter- and barcoded-ligated library was prepared using NS3 protease PCR products (555 bp), according to Ion protocol for long amplicon (>400 bp) library preparation and using Ion Xpress<sup>TM</sup> Plus Fragment Library kit. Briefly, PCR products were quantified by Qubit<sup>®</sup> fluorometer (Invitrogen), using Qubit<sup>®</sup> dsDNA BR Assay kit, and enzymatically fragmented for 4 min, using Ion Shear<sup>TM</sup> Reagents. Fragments were then purified using Agencourt<sup>®</sup> AMPure<sup>®</sup> XP Reagent. Fragmentation pattern was analyzed by semi-fluidic electrophoresis (Agilent<sup>®</sup> Bioanalyzer<sup>®</sup>, GE), using Agilent High Sensitivity DNA Kit. Barcoded adapters were ligated, and fragments were size-selected using E-gel<sup>®</sup> SizeSelect<sup>TM</sup> Agarose Gel. Emulsion PCR was performed using Ion OneTouch<sup>TM</sup> and Ion OneTouch<sup>TM</sup> 200 Template Kit. Ion sphere particle (ISP) enrichment was obtained using Ion OneTouch<sup>TM</sup> ES and Ion OneTouch<sup>TM</sup> 200 Template Kit. Templated ISPs were loaded onto 316<sup>TM</sup> or 318<sup>TM</sup> Chips and sequenced according to Ion PGM<sup>TM</sup> 200 sequencing kit protocol.

**Table 2**

Treatment outcome and number of synonymous and non-synonymous variants in NS3 HCV region in patients infected with HCV at baseline (PRE) and after 4 weeks treatment of triple therapy.

HCV type	Case	Response to previous treatment	Number of RAVs		Number of other non-synonymous variants		Number of synonymous variants		Outcome (triple therapy)
			PRE	Week 4	PRE	Week 4	PRE	Week 4	
1a	4	Naïve	2	*	7	*	47	*	SVR
	5	Naïve	0	*	6	*	44	*	SVR
	6	NULL	2	*	4	*	52	*	REL
	7	Naïve	1	*	5	*	88	*	REL
	8	Naïve	0	*	11	*	84	*	§
	12	Naïve	0	1	5	5	48	28	SVR
	13	Naïve	0	*	4	*	30	*	R
	22	NULL	0	2	3	8	51	33	R
1b	3	REL	1	3	8	17	59	47	SVR
	9	Naïve	0	0	7	10	62	38	R
	10	REL	0	*	3	*	49	*	R
	11	NULL	0	*	3	*	45	*	NR
	14	Naïve	1	*	4	*	53	*	R
	17	REL	0	4	2	20	47	59	R
	19	Naïve	1	*	*	*	67	*	R
	20	Naïve	1	0	9	6	47	54	NR

RAVs: resistance-associated variants; SVR: sustained viral response; REL: relapse; NR: non-responder; R: responder (undetected HCV RNA level after 48 weeks of treatment, but still incomplete 6 months follow-up).

\* Undetected viral load.

§ Deceased before end of treatment.

Sequences were analyzed with CLC Genomics Workbench v.7.5 software (CLC bio, Aarhus, Denmark) (<http://www.clcbio.com>). The CLC resequencing tool was used to compare generated sequences to HCV-1a (accession number AF009606) and HCV-1b (accession number D90208) references. Reads were size- (<30 or >250 bp) and quality-trimmed, and quality score >0.05 with maximum of 2 ambiguities was used. Synonymous and non-synonymous variants, with frequency lower than 1%, were discarded.

### 3. Results

We studied 16 patients infected with HCV genotypes 1a and 1b, naïve for protease inhibitor-based therapy. All patients, chronically infected, were selected by the Hepatology Department for triple therapy. Most of them had severe liver fibrosis at the beginning of treatment. Table 1 shows the demographic, clinical, and virological characterization of the population. Six patients had received previous therapy with

PEG-IFN and RBV, showing null ( $n = 3$ ) or relapsed response ( $n = 3$ ) (Tables 1 and 2).

After size- and quality trimming, an average of 182,093 reads (mean size of 157 bp) of the NS3 protease gene region were obtained for each sample. At 4 weeks of treatment, we obtained HCV-NS3 protease variants from 6 of the 16 patients studied, while 10 had no detectable HCV RNA. NGS sequencing was able to detect even low-frequency (<20%) variants. Several synonymous and non-synonymous substitutions were identified at baseline and 4 weeks of treatment (Table 2). Four of the six patients with detectable HCV-RNA levels at 4 weeks were infected with HCV-1b. In those patients, the total number of variants (synonymous and non-synonymous) at 4 weeks was either similar to or higher than at baseline. Triple therapy was discontinued before 48 weeks in three patients, either because of HCV RNA level above 1,000 IU/mL (considered non-responders, NR) or death (one case). All others completed the 48 weeks of treatment. Telaprevir-based therapy was effective in 11 cases: 4 patients showed sustained viral response (SVR, undetected HCV RNA level at 6 months after therapy),

**Table 3**  
Treatment outcome, HCV RNA level, and frequencies ( $f$ ) of non-synonymous variants in NS3 HCV region in patients infected with HCV genotype 1a at baseline (PRE) and after 4 weeks (W4) treatment of triple therapy.

Outcome	Case 4	Case 5	Case 6	Case 7	Case 8	Case 12	Case 13	Case 22
	SVR	SVR	REL	REL	§	SVR	R	R
	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4
<b>HCV RNA</b> (log IU/mL)	5.5-*	6.29-*	5.58-*	6.66-*	7.13-*	NI-<1.08	5.57-*	6.25-1.45
<b>Non-synonymous variants (<math>f</math>%)</b>								
A1E					11.5-0.0			
P2L		4.7-*		9.0-*	3.2-*			
I3F					3.4-*			
I3L		3.2-*						
T4R		1.1-*				2.7-0.0		
T4K		1.4-*				5.3-0.0		
A5P					12.3-*			
A5L					9.0-*			
Y6H	5.1-*		2.6-*		3.8-*		3.5-*	1.6-0.0
A7S							2.7-*	
L13F					5.7-*			
I18V	89.5-*							
G23S								
Q28E				98.8-*				
V33I				97.4-*				
<b>V36A</b>	<b>3.2-*</b>							
T40A						95.0-0.0		
A45T					2.0-*			
I48V				63.8-*				4.3-0.0
<b>T54S</b>			<b>2.8-*</b>			<b>0.0-99.4</b>		<b>0.0-98.8</b>
<b>V55I</b>			<b>2.7-*</b>					<b>0.0-94.5</b>
T61S						1.6-0.0		
R62K					34.6-*			
I64V					1.0-*			
S66T			2.5-*			4.4-99.6		0.0-99.5
P67S				95.9-*				0.0-1.1
P70L								0.0-1.1
<b>Q80K</b>	<b>3.3-*</b>							
S91A								98.4-97.1
<b>V107I</b>				<b>1.9-*</b>				
R109G	1.2-*							
V113I								0.0-97.1
R130Q			2.3-*					
A147S	5.6-*							
L153I	98.2-*	92.6-*	99.0-*		98.2-*	0.0-99.4	98.9-*	*-97.4
L153V								0.0-2.4
A157V	9.4-*							
N174H	11.3-*							
N174S		13.7-*						
M179L						0.0-96.8		0.0-96.4
R180S						0.0-92.1		
S181P						0.0-6.9		0.0-99.5

In bold: variants associated with resistance to protease inhibitors (underlined bold: variants associated with telaprevir resistance); NI: not informed.

§ Deceased before the end of treatment.

\* Undetected viral load.

while 7 responders (R) had undetected HCV RNA level at 48 weeks and are completing the 6 months follow-up to characterize SVR. Triple therapy was not effective for 2 of 3 patients with null response to previous PEG-IFN/RBV treatment, while the previously relapsed ones showed either SVR or undetected HCV RNA level at 48 weeks (Table 2).

Tables 3 and 4 show the frequency of variants with non-synonymous substitutions in HCV-NS3 protease region in sub-types 1a and 1b, before and at 4 weeks of PI-based therapy. Low-frequency variants corresponded to 74% and 35% of non-synonymous mutations identified

in HCV-1a and -1b, respectively. All patients, in both HCV genotypes, presented more than one HCV-NS3 protease non-synonymous mutation. On the other hand, most of the detected variants were identified in only one patient, while few (29% for HCV-1a and 42% for HCV-1b) showed high prevalence. Only 4.8% (out of 42 substitutions for HCV-1a) and 13.5% (out of 52 substitutions for HCV-1b) of the variants present at baseline remained at 4 weeks, showing no correspondence between variant profile mutations emerged under therapy and those at baseline.

In 10 out of 16 patients, we identified 9 non-synonymous substitutions in the NS3 protease variants previously associated with protease

**Table 4**

Treatment outcome, HCV RNA level, and frequencies (*f*) of non-synonymous variants in NS3 HCV region in patients infected with HCV genotype 1b at baseline (PRE) and after 4 weeks (W4) treatment of triple therapy.

Outcome	Case 3	Case 9	Case 10	Case 11	Case 14	Case 17	Case 19	Case 20
	SVR	R	R	NR	R	R	R	NR
	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4	PRE-W4
<b>HCV RNA</b> (log IU/mL)	5.10–1.08	5.79– < 1.08	6.20–*	5.98–*	5.86–*	6.21–NI	6.23–*	3.96–1.08
<b>Non-synonymous variants (f%)</b>								
S7A		0.0–25.6				46.2–0.0		40.8–0.0
I18V		0.0–1.4						
D30E	97.1–0.0	94.4–94.2			93.5–*			95.8–0.0
L36V	99.3–0.0	99.2–99.2			98.4–*			
T40A								0.0–1.5
S42T			98.1–*			0.0–70.0		
S42F								0.0–1.1
F43L						0.0–4.2		
T46A								1.1–0.0
V48A								99.1–0.0
V48I	99.0–96.0				98.8–*	0.0–92.9		
N49S		99.1–0.0						
<b>T54S</b>	<b>0.0–100</b>					<b>0.0–94.7</b>		
<b>V55I</b>	<b>0.0–97.6</b>					<b>0.0–94.7</b>		
Y56F		99.0–99.2	98.2–*	99.3–*		99.0–4.0		
S61T	0.0–98.5					0.0–96.0		
K62R	0.0–100							
L64M	0.0–100					0.0–96.4		
G66T	0.0–100					0.0–92.6		
K68N		0.0–97.8						
K68T								0.0–95.8
I71V	0.0–100					0.0–96.2		
T72I	0.0–98.5							
T72N								98.5–0.0
N77S		98.2–99.8				0.0–99.8		99.9–99.8
<b>Q80R</b>								<b>99.4–0.0</b>
W85R	0.0–1.1							
P89Q						0.0–94.2		
R92H						0.0–1.1		
P96Q					96.9–*			
M94I		1.8–0.0						
M94L	99.7–88.7	0.0–98.8				0.0–1.5		
V114I	0.0–96.1	0.0–96.6				0.0–98.3		
G120V						0.0–1.2		
D121E	0.0–11.0					0.0–4.5		
P131S	1.5–0.0							
<b>I132V</b>	<b>99.7–0.0</b>					<b>99.3–*</b>	<b>0.0–1.2</b>	
L144F	0.0–1.2							
S147A	0.0–96.7					0.0–97.6		
S147L	85.5–0.0							
S147P						0.0–1.0		
V150A	0.0–99.5					0.0–98.8		
V151A				99.6–*				
<b>D168E</b>							<b>33.7–*</b>	
I170V	99.9–0.0	98.8–99.9	99.3–*					98.1–97.7
I170M	98.5–0.0			1.2–*				97.7–0.0
V172I								0.0–1.1
S174A								99.3–0.0
S174N	0.0–97.8					0.0–95.5		
<b>M175L</b>	<b>0.0–97.8</b>					<b>0.0–95.5</b>		
M179L	0.0–98.6							
S181P	0.0–99.6					0.0–99.2		

In bold: variants associated with resistance to protease inhibitors (underlined bold: variants associated with telaprevir resistance); NI: not informed.

\* Undetected viral load.

inhibitor resistance, such as V36A [associated with resistance to telaprevir (TVR), boceprevir (BOC), danoprevir (ITMN-191), paritaprevir (ABT-450)], T54S [TVR, BOC, simeprevir (TMC-435), faldaprevir (BI-201335)], V55I (BOC, ITMN-191, ABT-450), Q80K (TMC-435), Q80R (TMC-435), V107I (BOC), I132V (TVR), D168E [TVR, BOC, TMC-435, BI-201335, ITMN-191, asunaprevir (ASV)] and M175L (BOC) (Tables 3 and 4). Five of these patients presented more than one resistance mutation, most of which confer cross-resistance to more than one drug. For HCV-1a infected patients, resistance mutations had a low frequency (<4%) at baseline yet high frequency (>94%) at 4 weeks. Conversely, the frequency of most resistance mutations in HCV-1b infected patients was high at both time points. It is noteworthy that, for both genotypes, virus resistance mutations identified at baseline did not persist at 4 weeks, whereas those detected at the latter time point were not present at baseline. Patients with resistant mutations at baseline showed diverse outcomes—from SVR to NR—suggesting no correlation between baseline profile and outcome. On the other hand, most patients presented also non-synonymous substitutions not yet described as RAVs, both at baseline and Week 4, which could potentially impact outcome (Table 3 and 4). The presence of high frequency telaprevir RAVs at baseline (cases 3, 14 and 19) did not predict therapy failure. Despite I132V variant high prevalence at baseline, it was not detected at week 4, while T54S, not detected at baseline, showed high frequency at week 4. Case 12, with no RAVs at baseline, presented telaprevir RAV-T54S variant at week 4, even though had SVR.

#### 4. Discussion

By using ultra-deep sequencing, we conducted a thorough assessment of HCV-NS3 protease variants in chronic PI-naïve patients infected with HCV-1a and HCV-1b under telaprevir-based triple therapy at baseline and after 4 weeks of treatment. Several synonymous and non-synonymous substitutions, including those at very low frequencies, were detected for both genotypes at both time points. However, there was no correspondence between resistance variants detected at baseline and at 4 weeks. Our results showed that triple therapy was effective for 11 patients (69%) since they showed SVR or had undetected HCV RNA level at 48 weeks of treatment (final sustained response will be available at 6 months after the end of treatment).

The observation that resistance mutations at baseline were not identified at 4 weeks is in contrast to previous suggestions that the widespread natural occurrence of HCV-resistant variants could explain the detection of resistance variants following PI monotherapy [16]. Non-synonymous resistance variants were detected at baseline in the peripheral blood of all patients, supporting the existence of circulating viral populations. The improved detection capacity of the NGS method used here may explain the observation of more highly prevalent and variable mutations at baseline in the peripheral blood than those detected by previous studies in Brazil [11,15,24,25] and other countries [12,18,26,27]. Our results therefore confirm and expand previous research, providing a comprehensive databank of non-synonymous HCV-NS3 variants induced by short-term therapy, which can be of potential importance for future drug resistance association studies involving approved and newly developed PI agents.

The predictive potential of baseline resistance variants remains controversial. Some authors still support routine baseline resistance mutation detection before PI therapy [18], while others report that resistant variants emerging during PI therapy are the same as those identified at baseline [19,20]. However, most studies have investigated emerging resistance mutations late in treatment or post-treatment, i.e., at the time of viral load re-elevation [19]. Although in our study only six patients had detectable HCV RNA level under treatment, resistance variants were detected already at 4 weeks, yielding early virus-diversity information that could be important for understanding viral-variant dynamics and guiding treatment.

Recent studies showed no association between the presence of resistance mutations before treatment and treatment outcome in samples of non-cirrhotic patients [7,21]. Our data potentially support and expand the previous observation to patients with severe liver fibrosis and cirrhosis since emerging resistance variants at 4 weeks were different from those present at baseline (see also [28]). On the other hand, recent study suggested that baseline variants profile could be predictive of outcome when accompanied by previous failure to classical PEG-IFN/RBV treatment [22]. Moreover, since resistance variants identified early in treatment were not concordant with baseline profile, the predictive prognostic power of those therapy-induced substitutions should be replicate in other cohorts to further support our interpretation.

In conclusion, our findings support the concept that HCV-NS3 resistant variants at baseline are not reliable predictors of resistant mutations that may emerge under PI treatment. Non-synonymous HCV-NS3 variants induced by short-term therapy, instead, could be important for future drug development as well as for better understanding viral-variant dynamics and guiding treatment. In addition, our study showed a large number of non-synonymous substitutions not yet described in HCV-NS3 protease region from patients both at baseline and short-term telaprevir-based therapy that can be useful for drug resistance association studies.

#### Transparency Document

The Transparency document associated with this article can be found, in the online version.

#### Conflict of interest

None declared.

#### Ethical approval

The study was approved by the Ethics Committee of HUCCF, UFRJ (number 166/05).

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