Journal of Clinical Virology 78 (2016) 36–43

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Clinical relevance of the HCV protease inhibitor-resistant mutant viral load assessed by ultra-deep pyrosequencing in treatment failure

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ARTICLE INFO

Article history:
Received 1 October 2015
Received in revised form 19 February 2016
Accepted 23 February 2016

Keywords:
HCV
Ultra-deep pyrosequencing
Protease inhibitors
Mutant viral load

ABSTRACT

Background: The detection of low frequency mutants in patients with hepatitis C virus (HCV) receiving direct-acting antivirals (DAAs) is still debated. The clinical relevance of the mutant viral load has not yet been evaluated.
Objectives: To assess the viral load of resistance associated variants (RAVs) in patients at different time points, including the baseline, virological failure and one year after the cessation of therapy.
Study design: The study included 22 patients who were previously treated with protease inhibitors (PI) (with telaprevir and boceprevir). For each patient, three time points were assessed using ultra-deep pyrosequencing (UDPS).
Results: Baseline mutations were observed in 14/22 patients (64%). At virological failure, RAVs were detected in 18/22 patients (82%). Persistent RAVs were observed in four HCV GT 1a patients (18%). Persistence mutations were found only in HCV GT 1a patients. The baseline relative V36M, R155K, R155T and A156T mutation load of patients with persistent RAVs was significantly higher ($P < 0.001$) than those of patients without persistent RAVs.
Conclusion: The UDPS follow-up analysis demonstrated that the presence of BOC or TLP-RAVs persist one year after therapy cessation only in HCV GT 1a patients. The relative mutant viral load should be considered prior to any PI based re-treatment. This concept of the baseline mutation viral load must be validated using current therapy and must be validated on a larger cohort.

1. Background

To treat chronic HCV genotype (GT) 1-infected patients, telaprevir (TLP) or boceprevir (BOC), first generation protease inhibitors (PIs), were used in combination with pegylated-interferon (PEG-IFN) and ribavirin (RBV) triple therapy [1,2]. The main weaknesses of the first-generation PIs are their low genetic barrier to resistance and the fact that their effectiveness is limited to GT-1 patients [2–5]. A second-wave of first generation PIs has a higher barrier to resistance; better activity against multiple genotypes, except GT-3 [6]; more convenient dosing schedules; and improved safety and tolerance. Second-generation PIs are broadly active against all genotypes and against viral isolates that carry resistance mutations for first-generation PIs [7]. In combination with PEG-IFN and RBV, the new PIs appear to achieve greater sustained virologic response (SVR) rates than the first-generation PIs [8]. IFN-free options combining several direct-acting antiviral (DAA) regimens progressively replaced the use of PEG-IFN with a highly sustained virological response [9,10]. Not surprisingly, resistance has been

Abbreviations: HCV, hepatitis C virus; GT, genotype; PIs, protease inhibitors; TLP, telaprevir; BOC, boceprevir; PEG-IFN, pegylated-interferon; RBV, ribavirin; SVR, sustained virologic response; DAAs, direct-acting antivirals; RAVs, resistance associated variants; UDPS, ultra-deep pyrosequencing; VF, virological failure; MID, multiplex identifier; AVA, Amplicon Variant Analyzer; GS Junior, Genome Sequencer Junior; BLNP, variants analyser; BLP, baseline patients with persistence resistance variants; VFNP, virological failure patients with no persistence resistance variants; VFP, virological failure patients with persistence resistance variants.

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http://dx.doi.org/10.1016/j.jcv.2016.02.022
1386-6532/

Table 1
RT-PCR and Nested-PCR primers for NS3/4a HCV genome amplification.

Name	Polarity	Sequence primer (5' → 3')[a]	Position H77 (bp)
RT-PCR			
UDSNS3F1	Forward	ATGGARAAGAARRTYATYRTITGGG	3276–3300
UDSNS3F2	Forward	ATGGARAYYAAGVTYATYACITGGG	
UDSNS3R1	Reverse	CTYTTICCRCTICCIGTIGGIGCRTG	4026–4051
Nested-PCR			
NestUDSNS3F1	Forward	AdaptatorA-Key-MID-CTBCTSGGRCCRGCCGAT	3375–3392
NestUDSNS3R1	Reverse	AdaptatorB-Key-MID-GCCACYTGGWAKSTCTGSGG	3980–3999

Y = C or T, R = A or G, V = A or C or G, I = *d*-Inosine were used as "universal" nucleotides to replace A or G or C or T; bp, base pair.
[a] Adaptator (A or B)-Key-MID primer sequences not shown (454 technology).

demonstrated for DAAs in clinical tests, specifically for PIs and NS5A polymerase inhibitors [11]. To our knowledge, the possibility of the long-term persistence of NS3 resistance mutations and their impact on PI re-treatment is unknown.

Ultra-deep pyrosequencing (UDPS) techniques with a cut-off below the standard 20% threshold of Sanger sequencing may provide additional information regarding the evolution and importance of low-level variants for the prediction of the viral response to HCV inhibitors [12].

2. Objectives

The first aim of this study was to assess the prevalence of baseline, virological failure (VF) and the persistence of resistant variants using UDPS. The second aim was to evaluate the relative resistant mutant viral load. Importantly, analyses on RAVs before, during and after antiviral treatment have already been described, but the relative resistant mutant viral load concept has not been described.

2.1. Study design

2.1.1. Patients

Twenty-two patients were retrospectively collected from treatment-experienced HCV-infected patients with VF attending a European hospital (Marseille, France) for diagnosis or treatment monitoring with PEG-IFN/Ribavirin/TLP or BOC triple-therapy. The majority of the treatments were started between 2009 and 2013. Authorization for the use of these two anti-HCV PIs was obtained following the French guidelines [3]. VF was defined as a viral load higher than 1000 copies/mL after adequate viral suppression (defined as a viral load of 400 copies/mL) in accordance with World Health Organization guidelines [13]. According to Article L1121-1 of the French Public Health law, non-interventional studies are not subject to a legal framework. Non-interventional studies are defined as actions that are routinely performed without any additional procedure or unusual diagnostics or monitoring. The viral RNA was extracted from 200 ml of plasma (previously stored at −80 °C) using the Nuclisens RNA extraction kit (Macherey-Nagel, Germany). The RNA was eluted in 50 ml of elution buffer according to the recommendations of the manufacturer.

2.1.2. UDPS error rate for mutation detection

To estimate the error rate of UDPS, PCR amplification and Genome Sequencer (GS) junior sequencing were performed in duplicate. The HCV plasmid DNA encoding for the NS3 region of the HCV reference H77 strain was synthesized by the Eurofins MWG Operon. The mutant plasmids were synthesized with the following 10 clinically relevant HCV drug resistance mutations: V36AM, Q41RP F43C, T54S, V55A, Q80K, R109K, R155K, A156T and V170A [14–16].

2.1.3. RNA amplification

cDNA synthesis was performed using 5 μL of eluted RNA at 50 °C for 30 min using one-step RT-PCR (Qiagen GmbH, Germany). The one-step RT-PCR Enzyme Mix contains enzyme for both reverse transcription and PCR. Reverse transcription and PCR are carried out sequentially in the same tube. One fragment of the HCV NS3/4A GT 1 was amplified using a nested-PCR approach. The first PCR was performed using two forward primers and one reverse primer (Table 1). To generate the sequencing fragment, a second PCR reaction was then performed. Twenty fusion primers were designed with amplicon adaptor sequences, multiplex identifier (MID) tags on both the forward and reverse primers and a sequence-specific primer (Table 1). The outer PCR was conducted using 0.4 μM of the two antisense primers and 0.8 μM of the antisense primer in a 12.5 μL reaction. PCR was performed using a temperature profile of 95 °C for 15 min and 45 cycles of 94 °C for 15 s at 53 °C for 1 min and 72 °C for 1 min, followed by a 4 °C cooling step. The inner PCR was performed on 1 μL of the outer PCR product and 0.2 μM concentrations of each primer. The inner PCR was performed using a temperature profile of 95 °C for 15 min and 35 cycles of 94 °C for 30 s at 59 °C for 1 min and 68 °C for 1 min, followed by a final extension at 68 °C for 5 min and a 4 °C cooling step.

2.1.4. Ultra-deep pyrosequencing (Fig. 1)

The nested PCR products were then purified using Agencourt AMPure XP magnetic beads (Agencourt, Beckman Coulter, USA) and were quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Molecular probe, USA). After amplicon mixing and equimolar pooling to 2 × 106 molecules/μL, emulsion-PCR was performed at a ratio of two molecules per bead. The GS Junior bead counter was used to evaluate the amount of enriched DNA beads. To ensure optimal picotitre plate loading, 500,000 enriched DNA beads were used in each well. The amplicons were then sequenced from both ends.

2.2. Data analysis

The GS Amplicon Variants Analyser software (AVA®) (Roche 454 Life Sciences, Branford, CT) was used for read alignment mapping using HCV reference H77 strain AF009606 variant calling and for de-multiplexing the twenty pooled patient data using the MID sequences. Data generated from AVA® were also analysed with DeepChek®-HCV software (ABL SA, Luxembourg) [17]. The amino acid substitutions associated with BOC and/or TLP resistance or possible resistance for GT 1 reported in the AVA® and DeepChek®-HCV software programs [11,18,19].

2.3. Persistence of RAVs

RAVs were defined as persistent if they were detected at baseline and/or at VF and one year after therapy cessation with a >0.5% frequency. The detection of HCV RNA was performed using the Roche COBAS AmpliPrep/COBAS TaqMan PCR systems (Roche Diagnostics,

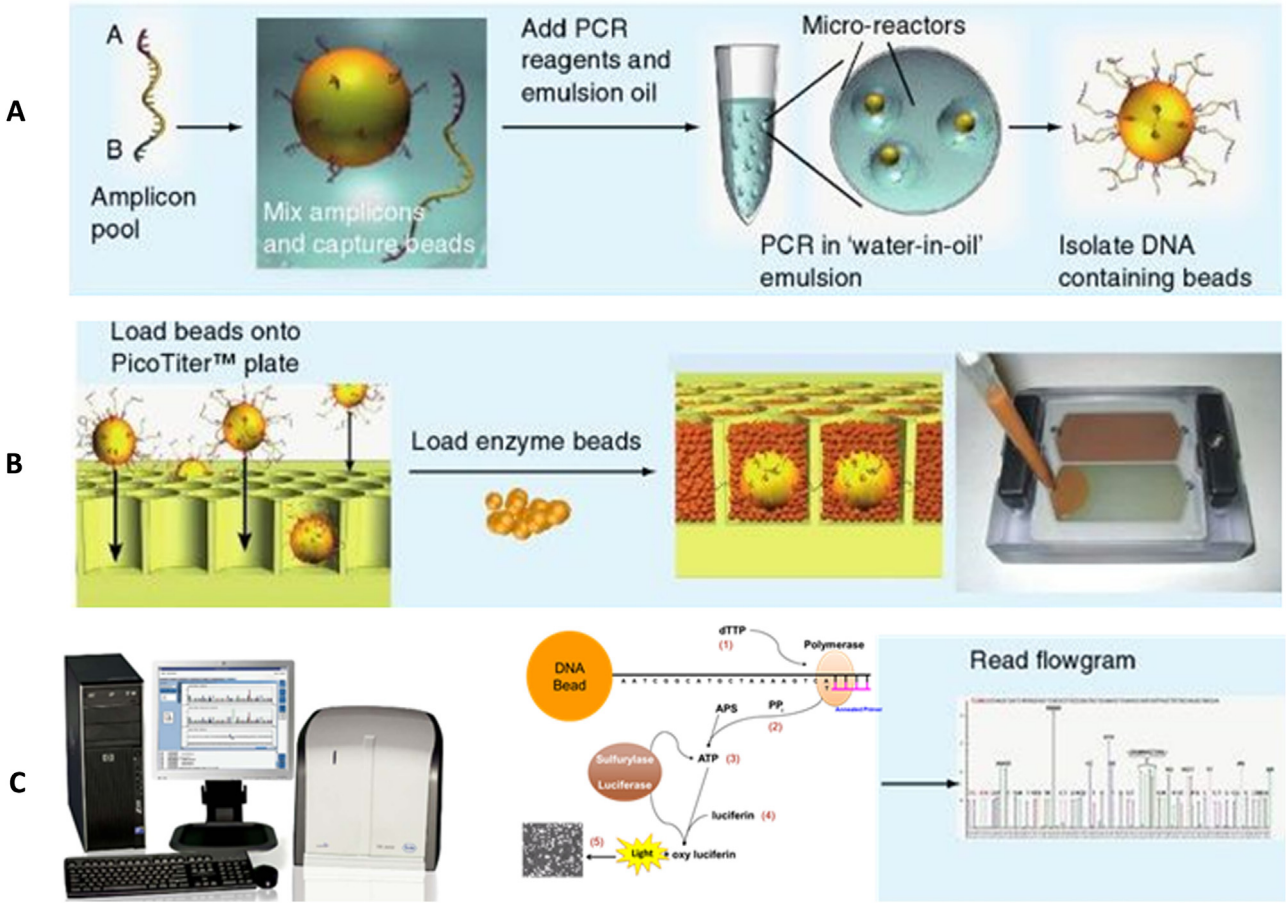

Fig. 1. Workflow of Genome Sequencer Junior System.
(A) EmPCR amplification with 2 molecules of library DNA per capture bead PCR reagents in water-in-oil microreactors. 2 molecules will yield a bead enrichment between 5% and 20%, and will generate satisfactory sequencing results. (B) Depositing 500.000 beads DNA beads into the PicoTiterPlate. The recommended input bead number for a GS Junior sequencing Run is 500,000 enriched beads. (C) Sequencing-by-synthesis on the GS Junior Sequencer.

Mannheim, Germany) in patient plasma as the three time points. Two groups of patients were defined for the baseline and VF time points according to the presence of persistent RAVs: (i) patients with no persistent RAVs (BLNP or VFNP) and (ii) patients with persistent RAVs (BLP or VFP) for baseline or VF, respectively. Only the mutant viral loads of persistent RAVs were selected (V36M, R155K, R155T and A156T).

2.4. Statistical analysis

The characteristics of BOC and TLP patients were compared using the nonparametric Wilcoxon–Mann–Whitney test. All of the calculations were performed using SAS V9.1 software (SAS Institute Inc., Cary, NC, USA). All p-values below α=0.05 were considered statistically significant.

3. Results

3.1. Patients

Table 2 shows the clinical and virological characteristics of the patients analysed in this study. Thirteen patients were infected with HCV GT 1a and nine patients with GT 1b. Among them, 10 patients were treated with TLP and 12 with BOC. Patients were previous non-responders to PEG-IFN/RBV dual therapy and naïve to any DAAs treatments.

3.2. UDPS error rate for mutation detection

The mean error rate was described using frequencies for categorical variables with the standard deviation. The mean error for the plasmid control was 0.25% ±0.11 base substitutions per NS3 base positions. As a result, mutations were considered significant at a frequency ≥0.5% among the total number of reads if they were present in sequences obtained from both directions. This threshold was selected on the basis of previous results [20].

3.3. Baseline and VF mutations

All of the mutations detected in patients using UDPS are summarized in Table 3. The UDPS data analysis showed that at baseline, 14 of 22 patients (64%) contained several detectable mutations. Several variants were detected with a frequency between 0.5% and 12% of the total viral population. The prevalence of minority mutations among these 14 patients was 22% (V36M), 5% (V55A), 18% (R155K), 14% (R155T), 18% (A156T) and 5% (A156V). At baseline if the absence of RAVs was similar between the HCV GT1a- and GT1b-infected patients, the number of RAVs detected in HCV GT1a infected patients was higher than in GT1b infected patients (Fig. 2A). Patients infected with GT1a and GT1b experienced a mean decline in HCV RNA of approximately 2.6 $\log^{10}$. The UDPS data analysis revealed that VF was associated in most cases with the presence of V36M or R155K/T mutations (7 of 12 BOC patients and 7 of 10 TLP patients). One patient with the V55A minority mutation (0.5%)

Table 2
Clinical and virological characteristics of the selected non-responders to telaprevir and boceprevir patients.

Characteristic	All patients	Non-responder to TLV ($n = 10$)	Non-responder to BOC ($n = 12$)
Gender			
Female—N(%)	6 (27.3%)	4 (40.0%)	2 (16.7%)
Male—N(%)	16 (72.7%)	6 (60.0%)	10 (83.3%)
Age, year—median (min–max)	56.9 (34–72)	54.4 (34–72)	58.6 (48–68)
Genotype			
1a—N(%)	13 (59.1%)	6 (60.0%)	7 (58.3%)
1b—N(%)	9 (40.9%)	4 (40.0%)	5 (41.7%)
Response to previous treatment			
Relapse—N(%)	9 (40.9%)	3 (30%)	6 (50.0%)
Partial response—N(%)	3 (13.6%)	1 (10%)	2 (16.7%)
Null response—N(%)	10 (45.5%)	6 (60%)	4 (33.3%)
HCV RNA, $\log_{10}$ IU/ml plasma			
Baseline—median (min–max)	6.1 (4.4–6.9)	5.9 (4.4–6.9)	6.1 (4.6–6.7)
Virological failure—median (min–max)	4.5 (1.1–5.5)	2.5 (1.1–3.7)	2.9 (1.7–5.5)
After therapeutic cessation—median (min–max)	5.9 (4.8–7.0)	5.8 (4.8–5.9)	6.9 (5.1–7.0)
Delay between VF and after therapeutic cessation (years)—median (min–max)	1.4 (1.1–1.6)	1.2 (1.1–1.5)	1.5 (1.2–1.6)

TLV—telaprevir; BOC—boceprevir; VF—virological failure.

Table 3
Variants associated with resistance to HCV protease inhibitors detected by UDPS at baseline, virological failure and one year after therapy cessation.

Patient	GT	Treatment	Baseline mutation (frequency %)	Virological failure mutation (frequency %)	Mutation after therapy cessation (frequency %)
01	1a	BOC	A156T (0.51)	V36M (88.9)	–
02	1a	BOC	–	R155T (65.15)	–
08	1a	BOC	V36M (0.5)	R155K (66.7), V36M (100)	R155K (4.24), V36M (99.0)
14	1a	BOC	A156T (0.5), R155T (1.0)	A156T (100)	–
20	1a	BOC	R155k (2.4)	NA	–
21	1a	BOC	–	V36M (100)	–
22	1a	BOC	–	V36L (89.3), R155K (75.6)	–
10	1a	BOC	A156T (12.4), R155K (0.5), R155T (11.6), V36M (0.5)	A156T (7.1), R155K (80.0)	A156T (5.2), R155K (30.2), R155T (20.4), V36M (1.2)
05	1a	TLP	Q80K[a] (100)	Q80K[a] (100), R155K (72.2), V36M (53.9)	–
11	1a	TLP	V36M (97.3), A156T (1.3)	R155K (96.7), V36M (100)	R155K (3.8), V36M (95.2)
04	1a	TLP	A156V (5.5), R155K (0.6)	V36M (23.3)	–
13	1a	TLP	–	T54S (99.5)	–
16	1a	TLP	V36M (0.9), A156V (1.5), R155K (2.2)	NA	–
19	1a	TLP	A156T (0.5), V36M (0.5)	V36M (100), R155K (98.8)	A156T (15.4), R155T (9.6)
12	1b	BOC	V36M (1.2)	NA	–
15	1b	BOC	A156V (37.5), R155K (2.4), R155T (5.4)	T54A (21.1), V55A (1.2)	–
17	1b	BOC	–	V36M (100)	–
03	1b	BOC	V36M (4.4)	R155K (50.0)	–
06	1b	TLP	V55A (0.5)	–	–
07	1b	TLP	–	A156T (1.9), R155K (85.9), R155T (2.8)	–
09	1b	TLP	–	R155K (1.6), V36M (37.5)	–
18	1b	TLP	–	V36M (89.8)	–

GT— genotype, BOC—boceprevir, TLP—elaprevir, NA—not applicable.
[a] Natural polymorphism.

at baseline did not express any mutation at the VF time point. All of the HCV GT1a infected patients and 87.5% of the HCV GT1b infected patients had RAVs at VF (Fig. 2B).

3.4. Variant persistence after therapy cessation

One year after the end of the therapy, the prevalence of RAV was not similar between GT1a- and GT1b-infected patients. Indeed, no resistant mutations were detected in the HCV GT1b-infected patients (Fig. 2**C**) whereas persistence variants were detected in four of the HCV GT1a patients (18%) (Fig. 3). Regarding patient 10, the resistance mutation at position A156T was detected at a low frequency at the baseline time point and also at week (W) 9 (12.4% and 7.1%, respectively). For the other three patients, the V36M mutation was present at low levels at the baseline and increased to a high frequency after VF. During the follow-up period for patients 8 and 11 at W49 and W47, the total percentage of the V36M variants was quite similar (99% and 95.3%, respectively), demonstrating the persistence of the resistance variants. In contrast, for the same patients, the R155K variant was persistent, but at low levels (4.2% and 3.8%, respectively). Concerning patient 10, A156T and V36M persistent RAVs were detected at frequency of 5.2% and 1.2%, respectively, and variants R155K/T were detected at a frequency of 30.2% and 20.4%. In addition, two mutations were detected as

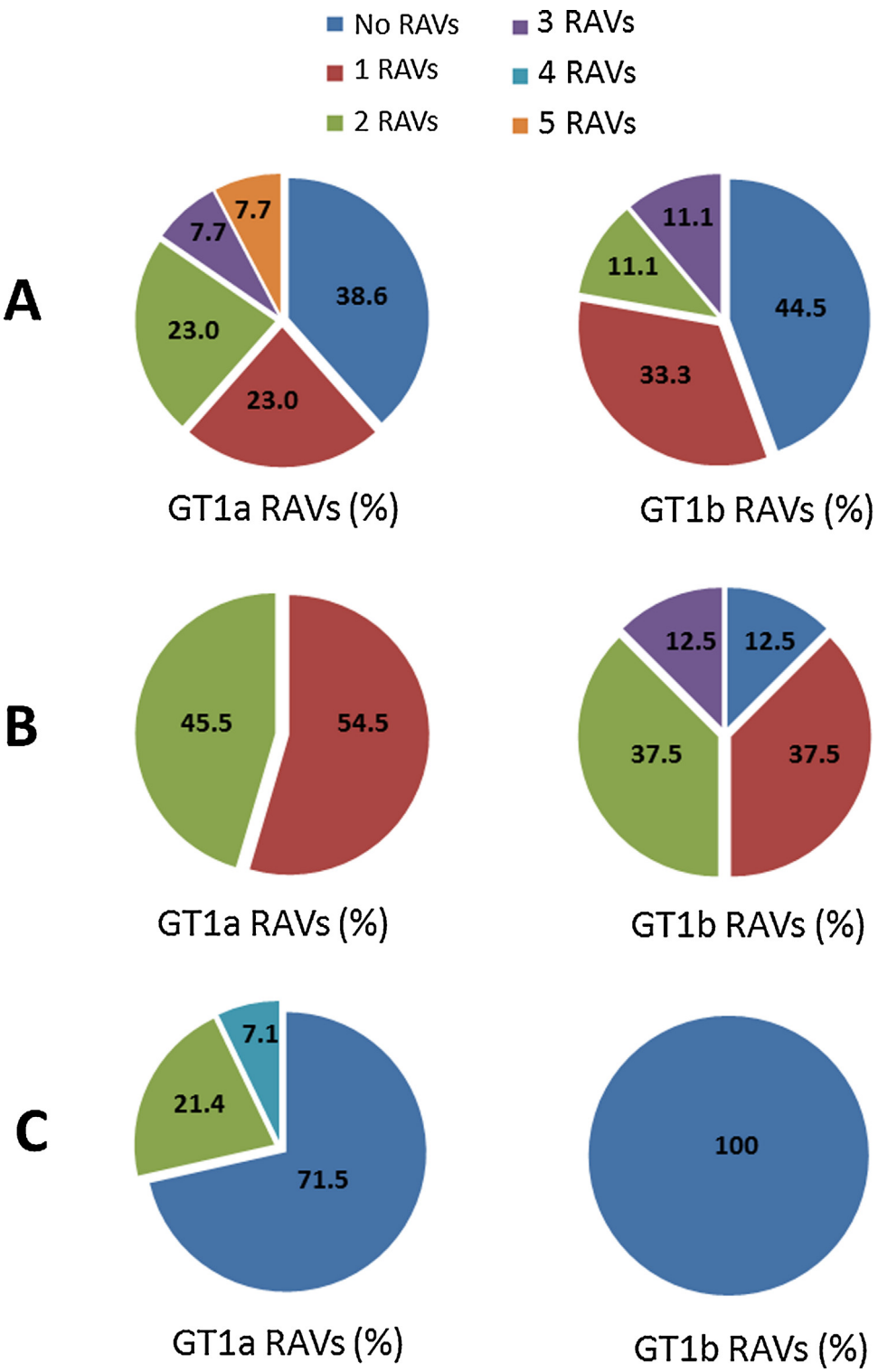

Fig. 2. Prevalence of the RAV numbers in the GT1a and GT1b patients at three time points. (A) baseline, (B) virological failure, (C) after cessation of treatment.

minority mutations at positions R155T (20.4%) and V36M (1.2%). V36M and R155K were simultaneously detected in three patients.

3.5. Impact of persistent RAVs viral load

Interestingly, the V36M, R155K/T and A156T mutations were found with a frequency of >20% at baseline and one year after therapy cessation. The relative mutation viral load ranged from 64 to 4,154,735 HCV RNA IU/ml plasma. The mean relative mutation viral load at baseline from BLNP vs BLP patients was 3.9 [3.4–4.2] and 5.4 [4.9–6.0] $\log^{10}$ (IU/ml) plasma, respectively. The mean relative mutation viral load at baseline was significantly different ($P < 0.001$) (Fig. 4). The presence of RAVs in this cohort impacted the persistence of RAVs. At VF, the mean relative mutation load from VFNP vs. VFP patients was 4.4 [3.7–5.0] and 4.2 [3.6–4.7] $\log^{10}$ (IU/ml) plasma, respectively. The difference of the mean relative mutation load at VF was not significant ($P = 0.47$) (Fig. 4).

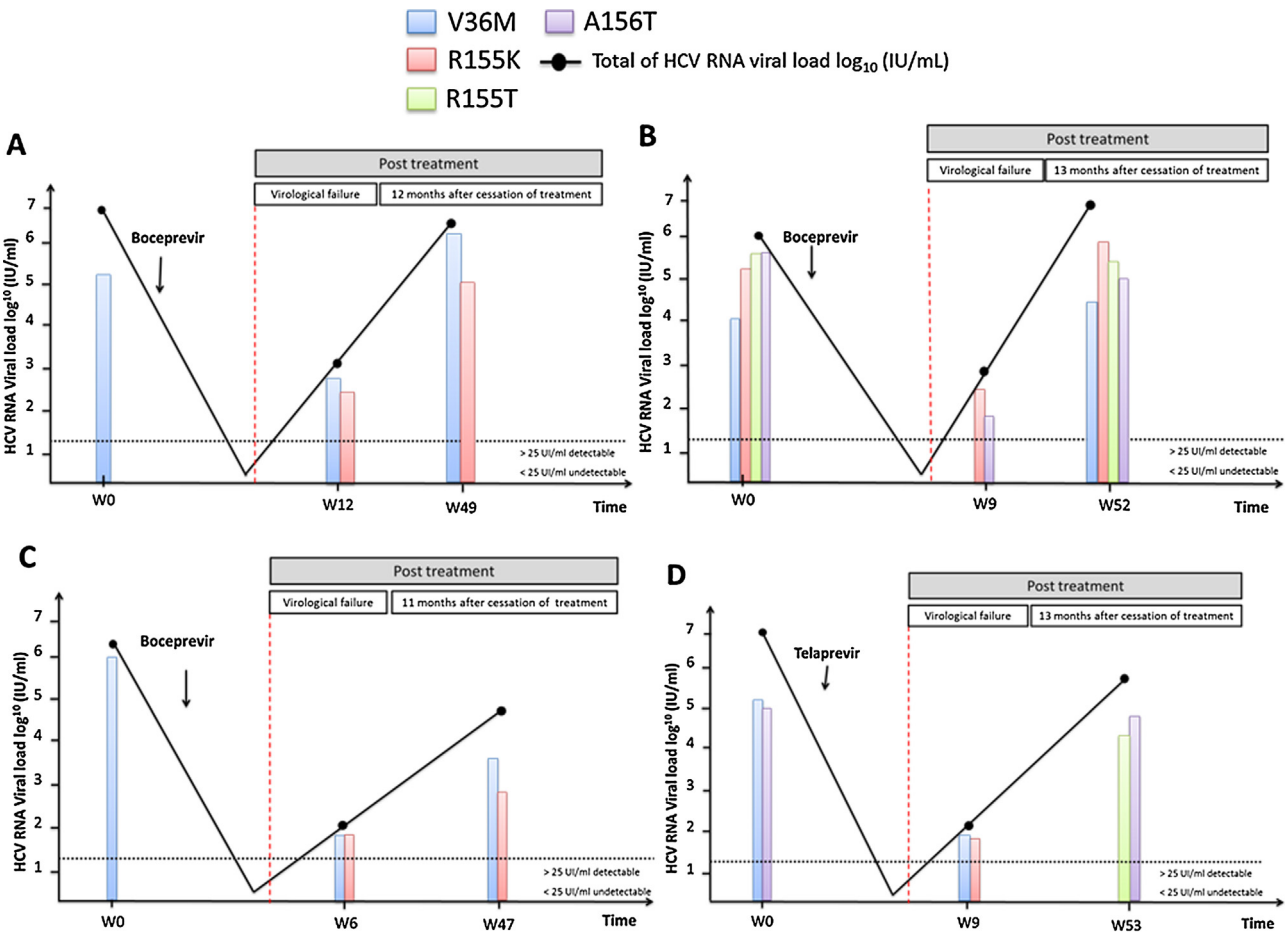

Fig. 3. Ultra-deep pyrosequencing analysis of the four patients presenting with a persistent resistant variant.
(A) patient 8, (B) patient 10, (C) patient 11, (D) patient 19. Only mutants detected at known positions to confirm BOC and TLP resistance are shown with the respective mutant viral load for each time point. W—week.

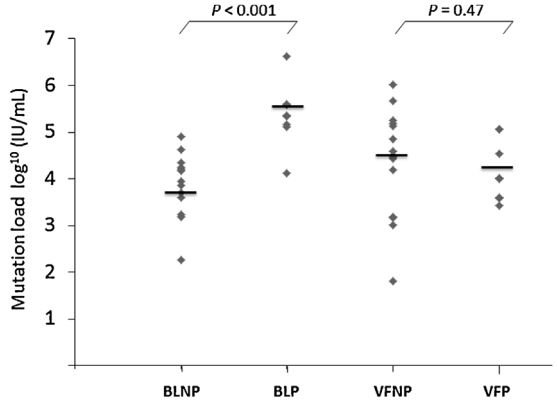

Fig. 4. Relative mutant viral load of the non-persistent and persistent resistant variants detected in patients at the baseline and virological failure time points.
BLNP and VFNP, Patients with no persistent RAVs at baseline and virological failure, respectively; BLP and VFP, Patients with persistent RAVs at baseline and virological failure, respectively. Dots represent the mutant viral load of the V36M, R155K, R155T and A156T persistent RAVs.

4. Discussion

In our study, the most prevalent minority mutations were V36M, R155K and A156T, and all of these mutations were detected with a relatively high mutant viral load. Several studies have demonstrated the presence of natural minority mutations associated with resistance to BOC and TLP among treatment-naïve HCV infected individuals [21–23].

TLP or BOC-RAVs (V36M, R155K/T or A156T) were detected after therapy cessation in 18% of patients (4/22) at a range of 1.2–99% of the viral population. The results from a previous study demonstrated that the frequency of TLP RAVs declined after treatment failure to low or undetectable levels and were no longer detectable in the majority of patients during follow-up using deep-sequencing [24]. Our study extends these results, as we demonstrated the presence of persistent variants in 18% of patients (4/22) after 1 year, as measured using the UDPS technology. Nevertheless, there are some limitations to our study that need to be considered. At VF, the results from three patients (patient 12, 16 and 20) were excluded from the analysis due to unsuccessful amplification or UDPS assay limitations. Although the persistence of NS3/4A variants was detected up to 11 months, additional follow-up data are necessary to determine the true duration or persistence of NS3/4A resistance. Moreover, to detect low frequency RAVs, it would be necessary to concentrate the RNA from higher volumes of plasma during extraction and to use a larger quantity of RNA for PCR to preserve viral diversity before amplification.

The key PIs resistance mutations, V36M, R155K or R155T, in GT 1a-infected patients were detected in baseline or follow-up samples [19,25]. This result suggests that in patients 8, 10, 11 and 19, the persistent variants sequenced during follow-up are naturally occurring variants that arose after treatment or VF. Previous studies have suggested that certain types of mutations, such as V36A/M or R155K/T/Q, could have an impact on TLP or BOC treatment failure

Table 4
HCV NS3/4a resistance associated variants detected at baseline and virological failure in several studies with boceprevir or telaprevir treatment.

References	Method	Treatment	Number of patient	RAVs detected at baseline	RAVs detected after > one year of cessation of therapy
[12]	UDPS	TLP	24	V36A/M; T54A/S; R155K/T/Q; A156S/T/V	V36A/M; T54A/S; R155K/T/Q
[23]	Clonal seq	BOC or TLP	82	V36A/M; T54A/S; V55A; R155K; A156T	V36A/M; T54A/S; V55A; T155K. A156T
[24]	UDPS	TLP	49	T54A/S; R155K	V36M/V; T54S; R155K
[25]	UDPS	TLP	15	V36I/L; T54A; R155S/P; A156G	V36M; T54S; R155G
[19]	Clonal seq	BOC	9	V55A	R155K, V55A, V36M, T54S

RAVs—resistance associated variants; UDPS—ultra-deep pyrosequencing; Clonal seq—Clonal sequencing; BOC—boceprevir; TLP—telaprevir.

(Table 4) [12,19,23–25]. In our study, the V36M, R155K, R155T and A156T baseline relative mutant viral loads of patients with persistent RAVs was significantly higher ($P < 0.001$) than those of patients without persistent RAVs. The presence of RAVs in this cohort significantly impacted the persistence of the RAVs.

Different SVR rates have been observed when comparing GT 1a- and 1b-infected patients receiving BOC or TLP therapy. The response rates in patients infected with GT 1b have generally been more favourable than in those with GT 1a [26]. In our study, persistence mutations were only observed in HCV GT 1a-infected patients. This is partially due to the lower genetic barrier of GT 1a versus 1b [14,27]. It would be interesting to study the distribution of frequency of the detected intra-host variants.

In conclusion, UDPS data could allow for an improved drug resistance data interpretation to ultimately help clinicians provide the appropriate treatment and improve personalized diagnosis in HCV infected patients. In addition, a 0.5% detection threshold allows for a better characterization of the viral population, thereby promoting the identification of additional resistance mutations and improving drug resistance interpretations. Finally, UDPS demonstrated that the frequency of BOC or TLP RAVs increases after treatment failure and that these variants persist for at least one year after therapeutic cessation, which can constitute a limit for the use of other PI treatment regimens. Therefore, the relative mutant viral load must be considered prior to any PIs based re-treatment. This concept of the baseline mutant viral load should be considered in the case of NS5A and NS5B inhibitor-based therapies and should be validated on a larger cohort.

Funding

Roche Diagnostics provides kits for ultra-deep pyrosequencing.

Conflict of interest

The authors declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript.

Ethical approval

According to Article L1121-1 of the French Public Health law, non-interventional studies are not subject to a legal framework. Non-interventional studies are defined as actions that are routinely performed without any additional procedure or unusual diagnostics or monitoring.

Author's contributions

Conceived and designed the experiments: SM, MB, SB, VO, PC, LC, DO, and PH. Performed the experiments: SM, CC, and HK. Analysed the data: SM, GP, DG, and CS. Contributed reagents/materials/analysis tools: SM and HK. Wrote the paper: SM, CC, and PH.

Acknowledgment

The authors thank Roche diagnostics (Grenoble, France) for kindly providing the reagents.

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