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Kinetics of hepatitis C virus RNA decay, quasispecies evolution and risk of virological failure during telaprevir-based triple therapy in clinical practice☆

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ABSTRACT

Background: The used first generation protease inhibitors may be hampered by virological failure in partially interferon-sensitive patients.
Aim: To investigate early hepatitis C virus (HCV)-RNA decay and quasispecies modifications, and disclose viral dynamics underlying failure.
Methods: Viraemia decay at early time-points during telaprevir treatment was modelled according to Neumann et al. (1998). NS3-sequences were obtained by population-sequencing and ultradeep-454-pyrosequencing.
Results: 13 treatment-experienced (8 non-responders, 5 relapsers), and two cirrhotic naïve patients, received telaprevir + pegylated-interferon-α + ribavirin.

Viraemia decay was biphasic. In all patients, first-phase was rapid and consistent, with a median [interquartile-range] viraemia decay of 2.8 [2.6–3.2] log IU/ml within 48 h. Second-phase decay was slower, especially in failing patients: 3/3 showed <1 log IU/ml decay between 48 h and 2 weeks, and HCV-RNA >100 IU/ml at week 2. Only one patient experiencing sustained viral response showed similar kinetics.

By pyrosequencing, mutational freeze was observed in all 15 patients within the first 24 h, but only in patients with sustained response afterwards. Indeed, 2/2 failing patients showed early resistance, as minor (V36A-T54A: prevalence <26% at 48 h) or major (V36M/A-R155K: prevalence, 99.8% at week 2) variants.
Conclusions: Following telaprevir administration, first-phase HCV-RNA decay is consistent with mutational freeze and limited/no viral replication, while second-phase is significantly slower in failing patients (with appearance of resistance), suggesting the usefulness of early HCV-RNA monitoring.

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1. Introduction

In hepatitis C virus (HCV) infection, the development of directly acting antiviral agents (DAAs) provides an outstanding opportunity to improve cure rates for patients with chronic hepatitis C, allowing hopes for a large-scale eradication of HCV infection. The introduction into clinical practice of first-generation HCV-1 protease inhibitors (PIs) improved treatment outcomes when added to pegylated interferon-α (pegIFN) and ribavirin (RBV) in both treatment-naïve and treatment-experienced patients [1–4]. Nevertheless, triple therapy, particularly for difficult patients, is often limited by safety and tolerability issues, which limit its use, along with a rapid onset of drug-resistance in virological failures. Paradoxically, drug-resistance is considered a hallmark of antiviral drugs, and de facto is both a major cause and consequence of virological failure [5,6].

Within an infected individual, the high in vivo HCV mutation rate ($2.5–3.2 \times 10^{-5}$ per nucleotide per genome replication round) [7], in synergy with a short half-life, and an extremely high daily viral production rate (10^{12} particles/day) leads to the generation of a swarm of genetically distinct but closely related viral variants, known as quasispecies [8]. In this heterogeneous viral population, the probability to have at least one viral strain harbouring one or two nucleotide mutations is close to 100% [9].

In an "ideal scenario", with completely wild-type and sensitive viral quasispecies, viral eradication by treatment follows a biphasic profile: in the first phase viral particle production is steeply blunted, completely blocking viral replication in the liver, then in the second phase all infected cells are cleared, either by cell-death or loss of replicative intermediated (i.e. "cell cure"). Mathematical models estimated that, in this ideal situation, 8 weeks of telaprevir-based therapy would be enough to cure 99% of HCV-infected patients [10].

Nevertheless, when an anti-HCV agent is used against an "actual scenario" of viral quasispecies, drug-effectiveness in blocking viral production may not be 100% and minority resistant variants in the liver can consequently get the chance to replicate. When replication under positive selecting pressure is allowed, it increases the risk of generating viral variants with better resistant profiles and viral fitness.

In the past, mathematical models of HCV dynamics have contributed in understanding the mechanisms of action of IFN, pegIFN and pegIFN + RBV, and consequently in better characterising viral behaviour under drug pressure [11–13]. Recently, dynamic parameters have been estimated in patients treated with DAAs, particularly in monotherapy [14]. However, so far no data are available on the effects of PI-based triple therapy in real clinical practice, often including difficult-to-treat patients who may actually have the greatest need of treatment, especially in Europe.

For all these reasons, the aims of this study were to describe the dynamics of genotype 1 HCV-RNA decay within the first hours and days of telaprevir-based triple therapy in treatment-experienced and/or cirrhotic patients, and to simultaneously evaluate the early modifications in the quasispecies population by mean of ultrasensitive techniques, correlating both factors with the clinical situation, plasma telaprevir concentration, and therapeutic outcome.

2. Materials and methods

2.1. Study population

Chronically infected HCV genotype 1 patients, starting a triple-therapy with PegIFN/RBV plus telaprevir between June 2012 and November 2012, who consented to frequent blood sampling were considered for inclusion. Exclusion criteria were age under 18 years and other concomitant chronic liver diseases. Treatment schedules and stopping rules followed telaprevir prescribing information [15].

The analysis was performed in 15 patients (Table 1). Of these, 13 had previously failed a pegIFN + RBV treatment as non-responders (7 null-responders and 1 partial-responder), or as relapsers ($N = 5$). The other 2/15 patients were cirrhotics naïve to treatment. Correlation of treatment outcome with viral kinetics was performed with a *per protocol* approach. This study was conformed to local ethical considerations and the principles outlined in the 1975 Declaration of Helsinki (6th revision, 2008). All patients allowed the participation to the study with a written consent for the use of samples for research purposes.

2.2. HCV-RNA quantification

Serum HCV-RNA was quantified by Roche COBAS® TaqMan® HCV Test v2.0 (lower limit of quantification: 25 IU/ml; LLOD, lower limit of detection: 15 IU/ml) at baseline, at very early time points after telaprevir + pegIFN + RBV start (1h–2h–3h–4h–5h–6h–8h–12h–24h–28h–48h–1w–2w) and during *per protocol* follow-up.

2.3. Mathematical modelling of HCV dynamics

A constant-effectiveness (CE) model was used to fit observed data [11]. In order to normalise fluctuations, baseline HCV-RNA values were calculated as a mean among 2–3 pre/post-therapy measurements (baseline–1h–2h after triple-therapy start).

Viral load data were also tested for fitting with a model with varying-efficacy or intracellular dynamics [16], but the sampling frequency did not allow enough information to make a significant difference respecting to the standard bi-phasic model.

2.4. Population sequencing

NS3-protease sequences (aa 1–181) were obtained by an home-made sequencing protocol [17] at baseline and at early time-points (8h–12h–24h–48h–1w–2w–4w), where plasma samples were available and HCV-RNA was detectable, and then in case of virological failure.

2.5. Ultra-Deep 454-Pyrosequencing (UDPS)

NS3-protease UDPS was performed in all patients at baseline. Tests at 8h–12h–24h–48h were performed in all available plasma samples with adequate HCV-RNA. Detailed information is reported in Appendix A. Briefly, 454 junior platform was used with 2 genotype specific protocols based on 2 overlapping amplicons spanning the entire NS3-protease-sequence. For each amplicon, reads were aligned and corrected using home-made Perl scripts and Shorah package [18]. By using a plasmid control, mutations found with frequency >0.1%, both in reverse and forward orientations were considered reliable. Global haplotype reconstruction was performed using Shorah package.

2.6. Genetic diversity analysis

Evolutionary divergence between sequences was calculated on NS3-sequences from population-sequencing and on UDPS-amplicons using the Tamura 3-parameter model. The variation rate among sites was modelled with a gamma distribution (shape parameter = 1). Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with <95% site coverage were eliminated. Phylogenetic-trees were contextually inferred. All phylogenetic analyses were performed with MEGA v5.1 [19].

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Table 1
Characteristics of the study population.

	Naïve cirrhotic patients (*N* = 2)	Treatment experienced patients (*N* = 13)	
		Non-responders (*N* = 8)	Relapsers (*N* = 5)
Age (years), Median (IQR)	59 (55–62)	52 (46–58)	66 (65–69)
Males, *N* (%)	1 (50.0)	7 (87.5)	3 (60.0)
IL-28B genotype, *N* (%)			
CC	0 (0.0)	1 (12.5)	0 (0.0)
CT	1 (50.0)	5 (62.5)	1 (20.0)
TT	0 (0.0)	1 (12.5)	0 (0.0)
Not determined	1 (50.0)	1 (12.5)	4 (80.0)
HCV-genotype, *N* (%)			
1a	1 (50.0)	4 (50.0)	0 (0.0)
1b	1 (50.0)	4 (50.0)	5 (100)
Patients with cirrhosis, *N* (%)	2 (100)	3 (37.5)	0 (0.0)
Baseline Log_{10} HCV-RNA (IU/ml), Median (IQR)	5.5 (5.1–5.8)	6.0 (5.5–6.7)	5.5 (4.5–5.8)
Baseline AST (IU/ml), Median (IQR)	72 (68–75)	71 (53–96)	89 (68–106)
Baseline ALT (IU/ml), Median (IQR)	81 (65–97)	105 (68–149)	118 (78–187)

HCV, hepatitis C virus; IU, international units; IQR, interquartile range; ALT, alanine transaminase; AST, aspartate transaminase.

Genetic complexity was also evaluated by Shannon entropy values, as described in [20].

2.7. Telaprevir concentration

Telaprevir was measured in plasma samples by ultra-performance liquid chromatography–tandem mass spectrometry (UPLCMS/MS). Detailed information is reported in Appendix A.

3. Results

3.1. Treatment outcome following telaprevir + pegIFN + RBV administration

Out of 15 patients included in the study, virological-failure was detected in 3 previous null-responders (1 = non-response; 2 = viral-breakthrough); 1 previous relapser dropped-out at week-8 for non-virological reasons (HCV-RNA at the time of interruption <LLOD, Table 2). The other 11 patients (2 cirrhotic-naïve; 4 relapsers; 1 partial-responder; 4 null-responder) reached End of Treatment (EOT) with undetectable HCV-RNA (target not detected, TND) and are now experiencing a Sustained Virologic Response (SVR) after at least 12 weeks of follow-up.

Treatment outcome was not compromised by the baseline presence of T54S (detected in PT12, SVR_{24}), nor by Q80K (detected in PT15 and PT8, SVR_{24} and SVR_{12} respectively).

A rapid viral response (RVR), defined as TND HCV-RNA at week-4 of triple-therapy, was observed in 11/15 (73.3%) patients. Out of 10 RVR patients who completed treatment, 9 (90.0%) obtained a SVR vs. 2/4 (50.0%) non-RVR patients ($P = 0.176$). All 4 patients who did not experience a RVR were previous null-responders.

3.2. Early HCV-RNA decay following telaprevir + pegIFN + RBV administration

An initial HCV-RNA decay was already visible in 11 patients at 4 h since first telaprevir administration (median [IQR] HCV-RNA $decay_{BL-4h} = 0.3$ [0.2–0.4] log IU/ml) (Fig. 1). HCV-RNA declined in all patients at 6 h (median [IQR] $decay_{BL-6h} = 0.6$ [0.6–0.8] log IU/ml) and at 8 h, before taking the second dose (median [IQR] $decay_{BL-8h} = 1.2$ [1.0–1.9] log IU/ml).

In a subgroup of 7 patients for whom 12 h HCV-RNA value was available, HCV-RNA decay showed its maximum value at this time-point (median [IQR] $decay_{BL-12h} = 2.1$ [1.5–2.2] log IU/ml), slowing down afterwards (median [IQR] $decay_{12h-24h} = 0.5$ [0.4–0.9] log IU/ml).

At 24 h after treatment start, median [IQR] HCV-RNA decline from baseline values was of 2.4 [2.2–2.7] log IU/ml (Fig. 1.). This decay was not affected by baseline viraemia (P = n.s. by linear regression analysis), nor by HCV-subtype 1a or 1b, previous treatment experience, stage of liver disease and final virological outcome to triple-therapy (Fig. 2).

The same result was obtained also analysing the baseline-to-48 h HCV-RNA decline (Fig. 2), thus indicating that the rough value of viral decay from baseline to 48 h seems not to be affected by baseline clinical and virological parameters, nor to affect the rate of virological success. Nevertheless, even if the rough value of HCV-RNA decay at 48 h was consistent among all patients, the median [IQR] slope of HCV-RNA decay between 24 and 48 h was significantly lower in failing-patients, respecting to SVRs (−0.4 [−0.7; −0.1] vs. −3.3 [−3.9; −2.9] log IU/ml/week, respectively; $P = 0.04$ by Mann–Whitney test) (Table 2).

Overall, failing-patients experienced a slowing down decay of HCV-RNA, starting from 24 h after treatment start. This different kinetic was maintained also during second-phase viraemia decline. Indeed, the 3 failing-patients were the only ones with <1 log IU/ml decline between 48 h and week-2 (Table 2), while all other patients had a median (IQR) $decay_{48h-2w}$ of 1.9 (1.4–2.3) log IU/ml ($P = 0.030$ by Mann–Whitney test). At week-2 of triple-therapy, all patients experiencing RVR (and afterwards SVR) had HCV-RNA values below LLOD ($N = 5$) or <100 IU/ml ($N = 4$). The only RVR patient (PT9) experiencing virological-failure had HCV-RNA at week-2 of 430 IU/ml (Table 2). Interestingly, 3/4 patients with HCV-RNA >100 at week-2 experienced virological-failure vs. 0/9 of those with HCV-RNA<100 IU/ml ($P = 0.003$ by Fisher Exact test) (Table 2).

At week-2, suboptimal viral-kinetic impacted also on rough HCV-RNA delta values from baseline, which was significantly lower in all patients experiencing virological-failure respecting to SVRs (median [IQR] HCV-RNA $decay_{BL-2w} = -3.1$ [−3.4; −0.5] vs. −4.6 [−4.8; −4.2] log IU/ml, respectively; $P = 0.014$ by Mann–Whitney test) (Fig. 2).

3.3. Viral load changes analysis using the standard model of viral dynamics

To verify whether an alteration of first-phase/second-phase viral decline occurs in patients experiencing virological-failure, a CE model for viral-kinetics was used.

In our dataset, the mean (SE) delay time t_0 assumed for pegIFN + RBV + telaprevir triple-therapy to be effective was of 10.7 (2.2) h, or 0.444 (0.092) days (Table 3). Higher t_0 values among

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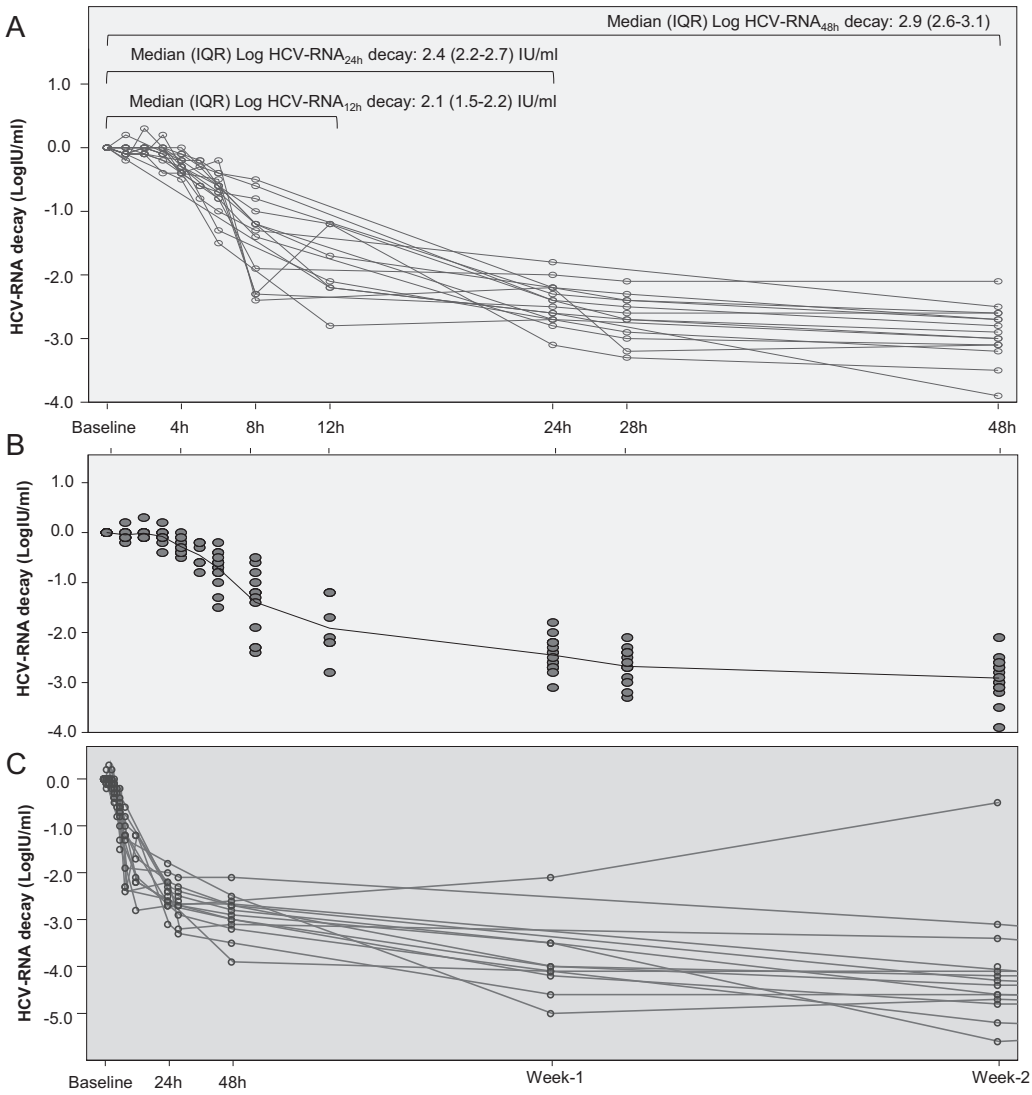

Fig. 1. Early patterns of HCV RNA decay during telaprevir-based triple therapy. The HCV RNA decay from baseline values (calculated as a mean of pre-therapy, 1 h and 2 h HCV-RNA values) within the first 48 h (A and B) and 2 weeks (C) of telaprevir-based triple therapy is reported for each patient. Line in panel B represents population mean. IU, international units; IQR, interquartile range.

patients were not related to treatment outcome nor to the stage of disease progression, since it was not significantly higher in cirrhotic patients (data not shown).

The mean free virions clearance rate (c), characterising first-phase HCV-RNA decline, was 9.4 day^{-1} (Table 3). It was higher than the 5.95 day^{-1} estimated with standard IFN, thus suggesting a lower duration of the first-phase of HCV-RNA drop (<48 h) with telaprevir.

Also the mean value of per capita rate of loss of infected cells (δ) was slightly higher than that reported with standard IFN (0.22 day^{-1} vs. 0.16 day^{-1}). Patients experiencing virological-failure tended to have lower mean δ values, and thus a slower second phase of HCV-decline, relative to patients achieving SVR (mean [SE] = 0.137 [0.093] day^{-1} vs. 0.244 [0.131] day^{-1}, respectively), even if the difference was not statistically significant. Also mean treatment-effectiveness ε was lower in failing patients relative to patients with SVR (88% vs. 94%) (Table 3) and linear regression analysis highlighted a correlation among treatment-effectiveness ε and δ ($P = 0.0133$), sustained by Spearman's correlation coefficient ($r = 0.671$). Overall, these data confirm a suboptimal second-phase decline in therapy-failing patients.

3.4. Telaprevir pharmacokinetics analysis in the context of cirrhosis and virological failure to triple therapy

To evaluate the factors potentially impacting on treatment-efficacy, telaprevir plasma concentration was calculated in all patients at different time-points (6h–8h–12h–24h–48h–1w–4w): in all cases, the concentration curve was always found to be above the recommended therapeutic-ranges. Nevertheless, the peak of plasma concentration at 6–8 h since treatment start tended to be lower in cirrhotic patients (median [IQR] plasma telaprevir-concentration = 2345 [1476–2692] ng/ml) in comparison to non-cirrhotic (2701 [1658–3318] ng/ml), even if the difference was not statistically significant ($P = 0.4923$ by Wilcoxon-Test), and did not correlate to previous treatment outcome (data not shown).

Analysing the peak (6–8 h) to trough (24 h) decline slope of plasma telaprevir-concentration, no differences were observed among previous relapsers or previous non-responders, if the patients were non-cirrhotic and excluding those experiencing virological-failure (Fig. 3). On the contrary, naïve cirrhotic patients tended to have lower telaprevir pharmacokinetics values at 24 h,

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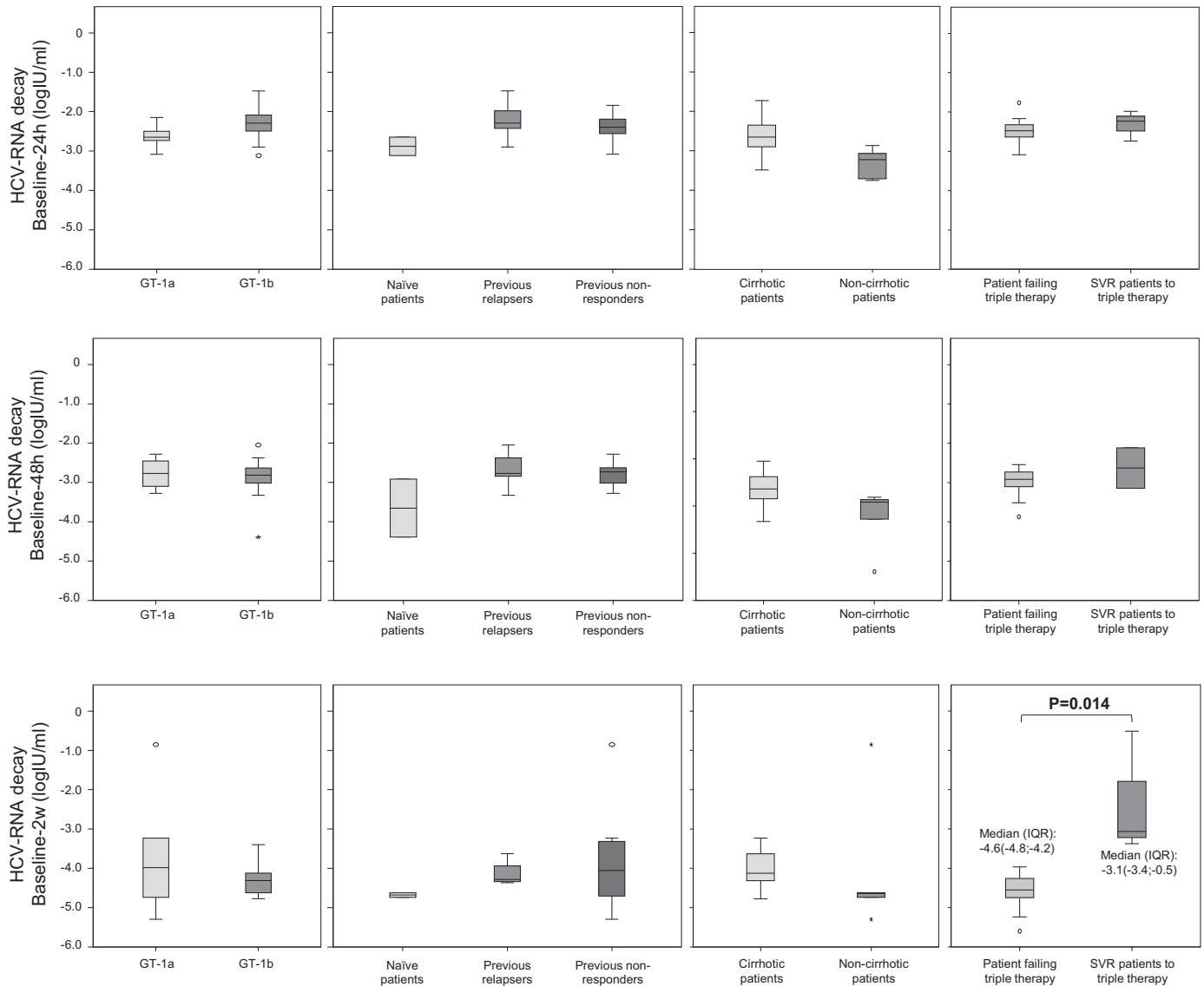

Fig. 2. Early HCV RNA decay in relation to clinical and virological patients' characteristics. Box plots representing HCV RNA decay from baseline values (calculated as a mean of pre-therapy, 1 h and 2 h HCV-RNA values) to 24 h, 48 h and week-2 of triple-therapy administration. Patients were stratified according to HCV-genotype, previous treatment experience, diagnosis of cirrhosis and virological outcome to telaprevir-based triple-therapy. P-value was calculated through Mann–Whitney test. IU, international units; SVR, sustained virological response; IQR, interquartile range.

such those who later experienced virological-failures, who also showed a decline in concentration respecting to the 6–8 h peak (Fig. 3).

Therefore, we confirmed an appropriate drug-exposure during the first period of triple-therapy administration, in all 15 patients included in the study.

3.5. *HCV quasispecies evolution and drug resistance detection in the early phases of telaprevir + PegIFN + RBV administration*

To assess HCV-quasispecies rearrangement at early time points during treatment, viral evolution by population-sequencing and UDPS was performed.

By population-sequencing, viral evolution in the first hours and days since treatment start (8h–24h–48h) was low, with a median (IQR) intrapatient evolutionary divergence, between baseline and last detectable early time-point, of 0.001 (0.000–0.003) nsubs/site. This corresponds to a median (IQR) of 1 (1–7) nucleotide variations on a total of 543 nucleotide positions analyzed.

Then, UDPS analysis was performed to better characterise the quasispecies dynamics and rearrangement. At baseline, patients experiencing virological-failure tended to have higher intra-patient nucleotide genomic complexity in comparison to SVR patients, both in terms of evolutionary-divergence (mean [SD] = 0.018 [0.009] nsubs/site vs. 0.013 [0.005], respectively, $P = 0.35$) and Shannon Entropy (mean [SD] = 0.029 [0.015] $\times 10^{-3}$ vs. 0.022 [0.010] $\times 10^{-3}$, respectively).

Within the first hours, viral-quasispecies detected at baseline, remained substantially stable at all early time-points (8h, 12h, 24h), in all patients studied for this purpose, with no rearrangements of species prevalence at both nucleotide and amino acid levels (data not shown). This suggests a mutational-freeze and a limited number of (or even absent) cycles of viral replication in this observation period.

After 48 h, still no quasispecies rearrangements were detected in SVR patients analyzed. On the contrary, in 2/2 failing-patients with available samples (PT7 and PT13), early development of resistance-associated variants (RAVs) was observed (Supplementary Table S1).

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Table 2
Early viral kinetics and treatment outcome.

Patient ID	Prior treatment experience	HCV genotype	IL28B	Fibrosis (Metavir)	Baseline HCV-RNA (log_{10} IU/ml)[a]	Decay slope (log_{10}/week)			HCV-RNA 2 w (IU/ml)	HCV-RNA 4 w (IU/ml)	Treatment length (weeks)	Treatment outcome
						0–24 h	24 h–48 h	48 h-2 w				
PT15	Naive	1a	n.a.	F4	5.8	−18.7	−1.9	>−2.8	TND	TND	24	SVR24
PT16		1b	CT	F4	5.1	−18.5	−8.9	>−1.2	TND	TND	48	SVR12
PT10	Relapsers	1b	n.a.	F2	5.7	−21.4	−3.0	−1.0	14	TND	24	SVR24
PT11		1b	n.a.	F1	5.4	−18.3	−2.9	>−2.4	TND	TND	24	SVR24
PT12		1b	n.a.	F3	5.9	−15.4	−4.0	−1.6	38	TND	24	SVR24
PT17		1b	n.a.	F0	5.6	−16.8	−3.4	n.a.	n.a.	TND	24	SVR16
PT2		1b	CT	F2	5.7	−15.2	−2.8	>−1.9	TND	TND	8	Drop out
PT3	Partial responder	1b	CT	F4	5.2	−15.6	−3.2	>−2.5	TND	TND	48	SVR12
PT1	Null responders	1b	CT	F4	6.0	−16.4	−3.3	−1.8	24	<15	48	SVR12
PT4		1a	CT	F4	7.0	−18.7	−3.8	−2.0	58	TND	48	SVR12
PT5		1b	CC	F3	6.2	−12.2	−5.4	−2.2	32	TND	24	SVR24
PT8		1a	CC	F3	6.7	−17.3	−0.17	−1.4	545	16	48	SVR12
PT7		1b	TT	F3	6.2	−15.6	0.2	−0.2	669	19	20	Breakthrough
PT9		1a	CT	F3	5.7	−13.6	−0.95	−0.9	430	TND	20	Breakthrough
PT13		1a	CT	F4	5.6	−19.3	n.a.	n.a.	122,528	200,000	6	Breakthrough

HCV, hepatitis C virus; IU, international units; n.a., not available; TND, target not detected (below the limit of detection); SVR, sustained virologic response; AE, adverse events.
[a] Baseline HCV-RNA values were calculated as mean among pre-therapy value, hour 1 and hour 2 values after triple-therapy start, in order to normalise fluctuations.

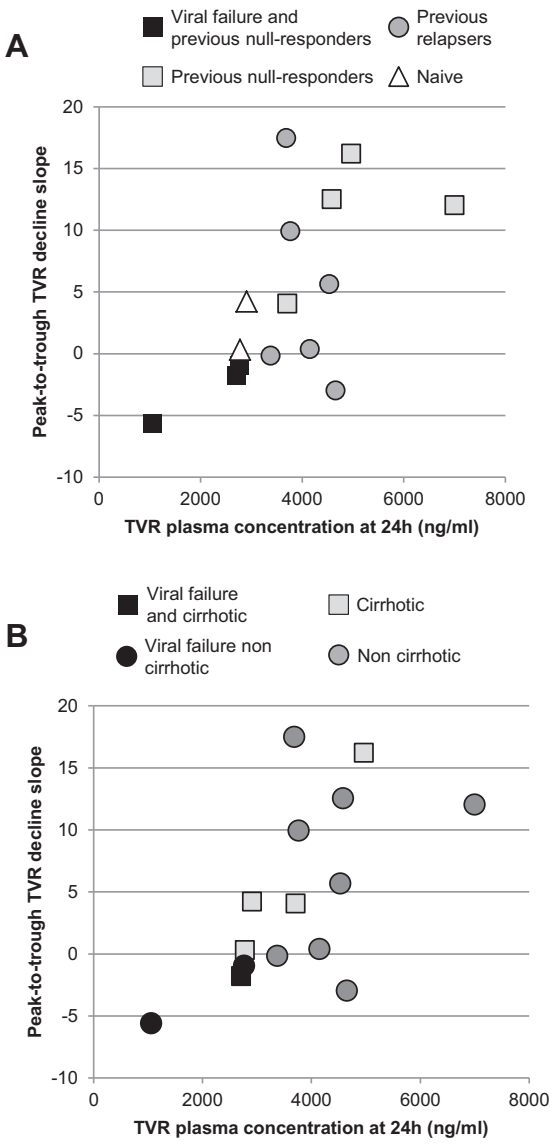

Fig. 3. Peak to trough decline slope of telaprevir plasma concentration. The peak (6–8 h) to trough (24 h) decline slope of telaprevir plasma concentration in relation to the 24 h value after triple-therapy start are reported for each patient. Patients were grouped according to triple-therapy outcome (black content for viral failures) and (A) previous response or (B) diagnosis of cirrhosis. TVR, telaprevir.

In PT13, the R155K accounted for 99.8% of infecting viral population already at 2 weeks of triple therapy, and it was associated with an increase in HCV-RNA (122,528 IU/ml vs. 728 IU/ml, from 24 h).

In PT7, experiencing late viral-breakthrough, UDPS analysis instead revealed, already at 48 h, resistant strains only as minority-variants: V36A (459/5244 reads, 8.7% prevalence, mutational-load = 2.0 log IU/ml) and T54A (1885/5248 reads, 26.1% prevalence, mutational-load = 2.5 log IU/ml). These RAVs were found later at failure, with a major prevalence of T54A (4726/5624 reads, 84.0% prevalence), and a minority contribution of V36A (49/5529 reads, 1.0% prevalence) and T54S (165/5624 reads, 2.8% prevalence).

4. Discussion

The kinetics of early HCV-RNA decline and quasispecies evolution under telaprevir-based triple-therapy were analysed in a small pivotal group of HCV-1 infected patients (failing previous IFN-based

Table 3
Estimation of viral dynamic parameters using constant effectiveness model.

Study ID	Treatment experience	Initial HCV-RNA (log IU/ml)	Delay (t_0, days)[a]	Virion clearance (c, day^{-1})[b]	Efficacy (ε, %)[c]	Infected cell death (δ, day^{-1})[d]	σ^2
Virological failure to telaprevir-based triple therapy							
PT7	Null responder	6.2	0.384	15.0	85.3	0.034	2.064
PT9	Null responder	5.7	0.317	12.5	83.2	0.162	0.778
PT13	Null responder	5.6	0.347	11.4	95.0	0.215	0.587
Sustained virological response to telaprevir-based triple therapy							
PT15	Naive	5.8	0.306	14.8	92.5	0.092	1.800
PT16	Naive	5.1	0.561	6.3	99.2	0.304	1.900
PT2	Relapser	5.7	0.532	5.5	97.1	0.445	1.183
PT10	Relapser	5.7	0.533	7.9	97.8	0.156	1.975
PT11	Relapser	5.4	0.372	10.1	96.0	0.226	1.495
PT12	Relapser	5.9	0.471	8.3	88.4	0.177	1.182
PT17	Relapser	5.6	0.542	6.5	97.1	0.250	1.310
PT3	Partial responder	5.2	0.513	5.8	96.2	0.449	0.822
PT1	Null responder	6.0	0.507	5.8	98.3	0.400	1.002
PT4	Null responder	7.0	0.518	7.0	98.5	0.233	2.160
PT5	Null responder	6.2	0.413	10.4	77.1	0.119	2.229
PT8	Null responder	6.7	0.347	13.6	88.5	0.079	2.038
Overall mean (SD)		5.9 (0.5)	0.444 (0.092)	9.4 (3.4)	92.7 (6.7)	0.223 (0.129)	1.502 (0.557)

HCV, hepatitis C virus; IU, international units; SD, standard deviation.
[a] t_0 is the pharmacological delay, representing the time after which treatment affects viral load.
[b] c is the clearance rate of free virions in plasma, calculated by non-linear fitting of HCV-RNA decay during the first 48 h of triple therapy to the mathematical model.
[c] ε is the effectiveness of therapeutic regimen in blocking virion production. Is assumed to have a value between 0 (no effect) and 1 (100% effective) and it was calculated by model fitting of the biphasic decline, using HCV-RNA decay during the first 48 h of triple therapy.
[d] δ is the rate at which infected cells are lost, calculated by non-linear fitting of HCV-RNA decay during the first 48 h of triple therapy to the mathematical model.

treatments, or with advanced liver disease), with frequent sampling at multiple time-points, including the very first hours of treatment. Our results show that first-phase HCV-RNA decline may be actually shorter than previously estimated with standard IFN-treatment [11], being comprised within the first 24 h since treatment-start. During this first-phase, all 15 patients experienced an effective and optimal HCV-RNA decline, with mutational-freeze, such as it occurs in more easily treatable patients, and independent from virological-outcome [10]. Vice versa, the second-phase of viral kinetics is in general slower than that found in easier patients [10], and even more compromised in those that experienced virological-failure.

Previous results on non-cirrhotic drug-naïve patients treated with either telaprevir-monotherapy or triple-therapy [10] have shown a much more rapid first phase and second phase HCV-RNA decline in comparison to dual IFN-based treatments [11,13].

In our population, the slope of first-phase decline was optimal and consistent with previous estimates [11,13,10], thus highlighting an independence of the first HCV-RNA decay from baseline clinical and virological characteristics. In particular, the slope of HCV-RNA decay was at its height at 24 h after treatment start. After this time-point, the decay rate suffered a slowdown, thus assuming an early start of the second-phase kinetic. The higher potency of telaprevir in blocking viral production, respecting to IFN, can account for the shortening of first-phase decline, as supported by the higher values of treatment effectiveness (ε) and free virions clearance rate (c) [11,13].

During this short first-phase, HCV-RNA decay was thus deep, rapid, and, notably, consistent in all 15 patients, including those that later failed. By UDPS, no rearrangement of viral quasispecies (nor emergence of RAVs, at the lowest limit of UDPS reliability) was detected within these first 24 h of treatment, indicating a mutational-freeze and a limited number of (or even absent) cycles of viral replication.

On the contrary, in all 15 patients, second-phase HCV-RNA decay kinetic was characterised by values of infected cell clearance rate (δ) lower than those previously defined in naïve and non-cirrhotic patients undergoing the same treatment [10]. In particular, the mean value of δ was much lower than the value of 1.19 day^{-1} reported using the same CE model in naïve patients treated with TVR-monotherapy or triple-therapy (whose determination was nevertheless considered inaccurate) and more close to the value of 0.58 day^{-1} calculated using a varying-effectiveness model [10]. In addition, while first-phase decay was identical among SVRs and failing-patients, after 24 h significant differences started to appear. Indeed, in all telaprevir-failing patients the second-phase viral-decline was characterised by a poor (or null) 24h–48h and 48h–2w HCV-RNA declines, and resulted in HCV-RNA values >100 IU/ml at week-2.

Independent from baseline viraemia, 3/4 patients with HCV-RNA >100 IU/ml at week-2 failed triple-therapy. In agreement with this result, in a larger dataset of telaprevir- and boceprevir-treated patients ($N = 83$), we recently reported that week-2 HCV-RNA decay is significantly lower in patients experiencing virological-failure than in SVRs (median [IQR] = −3.7 [−4.3; −3.2] vs. −4.6 [−5.2; −4.0] log IU/ml, $P = 0.007$) and that HCV-RNA >100 IU/ml at week-2 is significantly associated with virological-failure (adjusted OR: 27.955; 95% C.I.: 1.885–414.500; $P = 0.015$), after correction by baseline/early RAVs, HCV-subtype, cirrhosis, unfavourable IL28B and previous null-response [21]. Thus, assessment of HCV-RNA at week-2 may actually represent a good proxy for virological-response, promptly identifying patients at higher risk of virological-failure if higher than 100 IU/ml.

The suboptimal second-phase HCV-RNA decline observed in failing-patients can be associated to a lower δ, and/or to a lower ε. As previously reported [10], we indeed observed a direct correlation among ε and δ. This indicates that, within biological limits, the second slope of viral decline is improved as drug-effectiveness improves. The base mechanism of this phenomenon lies in the hypothesis that the second-phase HCV-RNA decline may be driven not only by loss of infected cells, but also by a continuous decline in viral production and "cell cure" via riddance of intracellular replication complexes [16]. Therefore,

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suboptimal treatment-effectiveness can slower the second-phase decline independently of immune response, increasing the risk of virological-failure.

A lower treatment efficacy is probably dependent by multiple factors, such as: (a) the suboptimal action of pegIFN + RBV backbone, supported by the fact that all 3 failing-patients were previous null-responders and with unfavourable IL28B genotype; (b) by the poorer telaprevir-pharmacokinetics (indeed observed in all 3 failing patients); and (c) selection or development of RAVs, further affecting drug-effectiveness during the course of treatment [6].

Whatever the mechanism, lower ε and δ values in both patients experiencing late viral-breakthrough corresponded to the lowest 24h–2w HCV-RNA decay slopes. Assuming a complete therapy-mediated control of viral replication ($\varepsilon = 100\%$), this decay pattern could simply represent a delay in infected cell clearance, and thus just underlie the utility of a longer treatment. Since also ε is reduced, however, the chance of low-level viral replication under drug-pressure is high, possibly allowing the development/selection of minority RAVs. Indeed, early rearrangement of viral quasispecies occurred exclusively in failing-patient, and it was associated with the development of RAVs at failure. During second-phase viral decline, mutational-freeze was thus no longer observed in patients experiencing virological-failure, since RAVs were detected in 2/2 analyzed patients between 48 h and 2 weeks, though with different prevalence.

In PT7, experiencing late viral breakthrough, RAVs were already detected at 48 h just as minor variants, and became the most prevalent viral population only at the moment of failure (week-20). These 48 h minority RAVs were completely absent at earlier evaluations (8 h and 24 h), suggesting a process of development and selection, rather than pure selection of pre-existent RAVs, at least when late failure occurs.

On the other hand, in PT13, RAVs detected at week-2 accounted for 99.8% of infecting viral population, and indeed determined an increase in HCV-RNA from previous values. This pattern may suggest in this patient the pre-existence of minority RAVs. Unfortunately, no 24h–48h samples were available for PT13 and, at baseline, no evidences of resistance were found. Nevertheless, the absence of RAVs detection at baseline doesn't exclude their minor presence, below the sensitivity-limit of UDPS (<0.1% prevalence). In PT13, for instance, 0.1% of baseline 440,465 IU/ml would correspond to 440 IU/ml, thus still 2.6 log IU/ml of circulating potentially resistant strains, even if this hypothesis needs to be confirmed by means of more sensitive techniques (such as illumina).

For the present study, week-2 100 IU/ml threshold was evaluated by Roche COBAS® TaqMan® HCV Test v2.0 assay. Although this assay and Abbott RealTime HCV assay have different sensitivity, they show high clinical concordance [22]. Indeed, in another study on a larger population we confirmed the same threshold with both tests [21].

In conclusion, now more than ever, clinical management of HCV-infected patients treated with first-generation PIs may benefit from a closer and accurate early virological-monitoring, given the rapid kinetics of HCV-RNA decline and the risk of resistance-development. For this reason HCV-RNA measurements performed during second-phase HCV-RNA decline (i.e. at week-2) under triple-therapy may provide relevant clinical information to monitor treatment efficacy. Suboptimal early HCV-RNA decline from 48 h to the first 2 weeks of treatment may indeed be the first hint for an ongoing residual viral replication under pharmacological-pressure, potentially leading to development and selection of resistance, and, eventually, virological failure.

Conflict of interest

P. Navarra, M. Angelico, C.F. Perno and F. Ceccherini-Silberstein have received funds for attending symposia, speaking, organizing educational activities, grant research support, consultancy and advisory board membership, from ViiV and Merck Sharp & Dohme (PN), from Roche, Gilead, Merck Sharp & Dohme, Janssen Cilag (MA), form Abbott, Boehringer Ingelheim, Bristol Myers Squibb, Gilead, Merck Sharp & Dohme, Janssen Cilag, Pfizer, Tibotec, Roche, and ViiV (CFP), and from Abbvie, Merck Sharp & Dohme, Gilead, Janssen Cilag, Roche, Bristol Myers Squibb, and ViiV (FCS).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dld.2014.12.004.

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