JCM
Journals.ASM.org

Ultradeep Pyrosequencing of NS3 To Predict Response to Triple Therapy with Protease Inhibitors in Previously Treated Chronic Hepatitis C Patients

Sylvie Larrat,[a,b] Om Kulkarni,[c] Jean-Baptiste Claude,[d] Réjane Beugnot,[a] Michaël G. B. Blum,[c] Katia Fusillier,[a] Julien Lupo,[a,b] Pauline Tremeaux,[a,b] Agnès Plages,[e†] Alice Marlu,[e] Hervé Duborjal,[f] Anne Signori-Schmuck,[a] Olivier Francois,[c] Jean-Pierre Zarski,[e,g] Patrice Morand,[a,b] Vincent Leroy[e,g]

Laboratoire de Virologie, Département des Agents Infectieux, Pôle Biologie, Centre Hospitalier Universitaire Grenoble, CS10217, Grenoble, France[a]; Unit of Virus Host Cell Interactions UMI 3265 UJF-EMBL-CNRS, B.P. 181, Grenoble, France[b]; Laboratoire TIMC-IMAG/University of Grenoble Alpes/CNRS/UMR 5525, Grenoble, France[c]; Genostar—Bioinformatics Solutions, Montbonnot Saint Martin, France[d]; Clinique Universitaire d'Hépato-gastroentérologie, Pôle Digidune, Centre Hospitalier Universitaire Grenoble, CS10217, Grenoble, France[e]; Floralis, Gieres, France[f]; Unité INSERM/University Grenoble Alpes U823, IAPC Institut Albert Bonniot, Grenoble, France[g]

Despite the gain in sustained virological responses (SVR) provided by protease inhibitors (PIs), failures still occur. The aim of this study was to determine if a baseline analysis of the NS3 region using ultradeep pyrosequencing (UDPS) can help to predict an SVR. Serum samples from 40 patients with previously nonresponding genotype 1 chronic hepatitis C who were retreated with triple therapy, including a PI, were analyzed. Baseline UDPS of the NS3 gene was performed on plasma and peripheral blood mononuclear cells (PBMC). Mutations conferring resistance to PIs were sought. The overall diversity of the quasispecies was evaluated by calculating the Shannon entropy (SE). Resistance mutations were found in plasma and PBMC but were not discriminating enough to predict an SVR. NS3 quasispecies heterogeneity was significantly lower at baseline in patients achieving an SVR than in those not achieving an SVR (SE of $26.98 \pm 16.64 \times 10^{-3}$ versus $44.93 \pm 19.58 \times 10^{-3}$, $P = 0.0047$). With multivariate analysis, the independent predictors of an SVR were fibrosis of stage F ≤2 (odds ratio [OR], 13.3; 95% confidence interval [CI], 1.25 to 141.096; $P < 0.03$) and SE below the median (OR, 5.4; 95% CI, 1.22 to 23.87; $P < 0.03$). More than the presence of minor mutations at the baseline in plasma or in PBMC, the NS3 viral heterogeneity determined by UDPS is an independent factor for an SVR in previously treated patients receiving triple therapy that includes a PI.

In the treatment of patients with chronic hepatitis C of genotype 1, the addition of a protease inhibitor (PI) (boceprevir [BOC] or telaprevir [TPV]) to pegylated interferon alpha (PegIFN-α) and ribavirin (RBV) has increased the rate of sustained virological responses (SVR) compared to that with dual therapy (PegIFN-α/RBV), both in naive and treatment-experienced patients (1–4). The role of the PIs is crucial, and failures of triple therapy are mainly due to mutations in the viral NS3 protease gene (5). Mutations are favored by the high rate of error of the hepatitis C virus (HCV) RNA-dependent RNA polymerase, estimated to be between 10^{-3} to 10^{-5} per copied base pair (6), combined with the high rate of viral replication (up to 1×10^{12} particles produced per day) (7), resulting in one mutation for every genome copied (8). Consequently, the large number of viral quasispecies generated constitutes a reservoir for the emergence of resistant strains. The early appearance (<15 days) of resistance mutations in patients treated with PIs alone strongly suggests that quasispecies harboring drug resistance mutations preexist at low frequencies (9). PI-resistant mutations have been detected as the majority strain in blood of 2% to 19% of patients before treatment, according to the study and the number of mutations considered (10–15). The association between these mutations found in plasma and the failure of triple therapy is not clear. Moreover, data on other compartments such as peripheral blood mononuclear cells (PBMC) are lacking.

Next-generation sequencing (NGS) technologies have demonstrated their ability to detect minority variants in some patients at baseline (16) or very shortly after the onset of therapy (17). Additionally, this approach allows the assessment of more macroscopic parameters that reflect the overall diversity of viral populations, such as the Shannon entropy (SE). We hypothesized that such fine analysis might be useful in the search for virological predictors of responses to antiviral drugs.

Therefore, the aims of this study were to comprehensively analyze the NS3 protease viral region in plasma and PBMC using an NGS technology, the 454 Roche ultradeep pyrosequencing (UDPS) platform, and to assess its impact on clinical outcomes of patients, including responses to triple therapy.

MATERIALS AND METHODS

Patients. Patients with a chronic HCV genotype 1 infection consecutively seen at Grenoble University Hospital between January 2011 and May 2012 for an indication of triple combination therapy, including PegIFN-α/RBV

Received 4 September 2014 **Returned for modification** 14 October 2014
Accepted 28 October 2014

Accepted manuscript posted online 19 November 2014

Citation Larrat S, Kulkarni O, Claude J-B, Beugnot R, Blum MGB, Fusillier K, Lupo J, Tremeaux P, Plages A, Marlu A, Duborjal H, Signori-Schmuck A, Francois O, Zarski J-P, Morand P, Leroy V. 2015. Ultradeep pyrosequencing of NS3 to predict response to triple therapy with protease inhibitors in previously treated chronic hepatitis C patients. J Clin Microbiol 53:389–397. doi:10.1128/JCM.02547-14.

Editor: M. J. Loeffelholz

Address correspondence to Sylvie Larrat, slarrat@chu-grenoble.fr.

† Deceased January 2014.

doi:10.1128/JCM.02547-14

and BOC or TPV, were considered for inclusion. The main inclusion criterion was failure of a previous optimal course of PegIFN-α/RBV dual therapy. The exclusion criteria were age under 18 years, other chronic liver disease, HIV or hepatitis B virus (HBV) coinfections, and alcohol consumption of >50 g/day. Patients who stopped triple therapy early for any reasons other than virological breakthrough or according to the stopping rules were also excluded. The treatment schedules and stopping rules followed the guidelines of the French Association for the Study of the Liver (18). The choice between a TPV- and a BOC-based regimen was at the investigator's discretion. A total of 25 patients had a lead-in phase, because some patients treated with TPV required this to evaluate their tolerance to dual therapy. In these cases, baseline samples correspond to samples taken on the day the triple therapy began.

No nonstandard investigations were performed for the purpose of this study, which conformed to local ethical considerations (including written consent from patients for the use of samples for research purposes and the sample use license granted by the Ministry of Education and Research) and the principles outlined in the Declaration of Helsinki.

Routine laboratory tests. Conventional laboratory monitoring was part of the therapeutic management of these patients. IL28B was determined using the LightMix kit IL28B TIB Molbiol (Roche Diagnostics, Meylan, France), HCV genotyping was performed using bidirectional Sanger sequencing (19), and fibrosis staging was performed using either FibroScan (Echosens, Paris, France), FibroTest (Biopredictive, Paris, France), or liver biopsy specimens interpreted by an expert pathologist. Interferon gamma-inducible protein 10 (IP10) values were determined with the human IP10 enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences, San Diego, CA, USA). Monitoring of the HCV RNA viral load using the Cobas AmpliPrep/Cobas TaqMan HCV quantitative test v2.0 (Roche Diagnostics) was done at week (W) 0, W2, W4, W8, W12, W16, W24, W36, W48, W60, and W72. Plasma and PBMC samples were collected and stored at −80°C at each visit.

HCV NS3 UDPS sample preparation and sequencing. EDTA samples were centrifuged for 20 min at 3,000 rpm. PBMC were isolated with Lymphoprep (Axis-Shield; Abcys, Paris, France). HCV RNA was extracted from cells using a QIAamp viral RNA minikit (Qiagen, Courtaboeuf, France) with an elution volume of 50 μl. HCV RNA was extracted from 1 ml of plasma using EasyMAG (bioMérieux, Marcy l'Étoile, France) with an elution volume of 25 μl. Extracted RNA (15 μl) was purified using Turbo DNase Ambion (Life Technologies, Cergy Pontoise, France), and cDNA was synthesized with the AccuScript high-fidelity kit (Agilent, Garches, France) using random primers.

For UDPS, the 454 GS Junior platform was used (Roche Diagnostics). The main part of HCV NS3 protease (nucleotides [nt] 3483 to 3982 according to reference HCV strain H77, i.e., amino acids 23 to 188) was amplified using Phusion Hot Start II (Finnzyme, Illkirch, France). For both 1a and 1b subtypes, the forward primers were a sample-specific multiplex identifier (MID) sequence and the HCV-specific sequence (5′-ACWGGYCGRGAYARRAAYCA-3′). The reverse primers comprised a matched sample-specific MID sequence and the HCV-specific sequence (5′-GARTTRTCYGWRAASACYGGRGA-3′). The PCR cycling conditions were initial denaturation at 98°C for 30 s followed by 50 cycles of denaturation at 98°C for 5 s, annealing at 58°C for 15 s and elongation at 72°C for 20 s with a final step of extension at 72°C for 5 min. The amplification efficacy was assessed using the Agilent DNA 1000 reagent kit and the Agilent 2100 expert bioanalyzer (Agilent, Les Ulis, France). The expected amplicon were obtained in plasma samples for all the patients and in PBMC for 18 patients out of 40 (45%). The amplicons were pooled equimolarly and purified using Agencourt AMPure XP reagents (Beckman Coulter, Roissy, France). A library on the amplicon pool was prepared using the GS Junior rapid library prep kit. An emulsion PCR was run with the GS Junior emPCR (Lib-L) kit. Sequencing was performed with a 454 GS Junior PicoTiterPlate to a target depth reading of 1,000×. Files were sent to Genostar for data analysis. A mean of 1,070 ± 51 (median, 890) reads per nucleotide was obtained with a median length of 457 nt.

TABLE 1 Demographic characteristics of the patients

Characteristic[a]	Patient data ($n = 40$)
Sex ratio (male/female)	1.5 (24/16)
Age (mean ± SD) (yr)	55 ± 8
Mode of HCV transmission (no. [%])	
i.v. drug injection	11 (27.5)
Transfusion	14 (35)
Other	6 (15.0)
Unknown	9 (22.5)
Duration of infection (mean ± SD) (yr)	34 ± 8[b]
Viral subtype	
1a	19 (47.5)
1b	21(52.5)
Viral load (mean ± SD) (IU/ml)	6.09 ± 0.81
Previous response to dual therapy (no. [%])	
NR	18 (45.0)
PR	14 (35.0)
RR	8(20.0)
RVR	
Yes	19 (47.5)
No	21 (52.5)
Antiprotease drug used	
BOC	18 (45.0)
TPV	22 (55.0)
IL28B genotype	
C/C	10 (25.0)
C/T	23 (57.5)
T/T	7 (17.5)
IP10 (mean ± SD) (pg/ml)	234.1 ± 228.2
ALT (mean ± SD) (IU/ml)	99 ± 68
Fibrosis stage	
F0, F1, F2	9 (22.5)
F3, F4	31 (77.5)

[a] i.v., intravenous; NR, null responders; PR, partial responders; RR, relapsers; RVR, rapid virologic response; BOC, boceprevir; TPV, telaprevir; ALT, alanine aminotransferase.
[b] $n = 28$.

Sensitivity of UDPS for mutation detection. A synthetic HCV NS3 protease RNA was transcribed from a TOPO TA plasmid carrying the HCV subtype 1a NS3 protease gene obtained by direct cloning of the NS3 amplicon (nucleotides 3483 to 3982 according to the reference HCV strain H77) of a chronic hepatitis C patient, according to the technical recommendations for TOPO TA cloning (Invitrogen/Life Technologies) and using the MEGAscript T7 kit and Turbo DNase (Ambion/Life Technologies). UDPS mutation analysis of this transcript showed a maximum mutation rate of 0.81% (22/2,725 reads) at amino acid position 39 (nt 115 of the amplicon), the second rate was 0.5% (13/2,719 reads), and the mutation rate fell rapidly below 0.3% with a total mean rate of 0.17% ± 0.09%. Based on these observations, the limit of detection of mutations was set at 1% for this study.

Mutation analysis. Trimming, assembly, and single nucleotide polymorphism (SNP) calling were performed with CLC Genomics Work-

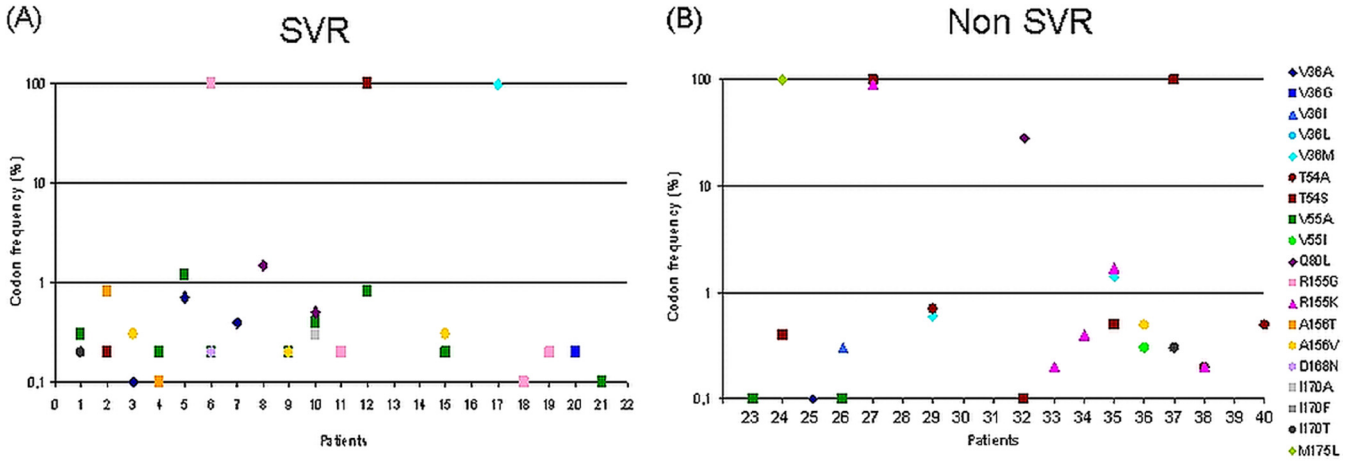

FIG 1 TPV- and BOC-resistant variants at baseline. UDPS was performed on plasma from 40 previous nonresponder patients who achieved an SVR ($n = 22$) (A) or had on-treatment virological failure or relapsed ($n = 18$) (B). Codon frequency (%) at NS3 positions 36, 54, 55, 155, 156, 168, and 170 are represented as colored dots. The 1% threshold is shown by a black line.

bench alignment software (CLC bio, Aarhus, Denmark). Reads belonging to a specific patient and time point were separated based on the sample-specific MID. Low-quality bases and failed reads were removed by trimming with classical parameters (minimum Phred score of Q20, minimum size of 50 nt, no ambiguous nucleotide allowed). Trimmed reads were aligned with the HCV reference strain H77 for each clinical specimen. Reads were accepted if more than half of the read length matched, with at least 80% sequence identity. Based on the above criteria, reads with ≥80% bases matching a particular position of the reference sequence were aligned. Each position of the viral genome was assigned a coverage depth, representing the number of times the nucleotide position, was sequenced.

Genetic variability analysis. The extent of viral quasispecies was evaluated by analyzing the genetic complexity of the NS3 protease gene based on the number of different sequences present in the population. Genetic complexity was determined by Shannon entropy values as described in reference 20,

$$Sn = -\frac{\sum_{i=1}^{n} fi(\ln fi)}{N}$$

where n is the number of different species identified, fi is the observed frequency of the particular variant in the quasispecies, and N is the total number of nucleotides studied (457 in this study). Only species detected with a minimum of 10 reads and a frequency of >0.1% were taken into account. Statistical comparisons of complexity were made using the Mann-Whitney U test, and its reproducibility was assessed using 19 plasma samples analyzed in two different runs. The intrapatient stability of this parameter in the absence of treatment was assessed in 10 patients using plasma samples collected in the 3 years preceding treatment.

Multivariate and assignment analysis to study compartmentalization between plasma and peripheral blood mononuclear cells. For each patient, the genetic structure of the viral population was analyzed using phylogenetic and multivariate data analysis methods. A multiple-sequence alignment combining PBMC and plasma HCV RNA samples was built with MAFFT software and the FFT-NS-2 method for large alignments (21). Multidimensional analysis was performed with the R computer package (R Core Team 2012, Vienna, Austria) after removal of RNA sites exhibiting >2% gaps. Principal component analysis was used to display a two-dimensional representation of the viral population genetic

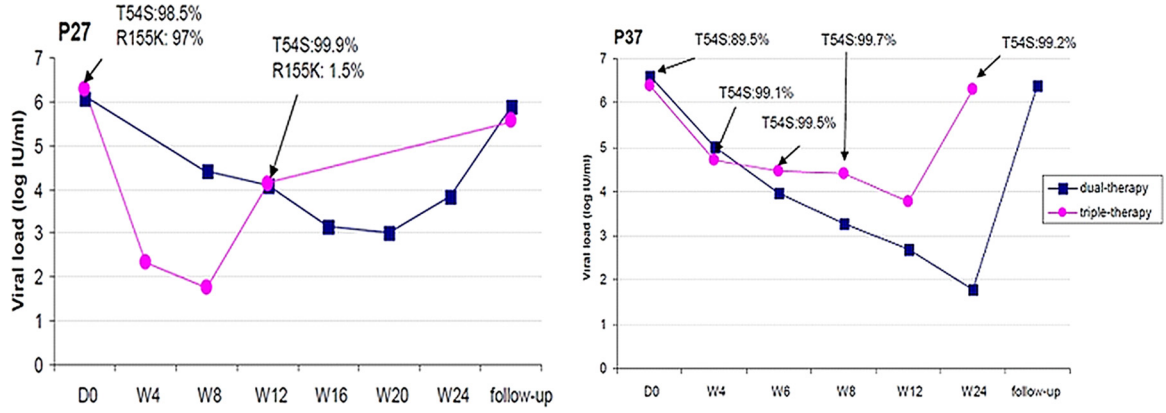

FIG 2 Viral load monitoring of triple-therapy nonresponder patients exhibiting plasmatic major mutation(s) at baseline. Results for patient 27 are presented in panel A and for patient 37 in panel B. Monitoring of the previous dual therapy is represented with the squares and blue lines, whereas triple therapy is represented with the circles and pink lines. In their second line of treatment, patient 27 was treated by a triple therapy, including TPV and patient 37 received 4 weeks of dual therapy before the addition of BOC. For patient 37, D0 corresponds to the beginning of the lead-in phase. Mutations found using UDPS at different points and their percentages are indicated with arrows. Dual therapy was stopped at W24 and triple therapy at W12 for the two patients according to the stopping rules.

TABLE 2 Summary of resistances found at baseline and failure

Patient no.	Previous response[a]	PI	Sample	Type (%) of mutation(s) found		
				UDPS baseline	UDPS failure	Sanger failure
SVR						
P1	RR	TPV	PL[b]	NMD[c]		
			PBMC	T54S (1.3)		
P5	NR	TPV	PL	V55A (1.2)		
P6	NR	TPV	PL	R155G (99)		
			PBMC	NMD		
P12	NR	TPV	PL	T54S (99.6)[c]		
			PBMC	T54S (98.5)		
P17	RR	TPV	PL	V36M (98.4)		
Non-SVR						
P24	NR	TPV	PL	M175L (9.7)	V36L (88.8) R155K (87.2)	V36L + R155K
P27	PR	TPV	PL	T54S (98.5) R155K (97)	T54S (99.9) R155K (1.5)	T54S
P29	NR	BOC	PL	V36M (0.6)[d,e]	V36 M (54.7) T54S (1) R155K (95.1)	V36 M + R155K
			PBMC	V36M (8) T54S (4.5) R155K (16.9)		
P35	PR	TPV	PL	V36M (1.4) R155K:1.7)	V36 M (91.7) V36L (6.7) T54S (1) R155K (99)	V36M + R155K
P37	PR	BOC	PL	T54S (89.52)[d]	T54S (99.2)	T54S
			PBMC	T54S (99.7)	T54S (99.7)	

[a] NR, null responders; PR, partial responders; RR, relapsers.
[b] PL, plasma.
[c] NMC, no mutation detected.
[d] Mutation was also found in the major strain sequenced before the lead-in phase.
[e] Mutations of <1% were interpreted according to the presence of the same mutation in PBMC and its development at failure.

data set. To test for compartmentalization in the viral sequences from the two sources of HCV RNA, Wilks' lambda, a statistic used in multivariate analysis of variance, was evaluated based on the combination of genotypes. In addition, linear discriminant analysis was used to evaluate the rate of misclassification for PBMC and plasma samples (22).

Statistical analysis. Results are expressed as mean values and ranges (minimum and maximum) or standard deviations. Baseline values were compared using the Mann-Whitney *U* test. Independent predictors of SVR were identified by multivariate analysis using logistic regression. The analysis was performed using SPSS 19.0 software.

RESULTS

Characteristics of patients. Forty patients were included in the study. Their characteristics are shown in Table 1. Overall, this was a difficult-to-treat population with 45% null responders and 78% of patients with stage F3 or F4 fibrosis. An SVR was achieved in 22 patients (55%) with a nonsignificant trend toward a better response in genotype 1b (67%) than in 1a (42%). Among the 18 patients who did not achieve an SVR, 14 were nonresponders with therapy stopped at W4 in 1 patient (viral load of >1,000 IU/ml), at W8 in 4 patients due to viral rebound, at W12 in 8 patients (viral load of >1,000 IU/ml, corresponding to rebound in 3), and at W24 in 1 patient (HCV RNA detectable). Mutations conferring resistance to PIs were detected by Sanger sequencing in all but 1 patient at the time of viral rebound. Other treatment failures corresponded to relapse observed in 4 patients (patients [P] 23, 28, 31, and 34) in whom no resistance was documented at the time of relapse.

Impact of baseline mutations in plasma on the response to triple therapy. UDPS of NS3 protease detected the presence of mutations above the significance threshold (fixed at 1% of the total viral population) in 5 patients with genotype 1a and 3 with genotype 1b. Among these 8 patients, 4 achieved an SVR and 4 did not (Fig. 1). In 2 SVR patients who were previously null responders (P6 and P12) and in a relapser (P17), the mutations R155G, T54S, and V36M, respectively, were found as the major strains. All patients were retreated with a TPV-based triple combination therapy and achieved undetectable HCV RNA by W8.

Mutations M175L, T54S plus R155K, and T54S were also found as major strains in 3 nonresponding patients (P24, P27, and P37, respectively). Two were previously partial responders and 1 was a null responder to PegIFN-α/RBV therapy. One of them (P37) was retreated with BOC-based triple therapy; the T54S mutation was still detected in plasma at the end of the lead-in phase, while HCV RNA declined by 1.7 log. Viral load monitoring of previous dual therapy was retrieved for 2 patients (P27 and P37). Figure 2 presents the viral kinetics obtained with dual and triple therapy. The HCV RNA declines were very similar between the two lines of treatment for patient 37, whereas a more pronounced decline was observed for patient 27, followed rapidly by a viral rebound at week 12. These results highlight the lack of efficacy of the added PI for these patients. We should note that three patients (P12, P29, and P37) presenting mutations at a rate of >1% received a lead-in phase, and the mutations detected at day 0 were always already present before the initiation of the dual-therapy phase.

Mutations at baseline in PBMC. UDPS was performed at baseline in PBMC from 18 patients, including 9 SVR and 9 non-SVR patients. For SVR patients, the mutation T54S was found as the major strain in cells in patient 12 and also in plasma (Table 2). Mutation frequencies of >1% were also detected in PBMC of 2 additional patients (P1 and P29). T54S was detected at 1.3% in PBMC of patient 1 but not in the corresponding plasma sample despite good coverage of NS3. Interestingly, in patient 29, the combined mutations V36M (8.0%) plus T54S (4.5%) plus R155K (16.9%) initially found in PBMC and at a very low level (0.6%) for

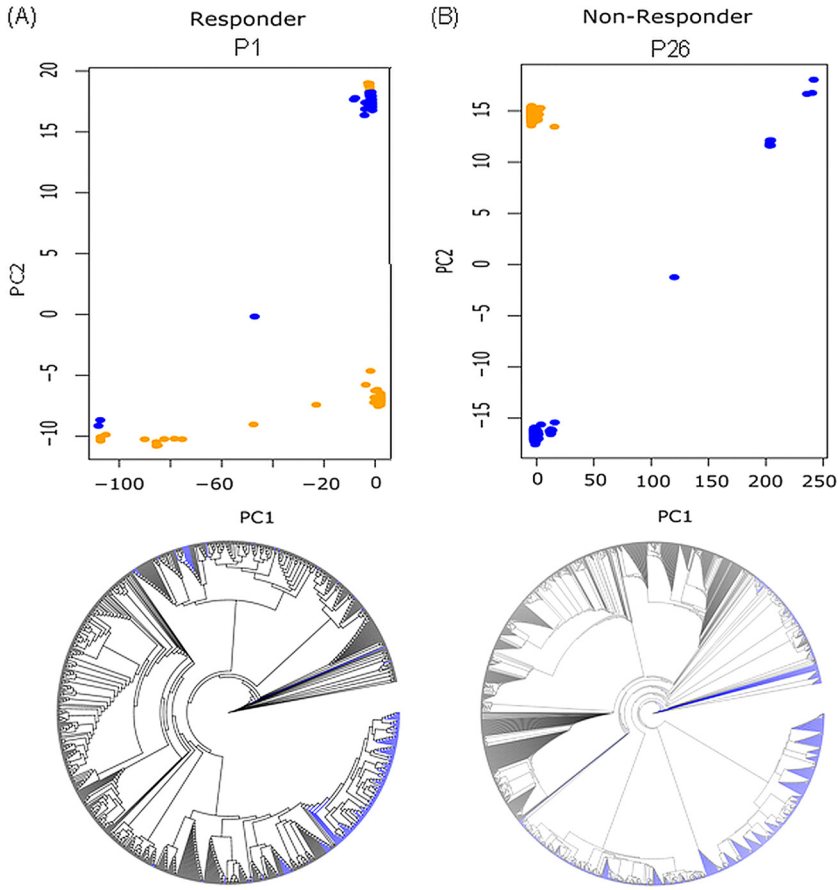

FIG 3 Principal component plots and circular phylogenetic trees for viral genetic diversity in responder and nonresponder patients. The plots and the associated circular phylogenetic trees represent clustering of sequences by type of sample showing the distribution of viral populations for PBMC (blue color) and plasma (orange for plots and black for phylogenetic trees) in two examples: (A) one responder patient (P1) and (B) a nonresponder patient (P26).

V36M in plasma became detectable in plasma (V36M [57.4%] plus T54S [1.0%] plus R155K [95.1%]) at the time of viral rebound.

Compartmentalization of NS3 quasispecies between plasma and PBMC. In order to further address the compartmentalization of NS3 quasispecies between plasma and PBMC, a linear discriminant analysis was performed for the 18 samples in which UDPS results were available for both compartments at baseline. The outputs of the principal component analysis clearly showed that PBMC and plasmatic NS3 protease sequences were segregated within each patient (Wilks' lambda test with Bonferroni's correction, $P < 10E{-}7$) (Fig. 3). When each sequence was assigned as PBMC or plasma using linear discriminant analysis, the misclassification rates were low (mean of 5.7% ± 5.5%). No significant difference in compartmentalization was observed between SVR and non-SVR patients.

Repertoire diversity evaluation using Shannon entropy measurement. We then took advantage of the UDPS technique to assess the overall genomic complexity of NS3, estimated by calculating the SE from frequencies of nucleotide substitutions measured at each position. The reproducibility of the assay was first assessed in 19 plasma samples analyzed in two different runs. The correlation coefficient was 0.84 (Fig. 4A), which was highly significant ($P = 0.0001$). Spontaneous variations over time were then analyzed in 10 plasma samples collected within a median time of 11.5 months (range, 3 to 28 months) before the start of triple therapy. The results demonstrated satisfactory intrapatient stability of the SE with a correlation coefficient of 0.56 (Fig. 4B) ($P = 0.006$). Moreover, SE calculated on 20 pairs of samples taken at the beginning of the lead-in phase and at the beginning of the triple therapy showed a correlation coefficient of 0.70646. Taken together, these results suggest that the overall genomic complexity estimated by SE is a robust parameter characterizing NS3. Then, we searched for relationships between SE and the clinical and virological characteristics of patients. No significant correlation was found with the viral subtype, viral load, or duration or severity of the disease. In contrast, a significant relationship was found with the response to the triple therapy. Indeed, SE at baseline was significantly lower in patients achieving an SVR than in nonresponders ($26.98 \pm 16.64 \times 10^{-3}$ versus $44.93 \pm 19.58 \times 10^{-3}$, $P = 0.0047$) (Fig. 4C). As shown in Fig. 4D, this difference persisted after stratification on the profile of the response to previous dual PegIFN-α/RBV therapy. The same difference was found in PBMC, with mean SE of $17.62 \pm 8.61 \times 10^{-3}$ in responding patients ($n = 9$) and $32.22 \pm 13.13 \times 10^{-3}$ in nonresponding patients ($n = 9$) ($P = 0.0152$).

Predictors of response to triple therapy. Finally, the ability of UDPS to provide predictive factors of response to the triple therapy at baseline along with more conventional predictors was evaluated. As shown in Table 3, a low IP10 concentration was associ-

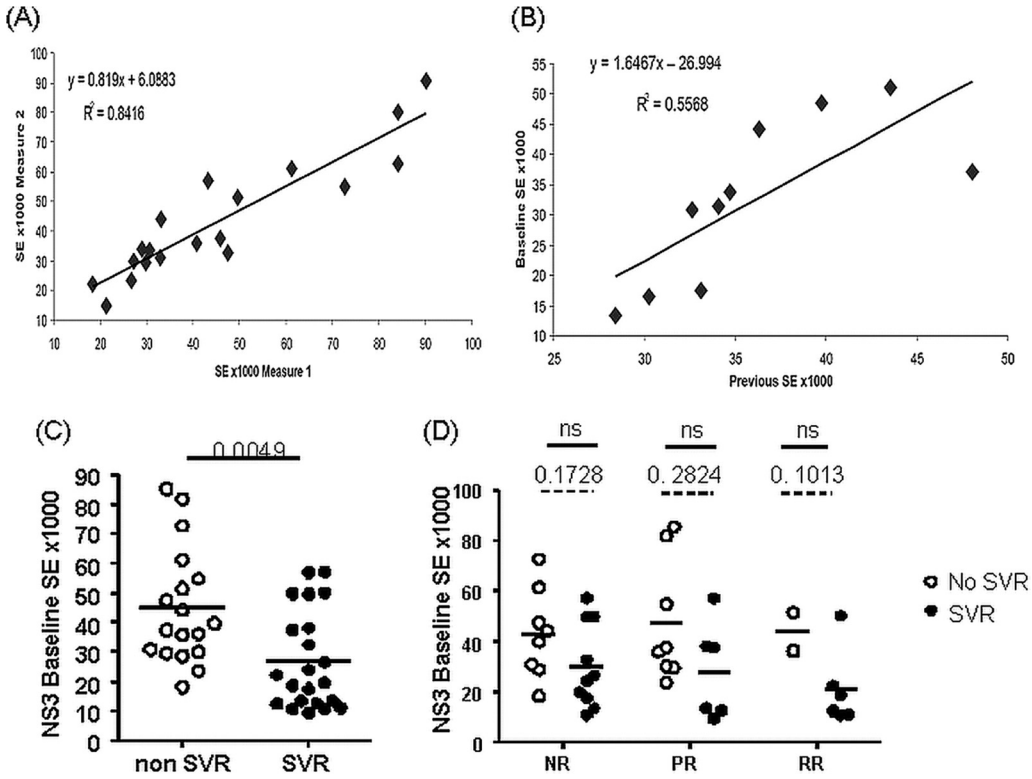

FIG 4 NS3 protease heterogeneity expressed as Shannon entropy (SE). (A) Reproducibility of SE measurement assessed with two measures on the same sample realized in two different runs for 19 samples. (B) Intrapatient stability of SE measures was assessed by measures at baseline and prior therapy for 10 patients. (C) SE at baseline in patients achieving an SVR on triple therapy ($n = 22$) and patients not achieving an SVR ($n = 18$), all patients being nonresponders to prior dual therapy. (D) SE according to the response profile under previous dual therapy. Black bars correspond to the mean values. NR, null responders; PR, partial responders; RR, relapsers; ns, not significant.

ated with SVR, whereas a nonsignificant trend was observed for a fibrosis stage of less than F3 versus F0, F1, or F2, genotype 1b versus 1a, and relapse versus partial response and null response to previous PegIFN-α/RBV therapy. From the UDPS results, the detection of baseline NS3 mutations had no impact on the response to treatment, while the SE value was associated with SVR. With multivariate analysis, the independent predictors of an SVR were fibrosis stage of F2 or lower (odds ratio [OR], 13.3; 95% confidence interval [CI], 1.25 to 141.096; $P < 0.03$) and SE value below the median, i.e., 0.03171 (OR, 5.4; 95% CI, 1.22 to 23.87; $P < 0.03$).

DISCUSSION

The failure of triple therapy with PegIFN-α/RBV and BOC or TPV in patients previously not responding to dual therapy is mainly associated with the emergence of PI-resistant variants (1, 4, 5, 23). The previous profile of response was one of the main predictive factors of an SVR to triple therapy but reflects only the lack of efficacy of PegIFN-α/RBV in these patients. We hypothesized that a fine analysis of the NS3 region using UDPS might provide pertinent additional information to better understand the sensitivity of the virus to the added PI. The main finding of our study is that the overall genomic complexity of NS3 protease estimated by SE is an independent predictor of SVR in patients in whom PegIFN-α/RBV dual therapy previously failed, whatever their previous profiles of viral load decline. The SE appeared as a robust parameter, because it had good reproducibility in the same sample between experiments and in the same patient over time. The calculation of SE to evaluate HCV gene diversity has already been used to study virus evolution (20, 24–27). Its reduction after 1 week of treatment was also reported to be predictive of the response to PegIFN-α/RBV dual therapy (20). A greater baseline quasispecies complexity of the hypervariable region 1 has also been described in patients who did not respond to dual therapy at W12, but no link was found with an SVR (28–31). Therefore, it might be hypothesized that a viral population with a greater complexity and heterogeneity stands up better under antiviral pressure, especially with direct antiviral agents, with a greater probability of developing mutations that drive treatment failure.

The use of this broad parameter is of particular interest since the detection of pretherapeutic point mutations, whatever their rate, does not seem sufficient to predict an SVR. We confirm in our study the detection of PI-resistant variants at baseline in both SVR and non-SVR patients as already reported by others using either population sequencing assays or NGS (10, 15, 17, 23, 32–34). Nevertheless, the association between these mutations and the profile of viral load decrease in dual therapy might also be of importance (35, 36). As suggested by Trimoulet et al. (16), in our study the mutation R155K was also present at baseline in 3 nonresponding patients (twice in plasma samples and once in PBMC) but was not found in any SVR patient. Moreover, we present here two cases of null responder patients, for whom the addition of a PI did not alter the viral kinetics, suggesting that resistance to PI was already conferred at baseline by the major mutations found (T54S

TABLE 3 Analysis of triple-therapy SVR predictive factors

Predictive factor[a]	SVR ($n = 22$)	No SVR ($n = 18$)	P[c]
Sex ratio (no. [%])			
Male	13 (54)	11 (46)	0.89
Female	9 (56)	7 (44)	
Age (mean ± SD) (yr)	56 ± 8	53 ± 7	0.17
Duration of infection (mean ± SD) (yr)	34 ± 9[b]	35 ± 6[b]	0.49
Viral subtype (no. [%])			
1a	8 (42)	11 (58)	0.11
1b	14 (67)	7 (33)	
Viral load (mean ± SD) (IU/ml)	5.99 (±0.93)	6.21 (±0.64)	0.62
Previous response to dual therapy (no. [%])			
NR	10 (56)	8 (44)	0.34
PR	6 (43)	8 (57)	
RR	6 (75)	2 (25)	
Antiprotease drug used (no. [%])			
BOC	9 (50)	9 (50)	0.57
TPV	13 (59)	9 (41)	
IL28B genotype (no. [%])			
C/C	8 (80)	2 (20)	0.15
C/T	10 (44)	13 (56)	
T/T	4 (57)	3 (43)	
IP10 (mean ± SD) (pg/ml)	182 ± 190	297 ± 258	**0.05**
ALT (mean ± SD) (IU/ml)	103 ± 80	93 ± 50	0.64
Fibrosis score (no. [%])			
F0, F1, F2	7 (78)	2 (22)	0.09
F3, F4	15 (48)	16 (52)	
Baseline PL mutations by UDPS (no. [%])			
Yes	4 (50)	4 (50)	
No	18 (56)	14 (44)	0.75
Shannon entropy (mean ± SD) (×1,000)	26.98 ± 16.64	44.93 ± 19.58	**0.0047**

[a] NR, null responders; PR, partial responders; RR, relapsers; RVR, rapid virologic response; BOC, boceprevir; TPV, telaprevir; ALT, alanine aminotransferase; PL, plasma.
[b] $n = 14$.
[c] Significant P values are in bold.

plus R155K for P27 and T54S for P37). Moreover, such previsions might be undermined by the fact that IFN sensitivity can vary from one treatment to another (37).

As recently demonstrated by the description of PI-resistant variants in the liver of naive patients, the investigation of HCV replication compartments may also be of interest (38). We found one case of mutations that were present in PBMC but not at >1% in plasma before treatment and that emerged during viral rebound in a nonresponding patient. However, mutations were also found in PBMC of a patient who responded to triple therapy, rending this parameter difficult to exploit. Nevertheless, we confirmed, using UDPS, the existence of a compartmentalization of viral strains between plasma and PBMC with two distinct populations and some common sequences, suggesting a possible interaction between the two compartments and the liver compartment. These data associated with the detection in plasma of mutations at 24 h or 48 h of treatment under monotherapy and not detected at baseline (17), might suggest the preexistence of drug-resistant variants generated in the liver and present at very low levels in plasma, not detectable even using NGS technologies. Therapeutic pressure encourages their growth and renders them detectable in blood by NGS in a few days. As PBMC are easier to collect than liver biopsy specimens, one can speculate that they might be an intermediate reservoir for these mutated strains (39–41).

The first limitation of our study is the small number of patients included, resulting in low statistical power to identify the predictors of SVR. For example, the profile of response to dual therapy, which is known to have a major impact on the SVR rate to triple therapy, was not a significant predictor. Thus, our study cannot rule out the possibility that the preexistence of major resistant variants in a null-responder population might decrease the overall probability of an SVR. However, to our knowledge, no other study has reported the use of NGS on a larger number of patients. The second limitation is directly linked to the use of first generation PIs, whereas several new direct antiviral agents that can be used in IFN-free combinations are now available in some countries (42). These new combinations are expected to give SVR rates of >90% in the real-life setting, but some failures associated with resistance will continue to occur, especially in cirrhotic patients. It will be then important to assess the relevance of UDPS to guide therapy in the most difficult-to-treat patients, including those with multiresistant viral strains.

In conclusion, the overall genetic diversity of NS3 assessed by the SE rather than the identification of resistant variants appears to impact the response to triple therapy with first generation PIs. Although these results need to be confirmed in larger series of patients, including those treated by the direct next-generation antiviral agents before implementation in clinical practice, they provide new insights into the relationships between viral diversity and risk of resistance.

ACKNOWLEDGMENTS

We thank Caroline Ashford for technical assistance and Alison Foote for English language editing.

The study was funded by the Direction de la Recherche Clinique du CHU de Grenoble, the Rhône-Alpes region, and in part by unrestricted grants from Janssen and MSD.

S.L. has received research grants from Janssen and MSD. J.-P.Z. has been a speaker and/or consultant for Boehringer Ingelheim, Bristol-Myers Squibb, Gilead Sciences, Janssen Pharmaceuticals, Merck Sharp & Dohme, Roche, and Siemens. V.L. has been a speaker and/or consultant for Bristol-Myers Squibb, Gilead Sciences, Janssen Pharmaceuticals, Merck Sharp & Dohme, Novartis and Roche. The other authors declare no conflicts of interest.

The authors' editor, Alison Foote, works for the Grenoble Clinical Research Centre and is funded by the Grenoble University Hospital.

REFERENCES

1. **Bacon BR, Gordon SC, Lawitz E, Marcellin P, Vierling JM, Zeuzem S, Poordad F, Goodman ZD, Sings HL, Boparai N, Burroughs M, Brass CA, Albrecht JK, Esteban R.** 2011. Boceprevir for previously treated chronic HCV genotype 1 infection. N Engl J Med **364:**1207–1217. http://dx.doi.org/10.1056/NEJMoa1009482.
2. **Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH, Marcellin P, Muir AJ, Ferenci P, Flisiak R, George J,**

Rizzetto M, Shouval D, Sola R, Terg RA, Yoshida EM, Adda N, Bengtsson L, Sankoh AJ, Kieffer TL, George S, Kauffman RS, Zeuzem S. 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med **364:**2405–2416. http://dx.doi.org/10.1056/NEJMoa1012912.

3. **Poordad F, McCone J, Jr, Bacon BR, Bruno S, Manns MP, Sulkowski MS, Jacobson IM, Reddy KR, Goodman ZD, Boparai N, DiNubile MJ, Sniukiene V, Brass CA, Albrecht JK, Bronowicki JP.** 2011. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med **364:**1195–1206. http://dx.doi.org/10.1056/NEJMoa1010494.
4. **Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, Roberts S, Focaccia R, Younossi Z, Foster GR, Horban A, Ferenci P, Nevens F, Mullhaupt B, Pockros P, Terg R, Shouval D, van Hoek B, Weiland O, Van Heeswijk R, De Meyer S, Luo D, Boogaerts G, Polo R, Picchio G, Beumont M.** 2011. Telaprevir for retreatment of HCV infection. N Engl J Med **364:**2417–2428. http://dx.doi.org/10.1056/NEJMoa1013086.
5. **Vermehren J, Sarrazin C.** 2012. The role of resistance in HCV treatment. Best Pract Res Clin Gastroenterol **26:**487–503. http://dx.doi.org/10.1016/j.bpg.2012.09.011.
6. **Duarte EA, Novella IS, Weaver SC, Domingo E, Wain-Hobson S, Clarke DK, Moya A, Elena SF, de la Torre JC, Holland JJ.** 1994. RNA virus quasispecies: significance for viral disease and epidemiology. Infect Agents Dis **3:**201–214.
7. **Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS.** 1998. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science **282:**103–107. http://dx.doi.org/10.1126/science.282.5386.103.
8. **Kieffer TL, Kwong AD, Picchio GR.** 2010. Viral resistance to specifically targeted antiviral therapies for hepatitis C (STAT-Cs). J Antimicrob Chemother **65:**202–212. http://dx.doi.org/10.1093/jac/dkp388.
9. **Halfon P, Sarrazin C.** 2012. Future treatment of chronic hepatitis C with direct acting antivirals: is resistance important? Liver Int **32**(Suppl 1)**:**79–87. http://dx.doi.org/10.1111/j.1478-3231.2011.02716.x.
10. **Bartels DJ, Sullivan JC, Zhang EZ, Tigges AM, Dorrian JL, De Meyer S, Takemoto D, Dondero E, Kwong AD, Picchio G, Kieffer TL.** 2013. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J Virol **87:**1544–1553. http://dx.doi.org/10.1128/JVI.02294-12.
11. **Bartels DJ, Zhou Y, Zhang EZ, Marcial M, Byrn RA, Pfeiffer T, Tigges AM, Adiwijaya BS, Lin C, Kwong AD, Kieffer TL.** 2008. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A protease inhibitors in treatment-naive subjects. J Infect Dis **198:**800–807. http://dx.doi.org/10.1086/591141.
12. **Besse B, Coste-Burel M, Bourgeois N, Feray C, Imbert-Marcille BM, Andre-Garnier E.** 2012. Genotyping and resistance profile of hepatitis C (HCV) genotypes 1-6 by sequencing the NS3 protease region using a single optimized sensitive method. J Virol Methods **185:**94–100. http://dx.doi.org/10.1016/j.jviromet.2012.06.011.
13. **Gaudieri S, Rauch A, Pfafferott K, Barnes E, Cheng W, McCaughan G, Shackel N, Jeffrey GP, Mollison L, Baker R, Furrer H, Gunthard HF, Freitas E, Humphreys I, Klenerman P, Mallal S, James I, Roberts S, Nolan D, Lucas M.** 2009. Hepatitis C virus drug resistance and immune-driven adaptations: relevance to new antiviral therapy. Hepatology **49:**1069–1082. http://dx.doi.org/10.1002/hep.22773.
14. **Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, Lee B, Heckerman D, Carlson J, Reyor LL, Kleyman M, McMahon CM, Birch C, Schulze Zur Wiesch J, Ledlie T, Koehrsen M, Kodira C, Roberts AD, Lauer GM, Rosen HR, Bihl F, Cerny A, Spengler U, Liu Z, Kim AY, Xing Y, Schneidewind A, Madey MA, Fleckenstein JF, Park VM, Galagan JE, Nusbaum C, Walker BD, Lake-Bakaar GV, Daar ES, Jacobson IM, Gomperts ED, Edlin BR, Donfield SM, Chung RT, Talal AH, Marion T, Birren BW, Henn MR, Allen TM.** 2008. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. Hepatology **48:**1769–1778. http://dx.doi.org/10.1002/hep.22549.
15. **Vallet S, Viron F, Henquell C, Le Guillou-Guillemette H, Lagathu G, Abravanel F, Trimoulet P, Soussan P, Schvoerer E, Rosenberg A, Gouriou S, Colson P, Izopet J, Payan C.** 2011. NS3 protease polymorphism and natural resistance to protease inhibitors in French patients infected with HCV genotypes 1−5. Antivir Ther **16:**1093–1102. http://dx.doi.org/10.3851/IMP1900.
16. **Trimoulet P, Pinson P, Papuchon J, Foucher J, Vergniol J, Chermak F, Wittkop L, Castaing N, Merrouche W, Reigadas S, Molimard M, Kann M, Fleury H, de Ledinghen V.** 2013. Dynamic and rapid changes in viral quasispecies by UDPS in chronic hepatitis C patients receiving telaprevir-based therapy. Antivir Ther **18:**723–727. http://dx.doi.org/10.3851/IMP2632.
17. **Svarovskaia ES, Martin R, McHutchison JG, Miller MD, Mo H.** 2012. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. J Clin Microbiol **50:**3267–3274. http://dx.doi.org/10.1128/JCM.00838-12.
18. **Leroy V, Serfaty L, Bourliere M, Bronowicki JP, Delasalle P, Pariente A, Pol S, Zoulim F, Pageaux GP.** 2012. Protease inhibitor-based triple therapy in chronic hepatitis C: guidelines by the French Association for the Study of the Liver. Liver Int **32:**1477–1492. http://dx.doi.org/10.1111/j.1478-3231.2012.02856.x.
19. **Sandres-Sauné K, Deny P, Pasquier C, Thibaut V, Duverlie G, Izopet J.** 2003. Determining hepatitis C genotype by analyzing the sequence of the NS5b region. J Virol Methods **109:**187–193. http://dx.doi.org/10.1016/S0166-0934(03)00070-3.
20. **Nasu A, Marusawa H, Ueda Y, Nishijima N, Takahashi K, Osaki Y, Yamashita Y, Inokuma T, Tamada T, Fujiwara T, Sato F, Shimizu K, Chiba T.** 2011. Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. PLoS One **6:**e24907. http://dx.doi.org/10.1371/journal.pone.0024907.
21. **Katoh K, Standley DM.** 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol **30:**772–780. http://dx.doi.org/10.1093/molbev/mst010.
22. **Mardia KV, Kent JT, Bibby JM.** 1979. Multivariate analysis. Academic Press, London, United Kingdom.
23. **De Meyer S, Dierynck I, Ghys A, Beumont M, Daems B, Van Baelen B, Sullivan JC, Bartels DJ, Kieffer TL, Zeuzem S, Picchio G.** 2012. Characterization of telaprevir treatment outcomes and resistance in patients with prior treatment failure: results from the REALIZE trial. Hepatology **56:**2106–2115. http://dx.doi.org/10.1002/hep.25962.
24. **Bull RA, Luciani F, McElroy K, Gaudieri S, Pham ST, Chopra A, Cameron B, Maher L, Dore GJ, White PA, Lloyd AR.** 2011. Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. PLoS Pathog **7:**e1002243. http://dx.doi.org/10.1371/journal.ppat.1002243.
25. **Gregori J, Esteban JI, Cubero M, Garcia-Cehic D, Perales C, Casillas R, Alvarez-Tejado M, Rodriguez-Frias F, Guardia J, Domingo E, Quer J.** 2013. Ultra-deep pyrosequencing (UDPS) data treatment to study amplicon HCV minor variants. PLoS One **8:**e83361. http://dx.doi.org/10.1371/journal.pone.0083361.
26. **Li H, Hughes AL, Bano N, McArdle S, Livingston S, Deubner H, McMahon BJ, Townshend-Bulson L, McMahan R, Rosen HR, Gretch DR.** 2011. Genetic diversity of near genome-wide hepatitis C virus sequences during chronic infection: evidence for protein structural conservation over time. PLoS One **6:**e19562. http://dx.doi.org/10.1371/journal.pone.0019562.
27. **Wang GP, Sherrill-Mix SA, Chang KM, Quince C, Bushman FD.** 2010. Hepatitis C virus transmission bottlenecks analyzed by deep sequencing. J Virol **84:**6218–6228. http://dx.doi.org/10.1128/JVI.02271-09.
28. **Abbate I, Lo Iacono O, Di Stefano R, Cappiello G, Girardi E, Longo R, Ferraro D, Antonucci G, Di Marco V, Solmone M, Craxi A, Ippolito G, Capobianchi MR.** 2004. HVR-1 quasispecies modifications occur early and are correlated to initial but not sustained response in HCV-infected patients treated with pegylated- or standard-interferon and ribavirin. J Hepatol **40:**831–836. http://dx.doi.org/10.1016/j.jhep.2004.01.019.
29. **Chambers TJ, Fan X, Droll DA, Hembrador E, Slater T, Nickells MW, Dustin LB, Dibisceglie AM.** 2005. Quasispecies heterogeneity within the E1/E2 region as a pretreatment variable during pegylated interferon therapy of chronic hepatitis C virus infection. J Virol **79:**3071–3083. http://dx.doi.org/10.1128/JVI.79.5.3071-3083.2005.
30. **Cortés KC, Zagordi O, Perlejewski K, Laskus T, Maroszek K, Bukowska-Osko I, Pawelczyk A, Ploski R, Berak H, Horban A, Radkowski M.** 2014. Deep sequencing of hepatitis C virus hypervariable region 1 reveals no correlation between genetic heterogeneity and antiviral treatment outcome. BMC Infect Dis **14:**389. http://dx.doi.org/10.1186/1471-2334-14-389.
31. **El-Shamy A, Hotta H.** 2014. Impact of hepatitis C virus heterogeneity on interferon sensitivity: an overview. World J Gastroenterol **20:**7555–7569. http://dx.doi.org/10.3748/wjg.v20.i24.7555.
32. **Akuta N, Suzuki F, Fukushima T, Kawamura Y, Sezaki H, Suzuki Y,**

Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2013. Prediction of treatment efficacy and telaprevir-resistant variants after triple therapy in patients infected with hepatitis C virus genotype 1. J Clin Microbiol **51:**2862–2868. http://dx.doi.org/10.1128/JCM.01129-13.

33. **Bartolini B, Giombini E, Zaccaro P, Selleri M, Rozera G, Abbate I, Comandini UV, Ippolito G, Solmone M, Capobianchi MR.** 2013. Extent of HCV NS3 protease variability and resistance-associated mutations assessed by next generation sequencing in HCV monoinfected and HIV/HCV coinfected patients. Virus Res **177:**205–208. http://dx.doi.org/10.1016/j.virusres.2013.08.001.
34. **Dierynck I, Thys K, Ghys A, Sullivan JC, Kieffer TL, Aerssens J, Picchio G, De Meyer S.** 2014. Deep-sequencing analysis of the gene encoding the hepatitis C virus nonstructural 3-4A protease confirms a low prevalence of telaprevir-resistant variants at baseline and the end of the REALIZE Study. J Infect Dis **210:**1871−1880. http://dx.doi.org/10.1093/infdis/jiu340.
35. **Pawlotsky JM.** 2011. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. Hepatology **53:**1742–1751. http://dx.doi.org/10.1002/hep.24262.
36. **Zhou Y, Muh U, Hanzelka BL, Bartels DJ, Wei Y, Rao BG, Brennan DL, Tigges AM, Swenson L, Kwong AD, Lin C.** 2007. Phenotypic and structural analyses of hepatitis C virus NS3 protease Arg155 variants: sensitivity to telaprevir (VX-950) and interferon alpha. J Biol Chem **282:**22619–22628. http://dx.doi.org/10.1074/jbc.M610207200.
37. **Jensen DM, Marcellin P, Freilich B, Andreone P, Di Bisceglie A, Brandao-Mello CE, Reddy KR, Craxi A, Martin AO, Teuber G, Messinger D, Thommes JA, Tietz A.** 2009. Re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha2b: a randomized trial. Ann Intern Med **150:**528–540. http://dx.doi.org/10.7326/0003-4819-150-8-200904210-00007.
38. **Maimone S, Musolino C, Squadrito G, Raffa G, Pollicino T, Raimondo G.** 2013. NS3 genetic variability in HCV genotype-1b isolates from liver specimens and blood samples of treatment-naive patients with chronic hepatitis C. Antivir Ther **18:**131–134. http://dx.doi.org/10.3851/IMP2326.
39. **Di Liberto G, Roque-Afonso AM, Kara R, Ducoulombier D, Fallot G, Samuel D, Feray C.** 2006. Clinical and therapeutic implications of hepatitis C virus compartmentalization. Gastroenterology **131:**76–84. http://dx.doi.org/10.1053/j.gastro.2006.04.016.
40. **Roque-Afonso AM, Ducoulombier D, Di Liberto G, Kara R, Gigou M, Dussaix E, Samuel D, Feray C.** 2005. Compartmentalization of hepatitis C virus genotypes between plasma and peripheral blood mononuclear cells. J Virol **79:**6349–6357. http://dx.doi.org/10.1128/JVI.79.10.6349-6357.2005.
41. **Vera-Otarola J, Barria MI, Leon U, Marsac D, Carvallo P, Soza A, Lopez-Lastra M.** 2009. Hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cells of treatment naive chronically infected patients. J Viral Hepat **16:**633–643. http://dx.doi.org/10.1111/j.1365-2893.2009.01112.x.
42. **European Association for the Study of Liver.** 2014. EASL recommendations on treatment of hepatitis C 2014. http://www.easl.eu/_newsroom/latest-news/easl-recommendations-on-treatment-of-hepatitis-c-2014.