



Inter and intra-host variability of hepatitis C virus genotype 1a hypervariable envelope coding domains followed for a 4–11 year of human immunodeficiency virus coinfection and highly active antiretroviral therapy

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ABSTRACT

The evolution of hepatitis C virus (HCV) quasispecies in patients with HIV-1 coinfection is not fully understood. The HCV-1a quasispecies heterogeneity was analyzed at inter and intra-host levels along 7.6 years in 21 coinfecting patients that showed different virological and immunological responses to highly active antiretroviral therapy (HAART). Two to nine serial samples were subjected to direct and clonal sequence analyses of the envelope glycoprotein 2 (E2) gene. E2-based phylogenies, intra-host HCV evolution and evolutionary rates, as well as dynamics of the quasispecies heterogeneity parameters were evaluated. Bayesian coalescent phylogenies indicated complex evolutionary histories, revealing some viral lineages that persisted along the follow up and others that were detectable at a single or some sampling times, suggesting the occurrence of emergence–extinction cycles. HCV quasispecies underwent very rapid evolution in HAART-treated patients ($\sim 3.1 \times 10^{-2}$ sub/site/year) following the recovery of the host immunocompetence irrespectively of the virological response to HAART.

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Introduction

About 30% of HIV-1-infected individuals in the Western world are coinfecting with hepatitis C virus (HCV) (Potter et al., 2010; Sherman et al., 2002). HIV-related immune suppression or immune dysregulation is associated with accelerated liver disease in individuals with HIV–HCV coinfection (Connoy et al., 2011; de Ledinghen et al., 2008; Verma et al., 2006). Accordingly, patients on highly active antiretroviral therapy (HAART) display slower progression of HCV disease and reduced HCV-related mortality rates (Pascual-Pareja et al., 2009).

At intra-host level, HCV exists as quasispecies defined by several closely related yet genetically distinct populations mainly because

of low viral RNA polymerase fidelity (Bittar et al., 2013; Caraballo Cortes et al., 2013). The mechanisms driving HCV quasispecies diversification are not completely understood. However, humoral pressure on the envelope gene hypervariable region (E2–HVR) seems to be involved in driving quasispecies evolution (Farci, 2011; Jackowiak et al., 2012; Mondelli et al., 1999). This genomic region includes three domains namely the hypervariable region 1 (HVR1), which is located at the N-terminus of E2 (Weiner et al., 1991), the second hypervariable cluster (HVR2) (Kato et al., 1992a, 1992b), and the third one (HVR3), which is positioned between HVR1 and HVR2 (Troesch et al., 2006). In vitro experiments have characterized three independent functional microdomains in HVR1 that play several critical roles for HCV cell entry (Guan et al., 2012).

The clinical relevance of the E2 genetic variation is also uncertain, but decreased variability in HCV quasispecies over time has been associated with more advanced liver disease (Chayama and Hayes, 2011; Gretch et al., 1996; Lopez-Labrador et al., 2006). Certain immunosuppressed states are associated with reduced quasispecies heterogeneity (Feliu et al., 2004; Gray et al., 2012; Kumar et al., 1994; Schramm et al., 2008). However, studies of HCV/HIV-coinfecting subjects have reported both increased

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(Sherman et al., 1996) and decreased (Shuhart et al., 2006) quasispecies heterogeneity, compared to subjects with HCV infection alone. Similarly, HCV exhibits greater quasispecies variability according to the magnitude of the immune pressure, as reflected by HIV-infected individuals with higher CD4 cell counts (Neau et al., 2003; Solmone et al., 2006) or delayed HIV disease progression (Lopez-Labrador et al., 2007). The effect of highly active antiretroviral therapy (HAART) in HCV variability over time in coinfecting subjects is still controversial.

Viral population dynamics can be inferred by a phylogenies combined with demographic and coalescence models (Pybus et al., 2000). This approach, in combination with new and sensitive methods for detecting selection pressures at the codon level (Kosakovsky Pond and Frost, 2005), may better reveal the evolutionary forces driving the viral heterogeneity in a single host during the infection.

We investigated the long-term evolution of HCV before and during HAART therapy in 21 HIV-coinfecting patients to determine the effect of HAART-responsiveness on viral dynamics. A longitudinal sampling strategy, spanning between 4 and 11 years previous and during antiretroviral therapy, was combined with phylogenetic methods to define the evolutionary relationships in the HCV population and the role played by both HIV-replication suppression and immunocompetence recovery in driving the long term evolution of HCV quasispecies.

Results

Characterization of the study group

The study population involved 14 males and 7 females; mean \pm SD age was 36.27 ± 6.6 years old. The length of the longitudinal sampling analysis (mean \pm SD) was similar between HAART responders (7.1 ± 1.9 years), and HAART non-responders (8.1 ± 1.6 years). Among all patients, the median (IQR) CD4 T-cell count was 397 (17–1384) cells/ μ L. Plasma HCV RNA was persistently detected in all patients from the baseline throughout the study, with a median (IQR) level of 5.85 (5.05–6.73) log IU/mL. These levels were similar before and during HAART therapy, as well as between therapy responders and non-responders. At baseline, the median (IQR) HIV viral load among all patients was 4.6 (3.9–5.4) log copies/mL and no differences were observed between group A (HAART-responders) and B (HAART non-responders) patients. Immunological restoration, which was defined as CD4 T-cell count increase more than 1.5 times from baseline to the

last sampling time under HAART (Bernini et al., 2011), was observed in both groups of patients, although it was more pronounced among HAART virological non-responders (group B) (3.3-fold vs. 1.9-fold increase from baseline, respectively; Table 1).

Analyses based on direct E2 nucleotide sequences

Phylogenetic analysis of the 102 E2 master sequences obtained from all sampling times confirmed that all these sequences belonged to genotype 1a. Furthermore, this analysis allowed the recovery of several monophyletic sequence groups that were consistent with the patient source, showing that they were more closely related to each other than to other patient isolates, and indicating the absence of cross-contamination (Fig. 1). No recombinant sequences were found after the GARD genetic algorithm analysis.

The comparison of the 102 partial E2 sequences (aa 364–554) obtained from the 21 patients at different time-points showed that amino acid mutations were located mainly at HVR1, and to a lesser extent at HVR3 (Fig. 2; Supplementary Table 1). The three functional microdomains of HVR1 showed dissimilar amino acid patterns. All patients exhibited amino acid variations at the second microdomain, while the presence of changes in the first and third microdomains was less prevalent. The three neutralization epitopes, which involve both linear and conformational protein structures, showed many amino acid substitutions. These changes appeared frequently at well defined positions, i.e. I414V, E431A/D, L438V/I, A531E/S/D, D533A/E. However, their presence did not appear to be related with neither HCV plasma viral load nor CD4 T-cell counts. All N-glycosylation sites were conserved. In addition, by using the sequence logo representation, we compared the relative amino acid positional frequencies within the E2 set of proteins for each group of patients according to HAART-response. It was primarily used to identify the highly conserved positions in the E2 amino acid sequences (e.g. E384; T388; W417; C432). Likewise, the comparison between baseline and latest sampling time sequence logos showed the similarities (e.g. Cysteine residues at positions 432, 439) and the differences (e.g. amino acid relative frequencies at positions from 390 to 405) between the two types of sequences, which were clearly visible in the sequence logos (Fig. 2).

When comparing the mean HCV genetic distance, complexity and immune pressure (measured as dN/dS ratio) between the two groups of patients, values were significantly higher in group B (HAART virological non-responders) than in group A during the follow-up (Table 2).

Table 1
Demographic, immunologic, and virologic characteristics of the patients studied.

Characteristic	HAART	Group A (n=12) HAART-responders [Pat1, 2, 5, 8, 13, 14, 17, 20, 21, 26, 27, 28]	Group B (n=9) HAART-non-responders [Pat3, 6, 7, 9, 18, 23,24, 25,29]	P*
Age, median (IQR)		42 (39.5–46.7)	43 (40.5–47.7)	NS
Sex (male/female)		8/4	6/3	NS
CD4+ T-cell count, median (IQR), cells/mm ³	Pre	207 (111–276)	154 (91–248)	NS
	Intra	386 (207–583)	509 (374–740)	
HCV RNA, median (IQR), log IU/mL ^a	Pre	5.8 (5.5–6.0)	4.4 (3.2–4.9)	NS
	Intra	5.8 (5.6–5.9)	4.92 (3.6–5.0)	
HIV RNA, median (IQR), log copies/mL ^b	Pre	4.5 (3.9–5.1)	5.0 (4.1–5.4)	NS
	Intra	ND	3.5 (3.0–4.1)	

ND: no detectable.

^a Bayer VERSANT[®] HCV RNA 3.0 Assay, range of 615–7,690,000 IU/mL).

^b VERSANT HIV-1 RNA version 3.0 bDNA Assay, Siemens Diagnostics range of 50–500,000 copies/mL.

* Student t test, NS: not significant.

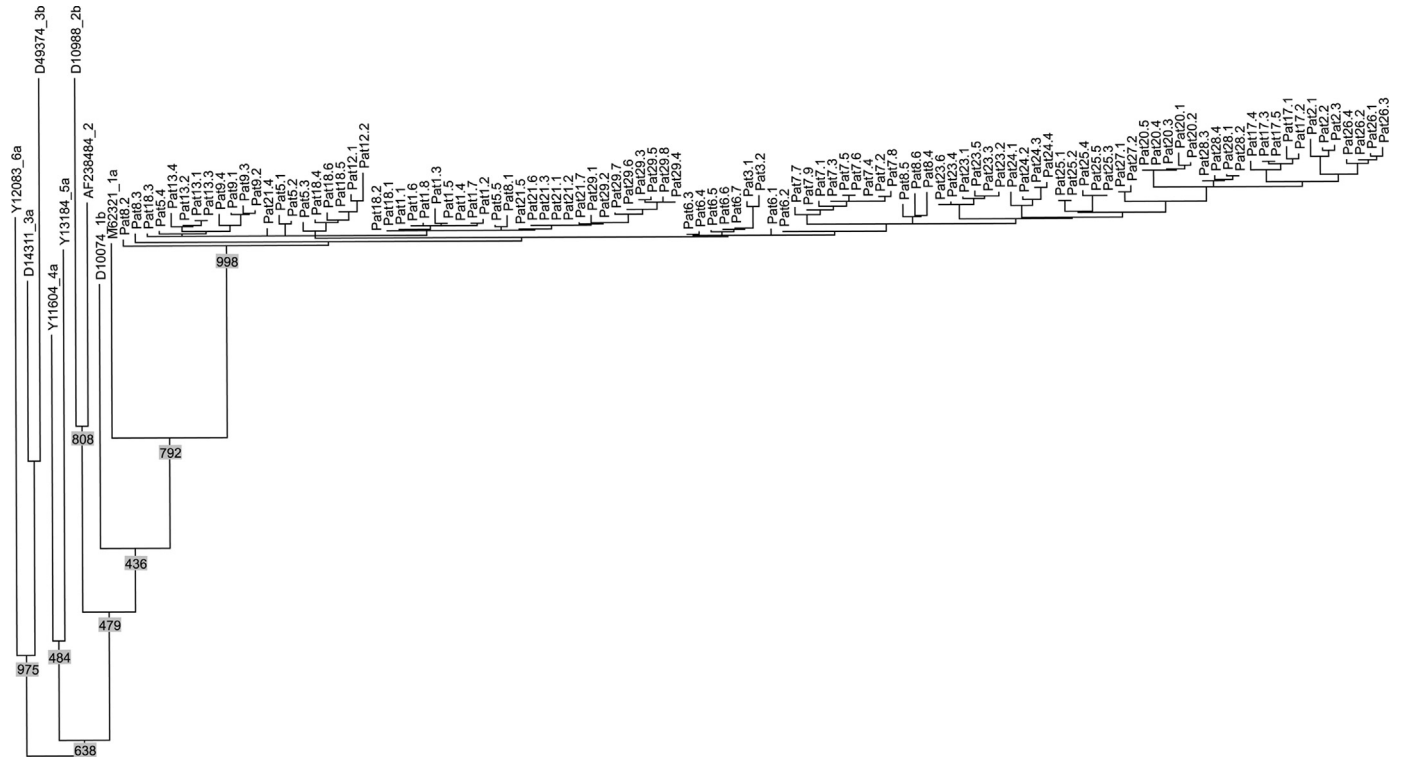


Fig. 1. Maximum likelihood tree of the 102 HCV E2 characterized from 21 HIV coinfecting patients during the 7.6 years longitudinal study. It was obtained by PhyML3.0 using GTR+ Γ +I as a model of nucleotide substitution as recommended by ModelTest 3.07 (Akaike Information Criterion). The sequences from each patient are numbered consecutively according to their sampling time. Reference sequences were named after their GenBank accession number and genotype/subtype. The numbers below the branches represent bootstrap support values (over 1000 pseudoreplicates).

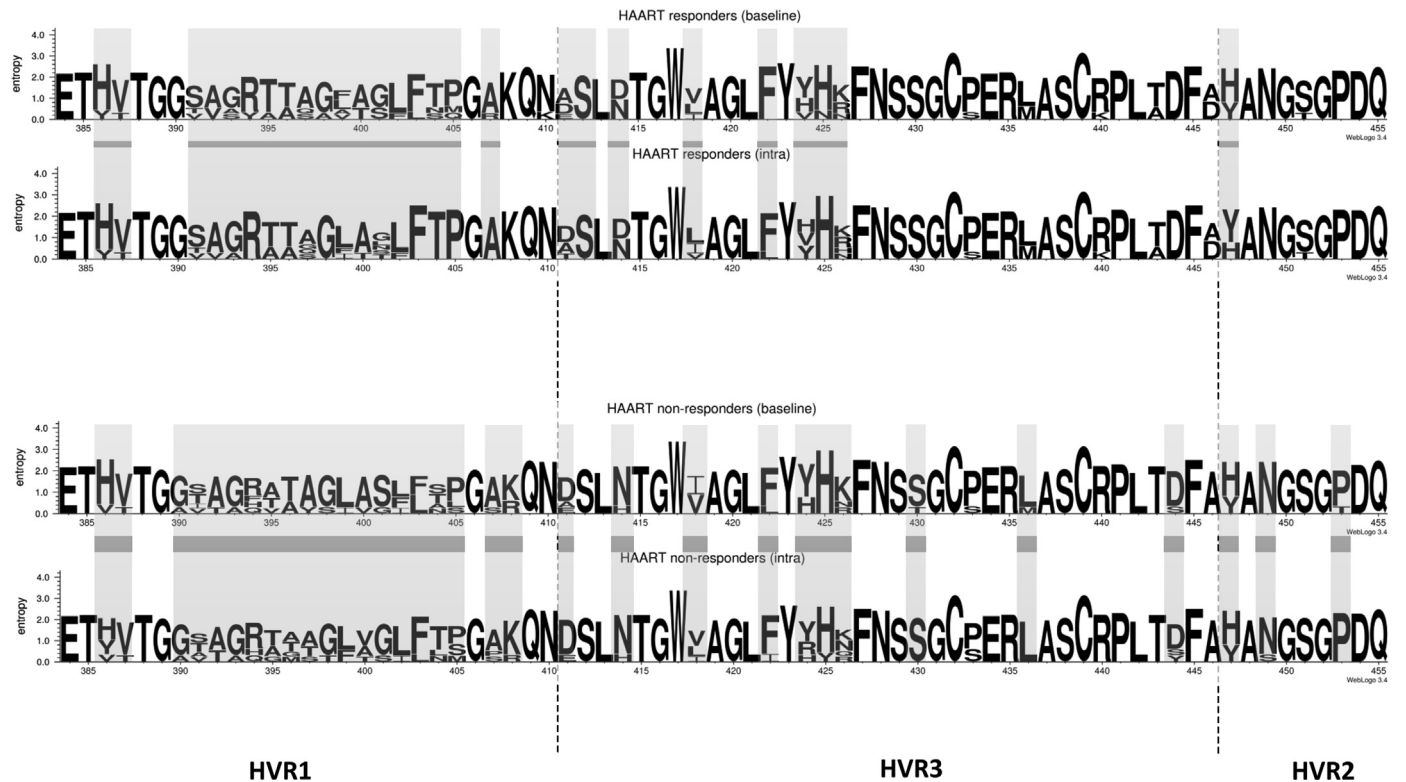


Fig. 2. Amino acid substitution patterns of hepatitis C virus (HCV) quasispecies studied in hypervariable regions of E2 (384–455). Four sequence logo (<http://weblogo.berkeley.edu>) representations based on sequence alignments from HAART-responders (upper paired logos) and non-responders (lower paired logos) are shown. For each condition, data were obtained by analyzing direct E2 amino acid sequences obtained before HAART initiation (“Baseline”) and at the latest sampling point during therapy (“Intra”). Consensus amino acid sequences are displayed on top of the graph, and the rest of amino acids recorded in the input sequence alignment appear below. The height of each letter indicates the relative frequency of the corresponding amino acid residue at each position. The overall height of the stack indicates sequence conservation, in bits. Amino acid positions exhibiting the highest levels of variation are shaded.

Table 2
HCV quasispecies heterogeneity analysis of paired sequence data.

Characteristic	Group A	Group B	p*
Diversity (mean genetic distance \pm SD)	0.025 (\pm 0.004)	0.045 (\pm 0.005)	0.02
Complexity (normalized Shannon' entropy \pm SD)	0.75 (\pm 0.05)	0.88 (\pm 0.03)	0.03
Duration of follow-up in months, median (IQR)	84 [48–132]	102 [60–120]	NS
dN/dS ratio, mean \pm SD	0.40 \pm 0.04	0.74 \pm 0.07	0.001

* Sequence results compared using the Student *t*-test or, the Mann–Whitney test.

Table 3
Sites under positive and negative selection previous and intra-HAART, dN/dS, complexity, diversity.

Patient/sampling time according HAART	Number of sites under positive selection (aa sites ^a)	Number of sites under negative selection	dN/dS	Complexity (as normalized Shannon entropy) ^b	Diversity (as mean genetic distance) ^b (mean \pm SD)	CD4 T cell count ^b (cell/mm ³)
1 Pre	0	5	0.35	0.899	0.022 \pm 0.013	307
1 Intra	2 (397 ; 476)	23	0.32	0.845	0.032 \pm 0.023	202 [17–538]
2 Intra	0	2	0.41	0.817	0.006 \pm 0.006	282 [152–427]
5 Pre	0	0	0.32	0.403	0.0028 \pm 0.0041	330
5 Intra	3 (397 ; 476; 529)	18	0.49	0.980	0.030 \pm 0.028	971 [720–1069]
6 Pre	0	7	0.62	1.000	0.075 \pm 0.079	223
6 Intra	13 (380; 397 ; 400 ; 405 ; 406 ; 425;439;447; 476; 481; 494; 501; 536)	29	0.63	0.989	0.053 \pm 0.027	278 [221–396]
18 Pre	1 (405)	4	0.71	1.000	0.045 \pm 0.024	200
18 Intra	7 (392 ; 397 ; 409 ; 499;502;529;550)	16	0.73	0.865	0.041 \pm 0.025	596 [306–740]
23 Intra	5 (392 ;442;506;522;539)	13	0.77	0.903	0.034 \pm 0.025	243 [154–332]

HCV E2-amino acid numbering is relative to the H77 reference sequence. HAART responding patients are in shadow rows.

^a Bold, underlined, and italics indicate amino acid positions within HVR1 (aa 382–410), HVR2 (aa 474–482), and HVR3 (aa 431–466), respectively.

^b Intra-HAART values were calculated as mean (for diversity and complexity) or median [IQR] (for CD4 T cell count) of the values during the follow-up measured under HAART.

Analyses based on E2 nucleotide sequences obtained by cloning

Regardless of the efficacy of HAART in terms of either immunological or virological response, HCV quasispecies showed similar patterns of evolution as indicated by the coalescent analysis. Dated maximum clade credibility trees showed that viral lineages could be detected along varying time periods (Fig. 4 and Supplementary Fig. 1). Fig. 4 displays the chronogram corresponding to patient 6, including a variety of such situations. The clade in red corresponds to a lineage that could be detected at all sampling times; i.e. persisted along the entire follow up. In contrast with the lineage in green, in Fig. 4 it was detected just at the beginning of the follow up. Intermediate situations were observed as well, such as the example the clade in blue in Fig. 4, which seems to have emerged around 95 months before the last sampling time and gone extinct around 45 months later. The viral populations from patients 1, 2, 5, 18 and 23 showed equivalent evolutionary patterns (Supplementary Fig. 1). This process was also observed at amino acid levels, where, at each time, at least two sequences with many amino acid differences at HVR-1 co-existed in the same sample, except for a HAART-experienced and responder patient (Pat2) who exhibited a homogenous viral population with most of the clones corresponding to almost identical HVR-1 amino acid sequences. However, taking into account that this patient was followed-up for a shorter period of time in relation to the other ones (4 vs. \geq 7 years; Supplementary Table 1) but with annual sampling-time frequency, the emergence of divergent sequences could not be ruled-out.

In agreement with this scenario of complex turnover of viral variants, the substitution rates behind this process was relatively high for both HAART-responder and non-responder patients (2.9 and 3.4×10^{-2} sub/site/year, respectively; see also Fig. 4 and Supplementary Fig. 1).

Taking into account the high substitution rates values inferred by coalescent analysis, quasispecies heterogeneity-related parameters were also evaluated for these six patients. Based on the analysis of a total of 778 HCV-E2 cloned sequences (Pat1:150; Pat2: 41; Pat5:87; Pat6:187; Pat18:152; Pat23:131), quasispecies diversity and complexity were obtained. The mean (\pm SD) genetic diversity ranged from 0.0028 (\pm 0.0041) to 0.075 (\pm 0.079) subs/site while the complexity varied from 0.403 to 1.00. These two parameters did not show correlation with intra-host CD4 T cell count variation (Table 3) but higher values were obtained more frequently early after HAART initiation when the CD4 T cell counts ongoing to increase among the four patients able to be studied previous and during therapy (Table 4).

During HAART, four patients who experienced a more prominent immune recovery (measured as increase in the CD4 T cell count) showed a significantly higher ($p=0.009$) selective pressure variation (measured as mean dN/dS ratio). They were the three HAART non-responders (Pat6, 18, and 23) and one HAART responder (Pat5) who also verified a 3.2 fold change in the absolute CD4 T cell count during the follow-up (Table 3; Supplementary Table 1). Only two patients (Pat5, Pat18) exhibited a higher HCV viremia level accompanying the CD4 T cell count increase (Fig. 3a).

As mentioned, after comparing the site-specific dN/dS rates by different evolutionary models (SLAC and FEL), a positive selection pressure was more evident among patients with higher immune restoration after HAART initiation, however the mean dN/dS ratio was < 1 , supporting the concept of positive selection at specific positions (Table 3). The most frequently positively selected codons were 397 and 476. In contrast, at baseline only one of them (Pat18) showed a single site (codon 405) under positive selection. Altogether, 22 different sites across the 6 patients studied displayed evidence of having been subjected to positive selection during the follow-up.

Table 4
Intra-host variations in the hepatitis C virus quasispecies heterogeneity-related parameters during the follow-up.

Pat1								
Time (months)	0	12	23	35	44	50	65	77
CD4 T cell count (cells/mL)	307	17	86	161	202	332	538	400
Complexity (as normalized Shannon entropy)	0.899	0.917	0.832	0.810	0.845	0.809	0.899	0.802
dN/dS	0.35	0.6	0.3	0.21	0.29	0.32	0.25	0.32
Diversity as mean genetic distance (mean ± SD)	0.022 ± 0.013	0.04 ± 0.038	0.02 ± 0.012	0.031 ± 0.023	0.042 ± 0.025	0.039 ± 0.03	0.021 ± 0.012	0.031 ± 0.023
Pat5								
Time (months)	0	12	24	48	57	71		
CD4 T cell count (cells/mL)	330	350	560	720	971	1069		
Complexity (as normalized Shannon entropy)	0.403	0.996	0.991	0.975	0.942	0.992		
dN/dS	0.32	0.8	0.69	0.45	0.15	0.31		
Diversity as mean genetic distance (mean ± SD)	0.003 ± 0.004	0.053 ± 0.024	0.05 ± 0.022	0.031 ± 0.026	0.017 ± 0.023	0.02 ± 0.022		
Pat6								
Time (months)	–12	0	7	18	20	37	79	91
CD4 T cell count (cells/mL)	NA	142	NA	NA	NA	752	952	1055
Complexity (as normalized Shannon entropy)	1.000	1.000	1.000	1.000	0.981	0.982	1.000	0.981
dN/dS	0.63	0.8	0.52	0.47	0.79	0.58	0.62	0.65
Diversity as mean genetic distance (mean ± SD)	0.026 ± 0.014	0.081 ± 0.033	0.055 ± 0.024	0.053 ± 0.029	0.077 ± 0.044	0.075 ± 0.079	0.035 ± 0.02	0.041 ± 0.024
Pat18								
Time (months)	0	24	48	63	73	87	108	
CD4 T cell count (cells/mL)	200	306	463	740	619	573	651	
Complexity (as normalized Shannon entropy)	1.000	1.000	0.896	0.874	0.885	0.852	0.702	
dN/dS	0.71	1.42	0.91	0.56	0.59	0.63	0.29	
Diversity as mean genetic distance (mean ± SD)	0.016 ± 0.01	0.068 ± 0.031	0.068 ± 0.038	0.059 ± 0.035	0.011 ± 0.008	0.045 ± 0.024	0.026 ± 0.027	

Six out of these 22 sites were located at the HVR1 region of E2, while two were at HVR2 and two at HVR3 (Table 3). These observations were also supported by sequence logo representations (Fig. 3).

Discussion

The long-term consequences of HAART-related changes in HCV quasispecies variability are not known among HIV coinfecting patients. Others have studied the HCV evolution during short term periods of HAART, and most of them were based on phenetic approaches (Babik and Holodniy, 2003; Bernini et al., 2011; Blackard et al., 2004; Shuhart et al., 2006; Solmone et al., 2006). Although their findings are partially conflicting, two main consistencies were found: a relatively low level of HCV heterogeneity before HAART and an increase after treatment initiation. In the present study we have analyzed the HCV quasispecies dynamics under HAART pressure, over a period ranged from 4 to 11 years, focusing on the E2 genomic region as the most variable due to its interaction with the immune system (Guan et al., 2012). The antiretroviral therapy could be considered a factor able to modify the host-HCV interaction resulting in altered HCV-E2 sequence evolution. For this goal, our study combines phenetic and phylogenetic approaches following a longitudinal sampling strategy. This study was conducted with a homogenous population, including only HCV genotype 1a/HIV coinfecting patients divided in two groups according to their virological response to HAART therapy. None of these subjects had undergone any prior interferon-based anti-HCV treatment. These subjects were analyzed for changes in immunological, virological and HCV quasispecies evolutionary parameters.

Regardless of the antiretroviral therapy conducted to quantify the immune restoration in HAART-responders and non-responders, it was more pronounced among the latter group. Antiretroviral therapy was prescribed to control HIV replication and to restore immune function. Surprisingly, in our study we observed a more pronounced immune restoration among non-responders than

responders to HAART. However, it is relevant to consider that our protocol definition of HAART response was very strict, and most of the patients included in the non-response group experienced blips during the follow up but finally achieved viral suppression. Many factors influence CD4 cell restoration despite optimal treatment with fully suppressed viral replication (Aiuti and Mezzaroma, 2006) including the hepatitis C coinfection (Antonucci et al., 2005; De Luca et al., 2002; Greub et al., 2000). Moreover, it is necessary to consider that the CD4 cell count in a given patient at any time is the result of production, destruction, and traffic between blood and lymphatic tissue. Thus, taking into account that host-dependent factors could influence the immunological response including the cell turnover and distribution, and the low number of patients studied may explain this paradoxical observation.

Among our patients, the overall HCV quasispecies genetic complexity and diversity as well as the selective pressure were significantly higher. As it was reported, early after HAART initiation several patients exhibited greater recovery of the immune response (Corbeau and Reynes, 2011). Coincidentally, we observed that more pronounced changes in the HCV evolution occurred during this period. However it was not always accompanied by a lower HAART-mediated HIV suppression and later, when a new HCV-host equilibrium was reached, the viral evolutionary dynamic was similar to the pre-HAART stage. However, this observation came from a small number of patients and further research is needed before definite conclusions can be drawn. Furthermore, these findings could be associated with HAART-related changes in T cell functions and consequently with the magnitude of the immune pressure, but not just with changes in T cell number (Shuhart et al., 2006). Another limitation of the present study is the general lack of functional immunologic data considering that we used increased CD4 cell counts as a surrogate marker of immune reconstitution. Even though, in the vast majority of this study population, the long term HAART did not appear to influence HCV replication measured as plasma viral load which was steadily high. These results are in contradiction with those

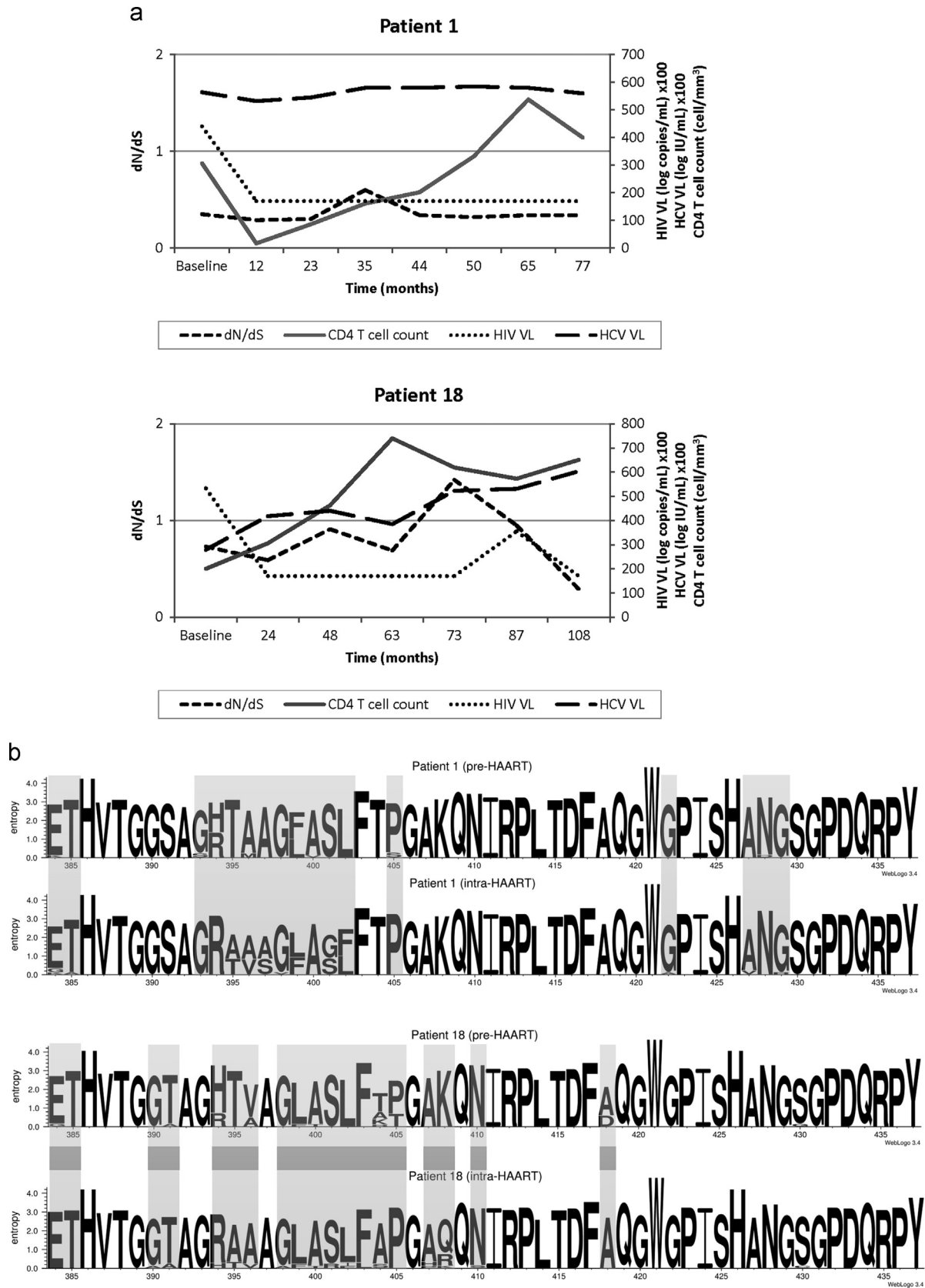


Fig. 3. (a) The dN/dS ratio (short dashed line), CD4 T cell count (continuous line; cell/mm³), HCV plasma viral load (long dashed line, log IU/mL \times 100) and HIV plasma viral load (dotted line, log copies/mL \times 100) dynamics during the study follow-up (expressed in months) for patient 1 (upper) and patient 18 (lower). (b) Four sequence logos are presented for partial E2 amino acid sequences (amino acid 384–437) obtained at both, pre and intra-HAART sampling times from these two patients. At intra-patient level, residues with significant differences in their frequency between the two datasets are shaded.

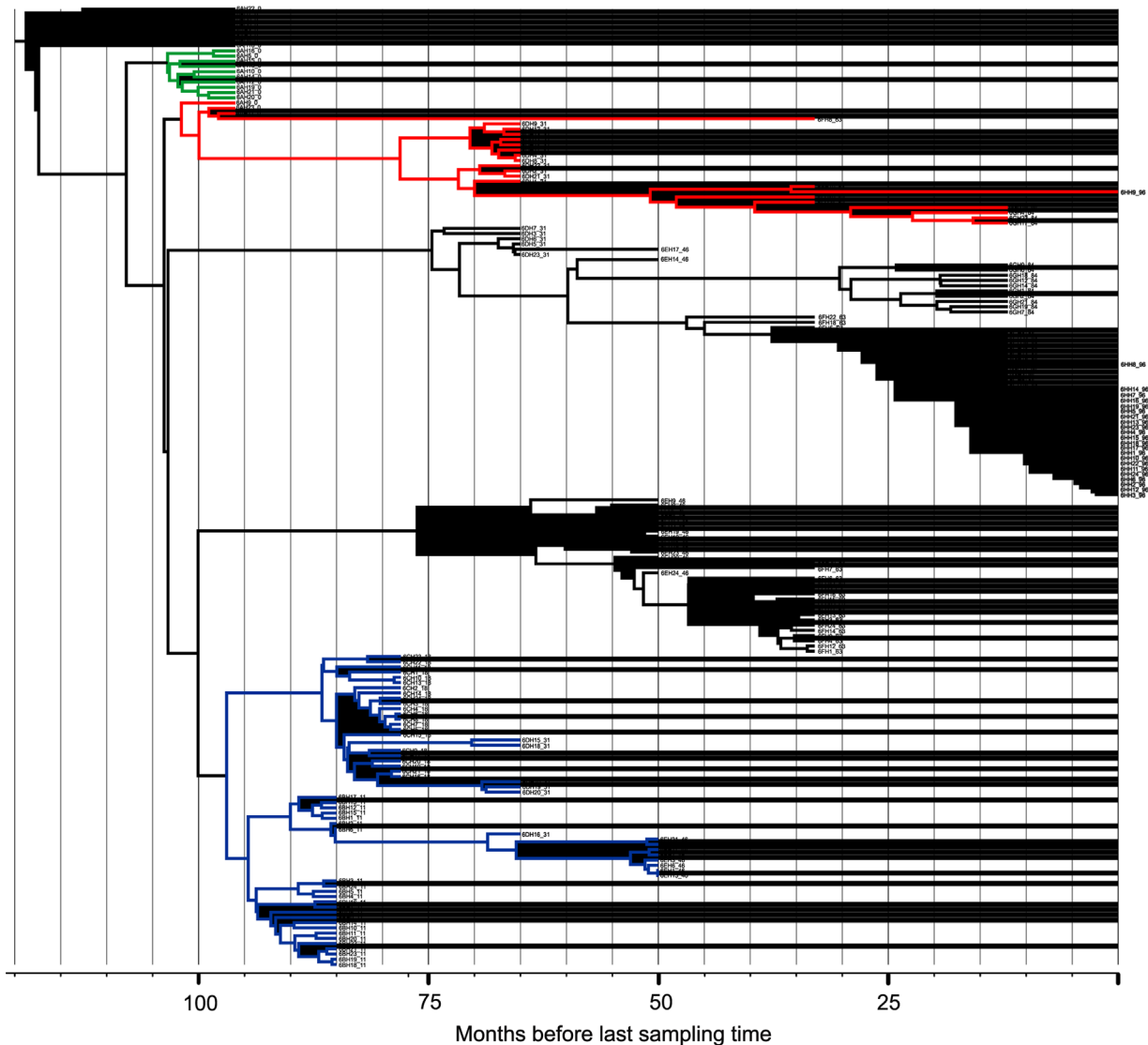


Fig. 4. Bayesian dated tree obtained from patient 6 sequences. The colors applied to some clades indicate lineages that persisted along the entire follow up (red), were observed at some sampling times (blue) or were detected just in one moment along the study (green).

published elsewhere (Bernini et al., 2011; Chung et al., 2002) that reported significant lower HCV viral load levels at baseline.

Hence, the vigor of the immune response may drive both the degree of HCV complexity and diversity during an ongoing process of continuous selection and the adaptation of the virus population in HAART virological non-responders. In contrast, the quasispecies dynamics in HAART virological responders seemed to be limited, probably by the need to maintain the defined E2 properties. Therefore, the HCV genotype 1a evolving in different HIV coinfecting patients, each with their genetic background, presents a different evolutionary pattern apparently modulated by the adaptive immune response which is modified by the antiretroviral therapy.

To better understand the HCV evolutionary process, a phylogenetic approach was performed using cloned E2 sequences in order to analyze the dynamics of viral variants at intra-host level over time. This approach was carried out by applying the coalescent theory to dated phylogenies that allowed estimating the rate of evolution of the E2 gene and reconstructing its trees on a real time scale using molecular clock models. Complex interactions among viral variants that coexist in each patient were recognized after the analysis of the time-annotated trees. In general, and

irrespective of the HAART response, our analysis showed the persistence of some variants along the infection, but also that new variants were generated continuously in HIV-coinfecting patients on antiretroviral therapy. These new variants showed either ulterior increase and dominance, or decrease and extinction. Such complex evolutionary scenarios could represent the impact of the host background on virus evolution, including HAART history and response but in some cases also could correspond to aleatory factors such as genetic drift. Most of the study population showed an emerging continuous process of new mutants, but in a HAART-experienced and responder patient, the HCV-1a probably reached an equilibrium point where immune evasion was not necessary, as most of the sequences cloned presented identical HVR-1 amino acid sequences. Hence, there is evidence that the HCV quasispecies could be related with increased HCV replication in HAART-treated individuals, though published studies have provided conflicting results (Caudai et al., 2005; Chung et al., 2002; Sherman et al., 2005; Solmone et al., 2006). The coalescent analysis showed a very rapid intra-host evolutionary rate regardless of the immunological response to HAART ($\sim 3.1 \times 10^{-2}$ sub/site/year). It appears higher than controls without HIV-coinfection (Gray et al., 2011). However,

it is well known that the reported estimation of HCV molecular evolution has been highly variable being inappropriate to compare directly due to the different methodologies, non-homogeneous populations and genome regions employed.

Based on an intra-host analysis, the HCV quasispecies evolution showed either complete or partial qualitative shifts in their composition after HAART-induced restoration of the immune pressure and HIV-suppression. This oscillatory variations of the HCV quasispecies diversity during the course of infection was already reported among HCV monoinfected (Ramachandran et al., 2011) and HCV–HIV coinfecting subjects (Babik and Holodniy, 2003).

Despite the limited number of patients included in this study, only a small median increase was observed without a significant association with either virological or immunological response or changes in HCV heterogeneity parameters. After measuring the HCV quasispecies diversity and complexity over time, we found no significant differences during the follow-up with various levels of CD4+ T cell counts, HAART initiation, and dissimilar therapy-mediated HIV suppression. Disparate findings were reported in other longitudinal studies either with similar (Babik and Holodniy, 2003; Solmone et al., 2006) or contrasting results (Blackard et al., 2004). However, these studies involved substantially shorter times of follow-up, and several similarities in their findings appeared as “snapshots” during our longitudinal observations. For instance, while the intra-host immune selection pressure measured as mean dN/dS ratio during the follow-up was < 1 for HVR-1, indicating a neutral selection pressure, the dynamics of this dN/dS ratio showed events of positive selection pressures acting on this region after HAART initiation for some individuals. The majority of the sites under positive selection identified here were located in the HVR1. This region contains epitopes targeted by neutralizing antibodies and that may also act as epitopes for helper and cytotoxic T cells (Wahid and Dubuisson, 2013; Wang et al., 2011). Several HVR1 amino acid variations were uniformly found in all HCV sequences on the second microdomain intended to function as immunological decoys to shield more conserved neutralization epitopes; instead, the other two microdomains were more randomly distributed.

Finally, our work demonstrates that HCV quasispecies undergo very rapid evolution in HAART-treated patients thus increasing their complexity and diversity following the recovery of the host immunocompetence irrespectively of the virological response to HAART. However, a much larger study population involving diverse hepatitis outcomes would be needed in order to find out whether the viral evolutionary process, as a reflection of the host immune response, really drives the outcome of chronic liver disease in persistent HCV/HIV coinfection.

Material and methods

Selection of patients

Twenty-one patients were chosen retrospectively from the database of patients followed up at the Instituto de Investigaciones Biomédicas en Retrovirus y Sida, Buenos Aires. These patients were previously identified as seropositive for both HIV and HCV, infected with HCV-genotype (Gt) 1a, and with persistently detectable HCV RNA. None of them were treated with anti-HCV interferon plus ribavirin-based therapy during the entire follow-up (Quarleri et al., 2007). They could be differentiated based on HAART virological response during the entire follow-up (Raymond et al., 2014): (A) subjects achieving sustained optimal virological responses to HAART with undetectable HIV-1 RNA (HAART responders; $n=12$); and (B) subjects exhibiting at least one episode of detectable HIV-1 RNA (HAART-non-responders; $n=9$) (Table 1). Samples were collected annually during the course of routine clinical follow-up and

stored at -80°C until use. For each patient, we tested plasma samples collected longitudinally across at least 4 to 11 years, beginning at the earliest time-point before HAART initiation. The protocol used was evaluated and approved according to the ethical guidelines of the 1975 Declaration of Helsinki by the Fundación Huésped Ethics Board Committee.

Amplification and direct sequencing of HCV-E2 gene

Viral RNA was isolated from plasma samples with Trizol LS following the manufacturer instructions (GIBCO, Life Technologies, USA). The HCV-E2 encoding sequence was amplified using primers EF1 (5'CACTGGACCACCCAGGATTGCA3', position 1171–1196) and E2R1 (5'TGAACAGTACCGGGTTCACCAA 3', position 1947–1967), and then primers E2F2 (5'CACTGGGGTGTCTCGTGGG 3', position 1356–1375) and E2R2 (5'TGGATGAACAGTACCGGGTTC3', position 2037–2058) in a nested reverse transcriptase polymerase chain reaction (RT-PCR) generating a 660 bp amplicon. The RT program included 60 min at 50°C followed by enzyme deactivation at 85°C for 5 min. The first PCR program included 35 cycles each of 30 s (s) at 94°C , 45 s at 54°C and 2 min at 72°C using a Taq DNA polymerase (Invitrogen, Argentina), followed by a final extension at 72°C for 10 min. The PCR product was used in the nested PCR and the program included 35 cycles of 30 s at 94°C , 30 s at 58°C , 1 min at 72°C and a final extension of 72°C for 10 min. PCR products were resolved by electrophoresis on 1% agarose gels, and the nested-PCR products were directly subjected to double-stranded sequence analysis with dye-labeled dideoxy terminators with both E2F and E2R primers (ABI PRISM 3100 automated sequencer, Applied Biosystems, Foster City, CA, USA), obtaining the quasispecies master sequence, or most-commonly-observed sequence (Cabot et al., 1997; Jones et al., 2002).

Cloning, plasmid purification and sequencing of HCV-E2 gene

A clone-based analysis was carried out on samples collected at all sampled time-points from six randomly selected individuals including HAART-responders (Pat1, Pat2, Pat5) and HAART-non-responders (Pat6, Pat18, and Pat23). Each purified envelope fragment was first ligated into the pGEM-T plasmid (Easy vector, Promega, Wisconsin, USA) according to the user manual and the generated construct (pGEM-T-E2) was used to transform DH5 α *Escherichia coli* competent cells. Approximately 20 clones were selected at each time point from each patient for sequence analysis. White colonies were confirmed by colony PCR, and PCR-positive ones were subjected to small-scale plasmid preparation using the GeneJet plasmid DNA miniprep kit (ThermoFisher Scientific, USA). The purified plasmid was subjected to automated sequencing from both directions using the HCV-E2 sequence specific forward (EF2) and reverse (ER2) primers. The obtained sequences were submitted to GenBank (accession numbers to be given).

Phylogenetic analysis of HCV-E2 nucleotide sequences

The E2 quasispecies master sequences determined by direct sequencing as well as those obtained after molecular cloning from all sampling time points were edited and assembled using Sequencher software v.4.10.1 (Gene Codes Corporation, Ann Arbor, MI USA) and aligned using Mafft program (Katoh et al., 2009). Maximum likelihood phylogenetic trees were performed for E2 nucleotide sequences obtained directly from each patient throughout the longitudinal study. Maximum-likelihood phylogenies were inferred with PhyML (Guindon et al., 2009). A general time reversible (GTR) model of nucleotide evolution and gamma-distributed rate variation among sites were set, with molecular clock not enforced. A total of 1000 bootstrap replicates were

performed to assess the statistical support for the topologies. Trees were visualized and annotated in FigTree v.1.3 and rooted according to the “best root” option in Pathogen v.1.2 (<http://tree.bio.ed.ac.uk/software/pathogen>). For the purposes of HCV subtyping, patients sequences were aligned with nine HCV reference sequences retrieved from the GenBank database (accession numbers in brackets): HCV1a (M62321); HCV1b (D10074); HCV2a (AF238484); HCV2b (D10988); HCV3a (D14311); HCV3b (D49374); HCV4a (Y11604); HCV5a (Y13184); and HCV6a (Y12083).

Evolutionary rate estimation and demographic analysis

Bayesian coalescent analysis was performed with the BEAST program (Drummond and Rambaut, 2007). Six different HCV-E2 sequences datasets were used for Bayesian coalescent analyses. Each of them contained all of the sequences obtained by molecular cloning from each selected patient and time points.

Evolutionary rates were estimated using a relaxed molecular clock model. We compared the constant size demographic model against the Bayesian Skyline Plot (BSP) one using a Bayes factor approach, which favored decisively a constant population size. The obtained Bayes factors were 682, 20, 113, 5, 224 and 75 for the sequences corresponding to patients 1, 2, 5, 6, 18 and 23, respectively. Thus, the model employed to reconstruct trees and calculate evolutionary rate implemented a constant population size. The MCMC chains were run for at least 100 million generations, and sampled every 10,000 steps. Convergence was assessed on the basis of the effective sampling size (ESS) after a 10% burn-in using Tracer software version 1.3 (<http://tree.bio.ed.ac.uk/software/tracer/>). Only ESSs of > 200 were accepted. Uncertainty in the estimates was indicated by 95% HPD intervals. The trees were summarized with a maximum clade credibility tree using the Tree Annotator program included in BEAST after discarding the burnin samples. This tree was then visualized using the program FigTree 1.2 (available at <http://tree.bio.ed.ac.uk/software/figtree/>).

HCV quasispecies heterogeneity and site-specific selection pressure analyses

The amino acid sequences of HCV-E2 protein were analyzed to assess both inter- and intra-patient quasispecies heterogeneity dynamics during the follow-up. Such analysis involved: (i) complexity, measured as normalized Shannon entropy (Pawlotsky et al., 1998); (ii) diversity, as the mean genetic distance of amino acid sequences calculated by PROTDIST using Jones–Taylor–Thornton matrix [<http://caps.ncbs.res.in/iws/protdist.html>]; (iii) the dN/dS ratio (as a surrogate indicator for immune pressure) according to the Nei–Gojobori method with the Jukes–Cantor correction (Nei and Gojobori, 1986) by using the Synonymous Non-synonymous Analysis Program (SNAP v1.1.1)(Korber, 2000) [www.hiv.lanl.gov]; and (iv) positive selection using the single likelihood ancestor counting (SLAC) and the Fixed Effects Likelihood (FEL) methods that enable detection of non-neutral selection. These methods were chosen for their applicability to large (> 50 taxa) datasets and short sequences. The Hyphy software was used for these analyses (<http://www.datamonkey.org/>) (Pond et al., 2005). Only sites that were identified by SLAC ($p \leq 0.05$ significance) were included in the final result (Kosakovsky Pond and Frost, 2005). The presence of recombination events was investigated using GARD, a genetic algorithm implemented in Datamonkey to identify non-recombinant fragments; recombinant sequences were excluded from the subsequent analyses.

E2-amino acid frequency analysis and qualitative mutational analysis

The relative amino acid frequencies at each E2 position were represented by sequence logo. To this aim a web based application

was used (<http://weblogo.berkeley.edu>) that allows distinguishing residue frequency differences between two given datasets (in our case, HCV E2 amino acid sequences at baseline and at the latest sampling time for each group of patients) (Crooks et al., 2004). To further evaluate the importance of the identified E2 amino acid exchanges, an analysis was conducted according to their functional properties. The properties under analysis included the three functional HVR1 microdomains (Guan et al., 2012; Wang et al., 2011).

Statistical analysis

Statistical analysis of quantitative variables was performed using the Student *t* test. Differences between mean genetic distances were assessed using non-parametric Kruskal–Wallis's and Dunn's multiple-comparison tests. Correlations between virological, immunological and quasispecies parameters were evaluated by Spearman's coefficient. A *p* value < 0.05 was considered significant. All the analyses were performed with SPSS version 12.0 (SPSS, Inc., Chicago, IL).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2014.09.016>.

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