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Short Communication

Previous failure of interferon-based therapy does not alter the frequency of HCV NS3 protease or NS5B polymerase inhibitor resistance-associated variants: longitudinal analysis in HCV/HIV co-infected patients

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ABSTRACT

Since 2011, treatment of chronic hepatitis C virus (HCV) includes direct-acting antivirals (DAAs) in addition to pegylated interferon-α (peg-IFN) and ribavirin (RBV). IFN-based treatment induces strong cytotoxic T-lymphocyte activity directed to the protease- and polymerase-derived epitopes. This enhanced immunological pressure could favour the emergence of viral epitope variants able to evade immune surveillance and, when resistance-associated variants (RAVs) are implicated, could also be co-selected as a hitchhiking effect. This study analysed the dynamics of the frequency of protease and polymerase inhibitor RAVs that could affect future HCV treatment in human immunodeficiency virus (HIV) co-infected patients on stable antiretroviral therapy with previous IFN-based treatment failure. HCV genotype 1a RNA was extracted from plasma samples of 18 patients prior to and during (24 h and 4, 12, 24 and 48 weeks) therapy with peg-IFN+RBV. Next-generation sequencing was performed on HCV-RNA populations using NS3 and NS5B PCR-amplified coding regions. Two measures of genetic diversity were used to compare virus populations: average pairwise nucleotide diversity (π) and Tajima's *D* statistic. Several protease and polymerase RAVs were detected in all subjects at very low frequencies (<5%), and in most cases their presence was not constant during follow-up. Only samples from two patients for each region exhibited Q80R/K/L and A421V as highly predominant variants. No significant differences were observed among sampling times for either π or *D* values. In conclusion, previous therapy and failure of peg-IFN+RBV were not associated with an increase in DAA-targeting NS3 or NS5B RAVs that naturally exist in HIV co-infected subjects.

1. Introduction

Due to shared routes of transmission, co-infection with hepatitis C virus (HCV) is common in human immunodeficiency virus (HIV)-infected patients. Approximately 10–15% of HIV-infected individuals worldwide are co-infected with HCV. Until 2011, the standard of care in HCV treatment for HCV/HIV co-infection was pegylated interferon-α (peg-IFN) and ribavirin (RBV) for 24 weeks or 48 weeks aimed at achieving virological clearance. However, only 40% of patients monoinfected with HCV, and 20% of those co-infected with HCV/HIV, achieved this goal. In this context, direct-acting antiviral (DAA) agents are one of the major advances in HCV medical treatment allowing improved rates of sustained virological response. Several DAAs have thus far been approved for use in the USA, Europe and Japan, targeting the viral NS3 protease and NS5B polymerase [1].

Virological failure in patients receiving DAA regimens is associated with the emergence of resistance-associated variants (RAVs) [2–4]. HCV NS5B polymerase misincorporates nucleotides at a rate of 1 per 10 000 bases copied. This low fidelity of replication, in addition to a high replication rate that can result in the production of up to 10^{12} virions per day, results in HCV existing as a diverse collection

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of closely related variants called quasispecies. Hence, according to theoretical considerations, emergent resistant variants represent pre-existing minor quasispecies that become dominant under the selective pressure of drugs [5].

As a result of IFN-enhanced immunological pressure, HCV variants carrying specific NS3- and NS5B-derived epitopes could have been selected in order to avoid the host immune response and consequently some resistant variants could also be co-selected as a hitchhiking effect [6,7].

The aim of the present longitudinal study was to analyse the dynamics of the frequency of protease and polymerase inhibitor RAVs that could affect future HCV treatment in HIV co-infected patients on stable antiretroviral therapy with prior IFN-based treatment failure.

2. Materials and methods

2.1. Patients and samples

This was a retrospective study of 18 HIV patients co-infected with HCV genotype 1a (VERSANT HCV Genotype Assay INNO-LiPA HCV II; Bayer HealthCare, Éragny, France) from a hospital in Buenos Aires (Argentina) treated with peg-IFN+RBV between 2007 and 2009. Samples were store at −80 °C and were obtained from patients not responding to dual therapy. The absence of response to peg-IFN+RBV therapy was defined according to treatment guidelines [8]. Samples were obtained prior to peg-IFN+RBV initiation (baseline) and at 24 h and 4, 12, 24 and 48 weeks during therapy. In some patients, an additional sample was collected 24 weeks after the end of treatment (72 weeks). The protocol was approved by the Ethics Committee of the Huesped Foundation (Buenos Aires, Argentina).

2.2. Nested reverse transcriptase PCR and ultra-deep pyrosequencing (UDPS)

RNA extraction and cDNA synthesis were performed using a QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and Superscript II (Invitrogen, Waltham, MA), respectively, according to the manufacturers' instructions. For the first round of PCR, amplification of the target regions was carried out using specific primers. Subsequent nested reactions were performed using primers NS3-nf (5′-GACAAAAACCARGTGGAGGG-3′, H77c nucleotides 3492–3511) and NS3-nr (5′-CCGGGGACCTCATGGTTGTC-3′, H77 nucleotides 3947–3966) and NS5B-nf (5′-TACGACTTGGAGCTCATAACA-3′, H77 nucleotides 8673–8693) and NS5B-nr (5′-GCGCGGACRCT-CCGGGCCCGG-3′, H77 nucleotides 9107–9127). PCR amplicons from three reactions were pooled for each sample.

UDPS was performed on a Genome Sequencer-FLX (Roche/454 Life Sciences, Penzberg, Germany) according to the manufacturer's instructions. The technical error rate for the UDPS run was estimated by cloning (Easy Vector; Promega, Fitchburg, WI), amplifying and sequencing (ABI PRISM 3100; Applied Biosystems, Foster City, CA) one randomly selected sample for each analysed region. Each UDPS read was aligned to the corresponding plasmid sequence using Mafft (http://mafft.cbrc.jp/alignment/software/) and the number of mismatches was counted.

2.3. Quality control

The data obtained were filtered based on sequence length, base calls quality and frame shifts. Strict parameters were selected to ensure proper filtering of the data. Reads with lengths either shorter than 300 bases or longer than 540 bases were filtered out. Also, reads containing at least one base displaying a base call value ≤10 were discarded, and no sequence with Ns was kept in the processed data set. In addition, an average (mean) quality value ≥30 was required for each read in order to pass the quality control. Finally, low-quality read ends were trimmed out. All of these pre-processing steps were done using PRINSEQ v.0.15 [9].

A limitation of this first pre-processing quality-based analysis is that it is unable to correct errors inherent to the presence of homopolymer regions, i.e. extension (insertions), incomplete extensions (deletions) and carry forward errors (insertions and substitutions), which could have associated high quality values. Thus, the obtained data set from each sample processed by PRINSEQ was aligned using isolate H77 as a reference sequence (GenBank accession no. AF009606), then the insertion/deletion within homopolymer regions were corrected, the carry forward errors were amended and reads with frame shifts were removed.

2.4. Inhibitor resistance-associated variants

NS3 and NS5B nucleotide sequences obtained by UDPS that passed the quality control were analysed to evaluate the presence of RAVs (Supplementary Table S1) as well as their frequencies with time for each patient. In addition, the mean RAV frequency for each position and the proportion of patient showing them were obtained. For these last calculations, if a position for any patients exhibited RAVs with frequencies >20%, they were not considered in order not to distort the analysis of minority variants.

2.5. Viral population diversity

Two measures of genetic diversity were used to compare virus populations, average pairwise nucleotide diversity (π) [10] and Tajima's D statistic [11]. An illustrative sliding window analysis of Tajima's D was done for patients with reads for each sample time between baseline and 24 weeks of treatment (seven patients for NS3 and NS5B, respectively). Both measures and sliding window analysis (50 nucleotide window with 10 nucleotide step size) were computed using DnaSP v.5.10.01 [12].

2.6. Statistical analysis

Differences in mean diversity indices were tested using Kolmogorov–Smirnov test. Correlations between nucleotide diversity indices and sequencing depth were evaluated by Spearman's correlation. A P-value of <0.05 was considered statistically significant. All analyses were performed with SPSS v.12.0 (SPSS Inc., Chicago, IL).

3. Results

The demographics and clinical characteristics of the patients as well as safety laboratory parameters at baseline are given in Table 1.

From 71 PCR products obtained from 18 HCV/HIV co-infected patient samples during follow-up, UDPS yielded 63 674 reads for the NS3 region (mean coverage 897, range 157–4202) and 48 127 reads for the NS5B region (mean coverage 678, range 288–1777). The reads obtained were processed according to the quality control requirements as described in Section 2.3. The median mismatch error rate or technical error rate was 7×10^{-4} for NS3 and 6×10^{-4} for NS5B.

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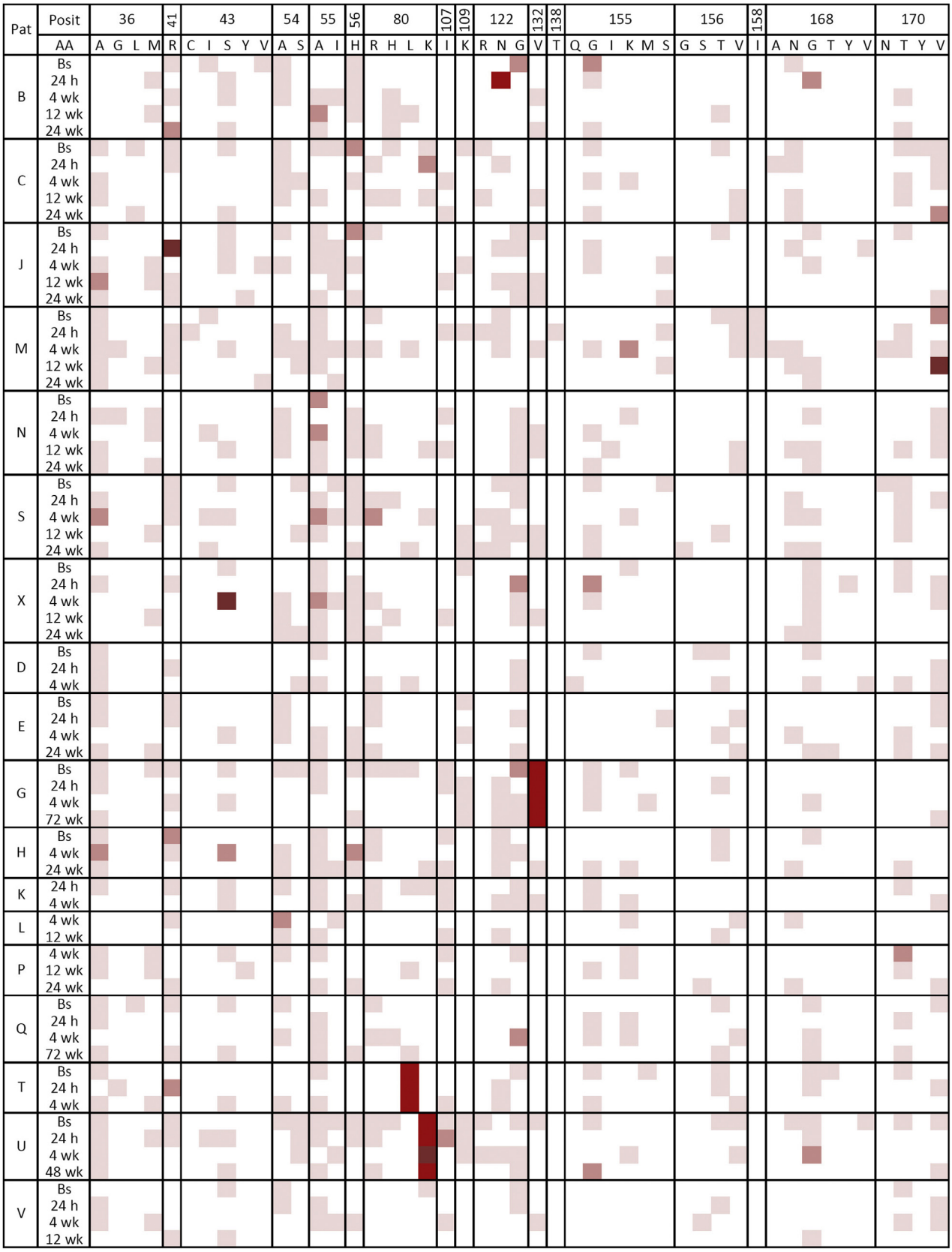

Fig. 1. Heat map showing the presence and relative frequencies of individual NS3 resistance-associated variants (RAVs) across virus populations from HCV/HIV co-infected patients ($n = 18$). Bs, baseline. The evaluated amino acid positions with no RAVs were omitted. ( ) 0%; ( ) <1.5%; ( ) 1.5–5%; ( ) 5–25%; ( ) >60%.

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Table 1
Demographic and clinical characteristics as well as safety laboratory parameters at baseline for 18 HIV patients co-infected with HCV genotype 1a.

Parameter	Baseline value[a]
Age (years)	41.3 ± 5.1
Baseline weight (kg)	70.7 ± 11.8
Baseline CD4+ T-lymphocyte count (cells/μL)	583 ± 312
Baseline CD4+ T-lymphocytes (%)	26 ± 10.6
ΔCD4 T-lymphocytes (cells/μL)[b]	301 ± 256
Male [*n* (%)]	17 (94.4)
Known time of HCV infection (years)	10.8 ± 6.5
Known time of HIV infection (years)	10.9 ± 5.6
Baseline HIV VL <50 copies/mL [*n* (%)]	18 (100)
Baseline HCV VL (log_{10} IU/mL)	5.9 ± 0.51
Fibrosis METAVIR 3–4 score [*n* (%)]	8 (44.4)
IDU [*n* (%)]	14 (77.8)
Baseline ALT (IU/mL)	84 ± 56

HIV, human immunodeficiency virus; HCV, hepatitis C virus; VL, viral load; IDU, intravenous drug user; ALT, alanine aminotransferase.
[a] Data are the mean ± standard deviation unless otherwise stated.
[b] ΔCD4 = baseline CD4 count − nadir CD4 count.

3.1. Protease (NS3) and polymerase (NS5B) inhibitor resistance-associated variants

The frequencies of resistance variants distributed through the follow-up of each patient are shown as heat maps in Fig. 1 and Supplementary Fig. S1.

3.1.1. NS3 protease

Among the patients, several RAVs with frequencies of <5% were detected. They clustered around the protease active site (catalytic triad, His57, Asp81 and Ser139) and their appearance exhibited no specific pattern. Instead, RAVs appeared to emerge and disappear irrespective of peg-IFN+RBV progress. Considering all patients, positions 138 and 158 looked like the most conservative, exhibiting a very low RAV mean frequency (Fig. 2). Conversely, the rest of the positions analysed, at least one sample time, presented RAV in ≥50% of the patients. Positions 36, 55, 122 and 170 were those exhibiting the higher RAV mean frequencies. In one patient (U), Q80K was the most prevalent variant (Fig. 1). This variant negatively impacts the therapeutic response to simeprevir [13]. However, it is a common polymorphism in genotype 1a virus. Another patient (T) showed an almost complete occurrence of Q80L at three of the four available sampling times (Fig. 1); only at Week 4 of treatment did Q80L decrease to 22%. Q80L is an observed polymorphism for genotype 1b isolates.

3.1.2. NS5B polymerase

The overall result was similar to that obtained for the NS3 region. Several resistance variants were detected at a low frequency (<5%) in all subjects without any pattern related to IFN-based treatment. Well defined positions showed absence (L392) or very low (L419) mean detection frequency of RAVs among patients. The A421V mutation is associated with a decrease in the effectiveness of BMS791325, a non-nucleoside NS5B polymerase inhibitor, which is being studied in combinations with other drugs [14]. It was observed in two patients (P and V) as a highly predominant variant, whilst in another two individuals (J and N) it emerged 24 h after peg-IFN+RBV initiation (Supplementary Figs. S1 and S2).

3.2. Genetic diversity

UDPS sequences resulting from the correction pipeline were analysed to evaluate diversity and selective pressure exerted by IFN-based treatment. Two different genetic diversity measures were calculated, the average pairwise nucleotide diversity (π) and Tajima's *D* statistic. For NS3 and NS5B regions, mean values of both indices were compared among sampling times (Supplementary Table S2) and no significant differences were observed ($P > 0.05$). They were also compared between regions and no significant differences were observed ($P > 0.05$) except for the pairwise nucleotide diversity at Week 4 ($P = 0.011$). The Tajima's *D* for all sampling times and for both regions were less than −1.83 for all patients.

The over-time sliding window analysis did not reveal localised regions of increased or decreased Tajima's *D* mean within the linear sequence of the NS3 coding region. The lines for each sample time are intermingled along the analysed region of the protein showing no temporal time trend. In contrast, the first part of the linear NS5B sequence showed slower mean *D* values both at basal and 24 h sampling times than those obtained later under peg-IFN+RBV therapy (Supplementary Fig. S3). From this position to the end of the region analysed, the lines are intermingled as for NS3. Considering individual subjects for both NS3 and NS5B, the region of maximum and minimum Tajima's *D* values varied widely among them.

Pairwise nucleotide diversity and Tajima's *D* were not significantly associated with sequencing depth ($P > 0.05$, Spearman's correlation).

4. Discussion

Virological failure in patients undergoing DAA-based therapy is associated with the emergence of resistance variants [4]. DAA monotherapy results in rapid emergence of resistant variants, so DAAs must be used in combinations that present a high genetic barrier to resistance, although viral kinetics and fitness of multidrug-resistant variants remain poorly characterised [2]. There is no information about the behaviour of quasispecies under the pressure exerted by IFN-based regimens in HCV/HIV co-infected patients.

At both NS3 and NS5B regions, several low-frequency RAVs for any given position were detected in all subjects, emerging and disappearing without any defined pattern with IFN-based treatment using a next-generation sequencing method. The impact of low-level RAVs on DAA treatment outcome is not yet fully determined. Regarding treatment-naïve patients and re-treatment after peg-IFN+RBV treatment failure, the American Association for the Study of Liver Diseases (AASLD) recommends baseline resistance testing for Q80K among HCV genotype 1a, and alternative treatments to simeprevir should be considered if this mutation is present [15]. In the current data, five patients exhibited this RAV as a minority variant that emerged and disappeared throughout follow-up. Interestingly, one patient showed Q80L as the majority variant at three of the four sampling times.

The co-infected patients studied here had been on antiretroviral therapy for many years and all had suppressed HIV replication (<50 copies/mL). Under this condition, the persistent HCV viraemia did not appear to influence the CD4 recovery but its levels could fall with peg-IFN+RBV therapy. In order to longitudinally evaluate the enhanced immunological pressure exerted by IFN, two diversity coefficients were obtained for both protease and polymerase regions. Average pairwise nucleotide diversity (π) did not significantly change over IFN-based treatment and appears to be lower for NS5B than for NS3, but the difference was not significant, except for Week 4. A limitation of this last comparison is that the catalytic site was not comprehended within the analysed NS5B region as it was for NS3. For all patients, the values obtained for Tajima's *D* statistic were significantly negative for both regions; this fact could be explained by an excess of low-frequency polymorphisms suggesting recent selective sweeps, purifying selection and population expansion [3]. The haplotypes corresponding to these low-frequency polymorphisms could include RAVs, but without the selective pressure exerted by the drugs they are not offering

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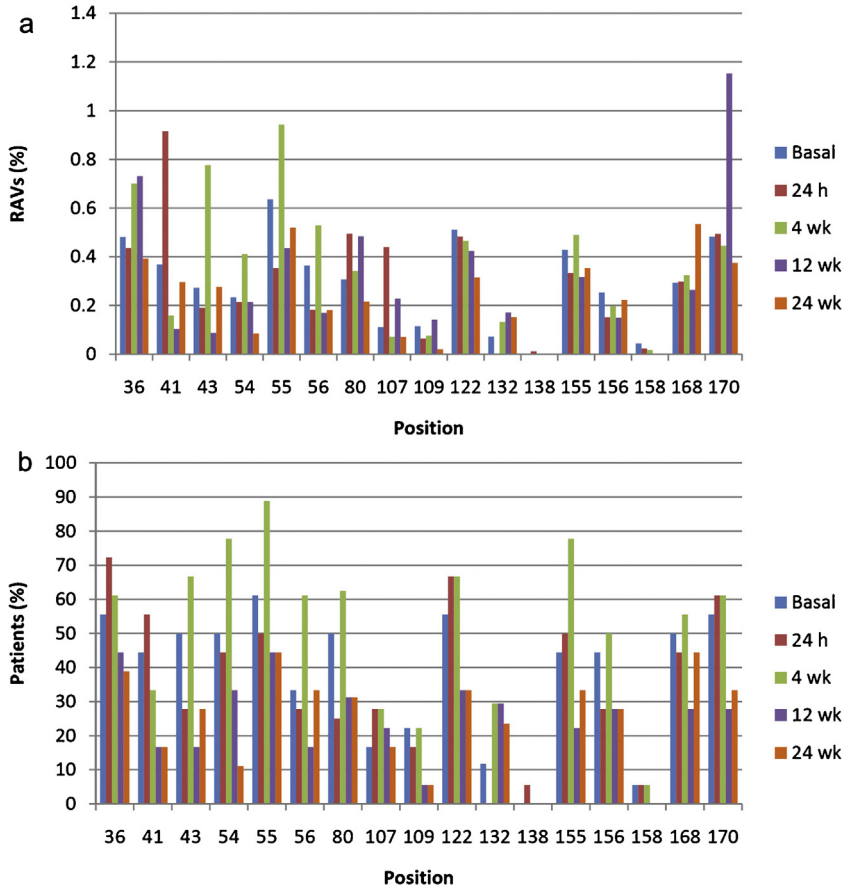

Fig. 2. NS3 inhibitor resistance-associated variants (RAVs) identified by next-generation sequencing in HCV/HIV co-infected subjects ($n = 18$). (a) Mean detection frequency of RAVs for each sampling time. (b) Percent of patients in which individual RAVs were identified.

any advantage or even could be detrimental to HCV fitness. Thus, they are negatively selected and therefore lead RAVs to remain or fluctuate at low frequency or even disappear. However, as HCV polymerase is error-prone and lacks proof-reading activity, RAVs can re-emerge. Finally, for the sliding window analysis and considering individual subjects, the region of maximum and minimum *D* values varied widely among them. This fact is in agreement with diversity values recently reported, suggesting host-specific differences in regional selective forces such as mediated by HLA-restricted T-cell responses as a possible explanation [3].

In conclusion, in this study we evaluated the hypothesis of whether RAVs could be accumulated or co-selected as a result of IFN-based treatments. An important limitation of this study is the small number of patients analysed as well as the lack of a comparable cohort of HCV monoinfected patients. However, the results suggest that previous therapy and failure of peg-IFN+RBV was not favourably associated with the increase in DAA RAVs that naturally exist in HCV/HIV co-infected subjects.

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Competing interests: None declared.

Ethical approval: The study protocol was approved by the Ethics Committee of the Huesped Foundation (Buenos Aires, Argentina).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2015.04.011

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