

## Specific mutations in HIV-1 gp41 are associated with immunological success in HIV-1-infected patients receiving enfuvirtide treatment

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**Objectives:** To investigate gp41 variability and correlation with viro-immunological parameters in 54 HIV-1-infected patients receiving enfuvirtide added as single active drug to a failing regimen.

**Methods:** One hundred and two HIV-1 gp41 sequences and clinical follow-up from 54 enfuvirtide-treated patients were analysed from baseline to week 36 of treatment. The association of mutations with viraemia/CD4 count was assessed by Mann–Whitney test.

**Results:** The addition of enfuvirtide to the failing regimen induced at week 4 a viraemia decrease from 5.1 to 4.3 log<sub>10</sub>/mL ( $P = 0.0002$ ) and a CD4 increase from 48 to 106 cells/mm<sup>3</sup> ( $P = 0.008$ ). While viraemia rebounded to 4.8 and 4.6 log<sub>10</sub>/mL at week 12 and 36, respectively, CD4 continued to increase to 136 cells/mm<sup>3</sup> at week 36. Enfuvirtide resistance mutations, rarely found at baseline, occurred in 45/54 (83.3%) enfuvirtide-treated patients. V38A/E were the most represented mutations at all time-points. The presence of V38A/E was significantly associated with a 4.5-fold CD4 increase from baseline to week 24 and with a 6-fold increase at week 36 ( $P = 0.004$  and  $0.02$  compared without V38A/E, respectively), without significant correlation with viraemia. In contrast, Q40H + L45M (present in six enfuvirtide-treated patients at week 36) correlated with CD4 loss from baseline to week 36 ( $P = 0.02$ ), without significant correlation with viraemia. Mutation N126K (observed in six enfuvirtide-treated patients, never found at baseline) abrogates the fourth gp41 glycosylation site and correlates with a 2.1-fold CD4 increase at week 24.

**Conclusions:** Specific enfuvirtide resistance mutations (V38A/E) are associated with a sustained CD4 increase, without remarkable effects upon viraemia. This CD4 recovery, due to virus- and immune-mediated mechanisms most probably not applicable to protease/reverse transcriptase inhibitors, is important for innovative therapeutic strategies.

Keywords: resistance, CD4 cell counts, viraemia, glycosylation

### Introduction

Fusion inhibitors represent a new generation of antiretroviral agents that bind the HIV-1 envelope glycoprotein gp41, essential for mediating the fusion between the viral and host cell

membrane.<sup>1,2</sup> The gp41 subunit is divided by the transmembrane region into an endodomain and an ectodomain; the latter contains a hydrophobic amino-terminal fusion peptide, followed by an amino-terminal and a carboxy-terminal leucine/isoleucine heptad repeat domain with a helical structure (HR1 and HR2,

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respectively).<sup>3–7</sup> The ectodomain of HIV-1 gp41 also contains at least four consensus sites [asparagine-X-serine/threonine (N-X-S/T) at positions 100–102, 105–107, 114–116 and 126–128] for the incorporation of N-linked carbohydrates; their presence decreases the portion of gp41 surface serving as an immunogenic target.<sup>8,9</sup>

Enfuvirtide (ENF/Fuzeon/T-20) (the first approved fusion inhibitor) is a synthetic peptide (36 amino acids) corresponding to residues 643–678 of the gp41 HR2 domain, characterized by a good antiviral activity and a low toxicity profile.<sup>10,11</sup> Enfuvirtide blocks the formation of the six-helix bundle, a coiled-coil structure composed of the internal triple-stranded HR1 paired with antiparallel outer HR2 domain.<sup>12</sup> A few studies have explored the enfuvirtide resistance profile and defined mutations at positions 36–45 in the HR1 domain that sharply decrease the affinity of the HR1 domain of HIV-1 gp41 for enfuvirtide. The clinical implications of this discovery are so far limited, for a number of reasons: (i) the number of patients analysed is limited; (ii) the methodology of mutation assessment is not standardized, since the majority of the studies evaluate only a short region of HR1 (amino acids 36–45) where mutations have been found; (iii) combination of mutations and the definition of mutations more relevant from a clinical point of view have not been exploited; (iv) last, but not least, the potential enfuvirtide-mediated modulation of glycosylation sites of gp41 (essential for masking the outer viral proteins to the immune system) has not yet been studied. This latter point may be particularly relevant, in view of the possibility that mutations of the sequence of gp41 (and gp120) induced by antiviral compounds may modulate the immunogenicity of HIV proteins and thus ultimately affect the immune response against the virus.

The objective of the present study was to track the evolution of sequence changes in both HR1 and HR2 domains of gp41 associated with resistance to enfuvirtide, and the correlations with the viro-immunological parameters, in a patient cohort receiving enfuvirtide added to a failing regimen.

## Patients and methods

### Patients

The study included 54 consecutive, non-selected, highly drug-experienced HIV-1-infected patients with a history of multiple virological failures, followed in three major centres in Italy. Enfuvirtide was mostly added as a single active drug to the last failing regimen. In 16 (29.6%) patients, minor modifications [i.e. changes limited to a single nucleoside reverse transcriptase inhibitor (NRTI) or protease inhibitor (PI) in the frame of extensive multidrug resistance] were made at the time of starting enfuvirtide. In 48 (88.9%) patients, enfuvirtide was administered together with 1–3 NRTIs + at least 1 PI; 44/48 (91.7%) patients received ritonavir-boosted PIs. Non-nucleoside RT inhibitors (NNRTIs) were used with enfuvirtide only in seven patients due to the high prevalence of mutations conferring full resistance to this class (present in 49/54 patients). In the remaining four patients, enfuvirtide was administered with only NRTIs. Plasma samples were collected from each patient at different time-points during enfuvirtide treatment. Overall, 102 plasma samples obtained between 4 and 36 weeks of enfuvirtide therapy were analysed. Baseline samples from 44/54 patients (81.5%) were also available for the analysis. As a control, another 11 patients, not treated with enfuvirtide, were included in the analysis of enfuvirtide-related mutations.

All patients carried HIV-1 B subtype, except two enfuvirtide-treated patients who carried HIV-1 C and F subtypes, respectively.

### Ethics

The study did not need ethics approval since it was a retrospective study in which the clinical data and the HIV gp41 sequences were obtained from plasma samples used for clinical routine.

### PCR amplification and sequencing

Viral RNA was extracted from plasma by use of the QIAamp Viral RNA kit (Qiagen), according to the manufacturer's instructions. The entire HIV-1 gp41 gene was amplified by RT-PCR with the primers Env-sense 10 (5'-CCAATTCCCACATACATTATTGT) and Env-antisense 9 (5'-GTC CCC CCT TTT CTT TTA AAA). Sequence reaction was performed by use of a big dye terminator v. 3.1 (PE) and eight sequencing primers: Env-sense 4 (5'-CCAATTCCCACATACATTATTGT), Env-antisense 4 (5'-CCCCTCCACAATTAATAA), Env-sense 5 (5'-GTTTTAATTGTGGAGGGGAAT), Env-antisense 5 (5'-CAGCCAGGACTCTTGCT), Env-sense 6 (5'-CTGACGGTACAGGCCAGA), Env-antisense 6 (5'-GAGTATCCCTGCCTAACT), Env-sense 7 (5'-GGCTGTGGTATATAAAAATAT) and Env-antisense 7 (5'-GTCCCCCTTTTCTTTTAAAA). The sequence was determined by capillary electrophoresis on an automated sequencing instrument and analysed using HIV-1 Seqscape v.2 software.

### Mutations

Consensus B was used as a reference strain for the definition of mutations [HIV Sequence Database (<http://hiv-web.lanl.gov>)]. We considered as enfuvirtide resistance mutations those previously described in patients treated with enfuvirtide plus an optimized antiretroviral regimen: G36D/S, I37V, V38A/E/M, Q39H, Q40H, N42T/D/Q/H, N43D/S/K/Q, L44M and L45M as well as those related to enfuvirtide monotherapy: Q32H/R, Q39R, R46M and V69I.<sup>13–17</sup>

Overall, mutation analysis was performed on samples from 55 enfuvirtide-naïve patients (44 later treated with enfuvirtide and with available follow-up, and 11 control HIV-infected patients) and from 54 enfuvirtide-treated patients. For the patients for whom multiple gp41 sequences were available in the absence of enfuvirtide treatment, we used the closest sequence to the time at which enfuvirtide was started. While for the patients for which multiple gp41 sequences were available during enfuvirtide treatment, we used the latest sequence to the time at which enfuvirtide was started, based on the assumption that the greater is the length of a pharmacological selective pressure then the higher is the number of mutations selected.

We also estimated the probability to observe selected (and most common, see the Results) mutations at positions 38 or 40 + 45 over time. In particular, the frequency of selected mutations was calculated at each time-point and then smoothed by using the 'Lowess' algorithm implemented in the statistical software 'R'.<sup>18,19</sup> The smoothed frequency at each time-point was estimated by a robust locally weighted regression.<sup>18,19</sup> The smoothed frequency was assumed to be the estimated probability to observe a mutation at a specific time-point for the central limit theorem. For this kind of analysis we used gp41 sequences available at week 4, 8, 12, 16, 20, 24, 32 and 36.

### Statistical analysis

Wilcoxon tests were used to identify statistically significant differences in viraemia and CD4 cell count between different time-points.

To correlate the presence of specific enfuvirtide resistance mutations with change in viraemia and CD4 cell count, we calculated the difference in viraemia and CD4 cell count between baseline and week 24 or week 36 for each patient with a specific mutation/s and for each patient without such mutation/s. Then, we used Mann-Whitney tests to compare the differences in the two groups of patients. We performed this kind of analysis in the subset of patients whose gp41 sequences, viraemia and CD4 cell count were available at baseline, week 24 and/or week 36. The Benjamini-Hochberg method was used to correct for multiple testing, at a false discovery rate of 0.05.<sup>20</sup>

The  $\chi^2$  test of independence (based on a 2 × 2 contingency table) was used to compare the number of mutated positions in the enfuvirtide target region between enfuvirtide-naïve and enfuvirtide-treated patients.

Genotypic sensitivity

We used resistance testing information to calculate a genotypic sensitivity score (GSS).<sup>21</sup> The GSS was calculated with the Stanford HIV drug resistance database sequence analysis program (Stanford HIV Drug Resistance Database; <http://hivdb.stanford.edu>) using the HIV DB algorithm (version 4.1.9).<sup>22</sup>

Results

Patients' characteristics

Table 1 summarizes the main patient characteristics. All patients were heavily drug experienced, with resistance to multiple NRTIs,

Table 1. Demographic and baseline characteristics of 54 patients receiving enfuvirtide (ENF)

No. of patients	54
male, <i>n</i> (%)	45 (83.3)
Median age, years (IQR)	45 (38–48)
Risk factor, <i>n</i> (%)	
heterosexual	20 (37.0)
drug addict	13 (24.1)
homosexual	8 (14.8)
not known	13 (24.1)
No. (%) of patients at CDC stage C3	31 (57.4)
Median (IQR) viraemia (log copies/mL)	5.1 (4.7–5.6)
Median (IQR) CD4 cell count (cells/mm <sup>3</sup> )	48 (21–127)
Median (IQR) number of years since diagnosis	13 (10–15)
Median (IQR) number of years under treatment	9 (6–11)
Median number (IQR) of previously received drugs	
NRTI	5 (5–6)
NNRTI	1 (1–2)
PI	4 (3–5)
ENF co-administered drugs, <i>n</i> (%)	
NRTI	54 (100)
NNRTI	7 (12.9)
PI	48 (88.9)
Median number (IQR) of resistance-associated mutations <sup>a</sup>	
NRTI	5 (3–6)
NNRTI	1 (0–2)
PI	8 (6–10)

IQR, interquartile range.

<sup>a</sup>The drug resistance mutations considered are those listed by the International AIDS Society (IAS).

NNRTIs and PIs. They were failing their last antiretroviral regimen, with nearly stable viraemia averaging 5 log<sub>10</sub>/mL, and CD4 cell count in progressive decrease during the last 12 weeks prior to enfuvirtide therapy (Figure 1). The addition of enfuvirtide to the failing antiretroviral regimen induced at week 4 a significant decline in viraemia to 4.3 [interquartile range (IQR): 3.3–5.2] log<sub>10</sub>/mL (*P* = 0.0002) and a significant increase in CD4 cell count from 48 (IQR: 21–127) cells/mm<sup>3</sup> at baseline to 106 (IQR: 73–196) cells/mm<sup>3</sup> (*P* = 0.008). In four patients viraemia reached levels <2.6 log<sub>10</sub>/mL (400 copies/mL) and in three patients <50 copies/mL. While viraemia rapidly rebounded to 4.8 (IQR: 3.7–5.1) log<sub>10</sub>/mL and 4.6 (IQR: 4.4–5.2) log<sub>10</sub>/mL at week 12 and week 36, respectively, CD4 cell count continued to increase up to 136 (IQR: 57–195) cells/mm<sup>3</sup> at week 36 (Figure 1), an almost 3-fold increase compared with baseline (*P* = 0.04). No significant difference in terms of viraemia and

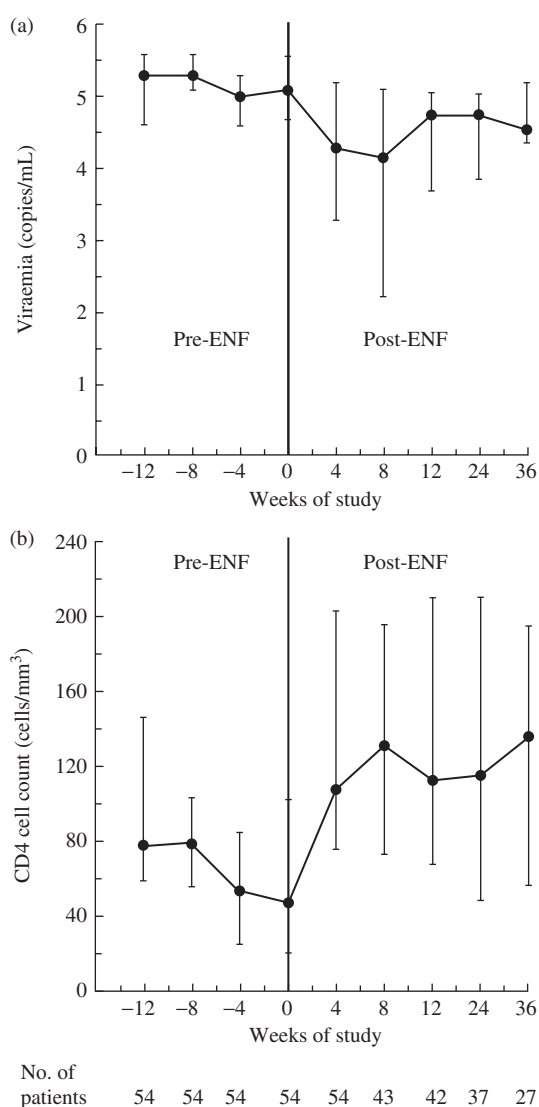


Figure 1. Evolution of viraemia (a) and CD4 cell count (b) in the cohort of 54 patients receiving treatment with an enfuvirtide (ENF)-based regimen. The number of patients, and the median and the interquartile range of viraemia (log<sub>10</sub>/mL) and CD4 cell count (cells/mm<sup>3</sup>) are shown at each time-point pre- and post-enfuvirtide treatment.

CD4 cell count was observed in those 16 patients with minor change of backbone regimen at the time of enfuvirtide addition, compared with patients whose backbone regimen remained unchanged at enfuvirtide addition. Thus despite the fact that the benefit in terms of viral load was transient, the addition of enfuvirtide induced a steady increase in CD4 cell count for at least 36 weeks (end of observation period).

#### *Pattern of HIV-1 gp41-HR1 mutations*

At baseline (without enfuvirtide treatment) the enfuvirtide key-target region of HIV-1 gp41, encompassing the amino acids 36–45, showed a high degree of conservation. In fact, polymorphic mutations at only two positions (Q39H, N42D/S) were observed in isolates from enfuvirtide-naïve patients (Figure 2a). The most frequent was N42S, present in nine (16.4%) patients (one of them carrying a HIV-1 C subtype). No mutations were found in the highly conserved three-amino-acid motif at codons 36–38 (GIV) that are important determinants of viral susceptibility to enfuvirtide.

After treatment, the degree of conservation of this 36–45 region of HR1 showed a significant ( $P = 0.007$ ) reduction compared with baseline. Out of 54 (83.3%) enfuvirtide-treated patients, 45 carried mutations reported to be associated with enfuvirtide resistance (Figure 2a).<sup>13–17</sup> V38A, present in 15 (27.8%) patients at week 24, was the most common sign of enfuvirtide failure. Enfuvirtide resistance mutations generally occurred alone (data not shown). Only Q40H and L45M always occurred together, with the exception of a single patient carrying Q40H + V38E.

In enfuvirtide-naïve patients, the HR1 domain flanking the region encompassing the amino acids 36–45 showed a natural degree of variability, far greater than that of the enfuvirtide target region; in fact, 54/54 patients showed mutations in this flanking region, with 15/39 (38.5%) positions mutated in at least 1 enfuvirtide-naïve patient (Figure 2a). After enfuvirtide treatment, new mutations, never present at baseline, appeared: A30T (5 patients), L33V (1 patient), L34V (1 patient), S35A (2 patients), E49G (1 patient), E49K (1 patient), Q66P (1 patient), A71V (1 patient) and R74K (1 patient).

#### *Association of enfuvirtide resistance mutations with viro-immunological outcome*

The high conservation of residues 36–45 in enfuvirtide-naïve patients suggests that enfuvirtide resistance mutations in the HR1 domain may have an impact on HIV-1 replicative capacity and/or on its cytopathic effect. According to this hypothesis, our analysis showed that the viro-immunological outcome of enfuvirtide-treated patients remarkably varied according to which gp41 mutation occurred during enfuvirtide treatment. In particular, in the presence of V38A or V38E, the median fold change in CD4 cell count from baseline to week 24 was 4.5-fold higher than that observed in the absence of these mutations ( $P = 0.004$ ) (Table 2). The V38A/E-associated increase in CD4 cell count remained significant also at week 36 ( $P = 0.02$ ) (Table 2). In contrast, the presence of V38A/E at week 24 or 36 did not significantly affect viral load (Table 2).

This beneficial effect of mutations at position 38 of gp41 upon CD4 number is due to both V38A (the most common mutation appearing during enfuvirtide treatment) and V38E. Indeed, in the presence of V38A alone, the median fold change in CD4 cell

count from baseline to week 24 was 3.6-fold higher than that observed in the absence of this mutation ( $P = 0.01$ ) (Table 2). Similarly, in the presence of V38E, the median fold change in CD4 cell count from baseline to week 24 was 3.6-fold higher than that observed in the absence of mutations at position 38 (data not shown). Thus, specific and selected mutations in the highly conserved GIV motif (residues 36–38) of HR1 are associated with a significant and sustained increase in CD4 cell count at week 24 and week 36. This effect is not related to a residual efficacy of the backbone failing therapy. In fact, we observed that the GSS score was the same (0.4) among patients carrying or not carrying V38A/E. In addition, this effect may be not attributed to the use of the double-boosted PIs used by only one patient with V38A/E.

In contrast with the increased CD4 cell count associated with the appearance of mutations V38A/E, the paired mutations Q40H + L45M (developed in 6 patients) seem to have a deleterious effect on CD4 cell count: indeed, in the presence of Q40H + L45M, the median fold change in CD4 cell count between baseline and week 36 was significantly lower than that observed in the absence of these mutations ( $P = 0.02$ ). The presence of Q40H + L45M was not correlated with significant changes in viraemia (Table 2). None of the patients carrying the paired mutations Q40H + L45M carried mutation V38A/E.

All other mutations found in the HR1 domain of gp41 did not show statistically significant association with changes in viraemia and/or CD4 cell count in this model (data not shown). Thus, the overall increase in CD4 cell count described in the entire cohort has to be ascribed mainly (if not only) to the selected appearance of mutation V38A/E.

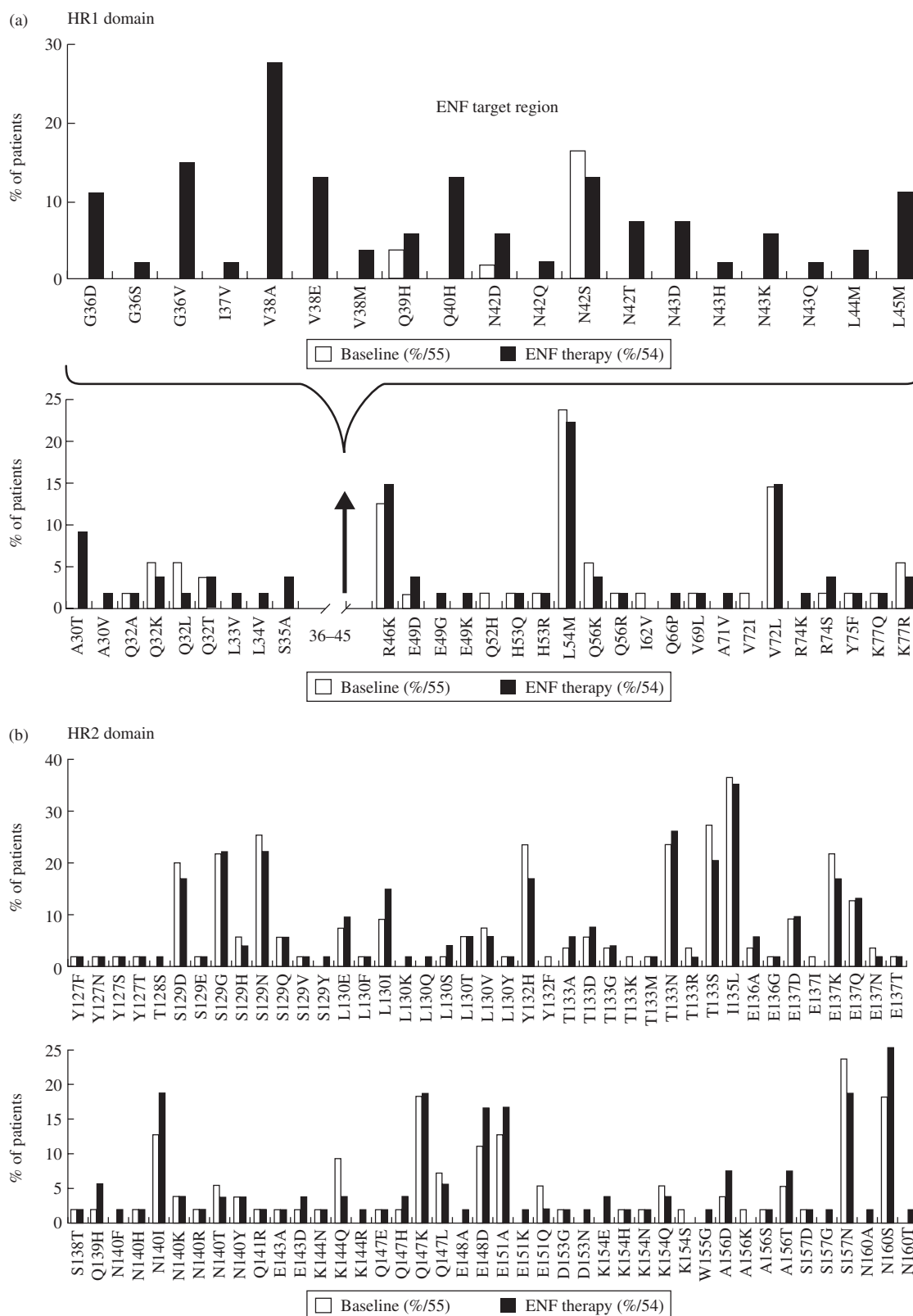
It is correct to mention that all results reported at week 24 remained statistically significant also after correction for multiple comparison. The statistical significance was not confirmed after the correction for multiple comparison for results at week 36. Thus, for results at week 36 a type 1 error cannot be excluded. Larger sample sizes are necessary to re-confirm the association of mutations with changes in CD4 cell count at time-points longer than week 24.

#### *Probability to observe mutations over time*

A further step in our analysis was to estimate the probability of appearance of enfuvirtide-related mutations over time. Our analysis showed that the estimated probability to observe the mutation V38A increases over time, from 27% at week 4 of enfuvirtide therapy to 40% at week 24 and finally to 50% at week 36 (Figure 3). In contrast, the estimated probability to observe mutations V38E or V38M is remarkably lower than V38A; slightly increases up to week 28, but has a plateau beyond this time-point at levels of 12% and 3%, respectively. Similarly, the estimated probability to observe mutations Q40H + L45M, significantly associated with a loss of CD4 cell count, increased up to 12% at 28 weeks of enfuvirtide therapy and then remained stable. None of the other mutations had an increased probability of appearance overtime during enfuvirtide treatment. Thus, the prevalence of V38A in enfuvirtide-treated patients (increasing over time more than other mutations) suggests that this mutation has a key role in the resistance to enfuvirtide; at the same time, the number of patients taking advantage of the beneficial effect of V38A upon CD4 cell count may increase over the course of enfuvirtide treatment.



Association of gp41 mutations with a CD4 increase



**Figure 2.** (a) Frequency of mutations in the HR1 domain of HIV-1 gp41. In the brace the frequency of mutations in the enfuvirtide (ENF) target region between the amino acids 36–45 is reported. (b) Frequency of mutations in the HR2 domain of HIV-1 gp41. The percentage of patients with a specific gp41 mutation was calculated in samples from 55 enfuvirtide-naïve patients and from 54 enfuvirtide-treated patients. Samples from 55 enfuvirtide-naïve patients included baseline samples from 44 out of 54 enfuvirtide-treated patients and samples from 11 enfuvirtide-naïve patients.

**Table 2.** Association of mutations at positions 38 and 40 + 45 with changes in CD4 cell count

Amino acid	Number <sup>a</sup>	Log <sub>10</sub> viraemia (median, IQR)				CD4 cells/mm <sup>3</sup> (median, IQR)			
		baseline	week 24 or 36	ratio <sup>b</sup>	<i>P</i> value <sup>c</sup>	baseline	week 24 or 36	ratio <sup>b</sup>	<i>P</i> value <sup>c</sup>
Significant association of V38A/E with a gain in CD4 cell count at week 24 of enfuvirtide therapy									
38A/E	15	4.9 (4.3–5.1)	4.4 (3.9–4.8)	0.9	0.97	48 (28–99)	142 (103–196)	4.5	<b>0.004</b>
38V/M	12	5.2 (4.9–5.8)	4.8 (5.2–4.8)			71 (36–174)	46 (28–197)		
38A	10	4.9 (4.3–5.1)	4.4 (4.0–4.9)	0.9	0.96	77 (38–134)	178 (114–207)	3.6	<b>0.01</b>
38V/M	12	5.2 (4.9–5.8)	4.8 (5.2–4.8)			71 (36–174)	46 (28–197)		
Significant association of V38A/E with a gain in CD4 cell count at week 36 of enfuvirtide therapy									
38A/E	9	5.0 (4.6–5.1)	4.7 (4.3–4.8)	1.0	0.97	53 (40–107)	186 (130–196)	6.0	0.02
38V/M	7	5.2 (4.8–5.7)	4.7 (4.6–5.5)			48 (31–65)	28 (22–30)		
38A	6	5.0 (4.4–5.1)	4.5 (4.1–5.2)	1.0	0.96	86 (46–134)	191 (144–255)	3.8	0.03
38V/M	7	5.2 (4.8–5.7)	4.7 (4.6–5.5)			48 (31–65)	28 (22–30)		
Significant association of Q40H + L45M with a loss in CD4 cell count at week 36 of enfuvirtide therapy									
40H + 45M	5	4.5 (3.3–5.3)	4.7 (4.6–5.4)	1.1	0.94	71 (48–229)	26 (23–115)	0.12	0.02
40Q + 45L	11	5.2 (5.0–5.5)	4.7 (4.7–5.3)			48 (33–102)	142 (87–235)		

Due to the low number of patients carrying HIV-1 strains with Q40H + L45M at week 24, the Q40H + L45M associated decrease in CD4 cell count is not reported in the table (*P* = not significant).

<sup>a</sup>The analysis was performed on patients whose viraemia, CD4 cell count and gp41 sequences at baseline and at week 24 (27 patients) or week 36 (16 patients) were available.

<sup>b</sup>The ratio was calculated between the median fold change in viraemia and CD4 cell count from baseline to week 24 or 36 in the presence and in the absence of reported mutations, respectively.

<sup>c</sup>*P* value was determined by Mann–Whitney test. Bold font, results that are significant at a false discovery rate of 0.05 following correction for multiple hypothesis testing by the Benjamini–Hochberg method.

### Pattern of HIV-1 gp41 HR2 mutations

The HR2 domain of HIV-1 gp41 showed a natural variability greater than that observed for HR1. Indeed, 24 out of 36 residues (66.7%) were variable in the absence of enfuvirtide pressure (Figure 2b). During the observation period considered, treatment with enfuvirtide was not associated with specific mutations, thus suggesting that HR2 can afford some degree of variability without detrimental effect on replication capacity.

### Glycosylation sites

Another and the final step of our research was to determine whether the pharmacological pressure imposed by enfuvirtide selects mutations at the N-linked glycosylation sites able to interfere with the glycosylation process, thus promoting the appearance of new gp41 epitopes. For this reason, the genetic variability of the four asparagine-linked glycosylation sites (at positions 100–102, 105–107, 114–116 and 126–128) was analysed over time in relation to the baseline sequences. A limited degree of natural variability at the four glycosylation sites occurred also in the absence of enfuvirtide selective pressure and was maintained during enfuvirtide treatment (data not shown). However, this variability does not affect glycosylation, since the substituted amino acids still allowed the addition of the sugar moiety, due to their general appearance at the second position of the glycosylation sites (that is conventionally variable) or at the third position (where a serine can be substituted by a threonine without effect on glycosylation process). In all these cases, the presence of the oxydrilic-group in the side chain of the amino acid, mandatory for the glycosylation process, is fully preserved.

Beyond this natural variability, the mutation N126K, never observed at baseline, occurred at the fourth glycosylation site

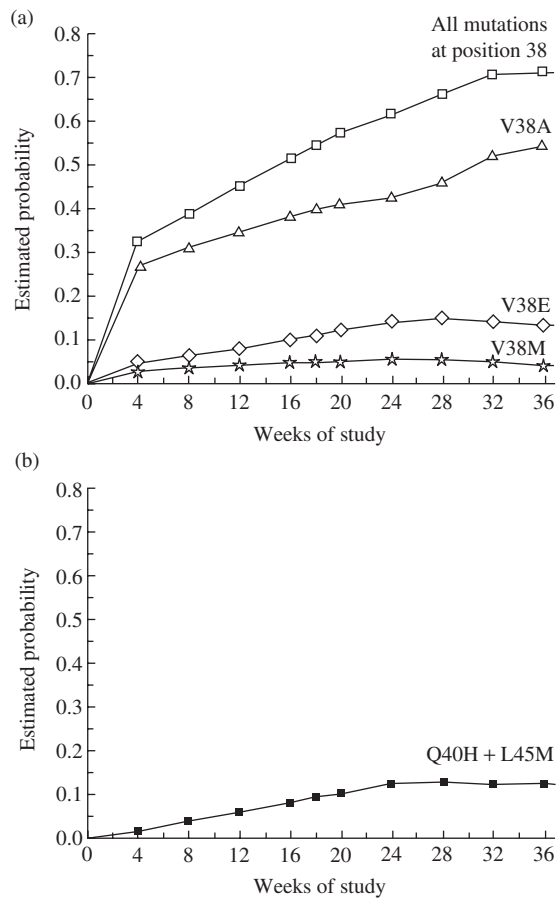
(NYT to KYT at positions 126–128) in six enfuvirtide-treated patients. This mutation changes the asparagine and thus interferes with the glycosylation process that cannot proceed; thus, no glycosylation can occur under these conditions. Of interest, our analysis showed that the presence of N126K was associated with a 2.1-fold increase in CD4 cell count from 86 (IQR: 66–124) cells/mm<sup>3</sup> at baseline to 181 (IQR: 143–198) cells/mm<sup>3</sup> at week 24 of enfuvirtide therapy. Mutation V38A/E was absent in these patients.

### Discussion

Our study, based upon one of the largest observational cohorts of enfuvirtide-treated patients, showed that the enfuvirtide target region encompassing the amino acids 36–45 in the HR1 domain of HIV-1 B subtype gp41 is highly conserved in enfuvirtide-naive patients and forced to change only under the selective pressure imposed by enfuvirtide. Moreover, our study identified specific enfuvirtide resistance mutations significantly associated with an increase in CD4 cell count despite virological failure.

This high degree of conservation suggests that the enfuvirtide target region plays a key role in maintaining the fusion-active state of gp41. This hypothesis is supported by our results, showing that the HR1 domain flanking the region encompassing the amino acids 36–45 has a natural degree of variability far greater than that observed for the enfuvirtide target region. Our findings are in agreement with several epidemiological studies where a low variability at residues 36–45 of HIV-1 gp41 region was observed. It also agrees with *in vitro* studies that demonstrated by site-directed mutagenesis that specific mutations in the enfuvirtide target region may reduce the membrane fusion activity of gp41

Association of gp41 mutations with a CD4 increase



**Figure 3.** Cumulative prevalence of mutations at position 38 (a) and of Q40H + L45M (b) at each time-point during enfuvirtide therapy. The frequency of mutations was determined at each time-point in patients whose HIV-1 gp41 sequence was available. Squares, all mutations at position 38; triangles, V38A; diamonds, V38E; stars, V38M.

(presumably by delaying the membrane fusion kinetic) and consequently the HIV replicative capacity.<sup>23–29</sup>

A recent study showed a natural variability of HR1 in 10.5% of samples from enfuvirtide-naïve patients. This result may be related to the large proportion of non-B subtypes analysed. In addition, the analysis was based on the number of samples and not on the number of patients, and this may overestimate the mutation prevalence. Finally Carmona *et al.*<sup>30</sup> also included mutations outside the 36–45 region (shown in our paper as being naturally variable), again favouring the overestimation of mutations. Both in Carmona’s paper and in our study, the natural polymorphism N42S is described in the absence of enfuvirtide therapy. This mutation has recently been associated with increased enfuvirtide susceptibility.<sup>31</sup> The comparative analysis of patients in our cohort carrying N42S, however, showed a viro-immunological outcome not significantly different from that of patients not carrying this mutation.

The degree of conservation of the enfuvirtide target region showed a significant reduction under enfuvirtide treatment. The resistance mutations in the enfuvirtide target region occurred alone (with the main exception of Q40H + L45M), thus suggesting the low genetic barrier of enfuvirtide to resistance.

Moreover, we did not observe the concurrent emergence of mutations in both HR1 and HR2 domains specifically

associated with enfuvirtide treatment, such as the mutation S138A, recently proposed as compensatory to mutations at position 43.<sup>32</sup>

One point that deserves attention in our study is the remarkable discrepancy between the direct inhibition of HIV replication by enfuvirtide, usually lost after some weeks of therapy, and the sustained increase in CD4 cell count. This latter effect is purely associated with enfuvirtide treatment, since enfuvirtide was added to a failing antiretroviral regimen in patients characterized by sustained and stable viral load and decreasing CD4 cell count (Figure 1). This suggests that the effect of enfuvirtide on CD4-count increase may not be related to a decrease in HIV-1 replicative capacity. Indeed, as shown in Figure 3(a), a steady increase in mutations at position V38 (mostly A, followed by E, and rarely M) occurred, with an estimated probability of appearance from 0% at baseline to ~30% of patients at week 4, 60% at week 24 and 70% at week 36 (Figure 3). This suggests that the ability of HIV to replicate in the presence of the drug (enfuvirtide) requires, in the majority of cases, a mutation at codon 38 of the GIV region (36–38) crucial for the correct folding and assembly of gp41 and gives reason for the decreased immune damage induced by V38-mutated strains of HIV (CD4 increase despite a limited effect on viral load). Consistent with our analysis, a recent observation showed the presence of V38A in isolates from enfuvirtide-treated patients who experienced a sustained increase in CD4 cell count despite viral rebound.<sup>33</sup> Moreover, our results have been recently fully confirmed in an independent dataset from the enfuvirtide Phase 3 clinical trials.<sup>34</sup> In fact, in such study it has been shown that substitution at position 38 is associated with a continued CD4 increase in patients failing ongoing enfuvirtide therapy independent of residual virological activity. It is conceivable that conformational changes induced by the mutations V38A/E in this key, highly conserved GIV motif of HIV-1 gp41-HR1 (never mutated in enfuvirtide-naïve patients) are closely related to a decreased ability to damage the immune system. This may facilitate CD4 recovery through mechanisms that can be virus-mediated such as loss of fusion efficiency upon cell targets, or decreased apoptosis induced directly by gp41 (as recently shown by Garg and Blumenthal<sup>35</sup>) and/or immune-response-mediated such as exposure of new epitopes not applicable to reverse transcriptase and protease inhibitors, and thus important for the design of innovative therapeutic strategies. Studies on large cohorts may provide more information on this important point.

Our study also showed that the beneficial effect of enfuvirtide on CD4 number is associated with selected mutations, while others, such as the co-presence of mutations Q40H + L45M, are associated with a significant loss in CD4 cell count, from baseline to week 36 of enfuvirtide therapy, without significant changes in viraemia. It is unclear whether the detrimental effect of the association Q40H + L45M on CD4 number is related to one more than to the other mutation, since they always appeared together, with a single exception of a patient carrying Q40H + V38E (whose CD4 lymphocytes increased from 48 to 97 cells/mm<sup>3</sup> at week 36). This negative effect of Q40H + L45M confirms that the viro-immunological outcome of enfuvirtide-treated patients remarkably varies according to the type and position of gp41-mutations occurring during enfuvirtide treatment. Algorithms evaluating the efficacy of enfuvirtide should therefore consider the different relevance of specific mutations on virological and immunological outcome.

Another result worth discussing is the mutation N126K at the fourth gp41 glycosylation site (NYT at positions 126–128), never found at baseline, and observed in six enfuvirtide-treated patients. As a confirmation of our analysis, a recent study showed that the gp41 C-HR-derived peptide (similar in structure to enfuvirtide) was able to select *in vitro* the mutation N126K.<sup>36</sup> As lysine substitutes asparagine at position 126, it abrogates the NXT/S triad required for the addition of sugar moiety. Although further studies are necessary, it is conceivable that the abrogation of the glycosylation site may determine the loss of the carbohydrate shield and consequently the appearance of new epitopes that may become targets of neutralizing antibodies. Consistent with our hypothesis, the presence of N126K in isolates from enfuvirtide-treated patients is associated with a 2.1-fold increase in CD4 cell count compared with baseline.

In conclusion, our study contributes to a better understanding of enfuvirtide resistance. It identifies specific enfuvirtide resistance mutations (V38A/E) significantly associated with an increase in CD4 cell count despite virological failure. This CD4 recovery, due to virus- and immune-mediated mechanisms most probably not applicable to protease/reverse transcriptase inhibitors, may provide the rationale for the design of innovative therapeutic strategies.

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## Transparency declarations

None to declare.

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