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HIV-1 Gag mutations alone are sufficient to reduce darunavir susceptibility during virological failure to boosted PI therapy

Oscar Blanch-Lombarte[1,2], José R. Santos [3,4], Ruth Peña[1], Esther Jiménez-Moyano[1], Bonaventura Clotet[1–5], Roger Paredes[1–5] and Julia G. Prado[1,2]*

[1]IrsiCaixa AIDS Research Institute, Badalona, Spain; [2]Germans Trias i Pujol Research Institute (IGTP), Badalona, Spain and Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain; [3]Lluita contra la SIDA Foundation, Hospital Universitari Germans Trias i Pujol, Badalona, Spain; [4]Infectious Diseases Department, Hospital Universitari Germans Trias i Pujol, Badalona, Spain; [5]Faculty of Medicine, University of Vic - Central University of Catalonia (UVic-UCC), Vic, Spain

*Corresponding author. E-mail: jgarciaprado@irsicaixa.es

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Background: Virological failure (VF) to boosted PIs with a high genetic barrier is not usually linked to the development of resistance-associated mutations in the protease gene.

Methods: From a cohort of 520 HIV-infected subjects treated with lopinavir/ritonavir or darunavir/ritonavir monotherapy, we retrospectively identified nine patients with VF. We sequenced the HIV-1 Gag-protease region and generated clonal virus from plasma samples. We characterized phenotypically clonal variants in terms of replicative capacity and susceptibility to PIs. Also, we used VESPA to identify signature mutations and 3D molecular modelling information to detect conformational changes in the Gag region.

Results: All subjects analysed harboured Gag-associated polymorphisms in the absence of resistance mutations in the protease gene. Most Gag changes occurred outside Gag cleavage sites. VESPA analyses identified K95R and R286K ($P < 0.01$) as signature mutations in Gag present at VF. In one out of four patients with clonal analysis available, we identified clonal variants with high replicative capacity and 8- to 13-fold reduction in darunavir susceptibility. These clonal variants harboured K95R, R286K and additional mutations in Gag. Low susceptibility to darunavir was dependent on the Gag sequence context. All other clonal variants analysed preserved drug susceptibility and virus replicative capacity.

Conclusions: Gag mutations may reduce darunavir susceptibility in the absence of protease mutations while preserving viral fitness. This effect is Gag-sequence context dependent and may occur during boosted PI failure.

Introduction

The introduction of active combined ART (cART) has led to the effective control of viral replication in HIV-1-infected individuals. Although integrase inhibitor-based cART treatments are currently the most widely used first-line regimens, darunavir/ritonavir and lopinavir/ritonavir are used in some simplification strategies and low-income settings.[1–5]

Despite the high genetic barrier of PIs to resistance development, the emergence of mutations at the active site of the protease leads to HIV-1 drug resistance and virological failure (VF).[6–9] The main pathways of drug resistance to PIs are well defined. They initiate with mutational changes in the active site of the viral protease,[10] followed by a step-wise accumulation of mutations surrounding the active site,[10–12] at cleavage sites[13–15] and at non-cleavage sites of the Gag-pol polyprotein,[15–17] which compensate for replication defects and increase phenotypic resistance. The emergence of protease-associated mutations is usually observed in patients who have experienced VF to unboosted or first-generation boosted PIs.[18,19] On the other hand, this rarely occurs in patients experiencing VF to cART based on the most recently developed boosted PIs as first-line regimens or in simplification strategies.[6,8,9] This observation raises questions about the mechanism leading to VF to boosted PIs. Although the answer remains elusive, increasing evidence points to the role played by the presence of mutations outside of the protease and other unexplored mechanisms of resistance.

In general with regard to HIV-1 resistance to PIs, several studies demonstrate the contribution of the Gag region to PI susceptibility in the absence of drug resistance mutations in the protease.[20–24] Mutations in and outside of Gag cleavage sites have been directly involved in resistance to PIs.[20,25,26] These data are also supported by co-evolutionary studies by our group and others that highlight a tight interdependence between Gag and protease during the development of PI resistance.[27,28] Similarly, a recent clinical study revealed associations between the presence of baseline mutations in Gag and gp41 with VF to PI-based regimens.[29] Also, the C-terminal region of gp41 has been previously associated with PI resistance in the absence of HIV-1 resistance mutations in the protease.[19] Taken together, these data support a multistep inhibitory mechanism of PI action at various levels of the viral replication cycle, indicating the complexity of the resistance mechanisms underlying VF to PIs. In particular for HIV-1 resistance to PIs in monotherapy, changes in Gag have been associated with treatment failure to monotherapy in the MONARK and SARA trials.[22,24] In addition, the baseline Gag sequence context in A, C and D subtypes has been linked to differences in PI susceptibility during monotherapy.[30,31]

With the aim of gaining new insights into the causes of VF to boosted PIs, we identified nine HIV-1-infected patients who had been receiving monotherapy with lopinavir/ritonavir or darunavir/ritonavir as a maintenance regimen but had experienced VF. Gag-protease genotyping revealed the absence of HIV-1 resistance mutations in the protease. We then successfully generated Gag-protease molecular clones in four patients and phenotypically characterized viral variants in terms of replicative capacity and drug susceptibility to lopinavir and darunavir. Moreover, to shed further light on the protein conformational changes present in clonal variants, we used 3D information on Gag p17 and p24 crystal structures.

Patients and methods

Study subjects

We performed a retrospective search among the 520 HIV-1-infected individuals who had been treated with lopinavir/ritonavir or darunavir/ritonavir monotherapy in a clinical setting between 2002 and 2009 at the Germans Trias i Pujol Hospital, to identify those who had experienced VF during treatment and for whom stored plasma samples existed. VF was defined as two consecutive viral loads of >50 HIV-RNA copies/mL during PI monotherapy. The search yielded nine patients, who had sustained virological suppression (<50 HIV-RNA copies/mL) for at least 6 months before the initiation of monotherapy and had recorded no VF to previous treatments containing PIs.

Ethics statement

All subjects provided their written informed consent for the purpose of research on biological samples taken from them. The project was approved by the institutional review board of Germans Trias i Pujol Hospital (PI-19-028). The study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Amplification of the HIV-1 Gag-protease coding region

Total HIV-RNA was extracted, reverse-transcribed and amplified by a second polymerization step as previously described.[27] Samples below 100 HIV-RNA copies/mL were ultracentrifuged at 72000 **g** for 90 min at 4°C

Construction of HIV-1 Gag-protease recombinant virus

We used pNL43CXS and p83-2 vectors to generate p83-2ΔGP. We digested these vectors with BssHII and AgeI (Thermo Fisher Scientific, Spain) at unique restriction sites and the Gag-integrase region from pNL43CXS was subcloned into p83-2. Then, the generated plasmid was digested with BssHII and ClaI (Thermo Fisher Scientific, Spain) to remove the Gag-protease region. Finally, we cloned a polylinker with BssHII, EcoRI, AgeI, HindIII and ClaI restriction sites to obtain the p83-2ΔGP vector. Then, Gag-protease regions were subcloned into p83-2ΔGP and the insert was identified by EcoRI digestion (Thermo Fisher Scientific, Spain) and subsequent sequencing. Viral stocks were generated by transfection and viral spread was monitored by determining the frequency of GFP-positive (GFP+) cells as previously described.[32] Supernatants were collected, filtered and stored at −80°C for further experiments. $TCID_{50}$ was determined as previously described.[33]

Sequence analyses and viral epidemiology signature analyses

All positive PCR reactions and recombinant clones were sequenced using the Sanger reaction (Macrogene, Netherlands). Sequences were aligned to the HXB2 sequence, and analysed using the neighbour-joining phylogenetic method as previously described.[27] We used the Stanford University HIV Drug Resistance Database for drug resistance interpretation.[34] In addition, we used Los Alamos National Laboratory's Viral Epidemiology Signature Pattern Analysis (VESPA) tool to identify signature mutations in the Gag coding region associated with VF to boosted PI therapy.[35] The VESPA tool calculates the frequency of each amino acid at each position of the alignment for the query (boosted PI in VF sequences) and the background set (treatment-naive B-clade sequences), and selects the position of the most common character in the query that differs from the background set. We retrieved 2757 treatment-naive B-clade Gag sequences from the Los Alamos database and excluded duplicated sequences using the ElimDupes tool. Sequences were then imported to VESPA and compared with the Gag bulk sequences obtained during VF to boosted PIs.[35]

Replication kinetics and drug susceptibility to PIs of Gag-protease recombinant virus

Jurkat cells (National Institutes of Health, USA) were infected with the WT virus or the Gag-protease recombinant virus in triplicate at an moi of 0.005. Cells were washed with PBS once, cultured in R10 (0.5×10^6 cells/mL) and GFP+ cells were monitored over 12 days as previously described.[32] The growth rate was calculated as the slope of GFP+ cells during exponential growth.[33,36] In addition, we measured virus susceptibility in the presence of 0.01, 1 and 100 nM of darunavir and lopinavir and GFP+ cells were monitored on days 1, 3, 6 and 10.[37] The fold change in darunavir and lopinavir was calculated as the ratio of GFP+ cells between the recombinant and WT virus during exponential growth.

Molecular modelling

Protein structures of p24 (PDB 1E6J) and p17 trimer (PDB 1HIW) were obtained from RCSB PDB.[38] The structures represent the B-clade virus and were modified using the PyMOL Molecular Graphics System, Version 1.8 (Schrödinger, New York, USA).

Results

Gag polymorphisms are predominant during VF to boosted PIs

As noted, out of 520 subjects treated with lopinavir/ritonavir or darunavir/ritonavir as a maintenance regimen, we identified nine

patients (1.7%) who fulfilled the inclusion criteria. Their baseline epidemiological and clinical characteristics are summarized in Table 1 and clinical follow-up and sampling are represented in Figure 1. Cross-sectional plasma samples (T1) were available at VF from Patients 1–9 (PT1–PT9) and longitudinal samples (T2) were available from PT3 and PT4. A total of nine samples from seven patients were successfully amplified for the Gag-protease HIV-1 coding region by RT-PCR as represented in the flow chart in Figure 2.

The amplified Gag-protease sequences confirmed B-clade subtypes and protease genotypes fully susceptible to lopinavir and darunavir at VF.[34] Protease genotypes are summarized in Table 2. Only non-polymorphic and polymorphic PI accessory selected mutations were found in PT1 (K20T), PT4 (A71V) and PT5 (L10V). Genotypes of the Gag region at cleavage site (CS) and non-cleavage site (NCS) mutations are summarized in Table 3. We calculated the frequency of mutations in the Gag proteins as the median of the number of mutations in relation to the total length of the protein in amino acids. The frequency of CS mutations ranged

Table 1. Epidemiological and baseline characteristics of the study group

Characteristic	Study group ($n = 9$)
Male gender, *n* (%)	8 (88.9)
Caucasian origin [*n* (%)]	9 (100)
Age (years), median (range)	46 (33–56)
Nadir CD4+ T-cell count per mm^3, median (IQR)	226.3 (14–397)
Time since diagnosis of HIV-1 (years), median (IQR)	8.45 (1.47–17.21)
Time on cART (years), median (IQR)	7.25 (0.1–17)
Time with virological suppression after PI monotherapy initiation (months), median (IQR)	20.27 (44–6)
Therapy on simplification	
lopinavir/ritonavir, *n* (%)	6 (66.7)
darunavir/ritonavir, *n* (%)	3 (33.3)

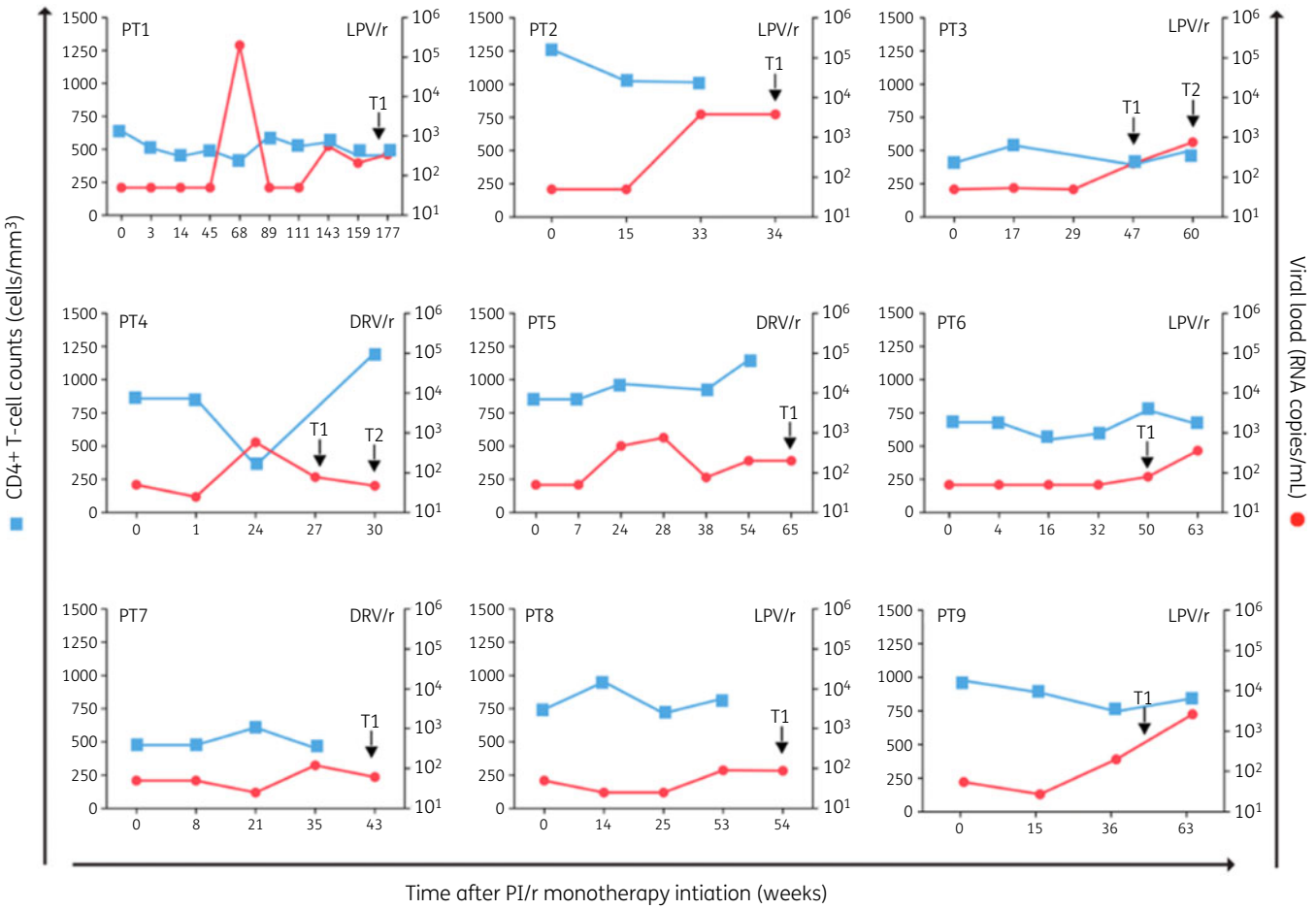

Figure 1. Clinical follow-up and sampling of study subjects. Blue lines indicate CD4+ T-cell counts (cells/mm^3) and red lines the HIV-1 viral load (RNA copies/mL) during follow-up after boosted PI monotherapy initiation. Arrows indicate samples analysed at corresponding timepoints (T1 and T2). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

from 0% in p24 to 7.14% in p2. We found Gag CS mutations associated with PI exposure *in vivo* at p2/p7, p7/p1 and p1/p6 in most of the patients (Table 3). At p2/p7, we found the mutation S373P in PT2 and PT6, the mutation A374S/P in PT3 and PT5 and the mutation T375A in PT4. Also, we identified changes in p7/p1 at K436R and I437V positions, respectively, in PT5 and PT7. In PT4, a single CS mutation was found at position S451N of p1/p6. For NCS mutations, the frequency of variations was 7.57% in p17, 3.47% in p24, 7.14% in p2, 3.63% in p7, 9.6% in p6 and 0% in p1. The frequency of NCS mutations correlated with the total frequency of amino acid variation in Gag ($P < 0.0001$; $r = 0.83$, Spearman correlation, data not shown). We observed Gag NCS mutations previously associated with exposure or resistance to PIs. The R76K mutation was present in 57% of the cases of VF and the E12K/D mutation was present in PT2, PT5 and PT6. Also, we found NCS mutations at positions V370A/M in p2 (PT3, PT5 and PT6) and I389T in p7 (PT2, PT3, PT4, PT5 and PT6). Other changes including CS and NCS mutations with unknown impact on drug resistance are also summarized in Table 3. These data indicate a relative predominance of Gag NCS mutations and an absence of protease resistance mutations during VF to lopinavir/ritonavir or darunavir/ritonavir.

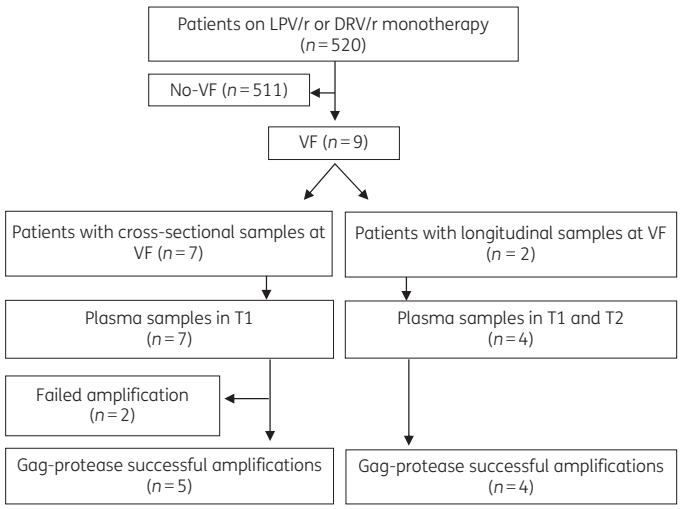

Figure 2. Flow chart of study subject identification, available plasma samples and successful Gag-protease amplifications.

Gag signature patterns during VF to boosted PIs

Next, we applied VESPA to identify Gag residues correlated with VF to boosted PIs in our sequences. VESPA compared the frequency of each Gag amino acid between our nine Gag bulk sequences with the background alignment of 2000 HIV-1 subtype B Gag sequences. As a result, two variations in Gag were noted. At position 95 in p17, we found K95R present in 44% of PI-treated patients at VF in comparison with 12% of naive sequences ($P < 0.01$). At position 286 in p24, we identified the mutation R286K with a frequency of 78% in PI-treated patients and 35.5% in naive sequences ($P < 0.01$). Thus, VESPA provided the signature K95R and R286K in Gag.

Preserved replication kinetics of Gag-protease recombinant virus at VF

To further evaluate the phenotypic characteristics of the HIV-1 variants during VF, we cloned the Gag-protease region into a p83-2ΔGP as detailed above. We obtained Gag-protease clonal plasmids from four patients: PT1 ($n = 10$), PT3 (T1, $n = 10$), PT3 (T2, $n = 8$), PT4 (T1, $n = 2$), PT4 (T2, $n = 3$) and PT6 ($n = 2$). The low viral loads may have limited the number of clones obtained at VF. Phylogenetic analyses confirmed sequence identity and lack of sample cross-contamination (Figure S1, available as Supplementary data at *JAC* Online). We observed low clonal diversity that is indicative of limited viral evolution during VF. In the case of PT3, we were able to detect a cluster of clonal sequences [C5, C6, C8 and C10 (Figure S1B)] containing drug resistance mutations I52V and V82A in the protease at T2. Clonal sequences were also analysed using the Stanford HIVdb database,[34] and clones were predicted to be PI susceptible, except the clones containing I52V and V82A, which were predicted to have intermediate resistance to lopinavir and be fully susceptibility to darunavir.

Viral stocks of Gag-protease clones were produced by co-transfection and replication kinetics were monitored as shown in Figure 3(a). Also, Table 4 summarizes in detail the genotypes of the

Table 2. Protease genotype in bulk sequences

				Protease genotype	
Patient	Timepoint	cART	VL (copies/mL)	DRM	polymorphism[a]
PT1	T1	LPV/r	340	–	**K20T**, R41K, H69R, V77I
PT2	T1	LPV/r	3800	–	R57K, L63P, E65D, V77I, I93L
PT3	T1	LPV/r	220	–	I15V, R41K, E53D, R57K, I62V, L63P
	T2	LPV/r	760	–	I15V, R41K, E53D, R57K, I62V, L63P
PT4	T1	DRV/r	78	–	E35D, N37T, L63P
	T2	DRV/r	47	–	E35D, N37H, R41K, I62V, L63P, **A71V**, I93L
PT5	T1	DRV/r	200	–	**L10V**, L63P, I93L
PT6	T1	LPV/r	80	–	N37S, I64V
PT7	T1	DRV/r	61	–	I15V, M36I, N37D

DRM, drug resistance mutation.
[a]Polymorphisms shown in bold are based on the Stanford HIVdb genotype–phenotype tool.[34]

Table 3. Gag genotype in bulk plasma sequences

Patient	Timepoint	cART	P17	P17/P24	P24	P24/P2	P2	P2/P7	P7	P7/P1	P1/P6	P6
PT1	T1	LPV/r	**E12D**[a], R15K, K30Q, S67A, **R76K**[b,c], L78V, R91K, K95R, K103R, N126R	–	A146P, S148T, V215M, T242N, N252S, R286K, T303V, E312D	–	–	–	Q386P, K411R	–	–	S465F, Q476P, K481Q, L483K, Y484S, S498*
PT2	T1	LPV/r	V7L, **E12K**[a], R15Q, K18R, K28Q, K30R, I34L, L61I, G62Q, **R76K**[b,c], **T81A**[b,c], T84V, E93D, S111I	–	E312D	–	N372G	**S373P**[b], T375N, M377L	**I389T**[b], R406K	–	–	S465F, T471I, E477D, P478S, R490K
PT3	T1	LPV/r	R15K, K30R, T84V, E93D, K113Q	–	V159I, S165N, Q199E, E203D, I223V, G248A, N252S, E260D, T280V, R286K, S310T, G357S	–	**V370A**[b]	**A374S**[b], T375V, I376M	**I389T**[b], K411R	–	–	T456S, S465L
	T2	LPV/r	R15K, K30R, T84V, E93D, K113Q	–	E203D, I223V, G248A, N252S, E260D, T280V, R286K, S310T, G357S	–	**V370A**[b]	**A374S**[b], T375V, I376M	**I389T**[b], K411R	–	–	T456S, S465L
PT4	T1	DRV/r	K28Q, E55G, G62E, **R76K**[b,c], R91K, E93D, K95R, K113Q, T122P, H124K	–	L268M, R286K, A326S	–	–	**T375A**[b] R380K, N382H	R387K, K388R	–	**S451N**[b]	S465F, E477G, I479T
	T2	DRV/r	K28Q, E55G, **R76K**[b,c], V82I, E93D, K95R, K113Q, T122P, H124K, S125N	–	L268M, R286K, A326S	–	–	R380K, N382H	R387K, K388R, **I389S**[b]	–	–	T456I, S465F, T470A, E477G, P478S, I479T
PT5	T1	DRV/r	**E12K**[a], R15K, K18R, K26N, K30R, S54A, E55D, R58K, G62E, Q69K, V82I, A83S, T84V, Q90K, R91N, E93D, K95T, E99A	–	S173T, E203D, V215M, M228I, G248T, N252S, P255A, T280V, E312D, A340G, M347S	–	**V370M**[b]	A374P	**I389T**[b], V390I, T401L, K411R, R429K	R429K, **K436R**[a,b,c]	–	T456S, E460A, R464K, S465F, T470A, I479P, D480E, P497Q, S498*
PT6	T1	LPV/r	**E12K**[a], **R76K**[b,c], R91G, K95R, K103R, K110C, Q117L	–	I147L, V159I, S173T, I223V, G248A, N252S, T280V, R286K, E312D, G357S	–	**V370A**[b]	**S373P**[b], M378V	**I389T**[b], K418R, D425E	–	–	S465F, E477G, P478T, L483I, L486W
PT7	T1	DRV/r	D121N	N131H	A179T, A209T, T318K	–	–	R380K	R384G, E398Q	**I437V**[a,b,c]	–	S465F, R490K

Mutations associated with exposure or resistance to PIs are shown in bold. An asterisk represents a stop codon.
[a]Exposure to PIs *in vitro*.
[b]Exposure to PIs *in vivo*.
[c]Associated with PI resistance.

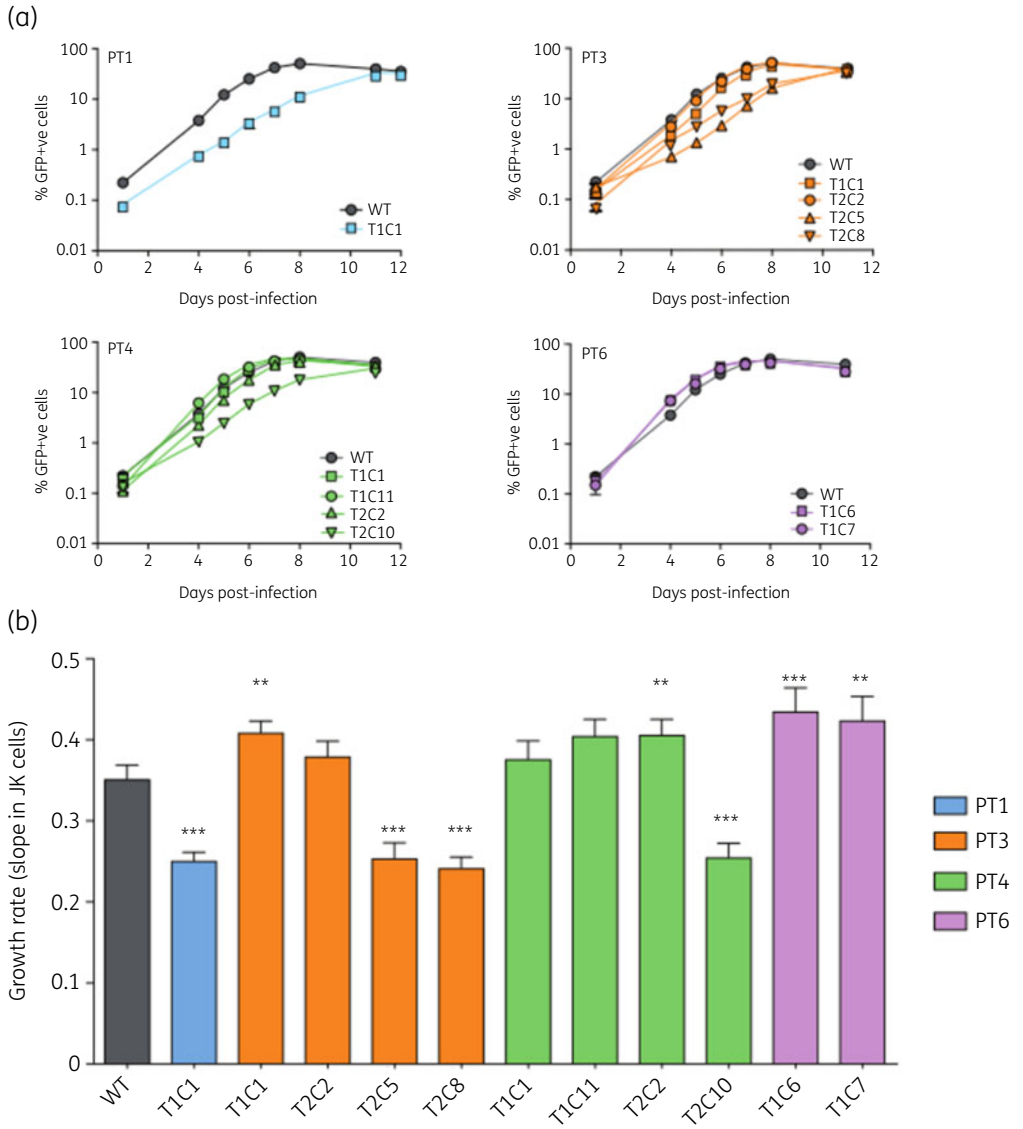

Figure 3. Replicative capacity of Gag-protease recombinant virus. (a) Replication kinetics of Gag-protease recombinant virus obtained from plasma samples from PT1, PT3, PT4 and PT6. Jurkat (JK) cells were infected in triplicate at an moi of 0.005. The kinetics of replication were monitored as the frequency of GFP+ cells by flow cytometry. Black circles represent the WT virus, coloured circles denote T1 virus and coloured triangles represent virus at T2. (b) The bar graph represents the growth rate of the virus calculated as the slope of GFP+ cells during the replication kinetics experiments. Bars represent the mean ± SD of three experimental replicates. The *P* values indicate comparisons between the recombinants and the WT virus. Only significant *P* values (**$P < 0.005$, ***$P < 0.0005$) are represented. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Gag-protease clonal virus tested. Most of the Gag-protease recombinant viruses did not show differences in replication kinetics compared with the WT (Figure 3b). A subgroup of viruses from T1 showed an increment in viral replication (PT3 and PT6; $P < 0.005$), and only one had a significant reduction (PT1; $P < 0.0005$) when compared with the WT. Moreover, in PT3 and PT4 with longitudinal clonal virus, we found a mixture of variants with preserved and low viral replication (Figure 3b). Of note, for PT4 only three mutational changes in Gag (E107G, T280A and E461G) differentiated the high and the low replicative variants at T2 (Table 4). Overall, these results suggest a general preservation of replicative capacity in the HIV-1 variants present at VF to lopinavir/ritonavir and darunavir/ritonavir.

Gag mutations alone reduce susceptibility to darunavir

Next, we tested drug susceptibility at increasing concentrations of darunavir or lopinavir (0.01, 1 and 100 nM). As shown in Figure 4(a), recombinant viruses from PT1, PT3 and PT6 were fully susceptible to darunavir in accordance with the high genetic barrier of the drug, even in the presence of I54V and V82A protease mutations, as predicted by Stanford for the T2 clonal viruses 5 and 8 from

Table 4. Gag and protease genotype in bulk and clonal plasma sequences

Patient	Timepoint	Sequence	P17	P17/P24	P24	P24/P2	P2	P2/P7	P7	P7/P1	P1/P6	P6	Protease
PT1	T1	B	**E12D**[a], R15K, K30Q, S67A, **R76K**[b,c], L78V, R91K, K95R, K103R, N126R		A146P, S148T, V215M, T242N, N252S, R286K, T303V, E312D				Q386P, K411R			S465F, Q476P, K481Q, L483K, Y484S, S498*	**K20T**[c], R41K, H69R, V77I
		C1	R15K, S67A, **R76K**[b,c], L78V, R91K, K95R, T97P, K103R, N126R		–				–			–	**–**
PT3	T1	B	R15K, K30R, T84V, E93D, K113Q		V159I, S165N, Q199E, E203D, I223V, G248A, N252S, E260D, T280V, R286K, S310T, G357S		**V370A**[b]	**A374S**[b], T375V, I376M	**I389T**[b], K411R			T456S, S465L	I15V, R41K, E53D, R57K, I62V, L63P
		C1	R15K, K30R, T84V, E93D, K113Q, A115V		V159I, S165N, V168I, Q199E, E203D, I223V, G248A, N252S, E260D, T280V, R286K, S310T, G357S		–	–	–			T456S, E461K, S465L	–
	T2	B	R15K, K30R, T84V, E93D, K113Q		V159I, S165N, V168I, Q199E, E203D, I223V, G248A, N252S, E260D, T280V, R286K, S310T, G357S		**V370A**[b]	**A374S**[b], T375V, I376M	**I389T**[b], K411R			T456S, S465L	I15V, R41K, E53D, R57K, I62V, L63P
		C2	–		–		–	–	–			T456S, E461K, S465L	–
		C5	–		–		–	–	–			–	**I54V**[c], **V82A**[c], I15V, R41K, E53D, R57K, I62V, L63P
		C8	R15K, R20Q, K30R, T84V, E93D, K113Q		–		–	–	–			–	**I I54V**[c], **V82A**[c], 15V, R41K, E53D, R57K, I62V, L63P
PT4	T1	B	K28Q, E55G, G62E, **R76K**[b,c], R91K, E93D, K95R, K113Q, T122P, H124K		L268M, R286K, A326S			**T375A**[b] R380K, N382H	R387K, K388R		**S451N**[b]	S465F, E477G, I479T	E35D, N37T, L63P
		C1	–		–			–	–		–	–	–
		C11	–		–			–	–		–	–	–
	T2	B	K28Q, E55G, **R76K**[b,c], V82I, E93D, K95R, K113Q, T122P, H124K, S125N		L268M, R286K, A326S			R380K, N382H	R387K, K388R, **I389S**[b]			T456I, S465F, T470A, E477G, P478S, I479T	E35D, N37H, R41K, I62V, L63P, **A71V**[c], I93L
		C2	K28Q, E55G, G62E, **R76K**[b,c], R91K, E93D, K95R, K113Q, T122P, H124K		–			**T375A**[b] R380K, N382H	R387K, K388R		**S451N**[b]	S465F, E477G, I479T	E35D, N37T, L63P
		C10	K28Q, E55G, G62E, **R76K**[b,c], R91K, E93D, K95R, E107G, K113Q, T122P, H124K		L268M, T280A, R286K, A326S			**T375A**[b] R380K, N382H	R387K, K388R		**S451N**[b]	E461G, S465F, E477G, I479T	E35D, N37T, L63P

Continued

Table 4. *Continued*

Patient	Timepoint	Sequence	P17	P17/P24	P24	P24/P2	P2	P2/P7	P7	P7/P1	P1/P6	P6	Protease
PT6	T1	B	**E12K**[a], **R76K**[b,c], R91G, K95R, K103R, K110C, Q117L		I147L, V159I, S173T, I223V, G248A, N252S, T280V, R286K, E312D, G357S		**V370A**[b]	**S373P**[b], M378V	**I389T**[b], K418R, D425E			S465F, E477G, P478T, L483I, L486W	N37S, I64V
		C6	–		I147L, V159I, S173T, I223V, G248A, N252S, T280V, R286K, E312D, C330F, G357S		–	–	–			–	–
		C7	–		–		–	–	–			–	–

Mutations associated with exposure or resistance to PIs are shown in bold. An asterisk represents a stop codon.
[a]Exposure to PIs *in vitro*.
[b]Exposure to PIs *in vivo*.
[c]Associated with PI resistance.

PT3.[34] Similarly, viruses from PT1, PT3 and PT6 were fully susceptible to lopinavir with the exception of the clonal viruses 5 and 8 from PT3 at T2 (Table 4), which carry I54V and V82A protease mutations able to replicate at 100 nM lopinavir (Figure 4b). In contrast, viruses from PT4 T1C1, T1C11 and T2C2 revealed low susceptibility to darunavir with a median of 9.6-, 6.7- and 13.58-fold increases in infectivity compared with the WT at 1 nM (Figure 4a). Concomitantly, the virus PT4 T2C2 had a median increase of 4.43-fold with 1 nM lopinavir, indicating a potential phenotype of darunavir and lopinavir cross-resistance. Replication kinetics in the presence of 1 nM darunavir for viruses T1C1, T1C11 and T2C2 from PT4 showed high replication and a significant increase in viral growth compared with the WT (Figure 4c, d). Sequences of T1C1, T1C11 and T2C2 from PT4 revealed identity in the Gag-protease genotype as shown in Table 4. Mutations R76K in p17, T375A in p2/p7 and S451N in p1/p6 were present, and have been previously associated with exposure to PIs *in vivo* but not directly with drug resistance to darunavir. Moreover, we observed three additional amino acid differences (E107G in p17, T280A in p24 and E461G in p6) in the low-replicating susceptible variant T2C10 from PT4 (Table 4). These data suggest sequence context dependency of Gag for darunavir resistance.

To further understand the nature of Gag mutational changes in clonal virus with low susceptibility to darunavir, we mapped the observed Gag mutational changes in solved p17 and p24 crystal structures. As shown in Figure 5, we mapped eight residues in p17 and three residues in p24. In p17, the residues were distributed at positions K28Q, E55G, G62E, R76K, R91K, E93D, K95R and K113Q (Figure 5a). Mutations were located between helix 1/2 (K28Q) and the globular domain of helix 3 (E55G, G62E), which are essential for protein structural stability and interactions with helix 4 (R76K) of p17. Also, we found variations at the helix 4/5 flap region (R91K, E93D and K95R) and helix 5 (K113Q). In p24, we mapped mutational changes at helix 7 (L268M), at the linker between helix 7 and 8 (R268K) and at the helix 9/10 flap region (A326S) (Figure 5b). Of note, all the darunavir-resistant clonal variants harboured the K95R and R268K signature mutations identified by VESPA and both positions are involved in structural flexibility of p17 and p24 tertiary structures. Altogether, these data suggest that susceptibility to darunavir can be affected by Gag mutations alone that have no impact on viral replication.

Discussion

The efficacy and safety of boosted PI-containing regimens supports their clinical use worldwide. However, the mechanism of virological escape from PI-based regimens remains not fully understood. In fact, the VF to boosted PIs occurs in most cases in the absence of drug resistance mutations in the protease. The emergence of mutations outside protease has been extensively described as one possible cause of this failure to boosted PIs.[20–26,39] These studies support the contribution of Gag and gp41 mutations in HIV-1 resistance to PIs[21,29,40] and underpin the complexity of the mechanisms that cause resistance. Nevertheless, the lack of protease resistance mutations leads to difficult clinical decisions, particularly in low income resource settings, where PIs are the second-line regimens recommended by the WHO.

Here, we focus on a homogeneous group of cART-experienced B-clade HIV-1-infected individuals with lopinavir/ritonavir or

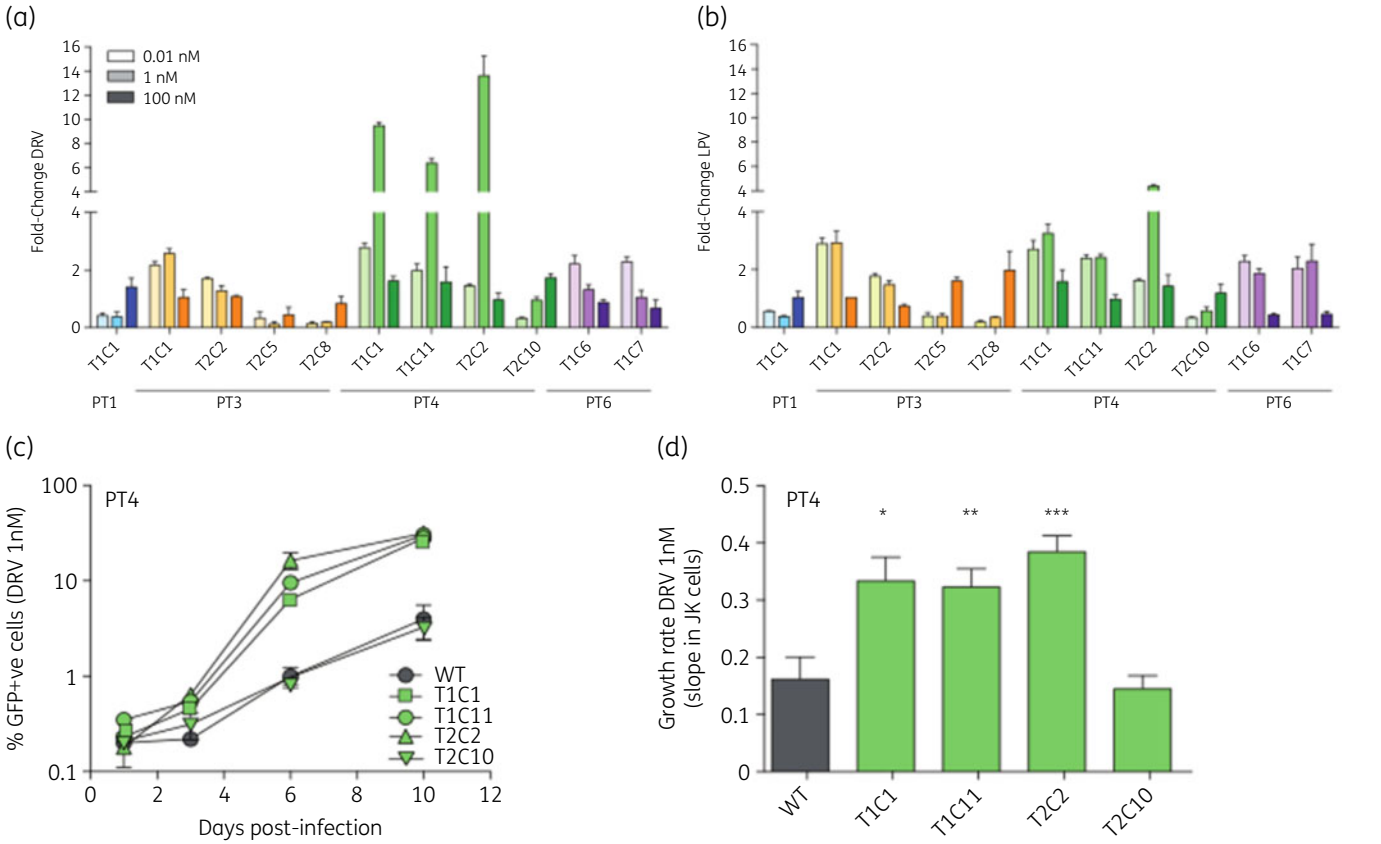

Figure 4. Drug susceptibility to lopinavir and darunavir of Gag-protease recombinant virus. (a) Fold change in darunavir (DRV) or (b) lopinavir (LPV). Jurkat (JK) cells were incubated with 0.01, 1 and 100 nM of darunavir or lopinavir for 4 h prior to infection with the Gag-protease recombinant virus. The fold change was calculated as the ratio between the percentage of GFP+ cells in the Gag-protease recombinant virus and the percentage of GFP+ cells at Day 6 after infection. Bars represent the mean ± SD of three experimental replicates. (c) Replication kinetics of PT4 Gag-protease recombinant virus in the presence of 1 nM of darunavir in Jurkat cells over 10 days in culture. (d) Bar graph representing the growth rate of WT and PT4 recombinant variants calculated as the slope of GFP-expressing cells by FACS during the replication kinetics experiments in the presence of 1 nM of darunavir. Bars represent the mean ± SD of three experimental replicates. The *P* values indicate comparisons of the recombinants with the WT. Only significant *P* values (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$) are represented. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

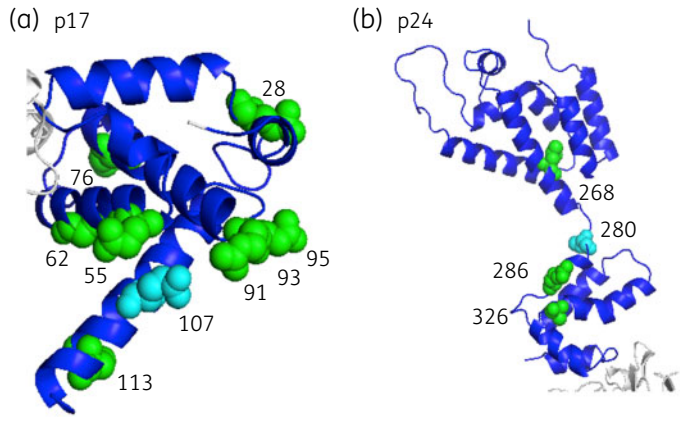

Figure 5. Gag protein structural mapping. Solved crystal structure of p17 (a) and p24 (b). The green spheres indicate positions with changes when compared with HXB2, whereas changes present only in T2C10 are indicated by cyan spheres. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

darunavir/ritonavir monotherapy who experienced VF. This design contrasts with previous studies that mainly focused on cART-naive individuals with a diversity of HIV-1 subtypes.[18,22,30] In this way, we avoid confounding effects due to cART or viral subtype. Moreover, we performed a detailed phenotypic analysis of clonally co-evolved Gag-protease viruses in terms of viral replication and drug susceptibility. Thus, our study provides novel insights on Gag signature mutations and phenotypic pathways towards VF to boosted PIs in the absence of protease drug resistance mutations.

Our findings agree with previous studies as we did not find drug resistance-associated mutations in the protease during VF.[16,25,26,39,41] Regarding Gag sequences, we observed changes in the CS, but predominantly in NCS locations. These data support our previous findings of the predominance of Gag NCS mutations during Gag-protease co-evolution.[27] The mutations in Gag CS and NCS are likely polymorphic changes relative to the consensus sequence in the absence of baseline sequences for each patient that could identify mutations within a patient over time.

At the CS, we observed mutations previously associated with exposure to PIs.[26,42–44] We observed mutations in p2/p7 (S373P,

A374S/P and T375A). S373P has been associated with a weaker virological response to saquinavir/ritonavir[42,43] and A374S/P and T375A have been found at increased frequency in PI-experienced individuals.[40] Also, we observed in p7/p1 the mutations K436R and I437V, both of which have been associated with PI exposure *in vivo* and *in vitro* and PI resistance in the absence of protease drug resistance mutations.[20,45] Moreover, in p1/p6 we identified S451N, which has been previously associated with PI exposure in non-B clade HIV-1 subtypes.[30,41] In addition, at NCS locations we found changes mainly in p17 (E12K, R76K and T81A), p2 (V370 A/M) and p7 (I389T) previously associated with resistance to PIs.[16,25,26,41,46] The R76K mutation has been previously associated with changes in viral fitness and susceptibility to PIs but always in the context of highly resistant HIV-1 proteases.[25,39] The E12K/D mutation has been previously detected *in vitro* during selection experiments with amprenavir in the presence of additional PI resistance mutations.[16,46]

Moreover, we identified two signature mutations (K95R in p17 and R286K in p24) in Gag NCS that were enriched in boosted PI-treated subjects during VF according to VESPA analyses. The R286K mutation has been recently described as an emerging mutation in two patients infected with the HIV-1 recombinant CRF02_AG during VF to darunavir.[47] The identification of K95R and R286K should be further confirmed in a larger number of sequences, but it could potentially help to classify patients with VF to boosted PI. However, we need to emphasize that our study group was receiving PI monotherapy, and this particular drug pressure may lead to a specific resistance profile.

In this study, we obtained 11 clonally co-evolved Gag-protease viruses across four patients and time. The low number of clones obtained may be associated with the low levels of viral load at VF. Phylogenetic analyses of clonal sequences identified low intrapatient diversity and close sequence identity to the bulk sequences. In addition, we detected a small cluster of sequences from PT3 at T2 with mutations I54V and V82A in the protease, which have previously been associated with the loss of viral fitness.[44,48,49] However, the contribution of these clonal variants to VF is limited based on the phenotypic information obtained.

In terms of functional characterization, the majority of clonal viruses demonstrated a conserved replicative capacity and full susceptibility to darunavir or lopinavir. Therefore, most of the variants at VF conserve the susceptibility to lopinavir and darunavir. In this scenario, we propose that Gag mutations favour protein structural changes in the absence of fitness cost and predispose to the development of resistance to PIs.

Furthermore, we identified one out of four patients with clonally co-evolved Gag-protease variants with high fitness and low susceptibility to darunavir. This is the first study to our knowledge reporting changes in darunavir susceptibility caused by a Gag effect alone. The low-susceptibility clones displayed total clonal identity in the Gag-protease region, differing by only three amino acid residues in Gag between the resistant and susceptible variants, thus indicating the importance of the Gag sequence context in the development of low susceptibility to darunavir, together with a fitness advantage for evolution towards resistance. Previous data support the accumulation of mutations in Gag with low fitness cost as a factor contributing to acquired PI resistance.[39]

Our observation of these viruses with low impact on viral replication and low susceptibility to darunavir agrees with the molecular modelling of p17 and p24 crystal structures. This model suggests an increase in protein flexibility mediated by K95R and R286K signature Gag mutations. We hypothesize that mutational changes in Gag present at VF are associated with protein flexibility and allow the preservation of viral replication while favouring accessibility for the cleavage of Gag or incorporation of *env* as previously suggested.[28,50]

Our results have some limitations. First, our data came from observations of bulk Gag-protease sequences with a scant follow-up over time and a limited number of clonal sequences. Next-generation sequencing data may provide additional information on the presence of minority resistance variants. However, limited sample availability, long periods between sample collection and testing and VLs <200 copies/mL at VF reduced the feasibility of this approach in our study.[51–53] Second, we did not directly evaluate the contribution of HIV-1 *env* as an alternative mechanism to modulate PI resistance in combination with Gag.[21,27–29]

In summary, our study provides for the first time evidence that mutational changes in Gag alone can reduce susceptibility to darunavir while preserving viral fitness. Signature mutations (K95R and R286K) in Gag may be crucial for the development of VF to boosted PIs. However, future site-directed mutagenesis and phenotypic analyses are needed to clarify the specific contributions of K95R and R286K to viral replication and darunavir susceptibility. The identification of signature mutations in Gag and their implication in darunavir resistance will be key to the improvement of patient classification for clinical decisions after VF in order to prevent the appearance of resistant variants or favour the reintroduction of PIs. Our findings may be particularly relevant in low-income settings where VF to second-line boosted PI-based regimens limits future therapeutic options.

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Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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