Evolution of protease inhibitor resistance in HIV-1-infected patients failing protease inhibitor monotherapy as second-line therapy in low-income countries: an observational analysis within the EARNEST randomised trial

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Summary: In patients failing protease inhibitor (lopinavir) monotherapy, intermediate/high level lopinavir resistance increased from 19% at failure to 68% 48 weeks later. Most retained darunavir

susceptibility. Viral load increased slowly after failure, driven by non-adherence and PI mutation development (particularly I47A).

Abstract

Background

Limited viral load (VL) testing in HIV-infected individuals on treatment in low-income countries often results in late detection of treatment failure. The impact of remaining on failing second-line, protease inhibitor (PI) containing regimens is unclear.

Methods

We retrospectively tested VL from 2,164 stored plasma samples from 386 patients randomised to receive PI-monotherapy (ritonavir-boosted lopinavir, after initial PI+raltegravir induction) in the EARNEST trial. Protease genotypic resistance testing was performed in samples with VL>1000 copies/ml. We assessed evolution of drug resistance mutations from virological failure (confirmed VL>1000 copies/ml) until discontinuation of PI-monotherapy and examined associations using Poisson and linear mixed-effects models.

Results

118 patients had a median 68(IQR 48-88) weeks on PI-monotherapy post-failure. At failure, 21/107(20%) had intermediate/high resistance to lopinavir. 40-48 weeks post-failure, 49/72(68%) and 36/71(51%) had intermediate/high-level resistance to lopinavir and atazanavir. Most remained susceptible to darunavir (12/72[17%] intermediate, no high resistance). Common PI mutations were

M46I, I54V, and V82A. On average, 1.7(95% CI 1.5,2.0) PI mutations developed per year; this increased after the first mutation developed, but decreased with subsequent mutations (p<0.0001). Modest VL changes were mainly driven by non-adherence (p=0.006) and PI-mutation development. I47A was associated with a larger increase in $\log_{10}$ VL(+0.53[+0.18,+0.87], p=0.003) than other PI mutations (+0.15[+0.07,+0.23] p<0.001; heterogeneity p=0.05).

Conclusion

Most develop intermediate/high-level lopinavir resistance within one year when lopinavir/ritonavir is exposed to sustained VL replication without protection from other drugs. Even in this extreme situation, annual VL testing (current WHO recommendation) would identify failure when most would still benefit from switching to darunavir.

Introduction

By 2020, 0.5-3 million HIV-infected individuals will be receiving second-line antiretroviral therapy (ART) in sub-Saharan Africa [1]. The World Health Organisation (WHO) recommends a second-line regimen of two nucleoside reverse transcriptase inhibitors (NRTIs) plus one boosted protease inhibitor (PI) [2]. Understanding the implications of failure on PI-containing regimens for third-line treatment is key to informing policy on second-line monitoring and choice of third line regimens in the public health approach. WHO does not recommend a particular third-line regimen but suggests including new drugs with minimal risk of cross-resistance [2].

At second-line viral load (VL) failure, patients commonly have no PI resistance mutations [3-6]. However, the relatively limited availability of VL monitoring in low-income countries results in late detection of treatment failure, leading to PI mutation development [4-6]. The effect of continuing on a failing PI-containing regimen is largely unknown; most studies have been cross-sectional with time since failure not known [4, 5, 7-9] or have been conducted in high-income countries, where patients spend little time on failing regimens [10, 11].

Here we explore the evolution of VL and PI resistance following virological failure in patients randomised to second-line PI-monotherapy in EARNEST, a pragmatic trial of second-line ART in sub-Saharan Africa. Although PI-monotherapy is clearly not an appropriate therapeutic option, PIs remain the cornerstone of second-line therapy. As the accumulation of viral resistance among patients on monotherapy is likely more rapid than among patients on combination therapy, our

analyses provide an upper bound on the impact of the latter. The specific aims were to determine whether darunavir retains sufficient activity to be a viable third-line treatment option; whether previous mutations affect the rate of development and nature of future mutations; and to determine the effect of mutations on viral load.

Methods

EARNEST randomized participants in Uganda, Zimbabwe, Malawi, Kenya, and Zambia failing first-line therapy (based on clinical, immunological and/or virological criteria, all confirmed by VL >400 copies/ml) to receive a PI (ritonavir-boosted lopinavir) plus 2-3 NRTIs, raltegravir, or alone as monotherapy following an initial induction with 12 weeks' raltegravir [12]. Patients were followed for 144 weeks from switch to second-line with scheduled visits every 4-8 weeks. Adherence was assessed by structured questions; additional adherence counselling (approach at the discretion of the clinical sites, tailored to the individual patient) was given when lapses were identified. Treatment was monitored clinically and with CD4 counts every 12-16 weeks. VL was measured retrospectively and monitored by the Data Monitoring Committee (DMC), but results were not available to clinicians. In May 2013, on reviewing week 96 data, the DMC recommended that patients on PI-monotherapy should resume combination therapy due to inferior viral suppression and greater PI resistance compared to PI+NRTI.

This study included patients randomised to PI-monotherapy from the start of PI-monotherapy (post raltegravir induction) to the earliest of their last clinic visit or stopping PI-monotherapy for >31 days.

Plasma samples were stored at weeks 4, 12, 24, 36, 48, 64, 80, 96, 112, 128, 136, and 144. VL was measured at weeks 4-48, 96, and 144 in all PI-monotherapy patients, additionally at weeks 64, 80, 112, and 128 in those with VL>400 copies/ml at weeks 48 and/or 96, and at week 112 or 128 when this was the last visit before return to combination therapy. VL was assayed retrospectively in batches using the Abbott RealTime HIV-1 assay in a central laboratory (Joint Clinical Research Centre (JCRC), Kampala, Uganda).

Protease genotyping was performed on all samples with VL>1000 copies/ml obtained at week 48, 96, or 144. Additional genotyping was performed on all samples after week 24 with VL>1000 copies/ml in patients with PI mutations at week 48 and/or 96, and on any week 112 or 128 samples with VL>400 copies/ml when that was the last sample before returning to combination therapy. In total, 337/363(93%) genotypes were performed at Virco (Beerse, Belgium) and 26(7%) (from one site with delayed shipping) at a WHO-designated and College of American Pathologist accredited laboratory (JCRC, Kampala, Uganda) (see supplementary text for details of the methods used at each laboratory). PI mutations were defined following the International Antiviral Society-USA [13], excluding minor PI polymorphisms not associated with drug resistance, following WHO recommendations [14]. Susceptibility was predicted using the Stanford algorithm version 7.0, on full sequence data [15]. Subtype was determined by the REGA algorithm version 3.0.9 [16]. Sequences were submitted to GenBank (MG549462-MG549824).

Statistical analysis

We defined virological failure as failure to supress VL<1,000 copies/ml by week 24 (12 weeks after stopping raltegravir induction), confirmed VL>1,000 copies/ml (first sample as date of failure), or final VL>1,000 copies/ml (unconfirmed).

Patients were PI-naive at trial entry (no protease genotypes performed at enrolment); we therefore considered PI mutations absent in genotypes as absent at previous time points when genotypes were not assayed since intermediate time points were only assayed if PI mutations were detected at weeks 48/96. Sensitivity analyses did not carry absence of PI mutations backwards or included only week 48, 96 and 144 samples.

We used univariable and multivariable logistic regression to explore associations between PI mutations at failure and adherence and viral subtype. Backward elimination (exit p>0.1) was used to adjust for confounders, including patient demographics (age, sex), characteristics at second-line switch (log_{10} VL, CD4, time on first-line ART), and characteristics on second-line ART before failure (proportion of samples with VL>50 copies/ml, log_{10} virological copy-years [17], time on PI-monotherapy). Adherence was defined by the proportion of previous visits on second-line with self-reported non-adherence or not adhering to the trial visit schedule. Fractional polynomials were used to incorporate non-linearity of continuous factors. This analysis included 72 patients with genotypes at failure (supplementary table 1) and 35 patients who did not have a genotype at failure but had no PI mutations in their first observed genotype (total=107).

After failure we analysed accumulation of PI mutations, including mutations that had been observed previously, assuming they were archived in the viral reservoir (so total mutation counts never decreased). Mutations present in one sample were rarely absent in subsequent samples; this occurred 49 times across 440 mutations with subsequent samples post-detection (mean 2.17 post-detection samples per mutation totalling 956 tests).

Associations between each mutation that appeared in at least 5 patients and the presence of other mutations in the previous sample were assessed using exact logistic regression.

We modelled the rate of mutation development using Poisson models, forcing into the model key exposures time since failure, adherence, subtype, and number of PI mutations in the previous sample. Backward elimination was used as above, including additional factors: VL at second-line failure, previous VL, and previous CD4. Adherence was time-updated. Interactions with time since failure were retained if $p<0.05$.

Change in $\log_{10}$ VL post-failure was modelled using linear-mixed-effect models with random effects for patient and time since failure (unstructured covariance). Associations were explored as for mutation development excluding previous VL and VL at switch to second-line. Viremia copy-years before failure and time on second-line before failure were collinear (Spearman's correlation=0.84) with opposing effects in the final model; only time on second-line before failure was retained due to its superior fit.

Results

We randomised 418 patients failing first-line therapy (43% with VL>100,000 copies/ml; 62% with CD4<100 cells/mm^3) to PI-monotherapy. 386(92%) had at least one VL from week-24 and were considered for inclusion in this analysis (supplementary figure 1). They spent a median 108(IQR 99-124) weeks on PI-monotherapy, with VLs obtained at 2164/2712(80%) scheduled visits (supplementary table 1; assays not run if suppression maintained).

Virological failure occurred in 134/386(34%) patients, with genotypes at or post failure for 118/134(88%). These 118 patients experienced virological failure after a median 36(IQR 12–68) weeks on PI-monotherapy and were followed for a median 68(IQR 48-88) weeks subsequently. 104(88%) reintroduced combination therapy at trial exit or following DMC recommendations and 8(7%) following clinical/immunological failure; 4 died (Table 1). Mortality, WHO4 clinical events and CD4 response was similar to the standard-of-care PI+NRTI arm at week 96/144 [12, 18]. 363/572(62%) genotypes were available from samples stored at or after failure (supplementary table 1), with a median 3(IQR 2-4) genotypes per patient.

Overall, 86/118(73%) patients developed at least one PI mutation. The most common were M46I, I54V, and V82A, present in 11/114(10%), 14/112(13%), and 13/113(12%) patients, respectively, at failure (figure 1), rising to 23/77(30%), 34/79(43%), and 31/77(40%), respectively, 40-48 weeks after failure (denominators vary due to assumptions that absent mutations were absent in previous ungenotyped samples and that mutations accumulate).

Development of I47A was associated with the absence of I54V in the previous sample (odds ratio [OR] =0.06 (95% CI NE(not estimable), 0.38) p=0.0008); conversely I54V was associated with the absence of I47A in the previous sample (OR=0.05 (NE, 0.30) p=0.0003). I47A was commonly followed by V32I (OR=26.91 (3.14, 292.28) p=0.002). Other associations (all p>0.01) are shown in supplementary table 2.

At failure, 21/107(20%) patients had intermediate/high level resistance to lopinavir (10% intermediate, 9% high); this increased to 49/72(68%) 40-48 weeks after failure (19% intermediate, 49% high, figure 2). Rates of intermediate/high-level resistance to atazanavir were similar: at failure,

15/107(14%) had intermediate/high level resistance (7% intermediate, 7% high), increasing to 36/71(51%) 40-48 weeks after failure (18% intermediate, 32% high). Most patients retained susceptibility to darunavir; one had intermediate- and none had high-level darunavir resistance at failure; 40-48 weeks after failure, 12/72(17%) had intermediate-level resistance and none had high-level darunavir resistance. One patient developed high-level darunavir resistance after failure (at 64 weeks, with mutations M46I, I47V, L76V, V82F, I84V, and I54V).

28/107(26%) patients had at least one PI mutation at failure (table 1). This was independently associated with longer duration of second-line before failure (OR per year 4.97 (95% CI 1.90,12.97) p=0.001) and weakly associated with viral subtype (overall p=0.06, OR(C vs A)=3.77 (1.28,11.09) p=0.02, OR(D vs A)=2.03 (0.41,10.02) p=0.38), but was not associated with adherence (p=0.17) (univariable associations in supplementary table 3).

After 40-48 weeks of failure, 13/58(22%) patients had no mutations, and 17/58(25%) had 4 or more mutation (figure 3). On average, patients developed 1.7(95% CI 1.5,2.0) new mutations per year. In a multivariable model (table 2), a second mutation developed faster than the first, but subsequent mutations took progressively longer to emerge than the second (p<0.0001). Mutations emerged faster in those with higher CD4 counts in their previous sample (p=0.03), who were older (p=0.0002), and with longer times since failure (p=0.01). There was no evidence of associations with adherence (p=0.67), and a weak effect of subtype (p=0.11) and VL at switch to second-line (p=0.07). There was no evidence that any specific mutation had a different effect than the average mutation effect (p>0.12) or of any effect changing over time (interaction p>0.08). Substituting resistance to lopinavir for number of mutations in the previous sample, there was borderline evidence that a higher level of

resistance to lopinavir in the previous sample reduced the rate of development of subsequent mutations (p=0.03, supplementary table 4).

The median VL at failure was 3,725(IQR 1,941-15,024) copies/ml (table 1), increasing by 0.51(95% CI 0.34, 0.67; p<0.0001) log_{10} copies/ml per year of post-failure follow-up. VL increased with each new PI mutation (p=0.0002, table 3, supplementary figure 2) with a significantly larger increase associated with I47A(p=0.003, heterogeneity vs other mutations p=0.05). Higher self-reported adherence(p=0.006), more time on second-line ART before failure(p=0.005), more time on first-line ART(p=0.02), and older age(p=0.03) were associated with lower VL. There was no association with subtype(p=0.45). VL plateaued after developing of I47A (-0.42 (-0.99, +0.14) log_{10} copies per year vs +0.29 (+0.12, +0.46)log_{10} copies per year without I47A (interaction p=0.01, supplementary table 5; no modification of effect on VL when I47A was combined with V32I, interaction p=0.95).

All results were similar not carrying the absence of mutations backwards (supplementary figures 3-4, supplementary tables 6-8).

Discussion

In this study of VL failure on lopinavir monotherapy, we found that patients often failed without PI mutations, but that resistance accumulated with longer time on the failing regimen. Most patients retained susceptibility to darunavir up to one year after failure and VL increases were modest.

~20% of patients had intermediate/high-level lopinavir resistance at virological failure, rising to ~70% one year post-failure. As the proportion with comparable atazanavir resistance was only ~10-

20% lower at the same time-points, there would be little advantage in switching empirically (i.e. without resistance testing) from lopinavir to atazanavir after treatment failure, other than for possible advantages of convenience or tolerability that might improve adherence and hence viral suppression. However, over half of all patients had virus that remained fully susceptible to darunavir one year after failure, supporting its suitability as a potential third-line option. Other studies have noted this [4, 5, 7], but our much larger sample size and more frequent sampling allowed more accurate assessment of resistance evolution and greater confidence in the findings. The benefit of resistance testing in this setting is uncertain [19]. Testing may identify some patients with no PI resistance who might re-supress with additional adherence counselling [20], delaying the need to switch to a costly third-line regimen. The few patients that have developed darunavir resistance may benefit from a non-standard third-line regimen, if available.

Many patients had virological failure without resistance, a well-known finding for PI-based regimens [4, 21-23]. Mutations in other viral regions (e.g. gag) [21, 24-26] and adherence [20] have both been suggested as causes. We found no evidence that adherence affected resistance at failure, and the low median VL at failure suggest the cause is unlikely to be due to complete non-adherence. Adherence support was provided throughout this trial [20] and importantly, most re-suppressed after NRTIs were reintroduced [18].

Our finding of low prevalence of mutations/resistance at failure followed by progressive accumulation is consistent with studies of PI-based combination regimens in high- and low-income countries [5, 10].Our study size allowed us to explore factors associated with mutation rate to a greater degree than previously possible. The main factor accelerating mutation development was the number of prior PI-mutations. This relationship was complex, with the rate increasing after the

first mutation, but then subsequently decreasing with additional mutations (i.e. reaching a "ceiling" effect). This may be mediated through the effects of mutations on variability in the virus population [25, 27]. It may also indicate the balance between maintaining replicative capacity and overcoming drug presence (a fitness effect, see below). Previous studies have noted this "ceiling effect" [28], and found the rate increased after the first mutation [5]; ours is the first study to combine these findings. The increase in mutation rate we found with higher previous-sample CD4 cell count (but not VL), could reflect a greater number of target cells for replication under drug pressure.

Despite varying subtypes, the pattern of resistance-mutations we found is similar to that in other studies of PI-based regimens [7, 23, 29-31]. I47A was often followed by V32I, and developing I47A was associated with a lower odds of developing I54V and vice versa. This has important consequences for third-line ART, as I47A confers intermediate-level resistance to darunavir and V32I is suggested as a key mutation for high-level resistance [32, 33], whereas I54V is not known to affect darunavir response [13]. We also frequently observed the V82A mutation¸ thought to improve susceptibility to darunavir [34].

We found that VL increased only modestly after failure, driven mostly by low adherence and development of PI mutations. Our finding of an initial relatively large increase in VL followed by a plateau in replication after I47A development is supported by in vitro evidence [33, 35]; contrary to this literature, we did not find a difference in virus that also had the V32I mutation, although numbers were small. Other PI mutations had comparatively modest effects as observed elsewhere [36], although not all studies have observed this effect [10]. The association between adherence and VL is unsurprising [37], but its impact was relatively small. Other factors associated with VL after

failure (age, time on first-line ART, time on second-line ART before failure) are possible surrogates for adherence.

This trial was designed pragmatically in 2008/9 to test second-line options in settings without regular VL monitoring, which still remains limited in many areas in sub-Saharan Africa. We included PI-monotherapy because, at that time, this was considered a potential second-line option for the public health approach [38, 39], albeit with some risk, that merited testing in a randomised controlled trial. We included monitoring of outcomes and the DMC stopped the arm when it had been demonstrated definitively that PI monotherapy was not viable in this setting. At least one subsequent trial in sub-Saharan Africa again confirmed high rates of VL rebound with PI-monotherapy and was stopped by their DMC [40]. Although PI-monotherapy is clearly not an appropriate therapeutic option, our findings remain relevant for patients on combination therapy in sub-Saharan African programs. The resistance profile resembles that seen with combination PI regimens and is unlikely to be influenced by NRTIs, but the overall rate of PI resistance development would likely be slowed with NRTIs (through direct effects on VL replication, or indirect effects on viral fidelity/fitness) [41]. Our results therefore represent a more extreme case than would likely been seen in practice. Here, the second-line PI was standardized to lopinavir/ritonavir. There are no comparable data for atazanavir in second-line therapy in program settings, although this is also a preferred PI in current WHO guidelines [2]. Whilst there are differences in mutation development between these PIs, our conclusion about likely success of an empirical switch to darunavir is also likely to apply to atazanavir failure in second-line.

Study strengths are its size (largest longitudinal study investigating PI resistance following failure in low-income countries), monitoring approach (VL testing was not influenced by patients' status,

removing a potential source of bias), and broad inclusion criteria. The key limitation is that patients were taking PI-monotherapy, an ineffective second-line regimen [12, 40, 42], as discussed above. Caution is needed in drawing conclusions beyond 40-48 weeks after failure due to smaller numbers in follow-up after this time and likely selective drop-out. We measured adherence by self-report, which may be inaccurate. We made assumptions around persistence/absence of resistance mutations to account for our sampling strategy, but results were similar in sensitivity analyses that explored alternative assumptions. Our sampling strategy may have led us to miss mutations that were selected and lost between weeks 48 and 96. However, mutations rarely disappeared in our data, suggesting that this is uncommon.

In summary, most patients develop virus with intermediate/high-level resistance within one year of failure when lopinavir/ritonavir is exposed to sustained VL replication without protection from other drugs. Even in this extreme situation, annual VL testing (current WHO recommendation) would identify failing patients at a time when most would likely still benefit from switching to darunavir as third-line.

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JH has received a grant for clinical trial support from NIH. AK has received person fees from Abbvie to attend an HIV meeting, Gilead Sciences to attend an HIV meeting and for speaking, and from Merck for speaking. JA has received personal fees for speaking and participation in an advisory board from Merck, ViiV, Gilead Sciences, Janssen, and Alexa. ASW has received funding from Gilead Sciences to her institution for lecturing and from Janssen for DSMB membership. NP has received personal fees for speaking (from Abbvie and Janssen), and chairing an advisory board (from Roche). NP has also received a grant from GSK for a TB trial, and provision of trial drugs from GSK and Sanofi. All other authors have no potential conflicts of interest.

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Table 1: Characteristics of patients on PI-monotherapy failing second-line ART who had one or more genotype test after failure

	N (%) / median (IQR)
Characteristic	**Total N= 118**
PI-mutations at failure carrying back absences of mutations (N=107) [a]	
None	79 (74%)
Major only	12 (11%)
Minor only	5 (5%)
Both	11 (10%)
PI-mutations at failure in those with genotype assayed (N=72)	
None	44 (61%)
Major only	12 (17%)
Minor only	5 (7%)
Both	11 (15%)
Country	
Malawi	11 (9%)
Uganda	82 (69%)
Zimbabwe	23 (19%)
Kenya	2 (2%)
Female	46 (39%)
Age (years)	38 (32-44)
Subtype	
A	56 (47%)
C	36 (31%)
D	16 (14%)
Recombinant	10 (8%)
First-line characteristics	

Years on first-line	3.6 (2.2-5.3)
CD4 at switch to second-line (cells/mm3)	
median (IQR)	66 (28-140)
<100 cells/mm3	78 (66%)
VL at switch to second-line (copies/ml)	
median (IQR)	142,320 (29,485-416,971)
>100,000 copies/ml	68 (58%)
Second-line characteristics	
Weeks to VL failure	36 (12-68)
VL at failure (copies/ml)	3,725 (1,941-15,024)
Weeks follow-up after VL failure	68 (48-88)
Reason for discontinuing monotherapy	
Trial exit/DMC decision	104 (88%)
Treatment failure	8 (7%)
Died [b]	4 (3%)
Patient decision/ missed visit/ pregnancy	2 (2%)

[a] 72 patients had a genotype at failure, 35 patients did not have a genotype at failure but had no mutations present in the next genotype so were assumed to have no mutations at failure. 11 patients had no genotype at failure but the subsequent genotype identified some mutations, so the genotype at failure could not be determined.

[b] 1 death of unknown cause (32 weeks post-failure), one due to HIV-related indeterminate cerebral disease (10 weeks post-failure) one due to intestinal obstruction (68 weeks post-failure), one intestinal perforation (100 weeks post-failure).

Note: subtype based on any available genotype, including baseline reverse transcriptase only.

Table 2: Independent predictors of mutation development after VL failure

Factor	**Unadjusted Rate Ratio (95% CI)**	**p value**	**Adjusted Rate Ratio (95% CI)**	**p value**
Weeks since failure [a]		0.13		0.01
16	1		1	
32	1.18 (0.95, 1.46)		1.37 (1.06, 1.76)	
48	1.25 (0.94, 1.66)		1.52 (1.09, 2.13)	

64	1.28 (0.93, 1.76)		1.60 (1.10, 2.34)	
Mutations in previous sample [b]		<0.0001		<0.0001
0	1		1	
1	2.07 (1.48, 2.88)		1.78 (1.25, 2.54)	
2	1.46 (1.09, 1.95)		1.15 (0.84, 1.59)	
3	1.01 (0.73, 1.39)		0.74 (0.52, 1.07)	
4	0.72 (0.48, 1.09)		0.51 (0.33, 0.80)	
5	0.54 (0.33, 0.90)		0.37 (0.21, 0.64)	
Adherence per 10% higher	1.03 (0.95, 1.11)	0.52	0.98 (0.90, 1.07)	0.67
Viral subtype		0.62		0.11
A	1		1	
C	0.98 (0.71, 1.36)		0.81 (0.57, 1.16)	
D	0.95 (0.65, 1.39)		0.65 (0.43, 0.99)	
Other	0.66 (0.35, 1.23)		0.55 (0.29, 1.04)	
CD4 in previous sample per 100 cell higher	1.13 (1.02, 1.25)	0.02	1.13 (1.01, 1.27)	0.04
VL at switch to second-line per $\log_{10}$ higher	1.17 (0.96, 1.42)	0.13	1.23 (0.98, 1.54)	0.07
Age [c]		0.0001		0.0002
20	1		1	
30	2.72 (1.63, 4.54)		2.81 (1.69, 4.69)	
40	3.15 (1.70, 5.84)		3.08 (1.66, 5.71)	
50	2.55 (1.34, 4.85)		2.21 (1.15, 4.24)	

Poisson model with time since failure, adherence and subtype forced into the model. Other factors selected using backward elimination (exit p>0.1). Factors not selected: age, sex, years on first-line, CD4 at switch to second-line, proportion of VL>50, viremia copy-years, or time on second-line before failure, VL in previous sample, VL or CD4 second-line failure.

[a] Included in model using fractional polynomials with 2 d.f as week $^{-1}$. P=0.03 for comparison to linear effect in final model

[b] Included in model using fractional polynomials with 4 d.f as previous-mutations $^{-2}$ + ln(previous-mutations). P=0.002 for comparison to linear effect in final model

[c] Included in model using fractional polynomials with 4 d.f as age $^{-2}$ + age ^{3}. P<0.0001 for comparison to linear

effect in final model

Table 3: Independent predictors of change in VL after VL failure

	Unadjusted		Adjusted	
Factor	**Effect on log_{10}(VL) (95% CI)**	**p value**	**Effect on log_{10}(VL) (95% CI)**	**p value**
Time since failure per year longer	+0.47 (+0.30, +0.64)	<0.0001	+0.25 (+0.08, +0.43)	0.004
Mutations in previous sample				
I47A	+0.56 (+0.22, +0.90)	0.001	+0.53 (+0.18, +0.87)	0.003
Other	+0.11 (+0.03, +0.18)	0.006	+0.15 (+0.07, +0.23)	0.0002
Adherence per 10% higher	-0.10 (-0.15, -0.04)	0.0004	-0.08 (-0.13, -0.02)	0.006
Subtype		0.23		0.44
A	1		1	
C	+0.07 (-0.19, +0.34)		+0.08 (-0.18, +0.34)	
D	+0.20 (-0.16, +0.56)		+0.15 (-0.18, +0.47)	
Other	+0.44 (-0.01, +0.88)		+0.31 (-0.09, +0.71)	
Time on second-line before failure per year longer	-0.40 (-0.59, -0.20)	0.0001	-0.34 (-0.57, -0.10)	0.005
Age per 10 years older	-0.12 (-0.23, -0.01)	0.03	-0.11 (-0.22, -0.01)	0.03
Time on first-line ART per year longer	-0.08 (-0.13, -0.02)	0.009	-0.06 (-0.11, -0.01)	0.02

Linear mixed-effect model with random effects (unstructured covariance) for intercept and change over time. Adherence, and subtype forced into the model as key exposures of interest; other factors selected using backward elimination (exit p>0.1). Factors not selected: sex, CD4 at switch to second-line, proportion of VL>50 copies/ml on second-line before failure, and CD4 at previous sample. Viremia copy-years before failure not included due to co-linearity with time on second-line before failure.

Figure 1: Cumulative prevalence of individual mutations at failure (darkest), 40-48 weeks (medium), and 80-120 weeks after failure (lightest). Denominators vary due to assumptions that absent mutations were absent in previous ungenotyped samples and that mutations accumulate. At failure: N=112-118, 40-48 weeks N=73-79, 80-120 weeks after failure N=26-28. See supplementary figure 3 for sensitivity analyses of missing data assumptions.

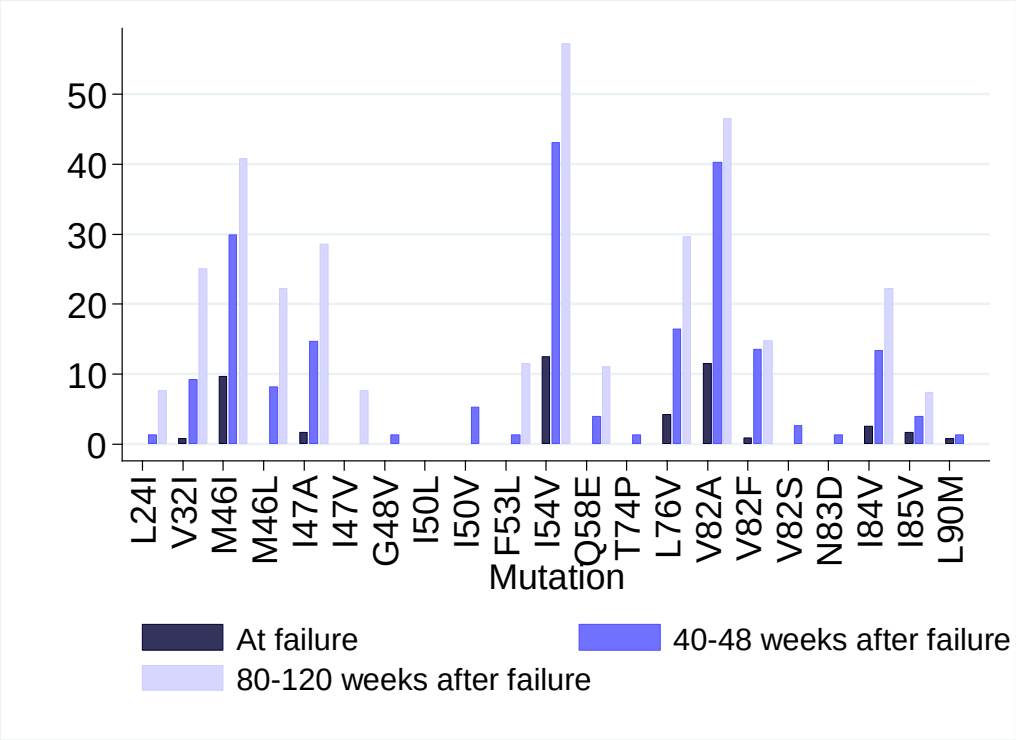

Figure 2: Predicted drug susceptibility of (a) lopinavir (b) atazanavir and (c) darunavir by time since second-line virological failure. Denominators vary due to assumptions that absent mutations were absent in previous ungenotyped samples and that mutations accumulate. See supplementary figure 4 for sensitivity analyses of missing data assumptions.

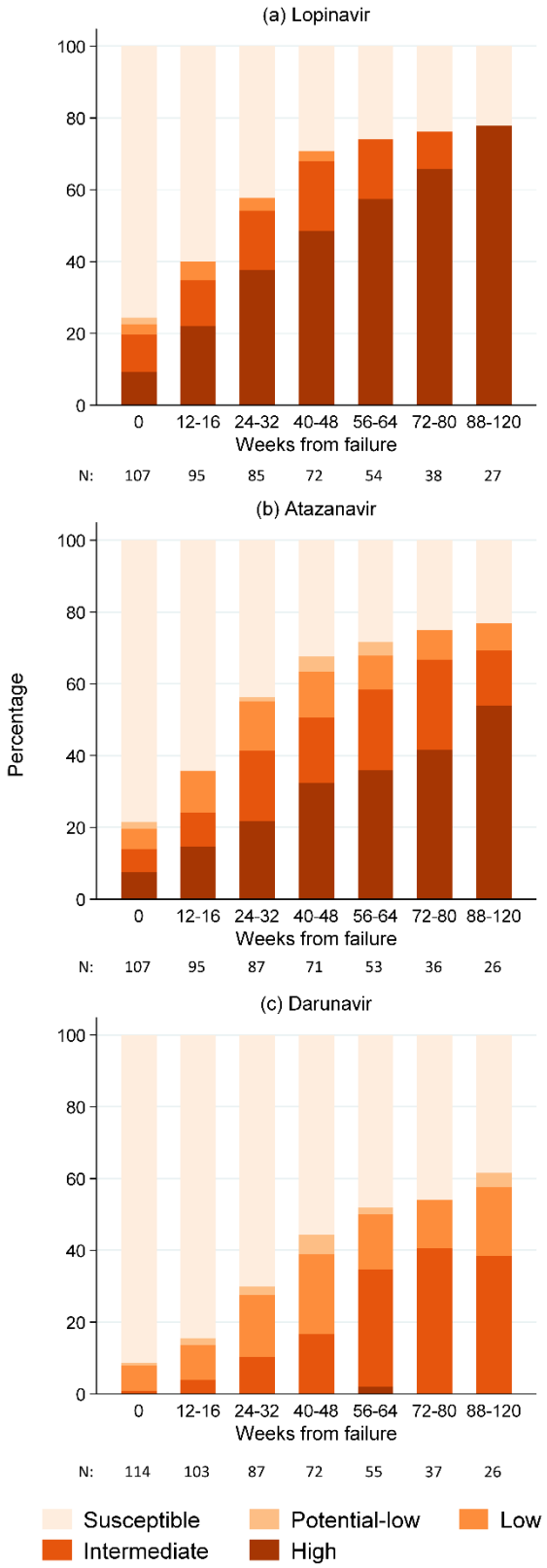

Figure 3: Number of PI-mutations present by time since second-line virological failure. See supplementary figure 5 for sensitivity analysis of missing data assumptions.