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Original article

Resistance profiles after different periods of exposure to a first-line antiretroviral regimen in a Cameroonian cohort of HIV type-1-infected patients

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Background: The lack of HIV type-1 (HIV-1) viral load (VL) monitoring in resource-limited settings might favour the accumulation of resistance mutations and thus hamper second-line treatment efficacy. We investigated the factors associated with resistance after the initiation of antiretroviral therapy (ART) in the absence of virological monitoring.

Methods: Cross-sectional VL sampling of HIV-1-infected patients receiving first-line ART (nevirapine or efavirenz plus stavudine or zidovudine plus lamivudine) was carried out; those with a detectable VL were genotyped.

Results: Of the 573 patients undergoing VL sampling, 84 were genotyped. The mean number of nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) mutations increased with the duration of ART exposure (P=0.02). Multivariable analysis showed that patients with a CD4$^+$ T-cell count ≤50 cells/mm^3 at ART initiation (baseline) had a higher mean number of both NRTI and non-NRTI (NNRTI) mutations than those with a baseline CD4$^+$ T-cell count >50 cells/mm^3 (2.10 versus 0.56; P<0.0001; and 1.65 versus 0.76; P=0.005, respectively). A baseline CD4$^+$ T-cell count ≤50 cells/mm^3 predicted ≥1 NRTI mutation (adjusted odds ratio [AOR] 7.49, 95% confidence interval [CI] 2.20–32.14), ≥1 NNRTI mutation (AOR 4.25, 95% CI 1.36–15.48), ≥1 thymidine analogue mutation (AOR 8.45, 95% CI 2.16–40.16) and resistance to didanosine (AOR 6.36, 95% CI 1.49–32.29) and etravirine (AOR 4.72, 95% CI 1.53–15.70).

Conclusions: Without VL monitoring, the risk of drug resistance increases with the duration of ART and is associated with lower CD4$^+$ T-cell counts at ART initiation. These data might help define strategies to preserve second-line treatment options in resource-limited settings.

Introduction

Increasing access to antiretroviral therapy (ART) in resource-limited countries [1] has raised concerns about resistance to initial regimens and its effect on second-line therapy [2]. The first-line regimen recommended by the World Health Organization (WHO) [3] includes the non-nucleoside/nucleotide reverse transcriptase inhibitor (NNRTI) plus lamivudine plus another nucleoside/nucleotide reverse transcriptase inhibitor (NRTI), which is often a thymidine analogue (TA) because of fixed-drug combinations (FDCs) and their low price. The lack of viral load monitoring limits the early detection of virological failure, thus favouring the accumulation of drug-resistance-associated mutations [4–6] and possibly accounting for the higher prevalence of NNRTI resistance mutations, M184V mutation and TA mutations [TAMs] in cohorts from resource-poor countries [7]. It has been found that surrogate markers of virological failure, such as CD4$^+$ T-cell counts or body weight, are poor predictive markers [8–10]. Furthermore, their use in deciding when to switch treatment is still controversial

[11–13]: if the decision is made too late, there might be an excessive accumulation of resistance and if it is made too early, it might lead to an unnecessarily premature start of a more expensive and inconvenient protease inhibitor (PI)-based regimen. Routine viral load testing has been advocated as the best strategy for avoiding extensive resistance [14], but its use is uncommon in resource-limited settings [15].

Little is known about the consequences of continuous suboptimal drug exposure in virologically failing patients in resource-limited settings because most of the published data have been prospectively collected by means of scheduled viral load sampling [16–20]. The most frequently observed mutations at the time of the failure of regimens containing an NNRTI, lamivudine and a TA are M184V (because of the low genetic barrier of lamivudine) and K103N/Y181C (because of the low genetic barrier of NNRTIs) [21], and it is highly probable that routine viral load monitoring prevents the accumulation of TAMs. When the switch to a second-line regimen is on the basis of an immunological failure, TAMs and other clinically relevant mutations are much more frequent [22–24]. Moreover, the accumulation of NNRTI resistance mutations might jeopardize the response to second-generation NNRTIs, such as etravirine, whose sensitivity sharply decreases after >2 etravirine resistance-associated mutations (RAMs) have been selected [25,26]. Recent preliminary data from Thai and Nigerian cohorts have highlighted increased resistance to etravirine in patients failing first-line NNRTI-based ART [27,28].

In order to explore the virological consequences of the absence of routine viral load monitoring, we undertook cross-sectional viral load sampling of all treated patients being actively followed-up in a Médecins Sans Frontières (MSF) project in Yaoundé, Cameroon, with the aim of investigating whether there was a relationship between the extent of resistance and the duration of ART exposure. We also sought baseline characteristics in treatment failing patients that might predict the frequency and extent of resistance.

Methods

Setting

In 2001, in collaboration with the PRESICA project (Institut de Recherce pour le Développement UMR 145, Montpellier, France), the Military Hospital in Yaoundé (Cameroon) and the Cameroon Ministry of Public Health (Yaoundé, Cameroon), MSF started a programme in which ART was offered on the basis of the WHO recommendations and national guidelines. At the start of ART (baseline), patients received an FDC of stavudine, lamivudine and nevirapine with alternatives being offered in the case of side effects (zidovudine instead of stavudine after the onset of peripheral neuropathy) or drug–drug interactions (efavirenz instead of nevirapine for patients receiving concomitant tuberculosis treatment). Follow-up visits took place every month for the first 6 months and every 2 months thereafter. $CD4^+$ T-cell counts were monitored every 6 months, but no individual viral load monitoring was available. Support for treatment adherence was provided at each visit by trained counsellors. In the case of treatment failure, a PI-based second-line treatment was introduced as recommended by the WHO.

Study population

All of the adults who had started the ART programme 12 ±2 months before the date of the study were eligible. Their medical background and follow-up information were routinely collected at each consultation and entered into the Follow-Up and Care of HIV Infection and AIDS (FUCHIA; Epicentre, Paris, France) monitoring database.

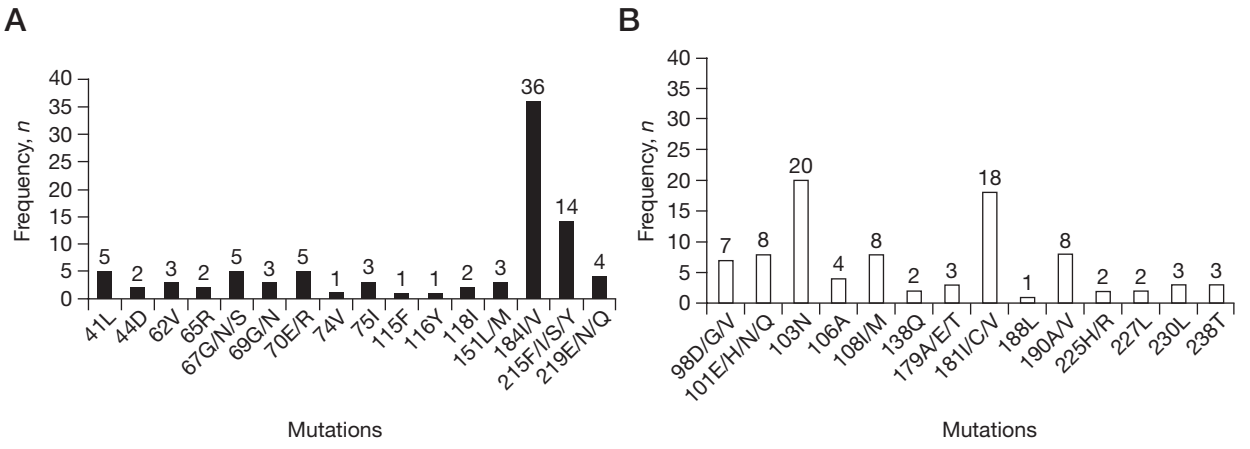
Cross-sectional survey

The cross-sectional survey was conducted from 15 September 2006 to 15 May 2007 with the aim of assessing the clinical, immunological and virological status of all eligible patients attending the clinic during the study period and their adherence to treatment.

At the survey visits, patient demographic data (age and gender), body height and weight were recorded, treatment adherence over the previous month was estimated using a modified visual analogue scale with a score ranging from one (poorest adherence) to six (highest adherence) [29] and a blood sample was taken. Patient characteristics at ART initiation (age, gender, body mass index [BMI], WHO stage and $CD4^+$ T-cell counts) were retrieved from the FUCHIA database and their $CD4^+$ T-cell counts were determined by flow cytometry (Facscount; Beckton Dickinson, Franklin Lakes, NJ, USA) at Centre Pasteur du Cameroun (Yaoundé, Cameroon).

Plasma HIV type-1 (HIV-1) RNA was quantified by means of second-generation long terminal repeat-based real-time reverse transcriptase (RT)-PCR (Biocentric, Inc., Bandol, France) [30]. In the case of a viral load more than the cutoff value of 250 copies/ml, RT and protease genes were reverse transcribed and amplified using the nested primer pairs MJ3/MJ4 and NE1/A35 for RT, and 5′prot1/3′prot1 and 5′prot2/3′prot2 for protease. Both strands were sequenced and aligned with the HIV-1 HXB2 reference genome using CEQ2000 software (Beckman–Coulter, Inc., Fullerton, CA, USA) and the nucleotide sequences were transmitted electronically to the Department of Infectious Diseases (San Raffaele Scientific Institute, Milan, Italy). Each sequence was analysed using the Stanford University resistance interpretation algorithm by means of online submission to the web-based HIVdb programme [31] and the output mutation list, subtype identification and resistance scores

Figure 1. Frequency of mutations in the 84 genotypes



Frequency of mutations conferring resistance to **(A)** nucleoside/nucleotide reverse transcriptase inhibitors and **(B)** non-nucleoside/nucleotide reverse transcriptase inhibitors.

were used in the analysis. Resistance to a specific drug was assumed when the resistance score was ≥30.

The study was approved by the MSF ethics review board, the ethics committee of Cameroon (Yaoundé, Cameroon), the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale of St Germain en Laye (Paris, France) and the Cameroon Ministry of Public Health (Yaoundé, Cameroon). All of the participants gave their written informed consent and received second-line ART on the basis of their individual genotypes.

Statistical analyses

Mutations were considered as outcomes in all of the statistical analyses; they were grouped into classes or considered individually when they were detected in ≥5 patients. The time of starting the first antiretroviral regimen was defined as baseline for the analyses.

The χ^2 or Fisher's exact tests were used to assess the relationships between discrete variables and the Cochran–Armitage test was used to assess trends over time (ART duration). The independent distributions of continuous variables were compared using the non-parametric Mann–Whitney U test or Kruskall–Wallis test and Pearson's correlation coefficients were calculated to assess linear correlations. In the multivariable analysis, the generalized linear model (GLM) and multiple logistic regression were used to estimate the independent contribution of baseline covariates in predicting the number of NRTI or NNRTI mutations, or the presence of different mutation patterns at the time of cross-sectional sampling. All of the significance tests were two-sided and a *P*-value ≤0.05 was considered statistically significant. The analyses were made using SAS software (version 8.2; SAS Institute, Inc., Cary, NC, USA).

Results

Study patients

Of the 950 patients who started first-line ART between January 2001 and November 2005, 573 attended survey visits during the study period: 476 (83.1%) patients had a viral load <250 copies/ml and 97 (16.9%) patients with a viral load >250 copies/ml were genotyped except for 4, whose RNA was not amplifiable. Of the 93 interpretable sequences, those from patients who had switched to a second-line PI-based regimen before the survey (*n*=7), who had started their first ART without lamivudine (*n*=1) or who were not treatment-naive at ART initiation (*n*=1) were excluded from the analysis thus leaving 84 analysed sequences, each corresponding with a different patient.

Figures 1 and 2 show the frequency of mutations and the distribution of the HIV-1 subtypes. The most prevalent subtype was CRF02_AG, which was in line with its regional distribution [32,33]. A few minor protease mutations were detected, most of which were polymorphisms of non-B subtypes [34]. Overall, there were 22 L10V/F/I/M, 1 V11I, 1 A71T and 1 G73S mutations detected. Few genotypes had major PI mutations (one I50M, one N83D and three N88K).

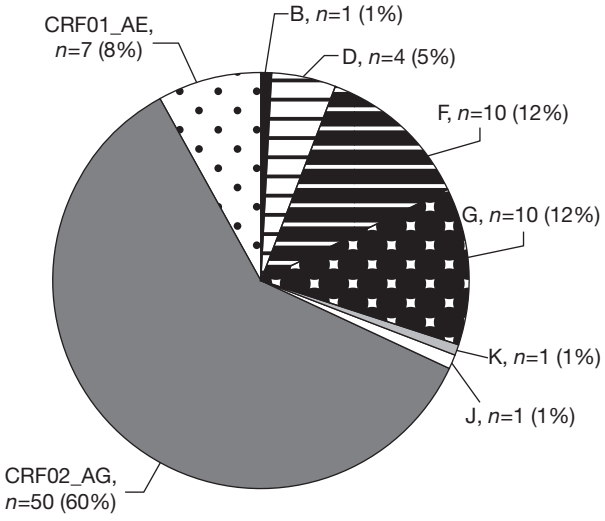
Table 1 shows the patient characteristics at the time of starting ART (baseline) and at the time of the cross-sectional sampling. Of the total patients, 70% were women. At the time of ART initiation, the median age was 33.2 years (interquartile range [IQR] 28.3–39.5), their median BMI was 22 kg/m^2 (IQR 20–25) and their median CD4$^+$ T-cell count was 102 cells/mm^3 (IQR 44.5–172) and 66.5% were in WHO stage III or IV. At the time of cross-sectional sampling, the median CD4$^+$

T-cell count was 226.5 cells/mm^3 (IQR 123–316) and median plasma HIV-1 RNA level was 4.53 $\log_{10}$ copies/ml (IQR 3.68–5.40). Overall, 73 patients (86.9%) were still receiving the first-line FDC of stavudine, lamivudine and nevirapine at the time of cross-sectional sampling.

Relationship between mutations and exposure to antiretrovirals

At the time of cross-sectional sampling of detectable virological failure, 39 of the 84 genotypes were wild-type (46.4%) and 45 (53.4%) had ≥1 mutation in the RT gene. The mean number of RT mutations was 2.13.

Figure 2. Distribution of the HIV type-1 subtypes



Of the 84 patients with available sequences, 44 (52.4%) patients had a viral genotype with no NRTI mutations, 19 (22.6%) had 1 mutation, 9 (10.7%) had 2 mutations and 12 (14.3%) had ≥3 mutations. The corresponding figures for NNRTI mutations were 40 (47.6%), 18 (21.4%), 16 (19.1%) and 10 (11.9%), respectively. A total of 39 (46.4%) patients simultaneously had ≥1 NRTI and ≥1 NNRTI mutation.

The median exposure to ART was 2.18 years (range 0.82–5.95). In order to investigate the relationship between the number of mutations and the duration of ART exposure, we divided the samples into four categories on the basis of the number of years from ART initiation: 6 patients had been on ART for 1 year, 39 for 2 years, 26 for 3 years and 13 for >3 years. The number of mutations increased over time and this trend was statistically significant for NRTI mutations (Cochran–Armitage P=0.019; Figure 3A), but not for NNRTI mutations (Cochran–Armitage P=0.174; Figure 3B).

A time trend was sought for all mutations detected in ≥5 patients (M41L, M184V, T215F, T215Y, A98G, K103N, V108I, Y181C and G190A), but only V108I showed a significant trend over time: it was not observed before 3 years of ART exposure (Cochran–Armitage P=0.001).

It is worth noting that the five women who had previously received nevirapine prophylaxis against mother-to-child transmission (MTCT) did not carry a higher number of NNRTI mutations.

Relationship between baseline CD4$^+$ T-cell counts and mutations

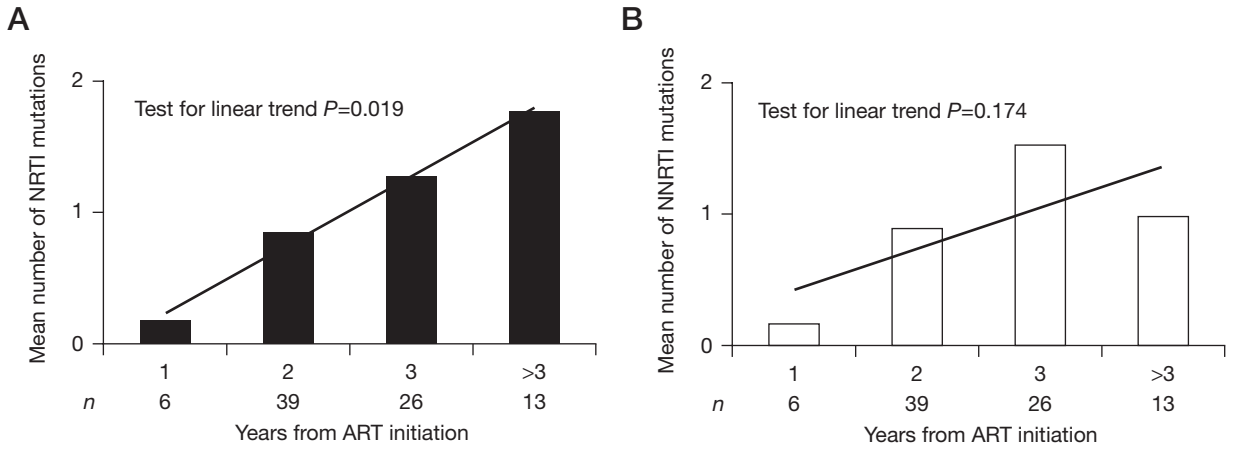
The lower the CD4$^+$ T-cell count at baseline, the higher the number of both NRTI (Pearson's

Table 1. Patient characteristics at the time of starting ART (baseline) and at the time of cross-sectional sampling

Characteristic	Baseline (n=84)	Sampling (n=84)
Female gender, *n* (%)	59 (70)	59 (70)
Median age, years (IQR)	33.2 (28.3–39.5)	35.5 (31–42)
Median body mass index, kg/m^2 (IQR)	22 (20–25)	23 (21–26)
WHO stage		
I, *n* (%)	16 (19)	–
II, *n* (%)	13 (15.5)	–
III, *n* (%)	41 (48.8)	–
IV, *n* (%)	14 (16.7)	–
Median CD4$^+$ T-cell count, cells/mm^3 (IQR)	102 (44.5–172)	226.5 (123–316)
Median plasma HIV-1 RNA, $\log_{10}$ copies/ml (IQR)	NA	4.59 (3.68–5.40)
Patients by drug regimen		
d4T+3TC+NVP, *n* (%)	65 (77.4)	62 (73.8)
AZT+3TC+NVP, *n* (%)	7 (8.3)	10 (11.9)
d4T+3TC+EFV, *n* (%)	10 (11.9)	9 (10.7)
AZT+3TC+EFV, *n* (%)	2 (2.4)	3 (3.6)

ART, antiretroviral therapy; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; HIV-1, HIV type-1; IQR, interquartile range; NA, not available; NVP, nevirapine; 3TC, lamivudine.

Figure 3. Mean number of NRTI mutations or NNRTI mutations by duration of exposure to antiretroviral therapy



Mean number of **(A)** nucleoside/nucleotide reverse transcriptase inhibitor (NRTI) mutations or **(B)** non-NRTI (NNRTI) mutations by duration of exposure to antiretroviral therapy (ART).

correlation coefficient r=-0.35; P<0.001) and NNRTI (r=-0.23; P=0.039) mutations. Patients with baseline CD4+ T-cell counts ≤50 cells/mm^3 had a higher mean number of NRTI and NNRTI mutations than those with CD4+ T-cell counts >50 cells/mm^3 (2.17 versus 0.66; Mann–Whitney U P=0.001; and 1.65 versus 0.84; Mann–Whitney U P=0.01, respectively) and were more likely to have mutations at the time that failure was detected (Table 2).

Relationship between self-reported adherence and mutations

With regards to adherence, 11 (13.6%) patients reported a score of 1, 6 (7.4%) patients a score of 2, 6 (7.4%) patients a score of 3, 8 (9.9%) patients a score of 4, 46 (56.8%) patients a score of 5 and 4 (4.9%) patients a score of 6; no adherence score was available for 3 patients. There were no statistically significant gender-related differences at any of the adherence levels, but it is interesting to note that 6 of 24 (25%) men, but only 5 of 57 (8.8%) women, had a score of 1.

Greater treatment adherence was associated with a lower viral load at the time of sampling (Pearson's correlation coefficient r=-0.52; P<0.0001), a higher number of CD4+ T-cells at the time of sampling (r=0.33; P=0.002), a greater increase in CD4+ T-cell counts from baseline (r=0.36; P=0.001) and a higher number of NRTI mutations (r=0.25; P=0.023). Table 3 shows that the patients with a low level of adherence (1–2) had fewer NRTI and NNRTI mutations, lower CD4+ T-cell counts at sampling, higher viral loads at sampling and a smaller gain in CD4+ T-cells from baseline to the time of sampling than those with intermediate (3–4) or high (5–6) levels of adherence.

The mean adherence of patients harbouring a virus with the M184V mutation was greater than that of those harbouring a virus without this mutation (4.63 versus 3.59; Mann–Whitney U P=0.006). The presence of the M184V mutation was also associated with a greater mean relative increase in CD4+ T-cell counts from baseline to the time of sampling (1072% versus 176%; Mann–Whitney U P=0.003).

Multivariable analyses

When adjusted for age, baseline BMI, baseline CD4+ T-cell counts of ≤50 or >50 cells/mm^3, gender and baseline WHO stage, patients with baseline CD4+ T-cell counts of ≤50 cells/mm^3 had a higher mean number of NRTI (GLM adjusted means 2.10 versus 0.56; P<0.0001) and NNRTI mutations (1.65 versus 0.76; P=0.005) and women had a higher mean number of NNRTI mutations (GLM adjusted means 1.60 versus 0.81; P=0.015).

At logistic regression, having a baseline CD4+ T-cell count of ≤50 cells/mm^3 predicted ≥1 NRTI mutation, ≥1 NNRTI mutation, ≥1 mutation in both classes and other specific mutations (Table 2), and being a woman predicted ≥1 NRTI mutation (odds ratio [OR] 4.05, 95% confidence interval [CI] 1.19–16.67; P=0.034) and ≥1 mutation in both classes (OR 4.95, 95% CI 1.44–20.83; P=0.017).

In order to assess whether other factors intervening after the start of ART might have affected the occurrence of mutations, we repeated the multivariable analysis, including ART duration and treatment adherence as covariates, and found approximately the same adjusted mean number of mutations and the same predictive values for the occurrence of mutations for baseline CD4+

T-cell counts of ≤50 cells/mm^3 and female gender (data not shown).

In relation to the effect of mutations on possible future NRTI (didanosine and tenofovir) and NNRTI (etravirine) options, we found that a baseline CD4$^+$ T-cell count of ≤50 cells/mm^3 was a strong independent predictor of resistance to didanosine and etravirine (Table 2).

Finally, we looked at the effect of mutations on second-line PI-based regimens, concentrating on the WHO-recommended NRTI backbones of tenofovir plus emtricitabine, abacavir plus didanosine and zidovudine plus lamivudine plus tenofovir and found that 5%, 5% and 55% of the patients with NRTI mutations, respectively, would benefit from two fully active drugs in these new backbones.

Discussion

The absence of routine viral load monitoring from many large-scale antiretroviral rollout programmes inevitably leads to a delay in detecting treatment failure. Moreover, the suboptimal drug exposure on a replicating virus favours a further accumulation of mutations and increased resistance extending to other drugs in the same class [35]. Our findings confirm this model as there was a linear relationship between the duration of ART exposure and a higher number of NRTI mutations; furthermore, although not statistically significant, the first 3 years showed a clearly similar trend with regard to mutations conferring resistance to NNRTIs.

Table 2. Relationships between baseline CD4$^+$ T-cell count of ≤50 cells/mm^3 or >50 cells/mm^3 and various types of drug resistance

		BL CD4$^+$ T-cell count ≤50 cells/mm^3	BL CD4$^+$ T-cell count >50 cells/mm^3	Univariable			Multivariable		
Outcome measure	Total[a]	*n* (%)[b]	*n* (%)[c]	OR	95% CI	*P*-value	AOR[d]	95% CI	*P*-value
≥1 NRTI mutation	40	17 (73.9)	23 (37.7)	4.68	1.68–14.57	0.004	7.49	2.20–2.14	0.003
≥1 NNRTI mutation	44	17 (73.9)	27 (44.3)	3.57	1.29–11.04	0.019	4.25	1.36–15.48	0.018
≥1 Mutation in both classes	39	16 (69.6)	23 (37.7)	3.77	1.39–11.14	0.011	6.10	1.80–25.53	0.006
Selection of M41L	5	4 (17.4)	1 (1.6)	12.60	1.30–120.00	0.007	NA	NA	NA
Selection of M184V	35	15 (65.2)	20 (32.8)	3.80	1.40–10.60	0.007	5.49	1.73–20.36	0.006
Selection of T215Y	6	4 (17.4)	2 (3.3)	6.20	1.05–36.60	0.026	NA	NA	NA
Selection of Y181C	16	10 (43.5)	6 (9.8)	7.00	2.20–22.90	<0.001	7.57	2.12–31.33	0.003
≥1 TAM	14	9 (39.1)	5 (8.2)	7.20	2.15–26.79	0.002	8.45	2.16–40.16	0.003
≥1 TAM-1[e]	9	6 (26.1)	3 (4.9)	6.82	1.62–35.12	0.011	7.12	1.46–45.80	0.021
≥2 TAM-1 or Q151M	4	3 (13.0)	1 (1.6)	NA	NA	NA	NA	NA	NA
Resistance to didanosine	11	7 (30.4)	4 (6.6)	6.23	1.67–26.41	0.008	6.36	1.49–32.29	0.016
Resistance to tenofovir	4	1 (4.3)	3 (4.9)	NA	NA	NA	NA	NA	NA
Resistance to etravirine[f]	24	12 (52.2)	12 (19.7)	4.45	1.60–12.84	0.005	4.72	1.53–15.70	0.008

Resistance was defined as a Stanford algorithm score of ≥30. [a]*n*=84. [b]*n*=23. [c]*n*=61. [d]Adjusted for age, baseline body mass index, gender and WHO stage. [e]Tam-1 is one of the following: 41L, 210W or 215Y. [f]Two patients had ≥3 etravirine resistance-associated mutations, whereas 24 had a Stanford score of ≥30 for etravirine resistance. AOR, adjusted odds ratio; BL, baseline; CI, confidence interval; NA, not applicable because of small sample size; NNRTI, non-nucleoside/nucleotide reverse transcriptase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor; OR, odds ratio; TAM, thymidine analogue mutations.

Table 3. Relationships between treatment adherence and the number of resistance mutations, CD4$^+$ T-cell counts and HIV-1 RNA level

	Treatment adherence levels			*P*-value[a]		
Measure	1–2	3–4	5–6	1–2 versus 3–4	1–2 versus 5–6	3–4 versus 5–6
Median number of NNRTI mutations (IQR)	0 (0–1)	1 (0–2)	1 (0–2)	0.034	0.018	0.628
Median number of NRTI mutations (IQR)	0 (0–0)	1 (0–2)	1 (0–2)	0.045	0.016	0.999
Median CD4$^+$ T-cell count at survey visit, cells/mm^3 (IQR)	120 (97–239)	212 (123–390)	262 (176–362)	0.153	0.003	0.460
Median HIV-1 RNA level at survey visit, $\log_{10}$ copies/ml (IQR)	5.5 (5.0–5.7)	5.2 (4.1–5.4)	3.8 (3.5–4.6)	0.092	<0.0001	0.020
Median change in CD4$^+$ T-cell count from baseline to survey visit, cells/mm^3 (IQR)	43 (-14–69)	66 (28–230)	156 (45–258)	0.321	0.002	0.194

Adherence was catergorized to levels 1–2 (poor adherence), 3–4 (intermediate adherence) and 5–6 (high adherence). [a]Comparisons made using the Mann–Whitney U test. The *P*-values were not adjusted for multiple testing. HIV-1, HIV type-1; IQR, interquartile range; NNRTI, non-nucleoside/nucleotide reverse transcriptase inhibitor; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor.

We thought that knowledge of the baseline factors possibly affecting the accumulation of mutations might help in implementing strategies aimed at preserving NRTI and NNRTI options for future treatment regimens in an ongoing scaling-up of ART delivery. Although the long-term risk of extensive virological failure after the start of therapy is small on the whole [36], we did find a clear and strong association between lower baseline CD4+ T-cell counts and drug-resistance-associated mutations. As previously reported for a smaller patient series from Tanzania [37], it can be speculated that this might reflect higher pre-ART plasma HIV-1 RNA levels but, unfortunately, we did not have any baseline sample to test. Moreover, previous studies have also shown a relationship between baseline CD4+ T-cell counts of ≤50 cells/mm^3 and virological failure on NNRTI-based regimens [38], and starting ART with CD4+ T-cell counts of ≥350 cells/mm^3 has been associated with a lower prevalence of mutations at the time of virological failure [39].

We explored the immediate and practical consequences of the accumulation of mutations by estimating the risk of compromising specific second-line treatment options. A Thai study found that the activity of tenofovir might be compromised by the duration of ART before failure and $\log_{10}$ HIV-1 RNA levels at the time of failure [40]. Only 4 of the 84 patients in our study showed intermediate- or high-level resistance to tenofovir according to the Stanford algorithm, and so the lack of any association with baseline characteristics might have been because of the small sample size. However, we did find an association between baseline CD4+ T-cell count and resistance to didanosine and etravirine. When considering the sensitivity of etravirine, we used a Stanford algorithm score of <30 rather than the presence of >2 etravirine RAMs because we believe that the latter might overestimate the activity of etravirine as this criteria is based on the DUET studies [25,26], in which all of the patients were also receiving darunavir as a new drug. It is unclear whether etravirine sensitivity determined on the basis of >2 etravirine RAMs is applicable to settings in which darunavir is not combined with etravirine [41]. Our findings might have implications for designing future second-line ART strategies in resource-limited settings [42]: if there is room for etravirine in ART sequencing after first-line treatment failure [43], the prevention of multiple NNRTI mutations becomes crucial because it has been shown that a regimen with etravirine plus two NRTIs after the failure of an NNRTI-based regimen is less efficacious than a boosted-PI based regimen [44].

We also found an interesting correlation between treatment adherence and viral load at the time failure was detected. It is, of course, known that adherence predicts therapeutic success [45], but other means of measuring adherence (pill counts and patient self-reports) have not revealed any statistically significant association with drug resistance in a similar setting [20]. Although our use of a modified visual analogue scale was quite a coarse means of assessing such a complex subject, it has the advantage of being very simple and could be taken into account in algorithms aimed at identifying selected patients for viral load testing [11] to avoid an unnecessary switch to second-line drugs when the lack of viral control is simply the result of poor adherence.

Another interesting finding was the relationship between treatment adherence and the presence of the M184V mutation at the time of the detection of virological failure. It is possible that the patients whose resistance test did not show the M184V mutation were not currently taking their medication: other studies of resistance after the failure of first-line regimens that include lamivudine, have found a higher prevalence of the M184V mutation ranging from 64.3% [20] to 89% [16], and it is known that the M184V mutation disappears soon after treatment interruption as it impairs viral fitness [46,47].

We observed a higher adjusted mean number of NNRTI mutations in women without any relationship with previous nevirapine exposure for MTCT prophylaxis. Although the numbers are too small to draw any definite conclusion, it is possible that the treatment adherence of the female participants might have been better as we found a gender-related difference in the very low levels of adherence that lead to a minimal chance of selecting mutations.

Our study has a number of limitations. First of all, its cross-sectional design prevented us from identifying the time of virological escape: when we detected a virological failure, we could not ascertain how long the patient had been failing. Nonetheless, there was a significant correlation between the duration of ART exposure and the extent of resistance. Furthermore, this is the first study specifically designed to investigate the accumulation of resistance over time in a resource-limited setting and particularly in the case of non-B and non-C subtypes. Another important limitation is that a considerable number of the 950 patients who started ART in our programme were lost to follow-up. The number was particularly high among those who joined the cohort at the time MSF handed over the management of the programme to the Ministry of Public Health, probably because of a lack of appropriate communication (a programme-related problem). As a result, our findings might underestimate the proportion of patients failing on their first-line regimen as they only take into account those who were actively followed-up. However, rather than estimating the exact prevalence of resistance, the aim of the study was to verify whether a critical accumulation of mutations is

related to the duration of ART exposure, and we found that it is. We think that this is a relevant finding in patients without the WHO criteria for treatment failure who would have continued their current regimen until immunological or clinical deterioration.

Finally, we probably underestimated the number of mutations, because we found an unexpectedly high number of wild-type genotypes. It can be hypothesized that selective drug pressure was not only insufficient in controlling viral replication, but also insufficient in selecting mutations in patients with very poor treatment compliance. HIV-1 RNA levels were inversely associated with adherence and the number of NRTI mutations was higher among the most compliant patients. It is likely that other mutations were present in the viruses of these non-adherent patients, but could not be detected by means of bulk sequencing because of the overgrowth of the wild-type strain in the absence of drug pressure.

In conclusion, we found that the risk of extensive drug resistance in patients failing on a regimen based on an NNRTI, a TA and lamivudine was generally relatively low. However, resistance was frequent when baseline $CD4^+$ T-cell counts were ≤50 cells/mm^3 and increased with the duration of ART exposure in the absence of virological monitoring. Our findings further support the need to start ART earlier, when $CD4^+$ T-cell counts are higher, without fear of rapidly exhausting the few available therapeutic regimens. In addition, viral load monitoring can be useful in preserving second-line options, at least in the case of patients at higher risk of developing extensive drug resistance.

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Disclosure statement

AL has acted as a consultant, participated in advisory boards or speaker bureaus or conducted clinical trials for Abbott Laboratories, Bristol–Myers Squibb, Gilead, Tibotec, Merck, Pfizer, Roche, GlaxoSmithKline and Boehringer Ingelheim. NG has acted as a consultant, participated in advisory boards or speaker bureaus or conducted clinical trials for Abbott Laboratories, Bristol–Myers Squibb, Gilead, Tibotec, Merck, Pfizer, Roche, GlaxoSmithKline and Boehringer Ingelheim. All of the other authors declare no competing interests.

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