Semi-sensitive Assessment of the Virologic Outcomes of Stopping and Restarting Non-Nucleoside Reverse Transcriptase Inhibitor-Based Antiretroviral Therapy

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Abstract

Background: Non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant mutants have been shown to emerge after interruption of suppressive NNRTI-based antiretroviral therapy (ART) using routine testing. The aim of this study was to quantify the risk of resistance by sensitive testing and correlate the detection of resistance with NNRTI concentrations after treatment interruption and virologic responses after treatment resumption.

Methods: Resistance-associated mutations (RAMs) and NNRTI concentrations were studied in plasma from 132 patients who interrupted suppressive ART within SMART. RAMs were detected by Sanger sequencing, allele-specific PCR, and ultra-deep sequencing. NNRTI concentrations were measured by sensitive high-performance liquid chromatography.

Results: Four weeks after NNRTI interruption, 19/31 (61.3%) and 34/39 (87.2%) patients showed measurable nevirapine (>0.25 ng/ml) or efavirenz (>5 ng/ml) concentrations, respectively. Median eight weeks after interruption, 22/131 (16.8%) patients showed ≤1 NNRTI-RAM, including eight patients with NNRTI-RAMs detected only by sensitive testing. The adjusted odds ratio (OR) of NNRTI-RAM detection was 7.62 (95% confidence interval [CI] 1.52, 38.30; p = 0.01) with nevirapine or efavirenz concentrations above vs. below the median measured in the study population. Staggered interruption, whereby nucleos(t)ide reverse transcriptase inhibitors (NRTIs) were continued for median nine days after NNRTI interruption, did not prevent NNRTI-RAMs, but increased detection of NRTI-RAMs (OR 4.25; 95% CI 1.02, 17.77; p = 0.03). After restarting NNRTI-based ART (n = 90), virologic suppression rates <400 copies/ml were 8/13 (61.5%) with NNRTI-RAMs, 7/11 (63.6%) with NRTI-RAMS only, and 51/59 (86.4%) without RAMs. The ORs of re-suppression were 0.18 (95% CI 0.03, 0.89) and 0.17 (95% CI 0.03, 1.15) for patients with NNRTI-RAMs or NRTI-RAMs only respectively vs. those without RAMs (p = 0.04).

Conclusions: Detection of resistant mutants in the rebound viremia after interruption of efavirenz- or nevirapine-based ART affects outcomes once these drugs are restarted. Further studies are needed to determine RAM persistence in untreated patients and impact on newer NNRTIs.

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Introduction

The SMART trial randomized HIV-1 infected patients with CD4 counts >350 cells/mm³ to take antiretroviral therapy (ART) either continuously or episodically, guided by the CD4 cell count [1]. Results showed that interrupting treatment carried a significant risk of morbidity and mortality. There remain circumstances when ART discontinuation may be required (e.g.,...
transmission of drug-resistant HIV.

in relation to both subsequent treatment outcomes and risk of resistance have not been conclusive, possibly due to small numbers of patients after treatment interruption and detection of NNRTI resistance in the two months following interruption [8]. Sanger sequencing fails to detect mutants present in the viral quasispecies at a frequency below approximately 20%, suggesting that an even greater proportion of patients may carry resistant mutants below this detection limit. The issue is especially relevant to NNRTI therapy. Low-frequency NNRTI-resistant mutants have been detected in both ART-naive and NNRTI-experienced patients with and without high-frequency mutants, and shown to impair responses to NNRTI-based ART [10,11]. Recommended strategies to minimize the potential risk of drug resistance after interruption of NNRTI-based ART include stopping the NNRTI first and continuing the remaining drugs in the regimen for a short period, commonly the nucleoside reverse transcriptase inhibitors (NRTIs) (staggered interruption), or replacing the NNRTI with a ritonavir-boosted protease inhibitor (PI/r) for a short period (switched interruption) [12]. There is limited evidence supporting one particular strategy. In a previous study using Sanger sequencing, no NNRTI-RAMs were detected in virologically suppressed children that stopped nevirapine or efavirenz according to either a staggered or a switched interruption modality [9]. Within SMART, we previously reported that both the detection of drug resistance after interruption (by Sanger sequencing) and re-suppression rates after restarting therapy were higher among patients with staggered or switched interruption relative to those with simultaneous interruption [8]. Expanding our previous observations, the aim of this study was to obtain a more accurate estimation of the risk of NNRTI resistance after interruption of NNRTI-based ART by sensitive testing with allele-specific (AS-)PCR and ultra-deep sequencing (UDS). We then investigated the correlation between detection of NNRTI resistance and NNRTI concentrations after treatment interruption, and analyzed the findings in relation to virologic responses after resumption of NNRTI-based ART.

Methods

Study Population

Eligible patients were receiving NNRTI-based ART, had a plasma HIV-1 RNA load (‘viral load’) <400 copies/ml, and were randomized to the drug conservation arm of SMART and thus to undergo a treatment interruption [1]. A total of 132/984 (13.4%) patients who interrupted suppressive NNRTI-based ART in SMART and had stored plasma samples available for testing were included in this sub-study. The modality of interruption was chosen by the treating physician, as previously described [8]. Therapy was re-started when the CD4 count decreased <250 cells/mm³ or at the occurrence of clinical events [1].

Drugs

Elavirenz and nevirapine concentrations were measured by validated highly sensitive high-performance liquid chromatography (HPLC) in plasma samples collected at week 4 (visit 1) after NNRTI interruption. The assay lower limit of quantification was 0.25 ng/ml for nevirapine and 5 ng/ml for efavirenz.

Drug Resistance

Elavirenz and nevirapine concentrations were measured by validated highly sensitive high-performance liquid chromatography (HPLC) in plasma samples collected at week 4 (visit 1) after NNRTI interruption. The assay lower limit of quantification was 0.25 ng/ml for nevirapine and 5 ng/ml for efavirenz.

Drug Resistance

Plasma samples collected 4–12 weeks after NNRTI interruption were used for resistance testing. Selection was based upon sample availability and viral load levels >3000 copies/ml to allow reliable testing by the sensitive assays. Samples underwent Sanger sequencing and AS-PCR as previously described [13–17]. The AS-PCR targeted the NNRTI resistance-associated mutations (RAMs) K103N, Y181C, and Y188L; samples showing K103N were also tested for G190A. In addition samples were screened for the presence of NRTI-RAMs, including thymidine analogue mutations (TAMs), K65R, Q151M and M184V/I. Mutation-specific interpretative cut-offs ranging from 0.3% to 1% were applied as previously described [15]. In a subset of 21 samples, UDS of the RT amino acid region 100 to 190 was performed as previously described [18]; samples were selected randomly from three subsets according to sample availability: samples with RAMs by AS-PCR; samples without RAMs by AS-PCR; and samples that failed the AS-PCR reaction. Briefly, viral RNA was extracted from 500 µl of plasma (EasyMag, Biomérieux, France) and reverse transcribed into cDNA using the AccuScript HF RT enzyme (Agilent, Santa Clara, USA) and random hexamers. The RT region spanning amino acids 100 to 190 was amplified by nested PCR, and pooled barcoded amplicons were sequenced on the GS-FLX instrument (454 Life Sciences, Roche, Branford, USA) according to the manufacturer’s standard protocol. The experiment was designed to reach on average a mutation detection sensitivity of 1% and an average coverage of 5,500 reads per position was obtained. Amplicons were sequenced from both ends (forward and reverse). The Amplicon Variant Analyzer (AVA) software (Roche) was used for read mapping and calculating variant frequencies at each nucleotide position relative to HIV-1 reference strain HXB2. The presence of relevant mutations was manually verified by inspection of the individual owgrams. A detection limit of 1% was chosen to avoid the high probability of technical artifacts below this threshold [19].
RAMs were assigned according to the International IAS-USA list (Nov 2011).

Statistical Analysis

Factors associated with the detection of RAMs by all testing modalities combined (n = 131) were investigated using standard univariable and multivariable logistic regression analysis. All factors of interest were stipulated a priori and included in the multivariable models. In the first model, the variables analyzed were age, gender, ethnicity, HIV-1 transmission risk group, nadir CD4 count, duration of ART before interruption, viral load and CD4 count at the time of interruption, and interruption modality. In a second model exploring factors associated with the detection of NNRTI-RAMs, the analysis also included nevirapine and efavirenz plasma concentrations (n = 70). To overcome the limitation related to the small number of observations, the drug concentration data were pooled and analyzed as categorical variables (either above or below the median concentration measured in the study population). This approach was stipulated a priori as there was insufficient statistical power to analyze the two drugs separately or assess the interaction between nevirapine and efavirenz in this model. The proportion of patients who regained virologic suppression <400 copies/ml after re-starting NNRTI-based ART was investigated using logistic regression analysis as an intention-to-treat switch = failure analysis. Only patients restarting ART without a PI and with at least one viral load measurement in the following 4–12 months were included (n = 90). All factors of interest were stipulated a priori and included in the multivariable models. The variable included were age, gender, duration of ART before interruption, viral load and CD4 count at the time of interruption, time between interrupting and restarting ART, the NNRTI restarted, the interruption modality and the presence of RAMs. P-values were not corrected for multiple comparisons. All statistical analyses were performed using STATA software (StataCorp. 2007. Stata Statistical Software: Version 10.2/SE, College Station, Texas, USA).

Results

Study Population

The analysis included 132 patients that interrupted efavirenz (n = 50, 60.6%), nevirapine (n = 41, 38.6%), or delavirdine (n = 1, 0.8%) in SMART. All patients were also receiving lamivudine (n = 86, 65.1%), tenofovir (n = 53; 41.7%), zidovudine (n = 48, 36.4%), emtricitabine (n = 22, 16.7%), abacavir (n = 20; 15.1%), didanosine (n = 19; 14.4%), or stavudine (n = 16, 12.1%). In addition, 13/132 (11.4%) patients were receiving a PI. The modality of interruption was simultaneous in 65/132 (47.7%) patients, staggered in 46/132 (34.8%) patients, and switched in 23/132 (17.4%) patients. The latter two groups comprising lamivudine (n = 20; 15.1%), didanosine (n = 19; 14.4%), or stavudine (n = 16, 12.1%). In addition, 13/132 (11.4%) patients were receiving a PI. The modality of interruption was simultaneous in 65/132 (47.7%) patients, staggered in 46/132 (34.8%) patients, and switched in 23/132 (17.4%) patients. The latter two groups according to the interruption modality was 13/62 (21.0%) for simultaneous interruption, 8/46 (17.4%) for staggered interruption, and 1/23 (4.3%) for switched interruption.

Drug Concentrations

NNRTI concentrations were measured in 70 patients with sufficient plasma samples available at week 4, the first study visit after NNRTI interruption. Median 32 days (IQR 27, 38) after interruption, nevirapine was detected in 19/31 (61.3%) patients at a median concentration of 2.2 ng/ml (IQR 1.0, 4.9). Median 30 days (IQR 27, 34) after interruption, efavirenz was detected in 34/39 (87.2%) patients at a median concentration of 21 ng/ml (IQR 11, 61). Overall median concentrations (calculated by assigning a value of zero to results below the assay cut-off) were 1.0 ng/ml for nevirapine (n = 31) and 16 ng/ml for efavirenz (n = 39).

Drug Resistance

Resistance testing was performed in samples collected median 8 weeks (IQR 4, 11) after NNRTI discontinuation. At the time of testing, the median viral load was 27,618 copies/ml (IQR 8,480, 76,200). Resistance results by were obtained in 131/132 (99.2%) samples, comprising 122 Sanger sequencing results, 124 AS-PCR results, and 21 RT100–190UDS results (Table 2). Overall, 116 samples had results by both Sanger sequencing and sensitive resistance testing. Six samples failed the AS-PCR reaction and had only Sanger sequencing results (including one sample with NNRTI-RAMs); two further samples failed the AS-PCR reaction and had both Sanger sequencing results (including one sample with NNRTI-RAMs) and RT100–190UDS results (no RAMs detected). Nine samples did not have sufficient volume and were tested only by AS-PCR (no RAMs detected). One sample failed to give a result by both Sanger sequencing and AS-PCR (RT100–190UDS not done). Of the 21 samples tested by both AS-PCR and RT100–190UDS, 21 yielded a result by RT100–190UDS and 19 yielded a result by AS-PCR.

The prevalence of ≥1 NNRTI-RAM by all testing modalities was 22/131 (16.8%) overall. Sensitive testing detected ≥1 NNRTI-RAM in 8/116 (6.9%) samples lacking NNRTI-RAMs by Sanger sequencing, and also increased the number of NNRTI-RAMs detected in each sample (Table 3). With 19 samples that underwent both tests, RT100–190UDS confirmed the AS-PCR results, with the exception of one sample that showed G190A by RT100–190UDS but not by AS-PCR; the frequency of the G190A mutant was 4% by RT100–190UDS. In addition RT100–190UDS detected NNRTI-RAMs not targeted by AS-PCR, including K70DR, L100I, and K101E. Detection of NNRTI-RAMs according to the interruption modality was 15/62 (21.0%) for simultaneous interruption, 8/46 (17.4%) for staggered interruption, and 1/23 (4.3%) for switched interruption.

The prevalence of ≥1 NNRTI-RAM by all testing modalities was 24/131 (18.3%) overall. The NNRTI-RAMs comprised TAMs in 20/24 patients, with mean 2.4 TAMs per patient; M184I/V in 15/24 patients; K65R in 3/25 patients; and L74V in 1/25 patient. Sensitive testing detected ≥1 NNRTI-RAM in 2/116 (1.6%) patients lacking NNRTI-RAMs by Sanger sequencing, and also increased the number of NNRTI-RAMs detected in each sample (Table 3). With 19 samples that underwent both tests, RT100–190UDS confirmed the AS-PCR results, with the exception of one sample that showed G190A by RT100–190UDS but not by AS-PCR; the frequency of the G190A mutant was 4% by RT100–190UDS. In addition RT100–190UDS detected NNRTI-RAMs not targeted by AS-PCR, including K70DR, L100I, and K101E. Detection of NNRTI-RAMs according to the interruption modality was 15/62 (21.0%) for simultaneous interruption, 8/46 (17.4%) for staggered interruption, and 1/23 (4.3%) for switched interruption.

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(28.3%) for staggered interruption, and 1/23 (4.3%) for switched interruption.

In total, 13/131 (9.9%), 9/131 (6.9%) and 15/131 (11.4%) patients had 1 NNRTI-RAM, both NRTI-RAMs and NNRTI-RAMs, and NRTI-RAMs only, respectively.

Predictors of Drug Resistance

Detection of NNRTI-RAMs was less likely in patients with a viral load recorded as <50 copies/ml at the time of treatment interruption with an adjusted odds ratio (OR) of 0.28 (95% confidence interval, CI 0.09, 0.91; p = 0.03) (Table 4). There was also a trend towards a reduced risk of NNRTI-RAMs after a switched interruption relative to a simultaneous interruption. NNRTI-RAMs were detected in 10/31 (32.3%) patients with week 4 NNRTI concentrations above the median level measured in the study population (1 ng/ml for nevirapine and 16 ng/ml for efavirenz), and 2/34 (5.9%) in patients with concentrations below the median level (p = 0.007). A separate multivariable model was used to assess the association between NNRTI-RAMs and drug concentrations, to account for the fact that drug concentrations were only available in 70 patients. In this analysis, NNRTI-RAM detection was more likely in patients with NNRTI concentrations above the median levels (1 ng/ml for nevirapine and 16 ng/ml for efavirenz) with an adjusted OR of 7.62 (95% CI 1.52, 38.30; p = 0.01). Detection of NRTI-RAMs was associated with duration of ART exposure prior to interruption with an adjusted OR of 1.26 for each year longer (95% CI 1.10, 1.45; p = 0.001); the nadir CD4 count with an adjusted OR 0.68 for each 50 cells/mm3 higher (95% CI 0.52, 0.87; p = 0.003); and staggered interruption relative to simultaneous interruption with an adjusted OR of 4.25 (95% CI 1.02, 17.77; p = 0.03).

Virologic Responses after Restarting NNRTI-based ART

Patients restarted ART median 29 weeks (IQR 13, 65) after interruption. The analysis of responses was restricted to 90 patients who restarted NNRTI-based ART (58 with efavirenz and 32 with nevirapine) without a PI and had at least one viral load measurement while on the NNRTI-based regimen in the 4–12 months after restarting. Overall, 73/90 (81.1%) patients regained virologic suppression <400 copies/ml; 50/90 (55.5%) had a viral load recorded as <50 copies/ml. The proportion of patients with viral load <400 copies/ml was 8/13 (61.5%) with NNRTI-RAMs, 7/11 (63.6%) with NRTI-RAMs only, and 51/59 (86.4%) without RAMs (p = 0.04); and 32/42 (76.2%), 23/29 (79.3%), and 18/19 (94.7%) following simultaneous, staggered, and switched interruption, respectively (p = 0.18). In multivariable analyses (Table 5), the adjusted OR of suppression <400 copies/ml was 0.18 (95% CI

Table 1. Characteristics of the study population that interrupted NNRTI-based ART in SMART, according to the interruption modality.a

| Total | Interruption modality |
|-------|-----------------------|
|       | Simultaneous | Staggered | Switched |
| n = 132 | n = 63 | n = 46 | n = 23 |
| Male gender, n (%) | 99 (75.0) | 47 (74.6) | 32 (69.6) | 20 (87.0) |
| Risk group, n (%) | MSM 61 (46.2) | 31 (49.2) | 18 (39.1) | 12 (52.2) |
| Heterosexual | 41 (31.1) | 20 (31.8) | 18 (39.1) | 3 (13.0) |
| Other/Unknown | 30 (22.7) | 12 (19.0) | 10 (21.7) | 8 (34.8) |
| Ethnicity, n (%) | Black 51 (38.6) | 22 (34.9) | 21 (45.7) | 8 (34.8) |
| White | 63 (47.7) | 34 (54.0) | 18 (39.1) | 11 (47.8) |
| Other/unknown | 18 (13.6) | 7 (11.1) | 7 (15.2) | 4 (17.4) |
| CDC category C, n (%) | 31 (23.5) | 11 (17.5) | 13 (28.3) | 7 (30.4) |
| HIV-1 RNA load <50 copies/ml, n (%)b | 67 (50.8) | 26 (41.3) | 29 (63.0) | 12 (52.2) |
| Age, median years (IQR) | 45 (39, 52) | 44 (39, 50) | 48 (41, 52) | 45 (41, 54) |
| CD4 count, median cells/mm3 (IQR) | 645 (475, 793) | 624 (475, 833) | 657 (461, 758) | 643 (527, 751) |
| Nadir CD4 count, median cells/mm3 (IQR) | 207 (90, 308) | 212 (115, 374) | 199 (67, 303) | 205 (70, 300) |
| Time on ART, median years (IQR) | 6 (3, 9) | 6 (3, 9) | 7 (3, 9) | 6 (3, 10) |

*Patients interrupted ART by simultaneously interrupting all drugs, continuing the nucleos(t)ide reverse transcriptase inhibitors (NRTIs) for a short period, or switching to a ritonavir-boosted protease inhibitor for a short period, referred to as simultaneous, staggered and switched interruption respectively.

*bIn 46 patients the viral load was measured by assays with a lower limit of quantification of either 75 or 400 copies/ml and results were “undetectable” below these cutoffs; 19 patients showed a viral load between 50 and 400 copies/ml. NNRTI = non-nucleoside reverse transcriptase inhibitor; ART = antiretroviral therapy; MSM = Men who have sex with men; IQR = interquartile range.

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Table 2. Summary of resistance results obtained by all methodologies.a

| Method | Number |
|--------|--------|
| SS+AS-PCR | 95 |
| SS+AS-PCR+UDS | 19 |
| SS+UDS | 2 |
| SS only | 6 |
| AS-PCR only | 9 |
| None | 1 |
| Total | 132 |

*aPlasma samples underwent testing by Sanger sequencing (SS), allele-specific PCR (AS-PCR) and ultra-deep sequencing (UDS) targeting mutations in HIV-1 reverse transcriptase.

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Stopping and Restarting NNRTI-Based ART

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Discussion

This study demonstrated RAMs in a substantial number of patients experiencing rebound viremia after stopping suppressive NNRTI-based ART. Interpretation of the findings should take two limitations into consideration. Viral load assays with different lower limits of quantification were used in SMART and viral load suppression <50 copies/ml could not be demonstrated in all patients at study entry. Furthermore, due to sample availability, NNRTI concentrations were obtained only in a subset of patients. Nonetheless, the data provide sufficient evidence to indicate that different factors influenced the detection of RAMs after ART interruption. NNRTI-RAMs were less likely in patients who had a viral load recorded as <50 copies/ml at the time of interruption, indicating a risk of selecting NNRTI resistance even at low levels of viremia between 50 and 400 copies/ml. In addition, NNRTI-RAMs were more frequent in patients showing higher plasma NNRTI concentrations at week 4 after interruption. These findings provide support to the notion that selection of NNRTI resistance can occur in patients experiencing slower NNRTI clearance after ART interruption. Two previous studies did not observe an association between NNRTI concentrations after interruption and detection of NNRTI-RAMs [6,7]. One study found that median efavirenz or nevirapine concentrations at day 15 after interruption did not differ significantly between 12 patients with NNRTI-RAMs (by Sanger sequencing) and 20 patients without RAMs [6]. Of note, the NNRTI was quantifiable in less than a third of available samples. A second study reported that median efavirenz concentrations and rate of efavirenz decline over 7–10 days after interruption did not differ significantly.
between 7 patients with NNRTI-RAMs (by Sanger sequencing) and 14 patients without RAMs. Thus, both the size of the study population, the timing of the assessment, and the sensitivity of the testing methods for both drug concentrations and NNRTI-RAMs differed in our study compared with the two previous reports. A previous study of 19 patients receiving intermittent efavirenz-based ART assay ed efavirenz concentrations and used AS-PCR to detect the NNRTI-RAM K103N during the off-therapy periods. Consistent with our findings, AS-PCR increased detection of NNRTI-RAM K103N during the off-therapy periods.

### Table 4. Predictors of detection of NNRTI resistance-associated mutations (RAMs) after interruption of NNRTI-based ART.

| Predictor                        | Univariable analysis | Multivariable analysis |
|----------------------------------|----------------------|------------------------|
| Gender                           |                      |                        |
| Female                           | 1.14                 | 0.83                   |
| Male                             | 1.00                 | 1.00                   |
| Age                              |                      |                        |
| Each 5 years older               | 1.00                 | 0.99                   |
| Ethnicity                        |                      |                        |
| White                            | 0.48                 | 0.41                   |
| Other/Unknown                    | 0.19                 | 0.18                   |
| Black                            | 1.00                 | 1.00                   |
| Risk group                       |                      |                        |
| Heterosexual                     | 0.94                 | 0.54                   |
| Other/Unknown                    | 0.68                 | 0.43                   |
| Nadir CD4 count                  |                      |                        |
| Each 50 cells/mm³ higher         | 0.94                 | 1.01                   |
| Time on ART pre-interruption     |                      |                        |
| Each year longer                 | 1.03                 | 1.05                   |
| HIV-1 RNA load at interruptionb |                      |                        |
| <50 copies/ml                    | 0.34                 | 0.28                   |
| Other                            | 1.00                 | 1.00                   |
| CD4 count at interruption        |                      |                        |
| Each 50 cells/mm³ higher         | 0.94                 | 0.93                   |
| Interruption modality            |                      |                        |
| Staggered                        | 0.71                 | 0.80                   |
| Switched                         | 0.19                 | 0.11                   |
| Simultaneous                     | 1.00                 | 1.00                   |

aDetection of NNRTI-RAMs by all testing modalities. NNRTI concentrations were analyzed in a separate model due to smaller numbers.

bAs noted above some patients had the viral load measured by assays with a lower limit of quantification of either 75 or 400 copies/ml. NNRTI = non-nucleoside reverse transcriptase inhibitor; ART = antiretroviral therapy; OR = Odds ratio; CI = confidence interval; MSM = Men who have sex with men.

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evidence that patients with slower nevirapine or efavirenz clearance have a greater risk of NNRTI resistance after interruption, although further studies are required to identify drug-specific cut-offs that are predictive of resistance. Further analyses of interest may also include the correlation between drug concentrations measured before and after treatment interruption. In addition, although we were unable to identify an association between detection of NNRTI-RAMs and the NRTIs used (data not shown), the different half-life of NRTIs has the potential to influence the risk for resistance development [7] and its effects warrant further investigation.

Detection of NRTI-RAMs was surprisingly common in this study. NRTI-RAMs were more likely in patients with a long previous ART history, suggesting re-emergence of resistant mutants archived during previous virologic failures, rather than, or in addition to, de novo selection during viral load rebound. In support of this hypothesis, most NRTI-RAMs were detected by Sanger sequencing, and there was a high prevalence of patients showing two or more TAMs. As TAMs are known to emerge in stepwise fashion during prolonged therapy with zidovudine or stavudine [21], it would seem that multiple TAMs were unlikely to arise for the first time solely as a result of treatment interruption. Detailed treatment histories and results of previous resistance tests would be required to corroborate this hypothesis. Interestingly, there was an increased detection of NRTI-RAMs in patients with a staggered interruption, suggesting a potential for selection or re-selection of mutants by the continued NRTIs.

We previously reported that patients who had undergone a simultaneous interruption showed reduced virologic responses after restarting ART compared with those with a staggered or a switched interruption [8]. Here we confirm the previous observation that simultaneous interruption should be avoided when possible. We further propose that a switched interruption may be
preferable to a staggered interruption both to offer improved protection against emergence of NNRTI-RAMs and reduce selection of NRTI resistance; the latter may be especially important in patients with previous NRTI experience.

In our previous study we used Sanger sequencing to detect RAMs after ART interruption [8]. Here we demonstrated that sensitive testing increased prevalence and spectrum of NNRTI-RAMs detected during viral load rebound. The data provide a measure of the potential risk of NNRTI resistance after ART interruption. It should be noted that at 16.8%, the overall prevalence of NNRTI-RAMs was lower than that observed in patients either failing NNRTI-based ART or receiving single-dose nevirapine for the prevention of mother to child transmission [10,22]. This may be explained by the consideration that both sufficient levels of virus replication and sufficient drug concentrations must co-exist to allow selection of drug resistance. The optimal “selection window” is likely to be narrower in patients stopping NNRTI-based ART with a suppressed viral load relative to patients receiving single-dose nevirapine in the presence of a fully replicating virus. A further consideration is that patients interrupting NNRTI-based ART in SMART had already achieved steady-state NNRTI pharmacokinetics through the induction of hepatic enzymes. In addition, testing samples collected several weeks after treatment interruption, while required to ensure adequate viral load levels, may have missed the earlier emergence of resistant strains. Finally, the AS-PCR method applies strict cut-offs for interpreting positivity.

The AS-PCR methodology employed in this study has undergone extensive validation [15–17]. In previous studies we demonstrated that low-frequency NNRTI-RAMs detected by AS-PCR were predictive of virologic failure among naive patients starting first-line NNRTI-based ART [11,16,17], and also influenced the detection probability and type of NNRTI-RAMs detected at the time of virologic failure [23]. One downside of AS-PCR is that it is mutation-specific and to a large extent clade-specific, and labor-intensive. In recent years, next-generation sequencing methodologies, including UDS, have become available that allow the quantitative detection of mutants with greatly enhanced sensitivity relative to Sanger sequencing (reliably down to a cut-off of about 1%) [19]. Direct comparisons of AS-PCR with UDS are limited. A previous study of 11 samples undergoing AS-PCR for K103N showed a good level of agreement with UDS [20]. Here, using a subset of 21 samples that underwent both AS-PCR and UDS, we found good concordance between the two techniques at the respective validated cut-offs for interpretation. Importantly, although the AS-PCR targeted a relatively small number of NNRTI-RAMs, these were the key RAMs associated with resistance to efavirenz or nevirapine, and the spectrum was only marginally expanded in the samples that also underwent UDS of the RT region spanning amino acids 100 to 190.

In summary, this study provides substantive evidence in support of the widely cited hypothesis that stopping NNRTI-based ART carries a risk of drug resistance. We show that viral load levels at the time of interruption, plasma NNRTI concentrations at week 4 after interruption, overall treatment history, and interruption modality combine to influence the risk of resistance and ultimately predict virologic responses when NNRTI-based ART is resumed. Further studies are required to assess the persistence of NNRTI-RAMs in patients remaining off therapy, the potential for their onward transmission, and the implication of these findings for efavirenz and rilpivirine use.

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Author Contributions
Conceived and designed the experiments: AMG AP ZF JB G. Tachedjian CL G. Touloumi. Performed the experiments: AMG JA JL AO LJS AP ZF JB.

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