Modest Nonadherence to Antiretroviral Therapy Promotes Residual HIV-1 Replication in the Absence of Virological Rebound in Plasma

Alexander O. Pasternak,1,3 Marijn de Bruin,5 Suzanne Jurriaans,2 Margreet Bakker,1,3 Ben Berkhout,1,3 Jan M. Prins,4 and Vladimir V. Lukashov1,3

1Laboratory of Experimental Virology and 2Laboratory of Clinical Virology, Department of Medical Microbiology, 3Center for Infection and Immunity Amsterdam, and 4Department of Internal Medicine, Division of Infectious Diseases, Tropical Medicine, and AIDS, Academic Medical Center, University of Amsterdam, Amsterdam, and 5Department of Communication Science, Wageningen University, Wageningen, the Netherlands

Background. Modern antiretroviral therapy (ART) regimens are widely assumed to forgive modest nonadherence, because virological suppression in plasma is common at adherence levels of >70%. Yet, it is unknown whether human immunodeficiency virus type 1 (HIV-1) replication is completely suppressed at these levels of adherence.

Methods. We longitudinally quantified levels of cell-associated HIV-1 RNA and DNA in 40 patients (median duration of successful ART before study initiation, 46 months), whose 1-week adherence to therapy prior to the sampling moments was measured electronically.

Results. Patients were constantly 100% adherent (the optimal-adherence group), demonstrated improving adherence over time (the improving-adherence group), or neither of the above (the poor-adherence group). Adherence never decreased to <70% in any patient, and no rebound in plasma virological levels was observed. Nevertheless, poor adherence but not optimal or improving adherence caused a significant longitudinal increase in cell-associated HIV RNA levels (P = .006). Time-weighted changes and regression slopes of viral RNA load for the poor-adherence group were significantly higher than those for the optimal-adherence group (P < .01).

Conclusions. Because ART only blocks infection of new cells but not viral RNA transcription in cells infected before therapy initiation, the observed effects strongly suggest that modest nonadherence can cause new cycles of HIV-1 replication that are undetectable by commercial plasma viral load assays.

Lifelong adherence to antiretroviral therapy (ART) by people living with human immunodeficiency virus (HIV) is vital for achieving and maintaining clinical success, because suboptimal adherence leads to inadequate suppression of virus replication, development of drug resistance, and treatment failure [1–4]. Whereas the early ART regimens, which were based on unboosted protease inhibitors (PIs), required >95% adherence for virological suppression, modern regimens based on nonnucleoside reverse-transcriptase inhibitors (NNRTIs) and boosted PIs are widely assumed to allow a certain degree of nonadherence while maintaining complete suppression of viral replication (ie, “forgiveness”) as a consequence of their higher potency and longer half-life [5]. This assumption is based on the fact that virological suppression, as measured by commercial plasma viral load assays, is common at adherence levels of >70% [6–10]. Yet, it is unknown whether HIV-1 replication is completely suppressed at these levels of adherence. Studies that analyzed whether adherence to ART is associated with transient HIV-1 viremia (ie, “blips”) have yielded conflicting results [11–13], and the association between adherence and HIV-1 load in the infected cells of ART-treated patients has, to our knowledge, not been studied.
It is unclear whether low-level replication of HIV continues in patients during ART, and a considerable debate on this matter is ongoing [14–18]. Here, we investigated whether residual replication could be promoted by modest nonadherence to ART. We studied the influence of slightly decreased adherence to therapy on the changes in levels of cell-associated viral markers (RNA and DNA). Surprisingly, even small deviations from 100% adherence, despite being forgiving for virological rebound in plasma, were found to be associated with significant longitudinal increase in the levels of cell-associated HIV-1 unspliced RNA (usRNA). Our findings suggest that constant optimal adherence may be necessary to stop all HIV replication and that forgiveness of ART may have been previously overestimated.

METHODS

Study Participants
We used longitudinally collected peripheral blood mononuclear cell (PBMC) samples from HIV-infected patients visiting the HIV outpatient clinic of the Academic Medical Center (Amsterdam, the Netherlands) between 2005 and 2007, in whom adherence to ART was measured electronically (MEMS-caps, Aardex) [1, 4] as part of a randomized controlled trial investigating the effects of an adherence intervention [19, 20]. The study was approved by the ethics committee of the Academic Medical Center, and all patients provided written informed consent. The study has been conducted in accordance with the ethical principles in the Declaration of Helsinki.

We selected 40 patients who showed optimal and reliable MEMS-data at all time points (no “pocket dosing” and no inaccurate use for any period were observed), who provided 3 longitudinal PBMC and plasma samples at 3–4-month intervals, and who were receiving successful ART (at least a 3-drug regimen, including a “backbone” of 2 nucleoside analogue reverse transcriptase inhibitors) at the start of the study. Success during ART was defined as an undetectable plasma viral load (ie, <50 copies/mL) in at least 2 consecutive measurements, divided by at least 3 months, prior to the start of the study.

Measurements of Viral Biomarkers
HIV-1 usRNA and total cell-associated viral DNA (vDNA) in PBMCs were quantified by seminested real-time polymerase chain reaction (PCR), as described earlier [21–23] (Supplementary Materials). The amounts of PBMC-derived HIV-1 DNA and RNA were normalized to total cellular inputs, which were quantified in separate real-time PCR assays, using the detection kits for either β-actin or ribosomal RNA, respectively (Applied Biosystems, Foster City, CA), and were expressed as the number of copies per 10^6 PBMCs (for vDNA) or the number of copies per microgram of total RNA (for usRNA). usRNA/vDNA ratios were calculated on the assumption that 1 μg of total cellular RNA corresponds to 10^6 PBMCs, as previously shown [24]. The sensitivity, reproducibility, and accuracy of these assays have been documented earlier, and in particular, assay variation was demonstrated to be low [21, 22]. Nevertheless, to exclude any interassay variation in the variances of longitudinal changes of usRNA and vDNA, all samples from any patient were always processed together, starting from the nucleic acid isolation, and quantified in one real-time PCR run.

Plasma viremia was quantified using a commercial ultrasensitive assay with a limit of detection of 50 copies/mL (Quantiplex bDNA 3.0, Bayer Diagnostics).

Statistical Analyses
For cellular HIV-1 load, statistical analyses were performed on log_{10}-transformed values. Undetectable levels of usRNA or vDNA were left-censored at the detection limits of corresponding assays. Linear mixed models were used to compare the changes of HIV-1 usRNA and vDNA levels between the adherence groups and to assess the longitudinal trends in the adherence groups. Changes in the usRNA level from time point 1 were compared with those for vDNA, using Wilcoxon signed rank tests. Longitudinal slopes of usRNA levels, vDNA levels, and the ratio of usRNA/vDNA levels in individual participants were calculated by linear regression, and time-weighted changes (TWCs) from time point 1 were calculated by linear trapezoidal integration. Slopes and TWCs of the viral parameters were compared between the adherence groups by Mann-Whitney U tests (for 2-group comparisons) or by Kruskal-Wallis tests (for 3-group comparisons). Linear mixed model analysis was performed by using PASW Statistics 18 (available at: http://www.spss.com/), and all other statistical tests were performed using GraphPad Prism 5.01 (available at: http://www.graphpad.com). All statistical tests were 2-sided. P values of <.05 were considered statistically significant.

RESULTS

Patients, Samples, and Adherence
In this study, we assessed the influence of longitudinal behavioral patterns of adherence to ART on the relative changes in cell-associated HIV-1 load. Levels of HIV-1 usRNA and vDNA in PBMCs were quantified in 40 HIV-infected individuals receiving ART, whose adherence to therapy was measured electronically. At the start of the study, these individuals had been receiving successful ART, with undetectable plasma viremia (except for incidental “blips,” defined as transient episodes of detectable plasma viremia measuring 50–1000 copies/mL) for a median of 46 months, and had a median CD4+ T-cell count of 620 cells/mm^3 (Tables 1 and 2). Viral nucleic acids were quantified in 3 longitudinally collected PBMC samples from every patient, taken at 3–4-month intervals (120
HIV-1 usRNA and vDNA loads were detectable in 91 samples (75.8%) and 116 samples (96.7%), respectively. The HIV-1 RNA load in plasma samples obtained at the same time that PBMC samples were collected was undetectable in 109 samples (90.8%); for the 11 samples (9.2%) with a detectable HIV-1 RNA load, its levels were <400 copies/mL for all samples and <100 copies/mL for all but 1 sample (Table 2). Thirty patients (75.0%) had undetectable plasma viremia at all 3 time points.

As a measure of adherence to therapy, we used the percentage of prescribed doses actually taken over the 1-week periods immediately prior to the PBMC sampling time points. At all 3 time points, patients demonstrated high adherence to ART (Table 2), with adherence never falling below 70% at any time point. Longitudinally, 3 main behavioral patterns were observed with respect to adherence. Twenty-three patients (57.5%) were 100% adherent at all time points (hereafter, the “optimal-adherence group”), 8 patients (20.0%) improved their adherence over time (<100% adherence at time point 1 but 100% adherence at the later time points; hereafter, the “improving-adherence group”), and the remaining 9 patients (22.5%) showed adherence that was decreasing, variable, or constantly <100% (hereafter, the “poor-adherence group”).

Association of Adherence and Longitudinal Trends of HIV-1 usRNA

No measurable virological rebound was observed in any of the patients, and detectability of low HIV-1 levels in plasma in the samples in total). HIV-1 usRNA and vDNA loads were detectable in 91 samples (75.8%) and 116 samples (96.7%), respectively. The HIV-1 RNA load in plasma samples obtained at the same time that PBMC samples were collected was undetectable in 109 samples (90.8%); for the 11 samples (9.2%) with a detectable HIV-1 RNA load, its levels were <400 copies/mL for all samples and <100 copies/mL for all but 1 sample (Table 2). Thirty patients (75.0%) had undetectable plasma viremia at all 3 time points.

As a measure of adherence to therapy, we used the percentage of prescribed doses actually taken over the 1-week periods immediately prior to the PBMC sampling time points. At all 3 time points, patients demonstrated high adherence to ART (Table 2), with adherence never falling below 70% at any time point. Longitudinally, 3 main behavioral patterns were observed with respect to adherence. Twenty-three patients (57.5%) were 100% adherent at all time points (hereafter, the “optimal-adherence group”), 8 patients (20.0%) improved their adherence over time (<100% adherence at time point 1 but 100% adherence at the later time points; hereafter, the “improving-adherence group”), and the remaining 9 patients (22.5%) showed adherence that was decreasing, variable, or constantly <100% (hereafter, the “poor-adherence group”).

Association of Adherence and Longitudinal Trends of HIV-1 usRNA

No measurable virological rebound was observed in any of the patients, and detectability of low HIV-1 levels in plasma in the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time Point 1</th>
<th>Time Point 2</th>
<th>Time Point 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 usRNA level, log₁₀ copies/µg total RNA</td>
<td>2.96 (2.29–3.39)</td>
<td>3.05 (2.57–3.69)</td>
<td>3.07 (2.50–3.51)</td>
</tr>
<tr>
<td>Range</td>
<td>1.85–4.38</td>
<td>2.01–4.05</td>
<td>1.76–4.22</td>
</tr>
<tr>
<td>HIV-1 vDNA level, log₁₀ copies/10⁶ PBMCs</td>
<td>2.90 (2.51–3.10)</td>
<td>2.90 (2.52–3.17)</td>
<td>2.96 (2.69–3.20)</td>
</tr>
<tr>
<td>Range</td>
<td>1.85–3.84</td>
<td>1.80–3.77</td>
<td>1.79–3.89</td>
</tr>
<tr>
<td>HIV-1 plasma viremia</td>
<td>Detectable (&gt;50 copies/mL)</td>
<td>20 (8/40)</td>
<td>7.5 (3/40)</td>
</tr>
<tr>
<td>Load, a copies/mL, median (range)</td>
<td>67.5 (54–366)</td>
<td>61 (58–78)</td>
<td>...</td>
</tr>
<tr>
<td>CD4⁺ T-cell count, cells/mm³</td>
<td>Median (IQR)</td>
<td>620 (425–765)</td>
<td>645 (467.5–765)</td>
</tr>
<tr>
<td>Range</td>
<td>200–970</td>
<td>190–990</td>
<td>260–1190</td>
</tr>
<tr>
<td>ART adherence</td>
<td>100%</td>
<td>75 (30/40)</td>
<td>90 (36/40)</td>
</tr>
<tr>
<td>&lt;100%</td>
<td>25 (10/40)</td>
<td>10 (4/40)</td>
<td>15 (6/40)</td>
</tr>
<tr>
<td>Adherence of those with &lt;100% adherence, %, median (range)</td>
<td>82 (71–93)</td>
<td>86 (71–93)</td>
<td>86 (86–93)</td>
</tr>
</tbody>
</table>

Data are % (proportion) of patients, unless otherwise indicated.

Abbreviations: HIV-1, human immunodeficiency virus type 1; IQR, interquartile range; PBMC, peripheral blood mononuclear cell; usRNA, unspliced RNA; vDNA, viral DNA.

a Data are for patients with detectable viremia.
minority of patients did not correlate with adherence (Table 3). Nevertheless, clear differences in longitudinal changes of cell-associated HIV-1 usRNA, but not of vDNA, were observed between the adherence groups (Figure 1A). Median changes in usRNA loads from time point 1 were −0.17 (interquartile range [IQR], −0.50 to 0.37), 0.14 (IQR, −0.50 to 0.36), and 0.62 (IQR, 0.36–1.40) log_{10} copies/μg total RNA for the optimal-adherence, improving-adherence, and poor-adherence groups, respectively (P = .001, by linear mixed models for the comparison between patients in the optimal- and poor-adherence groups). The longitudinal trend of usRNA in the poor-adherence group, estimated by fitting the linear mixed model, was significantly positive (median slope, 0.12 log_{10}/month [95% CI, .04–.20]; P = .006), meaning that poor adherence was associated with a significant longitudinal increase in the levels of usRNA, whereas no significant longitudinal trends in usRNA levels were observed for patients in the optimal- or improving-adherence groups (slopes, −0.02 [95% CI, −0.06 to 0.01] and 0.00 [95% CI, −0.08 to 0.08] log_{10}/month, respectively; P = .21 and P = .94, respectively) (Figure 1B). The fluctuations of the usRNA level, measured by the absolute values (moduli) of the changes in usRNA level from time point 1, were significantly higher than those of the vDNA level at both time points (P = .0010 and P = .0017, by Wilcoxon paired tests).

Next, 2 forms of longitudinal trends (linear regression slopes and TWCs) of usRNA and vDNA loads were calculated for every patient and compared between patients with different adherence patterns (Figure 2). Remarkably, a gradient of median TWCs in usRNA loads was observed that correlated with adherence patterns, as follows: −0.04 (IQR, −0.47 to 0.23), 0.06 (IQR, −0.10 to 0.23), and 0.45 (IQR, 0.30–0.97) log_{10} for the optimal-adherence, improving-adherence, and poor-adherence groups, respectively (P = .0023, by the Kruskal-Wallis test, for the 3-group comparison; P = .0009, by the Mann-Whitney U test, for the comparison between the optimal- and poor-adherence groups). A similar gradient was observed for usRNA slopes, with median values of −0.04 (IQR, −0.07 to 0.05), −0.01 (IQR, −0.08 to 0.07), and 0.10 (IQR, 0.02–0.21) log_{10}/month for the optimal-adherence, improving-adherence, and poor-adherence groups, respectively (P = .0023 for the 3-group comparison; P = .0009, by the Mann-Whitney U test, for the comparison between patients in the optimal- and poor-adherence groups). Again, no correlation with adherence was observed for the longitudinal trends in vDNA levels.

Table 3. Parameters Associated With Longitudinal Adherence Patterns, by Adherence Group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimal (n = 23)</th>
<th>Improving (n = 8)</th>
<th>Poor (n = 9)</th>
<th>Optimal vs Improving</th>
<th>Optimal vs Poor</th>
<th>Improving vs Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>49.6 (41.3–55.2)</td>
<td>42.9 (36.6–52.0)</td>
<td>40.8 (34.5–48.5)</td>
<td>.23</td>
<td>.029</td>
<td>.54</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>91 (21/23)</td>
<td>88 (7/8)</td>
<td>89 (8/9)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Female</td>
<td>9 (2/23)</td>
<td>12 (1/8)</td>
<td>11 (1/9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of suppressive ART, mo = b</td>
<td>54.3 (19.3–75.7)</td>
<td>36.1 (17.0–81.0)</td>
<td>44.3 (18.4–86.7)</td>
<td>.67</td>
<td>.87</td>
<td>.89</td>
</tr>
<tr>
<td>ART regimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNRTI</td>
<td>61 (14/23)</td>
<td>75 (6/8)</td>
<td>56 (5/9)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PI</td>
<td>30 (7/23)</td>
<td>25 (2/8)</td>
<td>33 (2/9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily dosing regimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 dose</td>
<td>61 (14/23)</td>
<td>63 (5/8)</td>
<td>67 (6/9)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2 doses</td>
<td>39 (9/23)</td>
<td>37 (3/8)</td>
<td>33 (2/9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T-cell count, cells/mm³</td>
<td>597 (463–743)</td>
<td>693 (542–842)</td>
<td>613 (407–902)</td>
<td>.23</td>
<td>.49</td>
<td>.74</td>
</tr>
<tr>
<td>Plasma HIV-1 viremia detected at any time</td>
<td>26 (6/23)</td>
<td>25 (2/8)</td>
<td>22 (2/9)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Data are % (proportion) of participants or median value (interquartile range). Adherence patterns are defined in the first subsection of Results. Abbreviations: ART, antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor.

a P values were calculated by Mann-Whitney U tests (for continuous variables) or Fisher exact tests (for discrete variables).

b Time with suppressed plasma viremia before study initiation.

c Defined as a level of >50 copies/mL.
As a measure of HIV transcriptional activity per infected cell, usRNA/vDNA ratios were then calculated for every patient at each time point, and TWCs and slopes of usRNA/vDNA ratios were produced. Similar to the usRNA values, gradients of TWCs and slope values, correlating with adherence patterns, were observed for usRNA/vDNA ratios (Figure 2): for the optimal-adherence, improving-adherence, and poor-adherence groups, median values were $-0.20$ (IQR, $-0.55$ to $0.10$), $0.17$ (IQR, $-0.34$ to $0.36$), and $0.59$ (IQR, $0.23$–$0.82$) log$_{10}$, respectively, for TWCs ($P = .0033$ for the 3-group comparison; $P = .0017$ for the comparison between the optimal- and poor-adherence groups) and $-0.07$ (IQR, $-0.15$ to $0.06$), $-0.03$ (IQR, $-0.09$ to $0.08$), and $0.10$ (IQR, $-0.01$ to $0.16$) log$_{10}$/month, respectively, for slopes ($P = .027$ for the 3-group comparison; $P = .011$ for the comparison between the optimal- and poor-adherence groups).

Among the demographic, immunological, virological parameters and ART regimen data, only age was significantly associated with longitudinal adherence patterns (Table 3). Adjustment for age did not change the significance of the association between trends in usRNA levels and adherence. None of the parameters was significantly associated with longitudinal trends of usRNA or vDNA levels (Supplementary Table 1).

**Figure 1.** Longitudinal changes of unspliced RNA (usRNA) and viral DNA (vDNA) loads in patients with different adherence patterns. **A,** Dots represent cumulative measurements of changes of usRNA (left panels) and vDNA (right panels) loads from time point 1 of patients with different adherence patterns at both follow-up time points. Colored lines on the $y$-axes depict the median changes of usRNA or vDNA loads in the corresponding adherence pattern groups. $P$ values of comparisons between the groups were calculated by fitting linear mixed models. **B,** Lines correspond to the individual longitudinal trends of usRNA (left panels) and vDNA (right panels) loads in each group. Arrows represent the directionality of longitudinal slopes in each group, estimated by linear mixed models. Thickness of arrows signifies the strength of corresponding longitudinal trends. An arrow is horizontal if the corresponding $P$ value is >.5.

We asked whether the association of longitudinal trends of usRNA levels with adherence, as observed in the full sample set, also holds for the 30 aviremic patients (ie, those with undetectable plasma viremia at all 3 time points). TWCs and slopes of usRNA levels were indeed also associated with
longitudinal adherence patterns in the aviremic patients (Figure 3A): the difference between median TWC usRNA values for patients in the optimal- and poor-adherence groups was 0.80 log$_{10}$ ($P = .0092$, by the Mann–Whitney $U$ test), and the corresponding difference for the slopes was 0.14 log$_{10}$/month ($P = .024$). Trends in usRNA or vDNA loads did not significantly differ between aviremic and viremic patients (Supplementary Table 1).

Next, we sought to determine whether adherence was associated with HIV-1 usRNA levels in patients with long and short virological suppression during ART. Patients were stratified into 2 groups according to the period of undetectable plasma viremia before study initiation. Four years of successful therapy was chosen as a cutoff because it was close to the median in this cohort: 21 patients had <4 years of continuous virological suppression before the start of this study (median duration, 1.6 years [IQR, 1.3–2.3 years]), whereas 19 patients had virological suppression for >4 years (median duration, 6.7 years [IQR, 5.7–8.3 years]). As shown in Figure 3B, adherence was significantly associated with longitudinal trends of usRNA levels in both of these patient strata, with the effects similar in both strata and mirroring those described above for the full sample set. The differences between the TWCs in usRNA loads for patients in the optimal- and poor-adherence groups were 0.43 log$_{10}$ ($P = .031$, by the Mann-Whitney $U$ test) for patients with short virological suppression and 0.74 log$_{10}$ ($P = .018$) for patients with long virological suppression. Remarkably, the association was even somewhat stronger in patients with long virological suppression (as an example, compare the associations of usRNA slopes with adherence in Figure 3B).
Finally, we asked whether the associations of usRNA load with adherence were similar in NNRTI- and PI-treated patients. Figure 3 shows that this was indeed the case. In the 25 NNRTI-receiving patients, TWCs and slopes of usRNA levels were significantly associated with adherence: the difference between median TWC usRNA values for patients in the optimal- and poor-adherence groups was 0.78 log₁₀\(^{10}\) (\(P = .0026\), by the Mann-Whitney U test), and the corresponding difference for the slopes was 0.15 log₁₀/month (\(P = .026\)). In the 12 PI-receiving patients, the associations were observed as a trend but did not reach statistical significance: the difference between median TWCs in usRNA values among patients in the optimal- and poor-adherence groups was 0.3 log₁₀\(^{10}\) (\(P = .18\), by the Mann-Whitney U test), and the corresponding difference for the slopes was 0.08 log₁₀/month (\(P = .21\)).

**DISCUSSION**

The goal of ART is suppression of the HIV RNA level in plasma to below the detection limit of the most sensitive clinical assay. In most ART-treated individuals, this goal is achieved, and therefore additional virological markers should be identified that correlate with infection progression and/or predict the therapy outcome. One of the candidate molecular
markers is the HIV-1 usRNA level. In the untreated infection, the usRNA level in PBMCs increases significantly faster than that of the plasma viral RNA level [23], and it is detectable in the majority of PBMC samples from patients receiving ART [21, 22, 25, 26], where its levels can predict treatment failure [22]. Here, we demonstrated that, in HIV-infected individuals who, during ART, had a clinical history of virological success that lasted a median of 3.8 years, longitudinal trends of HIV-1 usRNA levels were strongly associated with adherence to therapy. To the best of our knowledge, this is the first report demonstrating an association between adherence to ART and HIV-1 load in the infected cells of patients with plasma viremia undetectable by commercial assays. Remarkably, in terms of usRNA trends, patients with constant 100% adherence did the best, followed by those with improving adherence and, finally, by those with a poor longitudinal adherence pattern. This indicates that the HIV-1 usRNA level in PBMCs is a viral molecular marker with a significantly better sensitivity to modest changes in adherence than viral RNA in plasma, measured by an assay with a detection limit of 50 copies/mL.

The effects observed in this study were independent of time of previous virological suppression. Two recent studies demonstrated the dramatic decline in the risk of virological failure as the duration of therapy increased, even in low-adherence strata [27, 28]: by 6 years of virological suppression, the risk of failure was close to 0, even for patients with <40% adherence [27]. These results suggest that suboptimal adherence is becoming more forgivable the longer a patient is virologically suppressed. In contrast, in our cohort, “poor” (but still >70%) adherence caused a significant increase in the levels of usRNA irrespective of suppression time, including in a subset of patients with a median of 6.7 years of virological suppression. Our results suggest that constant optimal adherence to ART may be required lifelong to prevent the adverse effects of residual virus replication (see below). In addition, although NNRTI-based regimens may be more forgivable for suboptimal adherence than the PI-based regimens for virological rebound in plasma [6–8, 10], in our study they were still sensitive to small differences in adherence patterns in terms of trends of HIV-1 usRNA levels in PBMCs, because a significant association between usRNA level and adherence was observed for the NNRTI-treated patients, as well.

No association of longitudinal trends of HIV-1 DNA levels and adherence patterns was observed in this study. Levels of vDNA, which reflect the total size of the viral reservoir (ie, the total amount of HIV-infected cells), were more stable in time than those of usRNA, which reflect the size of the “active viral reservoir” (ie, the fraction of HIV-infected cells in which viral RNA is transcribed at a given moment). In other words, whereas the total amount of cells harboring HIV-1 provirus was stable and “indifferent” to adherence, the proportion of these cells actively transcribing viral RNA was fluctuating in time, and these fluctuations were strongly associated with adherence patterns. The finding that, despite a profound effect on usRNA levels, no effect of suboptimal adherence on vDNA burden was measured is in line with the evidence that only a small fraction of HIV DNA–positive cells is productively infected in ART-treated patients, but a productively infected cell can contain thousands of HIV RNA copies [29]. In accordance with this, HIV transcriptional activity per infected cell (the usRNA/vDNA ratio) also demonstrated a strong association with adherence, mirroring the effects observed for usRNA.

Low-level production of virus is observed in most patients during ART [30, 31], but it is controversial whether it reflects residual virus replication [16, 32–37]. Likewise, HIV-1 RNA has been repeatedly detected in peripheral blood and tissues of ART-treated patients [22, 25, 26, 38–41], but the mere presence of viral RNA in the infected cells also does not necessarily imply virus replication (ie, infection of new cells) and can be caused by reactivation of latently infected cells. However, a decrease in the level of HIV-1 usRNA in the ileum upon therapy intensification was recently reported [36], suggesting that residual replication may be ongoing in some compartments. We have previously demonstrated that higher levels of usRNA in PBMCs of ART-treated patients were predictive of future therapy failure [22]. That observation suggested that, in some patients, residual viral replication continues during ART and leads to therapy failure. Our present findings imply that also patients who do not experience virological failure during ART but are even modestly nonadherent may still have ongoing low-level residual HIV replication. ART only blocks infection of new cells, not HIV-1 RNA transcription in infected cells. Therefore, if increased levels of usRNA in PBMCs are associated with decreased ART pressure (eg, due to suboptimal adherence, as observed in this study), this strongly suggests infection of new cells. Notably, our study does not address the presence of ongoing replication in patients who are constantly 100% adherent. However, if an average ART-treated patient takes approximately 75% of the prescribed doses [5], it is reasonable to speculate that most patients experience bursts of residual replication at some point during treatment, albeit at levels that are probably not sufficient to cause rapid development of full-blown drug resistance and virological failure in the majority of patients. However, several studies have reported accumulation of drug resistance mutations at very high but not absolute levels of adherence (>90%) [42, 43]. Further studies, possibly involving genotyping of PBMC-derived viral RNA by deep sequencing, are necessary to determine whether the low-level residual virus replication facilitates the generation of drug-resistance mutants.

Furthermore, even if no virological rebound in plasma is observed, it is plausible that constant low-level viral replication would exert continuous pressure on the immune system and
cause additional morbidity as a result of persistent immune activation, inflammation, and immunosenescence [16, 44–46]. Although ART has dramatically increased the median survival time of HIV-infected individuals, several studies have found excess mortality rates in the infected and ART-treated population, compared with the general population [47–49]. It is unclear whether this excess mortality is due to the adverse effects of the antiretroviral drugs or to the effects of HIV infection itself. If the latter is true, an effort should be made to ensure that HIV-1 replication during therapy is maximally suppressed. On the basis of our present observations, we suggest that adherence to ART should be constantly monitored in the clinic and improved through behavioral intervention if necessary.

Several limitations of this study deserve comment. First, our usRNA assay does not distinguish between genuine intracellular HIV-1 usRNA and cell-associated virion RNA. However, it was previously shown [50] that in patients receiving ART with plasma viremia suppressed to <50 copies/mL, the extracellular fraction comprised, on average, 0.6% of total quantified usRNA level, which is negligible. Second, because unfractiated PBMCs were used, usRNA and vDNA values were normalized for total PBMC input but not for amounts of CD4+ T cells. It should be noted, however, that normalization of usRNA and vDNA values to the relative CD4+ T-cell content (assessed by the CD4+ T-cell counts) did not alter the effects observed in this study (Supplementary Figure 1). Third, because of insufficient sample size, we were unable to determine whether the observed effects were similar between patients receiving different ART regimens. We did determine, however, that NNRTI- and PI-treated patients showed similar outcomes (Figure 3C).

In summary, although it is unclear whether ART can completely stop HIV replication, our results strongly suggest that full adherence to modern ART is a prerequisite for this. Further studies are warranted to establish whether cell-associated HIV-1 RNA can be used in the clinic as a reliable surrogate marker of adherence to ART. Forgiveness of ART, defined as an ability to maintain complete viral suppression despite imperfect adherence [5], may require reevaluation in view of our results.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

**Acknowledgments.** We thank the study participants for their involvement.

**Financial support.** This work was supported by the Dutch AIDS Fonds (available at: http://www.aidsfonds.nl; grant 2004045).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**


19. de Bruin M, Hospers HJ, van Breukelen GJ, Kok G, Koevoets WM, Prins JM. Electronic monitoring-based counseling to enhance