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No Effect of Raltegravir Intensification on Viral Replication Markers in the Blood of HIV-1-infected Patients Receiving Antiretroviral Therapy

Rajesh T. Gandhi[1], **Robert W. Coombs**[2], **Ellen S. Chan**[3], **Ronald J. Bosch**[3], **Lu Zheng**[3], **David M. Margolis**[4], **Sarah Read**[5], **Beatrice Kallungal**[6], **Ming Chang**[2], **Erin A. Goecker**[2], **Ann Wiegand**[7], **Mary Kearney**[7], **Jeffrey M. Jacobson**[8], **Richard D'Aquila**[9], **Michael M. Lederman**[10], **John W. Mellors**[11], and **Joseph J. Eron**[4] **for the AIDS Clinical Trials Group (ACTG) A5244 team**

[1]Massachusetts General Hospital and Ragon Institute, Boston, Massachusetts, USA

[2]University of Washington School of Medicine, Seattle, Washington, USA

[3]Harvard School of Public Health, Boston, Massachusetts, USA

[4]University of North Carolina, Chapel Hill, North Carolina, USA

[5]National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA

[6]Social & Scientific Systems, Inc., Silver Spring, Maryland, USA

[7]National Cancer Institute, Frederick, Maryland, USA

[8]Drexel University, Philadelphia, Pennsylvania, USA

[9]Vanderbilt University School of Medicine, Cleveland, Ohio, USA

[10]Case Western Reserve University, Cleveland, Ohio, USA

[11]University of Pittsburgh, Pittsburgh, Pennsylvania, USA

Abstract

Background—Controversy continues regarding the extent of ongoing viral replication in HIV-1-infected patients on effective antiretroviral therapy (ART). Adding an additional potent agent, such as raltegravir, to effective ART in patients with low-level residual viremia may reveal whether there is ongoing HIV-1 replication.

Methods—We previously reported the outcome of a randomized, placebo-controlled study of raltegravir intensification in patients on ART with HIV-1 RNA <50 copies/mL that showed no effect on residual viremia measured by single copy assay (SCA). We now report the effects of

Corresponding author and address for reprints: Rajesh T. Gandhi, M.D., Massachusetts General Hospital, GRJ 504, 55 Fruit St, Boston, MA 02114. rgandhi@partners.org. Telephone: 617-724-9690. Fax: 617-726-7653.

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raltegravir intensification in that trial on other potential measures of ongoing HIV-1 replication: 2-LTR HIV-1 circles, total cellular HIV-1 DNA and T cell activation.

Results—Of 50 patients tested, 12 (24%) had 2-LTR-circles detected at baseline. Patients who were 2-LTR-positive had higher plasma HIV-1 RNA and HIV-1 DNA levels than 2-LTR-negative individuals. At week 12 of raltegravir intensification, there was no change from baseline in 2-LTR circles, in total HIV-1 DNA or in the ratio of 2-LTR circles to total HIV-1 DNA. There was also no change in markers of T cell activation.

Conclusions—In HIV-1-infected individuals on effective antiretroviral therapy, we find no evidence of ongoing viral replication in the blood that is suppressible by raltegravir intensification. The results imply that raltegravir intensification alone will not eradicate HIV-1 infection.

Keywords

Raltegravir; HIV-1; viral replication; reservoirs; 2-LTR circles; HIV-1 DNA; T cell activation

Introduction

Combination antiretroviral therapy (ART) lowers plasma human immunodeficiency virus (HIV)-1 RNA levels to below the detection limit of commercial assays, but most patients have residual low-level viremia when tested with more sensitive methods [1-3]. It is not known whether residual viremia arises from ongoing, complete cycles of viral replication and infection of new cells, sporadic or continuous virus release from stable reservoirs, or both. Further understanding of whether there is ongoing viral replication in HIV-1-infected patients on ART may inform eradication strategies[4-5] and approaches to reduce abnormal levels of immune activation in patients on therapy[6].

Several lines of evidence argue against ongoing cycles of viral replication as being the main source of residual viremia. Adding a potent antiretroviral agent, such as raltegravir, to an already suppressive regimen did not lower the level of residual viremia in several studies[7-11]. Moreover, there is no evidence for evolution of drug resistance mutations in patients on suppressive ART[12], which also suggests that HIV-1 replication is minimal.

By contrast, some studies have suggested that there is ongoing viral replication in patients receiving suppressive ART that is only revealed by measures other than viremia, such as persistent T cell activation or 2-LTR HIV-1 circles, a putative marker of recently-infected cells. For example, in a recent study, a transient increase in 2-LTR circles was seen at weeks 2 and 4 after raltegravir was added to effective ART[8]. Additionally, 2-LTR-positive patients had higher levels of CD8 cell activation than 2-LTR-negative individuals, and the level of CD8 cell activation declined in the 2-LTR-positive patients during raltegravir intensification. These findings were interpreted as evidence of ongoing HIV-1 replication, at least in a subset of patients. There is debate, however, regarding the decay rate of 2-LTR circles and their specificity as a marker of recently infected cells[13-14], especially in individuals on effective ART in whom the turnover of 2-LTR circles may differ from those not on treatment.

To address whether there is evidence for ongoing HIV-1 replication in patients on suppressive ART, we conducted a randomized placebo-controlled trial (ACTG A5244) of raltegravir intensification in patients receiving suppressive ART. The primary outcome of this trial, as previously reported[7], is that raltegravir intensification did not reduce plasma HIV-1 RNA measured by single copy assay (SCA). We performed additional analyses of this trial to address whether intensification with raltegravir affects other potential markers of HIV-1 replication, including 2-LTR circles, total HIV-1 DNA and T cell activation.

Methods

Study Population

The trial design and study population have been previously described[7]. All patients provided written informed consent for the study (NCT#00515827). The main inclusion criteria were: 1) HIV-1 infected adults receiving ART for ≥12 months with ≥2 nucleoside reverse transcriptase inhibitors (NRTI) and a ritonavir-boosted protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI); 2) plasma HIV-1 RNA levels below detection limits for ≥6 months using commercial assays; 3) CD4 cell count ≥200/mm^3; 4) pre-ART HIV-1 RNA >100,000 copies/mL. Patients who met these criteria underwent testing with a real-time PCR assay that can detect a single copy of HIV-1 RNA in a plasma sample. Patients who had detectable SCA HIV-1 RNA (>0.2 copies/mL) on a screening assay were eligible for intensification, and were randomized 1:1 to immediate-intensification (group A, raltegravir-first) or delayed-intensification (group B, placebo-first).

Study Procedures and Statistical Methods

Patients in the immediate-intensification group at week 0 added raltegravir 400 mg bid to their entry regimen; at week 12, they stopped raltegravir and added matching placebo bid for 12 more weeks. Patients in the delayed-intensification group at week 0 added placebo first and at week 12 stopped placebo and added raltegravir for 12 more weeks. At baseline, weeks 12 and 24 we measured plasma HIV-1 RNA by SCA, total HIV-1 DNA and extrachromosomal (episomal) HIV-1 DNA (2-LTR circles) in peripheral blood mononuclear cells (PBMC), and CD4 and CD8 cell activation (as defined by percentage that co-expressed CD38 and HLA-DR).

Data were excluded when subjects went off study treatment or experienced virologic failure (confirmed HIV-1 RNA >50 copies/mL). SCA measurements below lower limits of quantification (LLQ) were imputed as LLQ divided by 2. HIV-1 DNA values below LLQ were imputed a value of 10 copies/million PBMC and divided by CD4 cell percentage at the same time point as the HIV-1 DNA measurement. All baseline values were calculated as the average of pre-entry and entry except for HIV-1 DNA and SCA HIV-1, which used the geometric mean instead. Baseline 2-LTR circle status was defined as positive when either of the pre-entry or entry measurements was detectable. For analysis of 2-LTR circle to HIV-1 DNA ratio, when 2-LTR circles were undetectable, they were imputed a value of 0.1 copies/million PBMC.

The treatment groups and 2-LTR-circle-positive and –negative groups were compared using exact Wilcoxon rank sum tests for continuous endpoints, stratified Wilcoxon rank sum tests when there were adjustment variables and Fisher's exact tests for proportions. Rank-based (Spearman) correlations were used to assess associations between responses. All statistical tests were two-sided, exploratory without adjusting for multiple comparisons, and 0.05 was used for nominal level of significance.

Nucleic acid purification

Total HIV-1 DNA and 2-LTR circles were extracted from duplicate aliquots of 5×10^6 cryopreserved PBMCs. The quantity of extracted PBMC DNA was determined by Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA) and the number of PBMC for reporting purposes was derived using 6.25 micrograms of DNA/million PBMCs[15]. Extrachromosomal 2-LTR HIV DNA was extracted using the Qiagen Q1Aprep Spin Miniprep Kit (Qiagen, Santa Clara, CA) in 100 microliters of AE buffer containing 10 ng/microliter of carrier RNA[13, 16]. Immediately prior to DNA extraction, 300 copies of an internal pCRII-derived plasmid control (pNFC) containing a drosophila fly gene DNA insert, flanked by the HIV-1

gag HBX2 forward- and SK431 gag reverse-primer target sequences, was added to the PBMC aliquot, co-extracted with 2-LTR HIV-1 DNA, and detected by multiplex PCR[17]. Total HIV-1 DNA from 5×10^6 PMBC was extracted into FG3 buffer using the Qiagen FlexiGene kit. To minimize measurement variability, the respective nucleic acid extracts from each subject were tested in the same assay run.

Quantification of HIV-1 2-LTR circles

Quantification of extrachromosomal HIV-1 2-LTR circles used a single-step, real-time PCR in a 50 microliter PCR reaction mixture containing 12.5 microliters of the AE buffer extract (representing the extrachromosomal extract from 625,000 PBMCs) plus 37.5 microliters of TaqMan Platinum Quantitative PCR Super Mix-UDG with Rox Master Mix (Invitrogen), containing the following primers and probes (Invitrogen): 2-LTR forward primer, 5′-CGT CTG TTG TGT GAC TCT GGT AAC T-3′; 2-LTR reverse primer, 5′-GGA GTG AAT TAG CCC TTC CA-3′; HIV-1 gag (HBX2) forward primer, 5′-CAA GCA GCC ATG CAA ATG TT-3′ and HIV-1 gag (SK431) reverse primer, 5′-TGC TAT GTC ACT TCC CCT TGG TTC TCT-3′; 2-LTR probe, FAM-AGA-TCC CTC AGA CCC T-MGBNFQ and NFC probe, VIC-ATT GTA GTT GGT AGG AC-MGBNFQ. Appropriate HIV-1 2-LTR positive (HIV-1 Ba-L-infected PBMC) and negative PBMC controls were also run with each assay along with a dilution series of an external 2 LTR ssDNA standard curve (Invitrogen): 5′-GTG CCC GTC TGT TGT GTG ACT CTG GTA ACT AGA GAT CCC TCA GAC CCT TTT AGT CAG TGT GGA AAA TCT CTA GCA TGG AAG GGC TAA TTC ACT CCC AAA G-3′.

Quantification of total HIV-1 gag DNA

We used a single-step, real-time PCR assay to quantify total HIV-1 DNA in 50 microliters of PCR reaction mix containing 12.5 microliters of chromosomal HIV-1 DNA and 37.5 microliters of TaqMan Platinum Quantitative PCR Super Mix-UDG with Rox Master Mix (Invitrogen, Carlsbad, CA), containing the internal competitive control NFC primers, gag HBX2 forward and SK431 reverse primers, and NFC and gag probes as previously described[18]. An external standard curve was used[18]. All PCR amplifications were performed using an Applied Biosystems 7900 Real-time PCR system.

Detection limits and intra-assay precision

The lower limit of detection for 2-LTR circles was 1 copy/million PBMC. The lower limit of detection for total HIV-1 DNA was 10 copies/million PBMC. The intra-assay precision (standard deviation (SD) of $\log_{10}$ transformed copies/PCR reaction; coefficient of variation (CV, %) for non-$\log_{10}$ transformed data) for 2-LTR circles (SD, 0.10; CV 8.5%) and HIV-1 DNA (SD, 0.13; CV 28%) was adequate to reliably detect at least 3 or 4–fold change in DNA targets among the three time-points evaluated for each subject. The extraction and PCR amplification recovery for the NFC was consistently >90%; as such, neither 2-LTR circles nor total HIV-1 DNA were adjusted for the recovery of the NFC plasmid control. The reported values for 2-LTR circle and HIV-1 DNA were expressed as copies/million CD4 cells. The number of CD4 cells was calculated by multiplying total PBMC count by CD4 cell percentage.

Results

Baseline 2-LTR circle status

Of 50 patients tested, 12 (24%) had detectable 2-LTR circles (2-LTR^{pos}) at baseline. Among patients with detectable 2-LTR circles at baseline, the median number was 65 copies/million CD4 cells. Two-LTR^{pos} patients had higher SCA HIV-1 RNA than 2-LTR^{neg} individuals

(median 2 vs. 1 copy/mL, p=0.040) (Table). Two-LTR^{pos} patients also had higher baseline HIV-1 DNA levels than 2-LTR^{neg} individuals (median 18.1 vs. 10.4 × 10^3 copies/million CD4 cells, p=0.025) (Table). Two-LTR^{pos} and 2-LTR^{neg} patients were similar in terms of years since first HIV-1 RNA below detectable limits, baseline CD4 and CD8 cell activation, background regimen (PI vs. NNRTI), baseline CD4 cell count and pre-ART HIV-1 RNA level (Table).

Baseline Total HIV-1 DNA

Total HIV-1 DNA was measured in 49 patients' samples at baseline. We found trends for associations between higher total HIV-1 DNA and the following: higher SCA HIV-1 RNA (r=0.24, p=0.094) and higher pre-ART HIV-1 RNA (r=0.28, p=0.06). We did not find any association between total HIV-1 DNA and baseline CD4 cell count, CD4 or CD8 cell activation, years since first HIV-1 RNA below detectable limits by commercial assays or background antiretroviral regimen (data not shown).

Effect of Raltegravir Intensification on HIV-1 RNA by SCA

As previously reported[7], raltegravir intensification did not reduce low-level viremia either by comparing averaged week 10 and 12 SCA HIV-1 RNA between the group that added raltegravir and the group that added placebo or by examining the change in HIV-1 RNA from baseline. New analyses revealed that neither patients on PI-containing background ART nor those on NNRTI-containing ART had a decrease in SCA during intensification (raltegravir-first group, baseline to week 12; placebo-first group, week 12 to week 24): the median (Q1, Q3) change in SCA from pre- to post-intensification (copies/mL) was −0.22 (−1.02, 0.21) in patients on PI-containing regimens and −0.04 (−1.20, 0.93) in those on NNRTI-containing regimens.

Effect of Raltegravir Intensification on 2-LTR Circles

At week 12, the proportion of patients who were 2-LTR^{pos} was not significantly different in the placebo and raltegravir groups (Figure 1). Two-LTR circle levels also did not differ by treatment group. During raltegravir intensification, 3 patients (7%) who were initially 2-LTR^{neg} became 2-LTR^{pos} and 4 (10%) who were 2-LTR^{pos} became 2-LTR^{neg}. Similar changes were seen during the placebo period. Moreover, the proportion of never-detectable (2-LTR^{neg} at all time points, n=33) and ever-detectable (2-LTR^{pos} at any time point, n=17) subjects did not differ by treatment group or background regimen. Patients who were 2-LTR ever-detectable had a trend towards a shorter duration of virologic suppression than those who were 2-LTR never-detectable (median 4 vs. 6 years, p=0.055).

During intensification, patients who were 2-LTR^{pos} at baseline did not have a greater decline in SCA HIV-1 RNA than those who were 2-LTR^{neg}: those who were 2-LTR^{pos} had a median (Q1, Q3) change in SCA during intensification of −1 (−1, 0) copies/mL whereas those who were 2-LTR^{neg} had a change of 0 (−1, 1) copies/mL (p=0.18 for comparison). Similarly, no significant difference in change in SCA was seen during intensification between patients who 2-LTR never-detectable and those who were 2-LTR ever-detectable (data not shown).

Finally, patients who were 2-LTR^{pos} at baseline did not have a greater change in CD4 cell count during intensification that those who were 2-LTR^{neg} (data not shown).

Effect of Raltegravir Intensification on HIV-1 DNA and 2-LTR/total HIV-1 DNA ratio

Total HIV-1 DNA was assessed at baseline, week 12 and 24. One patient had a level below LLQ at baseline and week 12; these values were imputed to 10 copies/million PBMC (see Methods). HIV-1 DNA did not change in either treatment group (Figure 2). Combining

treatment groups (n=42), the median (Q1, Q3) change in HIV-1 DNA from pre- to post-intensification was 0.0 (−4.0, 2.5) × 10^3 copies/million CD4 cells (p=0.81). We did not observe a significant change in the 2-LTR circle to HIV-1 DNA ratio during intensification (data not shown). The 2-LTR^{pos} group had a greater decline in HIV-1 DNA from pre- to post-intensification than the 2-LTR^{neg} group: median change −4.2 (−9.9, 2.1) vs. 0.3 (−2, 3.6) × 10^3 copies/million CD4 cells, p=0.044.)

Effect of Raltegravir Intensification on T cell activation

If adding raltegravir inhibits ongoing HIV-1 replication that is inducing T cell activation, intensification might lower the level of CD4 and CD8 cell activation. As reported [7], we did not observe a significant decline during raltegravir intensification in CD4 or CD8 cell activation, defined as percentage that co-express CD38 and HLA-DR. New analyses showed that neither the 2-LTR^{pos} nor the 2-LTR^{neg} groups had a significant decline in percentage of CD4 or CD8 cells that were CD38+HLA-DR+.

Although there was no effect of 12 weeks of raltegravir intensification on T cell activation in the overall study population, we found that higher baseline total HIV-1 DNA was significantly associated with a greater decline in CD4 cell activation (% CD38+/HLA-DR+) (r = −0.39, p=0.015). We did not observe a significant association between HIV-1 DNA and change in CD8 cell activation (% CD38+/HLA-DR+) (r = −0.23, p = 0.16).

Discussion

In a randomized, placebo-controlled study, we found that 12 weeks of raltegravir intensification did not reduce SCA HIV-1 RNA, total HIV-1 DNA, or T cell activation. Moreover, there was no change in 2-LTR circles at week 12 nor was there an effect on 2-LTR circle to HIV-1 DNA ratio. Taken together, these results indicate that adding raltegravir to suppressive ART does not reduce several measures of HIV-1 burden, at least in blood or in compartments that freely communicate with the blood.

We found that a substantial proportion of patients in our study (24%) were 2-LTR^{pos} prior to adding raltegravir. This proportion is higher than was seen in a previous study by Buzon et al[8] (5 of 69, 7.2%), possibly because the current study required patients to have a pre-ART HIV-1 RNA >100,000 c/mL and to have a positive SCA to enter the study (neither was an inclusion criterion for the Buzon et al trial). In the current study, the 2-LTR assay had a lower limit of detection of 1 copy/million PBMC, which appears to be similar to that of the assay used by Buzon et al based on data shown in their paper. Interestingly, 2-LTR^{pos} patients had a higher SCA HIV-1 RNA and HIV-1 DNA than 2-LTR^{neg} patients. However, raltegravir intensification did not affect SCA HIV-1 RNA in either the 2-LTR^{pos} or in the 2-LTR^{neg} groups. These data suggest that 2-LTR circles may be a measure of residual virus burden in patients suppressed on ART rather than a marker of ongoing replication. We did observe a decline in total HIV-1 DNA with intensification in 2-LTR^{pos} patients. This may be a chance observation or regression to the mean in a small subset population. We cannot rule out a true effect, though the mechanism is unclear given no effect of intensification on SCA HIV-1 RNA or 2-LTR circles in this subset. We did not observe an association between PI use and 2-LTR positivity as was reported in a prior study[8].

We did not observe a change in 2-LTR circles 12 weeks after raltegravir intensification. However, it is important to stress that we did not measure 2-LTR circle status at weeks 2 and 4 after intensification, as was done in a study by Buzon *et al*[8]; for this reason, we cannot directly compare our results to those obtained in the previous study. In the trial published by Buzon et al, there was a significant increase in 2-LTR circles in some patients at time points between weeks 2 and 4 after intensification compared to baseline and a significant decrease

afterwards. Because we did not measure 2-LTR circles before week 12, we cannot rule out a transient effect of raltegravir on 2-LTR circles. (We note that in a recent small, single-arm study, there was no increase in 2-LTR circles 4 weeks after raltegravir intensification.[19]) Unlike Buzon *et al*, we did not observe a higher level of CD8 cell activation in the 2-LTR^{pos} group, nor did we see a decline in CD8 cell activation during 12 weeks of raltegravir intensification in the 2-LTR^{pos} group. However, we categorized patients' 2-LTR changes based on their results at baseline and week 12—and not based on results at baseline and earlier time points, as was done in the previous study. For these reasons, it is not possible to directly compare the results of the two studies. Another potential explanation for the different observations between the trials is that the study populations were, in fact, different. Neither study population is very large; it is plausible that a subset of the patients in the previous study had ongoing low-level viral replication that was not reflected in the SCA HIV-1 RNA levels but may have been revealed by early changes in 2-LTR detection after adding raltegravir.

Our study has other limitations. First, because we did not measure the effect of raltegravir on gut-associated lymphoid tissue (GALT) or other potential tissue reservoirs, our findings do not rule out the presence of cell-to-cell spread of HIV-1[20] or focal cryptic sites of replication in tissue compartments that do not contribute substantially to blood HIV-1 RNA levels; these reservoirs may be playing a role in the persistent immune activation seen in persons on suppressive ART. Therefore, studying the impact of treatment intensification on GALT, as has been done in a recent study[10], and on other compartments is critical to furthering our understanding of HIV-1 persistence. Second, the duration of intensification in our study was only 12 weeks; although this should be long enough to detect an effect on plasma viremia[7], it may not be long enough to detect an impact on immune activation. However, we note that a recently published study did not observe an effect of 24 weeks of raltegravir intensification on immune activation in the blood or gut[11]. Finally, the patients in our study had undetectable HIV-1 RNA levels on ART for a median of 5.5 years[7]; this duration of suppression is comparable to or longer than that of other recently published randomized intensification trials[8, 11] although not as long as in the study by McMahon et al (median duration of suppression of 9 years)[9], a single-arm trial which also found no effect on plasma HIV-1 RNA of adding raltegravir.

Nevertheless, the main finding of our study—that adding raltegravir to suppressive ART does not reduce plasma HIV-1 RNA or other measures of virus burden in the blood—has important implications. This finding is consistent with all other raltegravir intensification studies reported to date[8-11]. Despite difference between the studies, adding raltegravir did not reduce plasma HIV-1 RNA in any of the trials. The inability of this agent to reduce residual plasma viremia in patients on suppressive ART suggests that the source of plasma virus is from a site not reached by raltegravir or that residual viremia is not dependent on new rounds of viral replication—and is therefore insensitive to addition of raltegravir. In either case, the results imply that raltegravir intensification will not—at least by itself—affect HIV-1 persistence in the blood and will not eradicate HIV-1 infection. Although intensification of current standard therapy may be a necessary supplement to eradication strategies if there is cryptic viral replication in tissue reservoirs, interventions that directly target latently-infected cells and potentially other stable viral reservoirs will be needed to cure HIV-1 infection.

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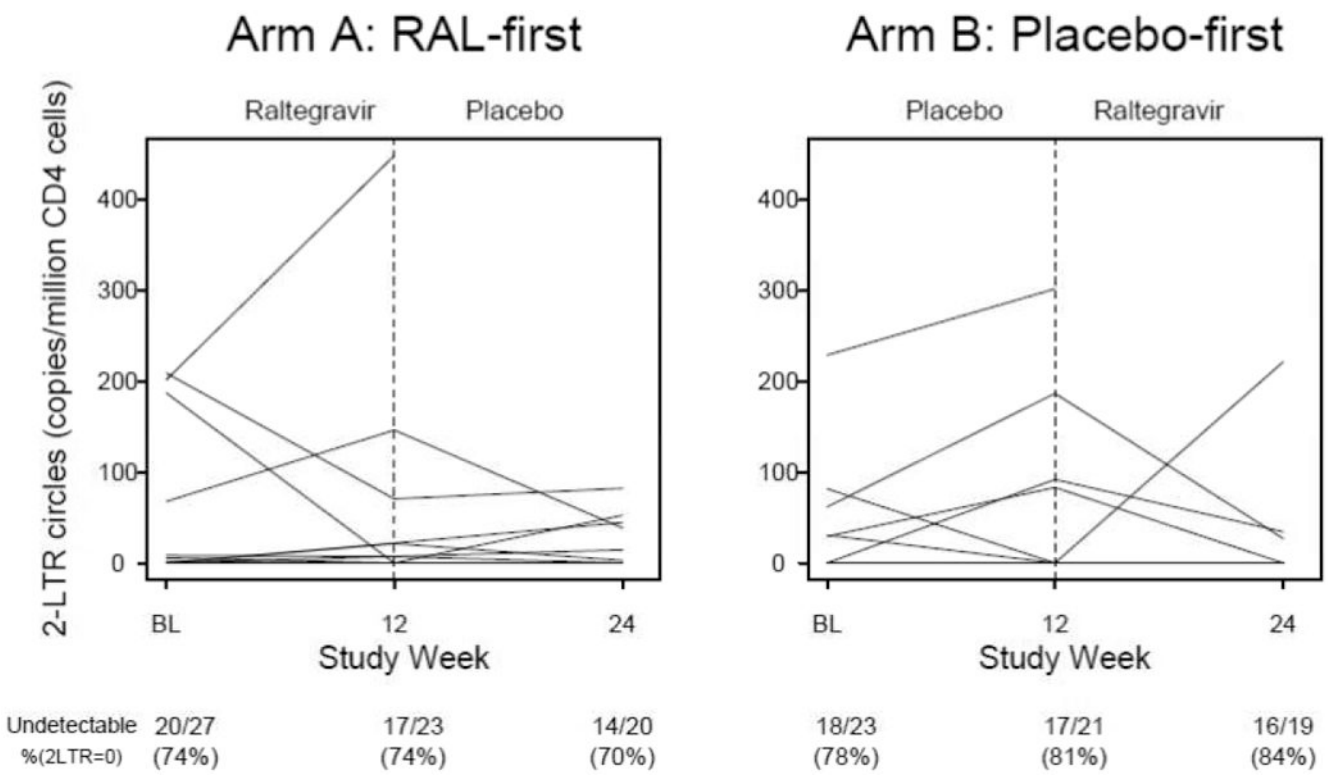

Figure 1. 2-LTR Circles During Raltegravir Intensification

2-LTR circles were measured at baseline, week 12 and 24 in patients who received raltegravir (RAL)-first or placebo-first; 12 weeks after initial treatment assignment, the two groups crossed-over to the other treatment (see Methods). BL: baseline.

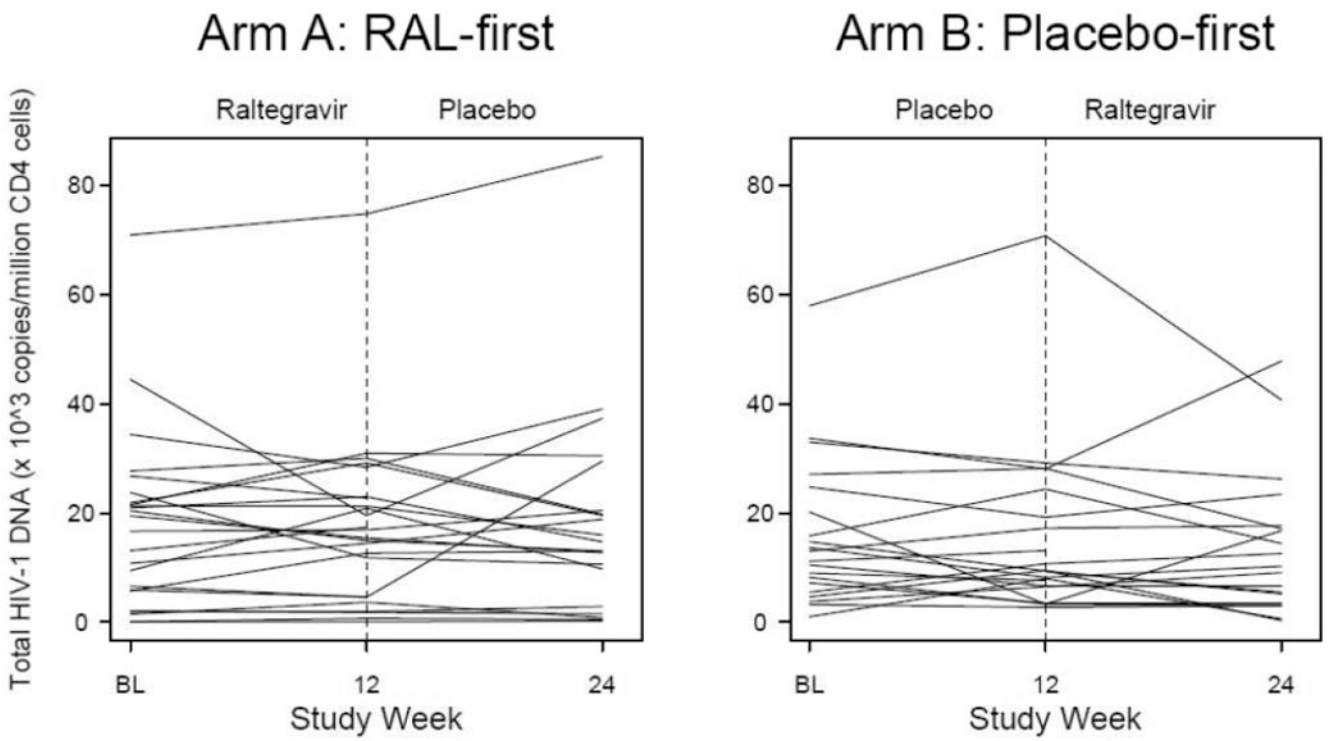

Figure 2. Total Cellular HIV-1 DNA During Raltegravir Intensification

HIV-1 DNA was measured at baseline, week 12 and 24 in patients who received raltegravir (RAL)-first or placebo-first; 12 weeks after initial treatment assignment, the two groups crossed-over to the other treatment (see Methods). BL: baseline.

Table

Baseline factors and 2-LTR circle status

Baseline Factor	2-LTR^{pos} Patients	2-LTR^{neg} Patients	P*
HIV-1 RNA by SCA (c/mL), median (Q1, Q3)	2 (2, 7) [n=12]	1 (0, 4) [n=37]	0.040
Total HIV-1 DNA (× 10^3 copies/million CD4 cells), median (Q1, Q3)	18.1 (13.4, 33.7) [n=12]	10.4 (5.4, 21.3) [n=37]	0.025
Years since first HIV-1 RNA value below detectable limits, median (Q1, Q3)	5 (3, 6) [n=12]	6 (4, 8) [n=37]	0.27
Regimen at Study Entry			
PI-containing [n=17]	5 (42%)	12 (32%)	0.73
NNRTI-containing [n=33]	7 (58%)	26 (68%)	
Baseline CD4 count (cells/mm^3), median (Q1, Q3)	538 (464, 678) [n=12]	599 (401, 771) [n=38]	0.70
CD4 activation (%CD38+ HLA-DR+), median (Q1, Q3)	7 (6, 10) [n=10]	6 (5, 8) [n=36]	0.14
CD8 activation (%CD38+ HLA-DR+), median (Q1, Q3)	13 (11, 23) [n=10]	14 (8, 20) [n=36]	0.53
Pre-ART HIV-1 RNA (c/mL), median (Q1, Q3)	254,940 (181387, 582060) [n=12]	289,734 (159405, 506608) [n=36]	0.93

* Wilcoxon rank sum test; Fisher's exact test for background entry regimen.