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Authors

Hatano, Hiroyu
Strain, Matthew C
Scherzer, Rebecca
et al.

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Increase in 2–Long Terminal Repeat Circles and Decrease in D-dimer After Raltegravir Intensification in Patients With Treated HIV Infection: A Randomized, Placebo-Controlled Trial

Hiroyu Hatano,[1] Matthew C. Strain,[4,5] Rebecca Scherzer,[1,3] Peter Bacchetti,[2] Deborah Wentworth,[6] Rebecca Hoh,[1] Jeffrey N. Martin,[2] Joseph M. McCune,[1] James D. Neaton,[6] Russell P. Tracy,[7] Priscilla Y. Hsue,[1] Douglas D. Richman,[4,5] and Steven G. Deeks[1]

[1]Department of Medicine and [2]Department of Epidemiology and Biostatistics, University of California, San Francisco, and [3]Department of Medicine, San Francisco VA Medical Center, and [4]Departments of Pathology and Medicine, University of California, San Diego, and [5]VA San Diego Healthcare System, California; [6]Division of Biostatistics, School of Public Health, University of Minnesota, Minneapolis; and [7]Departments of Pathology and Biochemistry, University of Vermont, Colchester

(See the editorial commentary by Chun and Fauci on pages 1356–8.)

Background. The degree to which human immunodeficiency virus (HIV) continues to replicate during antiretroviral therapy (ART) is controversial. We conducted a randomized, double-blind, placebo-controlled study to assess whether raltegravir intensification reduces low-level viral replication, as defined by an increase in the level of 2–long terminal repeat (2-LTR) circles.

Methods. Thirty-one subjects with an ART-suppressed plasma HIV RNA level of <40 copies/mL and a CD4$^+$ T-cell count of ≥350 cells/mm^3 for ≥1 year were randomly assigned to receive raltegravir 400 mg twice daily or placebo for 24 weeks. 2-LTR circles were analyzed by droplet digital polymerase chain reaction at weeks 0, 1, 2, and 8.

Results. The median duration of ART suppression was 3.8 years. The raltegravir group had a significant increase in the level of 2-LTR circles, compared to the placebo group. The week 1 to 0 ratio was 8.8-fold higher ($P = .0025$) and the week 2 to 0 ratio was 5.7-fold higher ($P = .023$) in the raltegravir vs. placebo group. Intensification also led to a statistically significant decrease in the D-dimer level, compared to placebo ($P = .045$).

Conclusions. Raltegravir intensification resulted in a rapid increase in the level of 2-LTR circles in a proportion of subjects, indicating that low-level viral replication persists in some individuals even after long-term ART. Intensification also reduced the D-dimer level, a coagulation biomarker that is predictive of morbidity and mortality among patients receiving treatment for HIV infection.

Keywords. HIV; raltegravir intensification; 2-LTR circles; ongoing viral replication; D-dimer.

One of the major controversies in human immunodeficiency virus (HIV) cure research is whether low-level viral replication persists in otherwise effectively treated HIV-infected individuals. This issue has potential significance not only for ongoing cure strategies, but also for interventions aimed at decreasing the residual immune activation [1–3], immune dysfunction [4, 5], and increased levels of morbidity and mortality [6] that persist in treated HIV-infected individuals.

Several recent studies have consistently shown that treatment intensification in HIV-infected patients receiving antiretroviral therapy (ART) does not decrease plasma HIV RNA levels, as measured by ultrasensitive assays [7–12]. However, some studies have suggested

that there may be ongoing, low-level viral replication in a proportion of individuals with ART-suppressed plasma HIV RNA levels, as detected by an early increase in the level of 2–long terminal repeat (2-LTR) circles [8], a decrease in immune activation in peripheral blood mononuclear cells (PBMCs) [13], or a decrease in immune activation and levels of cell-associated HIV RNA in gut-associated lymphoid tissue [9]. Moreover, despite relatively effective suppression of HIV replication, ART-treated adults have a higher risk of developing non–AIDS-associated diseases (including cardiovascular disease, liver disease, renal disease, bone disease, and cancer [14–17]), compared with HIV-uninfected adults. Many factors potentially contribute to this excess risk, including a higher prevalence of traditional risk factors, antiretroviral drug toxicity, and chronic inflammation [18]. Compared with age-matched controls, many markers of inflammation (eg, interleukin-6 [IL-6] and T-cell activation) and hypercoagulation (eg, D-dimer level) remain elevated in treated HIV disease [1, 19]. In fact, these biomarkers are strongly predictive of morbidity and all-cause mortality in ART-treated adults [6, 20, 21].

To determine whether HIV replication persists during ART and whether low-level viral replication contributes to persistent immune dysfunction, we conducted a randomized, double-blind, placebo-controlled study of raltegravir intensification in ART-suppressed individuals and assessed whether intensification led to an increase in the level of 2-LTR circles. We also examined whether intensification led to a reduction in biomarkers of inflammation and hypercoagulation in order to assess whether low-level viral replication has any potential clinical consequences and contributes to the increased non–AIDS-related morbidity and mortality observed among treated individuals.

METHODS

Study Participants

Thirty-one ART-suppressed, HIV-infected subjects with plasma HIV RNA < 40 copies/mL and CD4$^+$ T-cell count ≥350 cells/mm^3 for at least 1 year were randomly assigned to receive either raltegravir 400 mg twice daily or matching placebo for 24 weeks (clinical trials registration NCT00843713). Results of the primary virologic, immunologic, and cardiovascular end points have recently been reported elsewhere [12]. All subjects provided written informed consent. This study was approved by the University of California, San Francisco, Committee on Human Research.

2-LTR Circles

2-LTR circles were analyzed in triplicate from DNA extracted from 0.5 million cryopreserved PBMCs at weeks 0, 1, 2, and 8 by droplet digital PCR (ddPCR) [22]. Cellular DNA was extracted using a Qiagen DNA Blood Midi Kit. ddPCR was performed using the Bio-Rad QX-100 emulsification device. Primers to conserved regions of HIV-1 pol (HXB2 positions

2536–2662) and HIV-1 LTR (HXB2 positions 9585–51) were used. An RPP30 (RNAse P) primer/probe set was used to quantify host genomic DNA for purposes of normalization. DNA concentration was estimated from the A260/A280 absorbance ratio, using a NanoDrop 2000 spectrophotometer.

To compare the accuracy of HIV DNA quantification by real-time PCR (qPCR) and ddPCR, stored PBMC samples (n = 156) were analyzed by both qPCR and ddPCR in triplicate [22]. Measurements made by the 2 methods were linearly correlated (Pearson r^2 = 0.64), but it was not known a priori which assay was more accurate or precise. To evaluate the precision of the 2-LTR assay, the coefficient of variation among replicate wells was computed for each sample. To account for the expected decrease in assay precision at low copy numbers, the average assay variance was computed as a function of measured copy number (in 0.1 $\log_{10}$ bins). On average, the assay variance was 20-fold lower by ddPCR, compared with qPCR. In addition, the limit of detection of the ddPCR assay was evaluated [1]. A serial dilution of DNA isolated from infected CD4$^+$ T cells into uninfected PBMC DNA was tested with a number of replicate wells that increased as the reciprocal of the template number to a maximum of 36 wells. This allowed detection of both pol and 2-LTR circles at frequencies of 0.7 per million cells.

We compared 2-LTR rates by applying Poisson regression to event count raw data from ddPCR, with a fixed term (known as an "offset") included in the model to account for variations in the number of droplets tested and the total cellular input, determined by RPP30. The models included fixed person effects to focus on within-person changes between different times while fully controlling for person-to-person variability, as in a matched analysis. All results for 2-LTR circle comparisons are from regression parameters estimated in such models. Although Poisson regression mitigates the influence of extreme values because it models effects on the logarithmic scale, one subject had an extremely large increase in the 2-LTR count at week 1, so we also performed a sensitivity analysis that excluded this subject.

Plasma Biomarkers

Interleukin 6 (IL-6) and D-dimer levels were measured in stored fasting plasma samples for 25 of the 31 subjects (12 raltegravir recipients and 13 placebo recipients) at weeks 0, 4, and 24 at the Laboratory for Clinical Biochemistry Research at the University of Vermont. IL-6 levels were measured with a chemiluminescent sandwich enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), and D-dimer levels were measured with immunoturbidometric methods on the Sta-R analyzer, Liatest D-DI (Diagnostica Stago, Parsippany, NJ). Interassay coefficients of variation for a number of different control materials of different values averaged approximately 10% for both assays. IL-6 and D-dimer levels were $\log_2$ transformed because the distributions were right skewed. Between-

group differences in the change in the level of each biomarker from weeks 0 to 24 were calculated using analysis of covariance, with adjustment for the baseline level of the biomarker considered. Geometric means of biomarker levels at week 24 are also reported after adjustment for the baseline level. Statistical analyses were performed using SAS software, version 9.2.

RESULTS

Baseline Characteristics

At baseline, the median current and nadir $CD4^+$ T-cell counts were 703 and 210 cells/mm³, respectively (Table 1). The median duration since HIV diagnosis and of ART suppression were 19 and 3.8 years, respectively. Fifteen of the 31 subjects (48%) were receiving a protease inhibitor (PI) as part of their ART regimen.

As reported previously, raltegravir intensification did not have a substantial effect on plasma HIV RNA level (as measured by an ultrasensitive single-copy assay; $P = .16$), cell-associated HIV RNA level ($P = .90$), proviral HIV DNA level ($P = .51$), or $CD4^+$ T-cell activation ($CD38^+HLA-DR^+$; $P = .90$) and $CD8^+$ T-cell activation ($CD38^+HLA-DR^+$; $P = .91$) in PBMCs [12].

2-LTR Circles

The raltegravir group had a substantial increase in the level of 2-LTR circles, compared with baseline (estimated ratio of week

Table 1. Baseline Characteristics of 31 Human Immunodeficiency Virus (HIV)–Infected Study Subjects

Characteristic	Raltegravir (n = 15)	Placebo (n = 16)	P
Age, y	55 (49–59)	55 (51–60)	.464
Male sex, subjects, %	87	94	.600
$CD4^+$ T-cell count, cells/mm³	670 (591–819)	705 (629–808)	.812
Nadir $CD4^+$ T-cell count, cells/mm³	205 (74–318)	215 (99–400)	.549
Duration since HIV diagnosis, y	19 (17–23)	15 (9–20)	.072
Duration of HAART suppression, y	4.4 (3.2–5.7)	2.7 (2.1–4.4)	.166
PI-containing HAART			
Overall, subjects, no. (%)	9 (60)	6 (38)	.289
By PI(s), subjects, no.			
Atazanavir/ritonavir	4	1	
Atazanavir	2	0	
Fosamprenavir/ritonavir	0	3	
Fosamprenavir	1	0	
Lopinavir/ritonavir	1	1	
Lopinavir/ritonavir/saquinavir	0	1	
Saquinavir/ritonavir	1	0	

Data are medians (interquartile ranges), unless otherwise noted.
Abbreviations: HAART, highly active antiretroviral therapy; PI, protease inhibitor.

1 to week 0 values, 4.7 [95% confidence interval {CI}, 1.55–20.1; $P = .0045$]; ratio of week 2 to week 0 values, 3.4 [95% CI, 1.02–15.3; $P = .046$]; and ratio of week 8 to week 0 values, 3.6 [95% CI, 1.10–15.9; $P = .033$]; Figure 1). There were no substantial changes in the level of 2-LTR circles in the placebo group. The raltegravir group had a significant increase in the level of 2-LTR circles, compared with the placebo group. The week 1 to week 0 ratio averaged 8.8-fold (95% CI, 2.1–48.4-fold) higher in the raltegravir group, compared with the placebo group ($P = .0025$), and the week 2 to week 0 ratio averaged 5.7-fold (95% CI, 1.27–32.0-fold) higher in the raltegravir group, compared with the placebo group ($P = .023$). Nine of the 15 subjects receiving raltegravir had an increase in the level of 2-LTR circles at weeks 1 or 2, compared with 3 of 15 subjects in the placebo group ($P = .060$; 1 subject in the placebo group was excluded because of no follow-up 2-LTR circle measurements). When the 1 subject with an extreme increase in the level of 2-LTR circles at week 1 was excluded, the week 1 to week 0 ratio still averaged 2.9-fold (95% CI, .55–18.2-fold) higher in the raltegravir group, compared with the placebo group, although this was not statistically significant ($P = .21$).

The rise in the level of 2-LTR circles in the raltegravir group was more pronounced in subjects receiving a PI-based ART regimen. Among the subset of subjects in the raltegravir group receiving a PI, there was a marked increase in the level of 2-LTR circles, compared with baseline (estimated ratio of week 1 to week 0 values, 6.4 [95% CI, 1.80–40.5; $P = .0024$]; ratio of week 2 to week 0 values, 3.9 [95% CI, .97–26.0; $P = .055$]; and ratio of week 8 to week 0 values, 4.1 [95% CI, 1.06–27.3; $P = .04$]). In contrast, among the subset of subjects in the raltegravir group not receiving a PI, the effect was attenuated (estimated ratio of week 1 to week 0 values, 1.07 [95% CI, .04–27.3; $P = .96$]; ratio of week 2 to week 0 values, 2.3 [95% CI, .20–51.2; $P = .50$]; and ratio of week 8 to week 0 values, 2.3 [95% CI, .20–51.2; $P = .48$]).

Plasma Biomarkers

At baseline, the median levels of IL-6 and D-dimer were 1.78 pg/mL (interquartile range [IQR], 1.30–2.74 pg/mL) and 0.31 pg/mL (IQR, 0.20–0.39 μg/mL), respectively; these levels are comparable to IL-6 and D-dimer levels of HIV-infected individuals from other cohorts [6, 19]. The change in IL-6 levels at week 24 did not differ significantly between the raltegravir and placebo groups ($P = .39$). On the log_2 scale, the IL-6 levels increased by 0.31 pg/mL for the raltegravir group and by 0.01 pg/mL for the placebo group (difference, −0.29 pg/mL; 95% CI, −.99 to .41). Geometric mean IL-6 levels at week 24 for the raltegravir and placebo groups were 2.35 and 1.91 pg/mL, respectively.
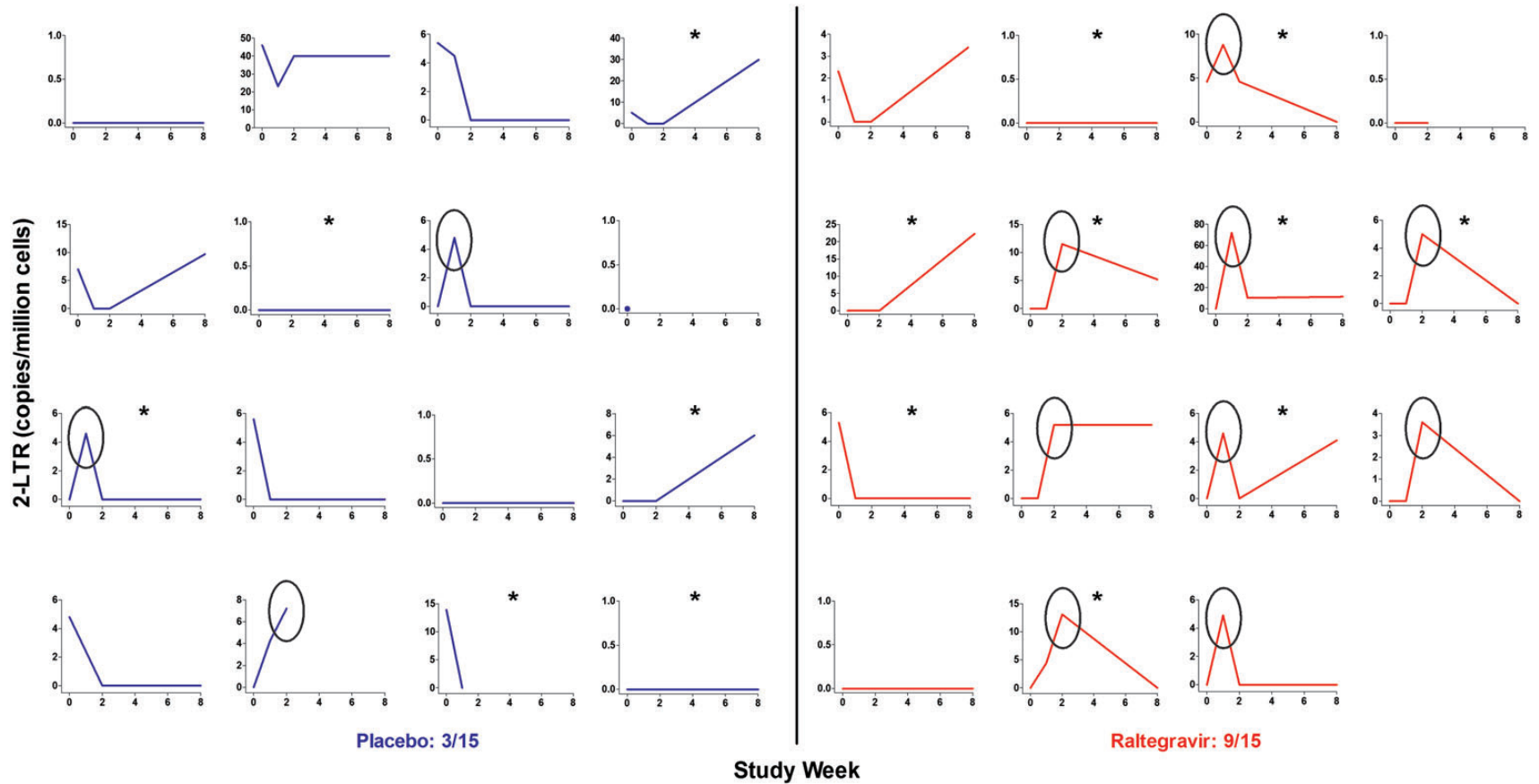
On the other hand, intensification led to a statistically significant decrease in D-dimer levels at week 24, compared with placebo (change in D-dimer levels, −0.26 log_2 μg/mL in the

Figure 1. Increase in the level of 2–long terminal repeat (2-LTR) circles in the raltegravir group. The raltegravir group (red) had a significant increase in the level of 2-LTR circles, compared with the placebo group (blue). The week 1 to week 0 ratio was 8.8-fold higher ($P = .0025$) and the week 2 to week 0 ratio was 5.7-fold higher ($P = .023$) in the raltegravir group, compared with the placebo group. Nine of the 15 subjects receiving raltegravir had an increase in the level of 2-LTR circles at weeks 1 or 2, compared with 3 of 15 subjects in the placebo group ($P = .060$; 1 subject in the placebo group was excluded because of no follow-up 2-LTR circle measurements). Black ovals indicate subjects who had an increase in the level of 2-LTR circles at weeks 1 or 2. Asterisks indicate subjects receiving a protease inhibitor as part of their antiretroviral therapy regimen.

raltegravir group and +0.49 log$_2$ µg/mL in the placebo group; $P = .045$; Figure 2). This corresponds to a 0.60-fold (95% CI, .36–.99-fold) decrease in D-dimer levels for the raltegravir group, relative to the placebo group. Geometric mean D-dimer levels at week 24 for the raltegravir and placebo groups were 0.24 and 0.41 µg/mL, respectively. The decline in D-dimer levels for the raltegravir group was evident at week 4 ($P = .039$ for the difference from the placebo group); geometric mean D-dimer levels at week 4 for the raltegravir and placebo groups were 0.19 and 0.40 µg/mL, respectively.
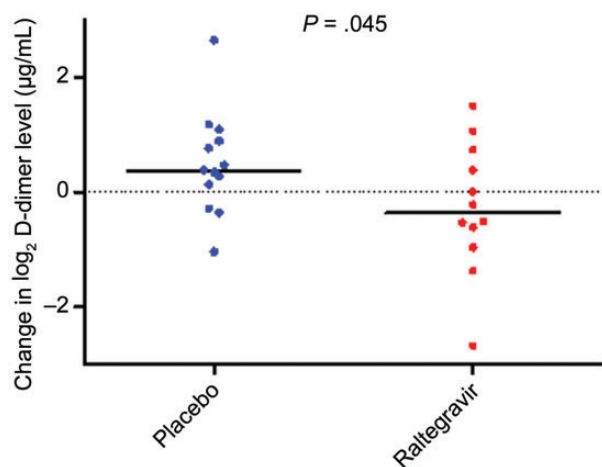
DISCUSSION

In this randomized, double-blind, placebo-controlled study, raltegravir intensification resulted in a rapid and transient increase in the level of 2-LTR circles in a proportion of patients with effectively treated HIV infection. Linear HIV complementary DNA (cDNA) is circularized by host DNA repair enzymes to form episomal cDNA (1- and 2-LTR circles) [23]. Raltegravir, an integrase inhibitor, prevents linear HIV cDNA from integrating into the host genome. Although there is some controversy over how long 2-LTR circles persist [24], several studies have shown that they are labile products of virus infection and can therefore be used as surrogate markers of recent viral replication events [23, 25, 26]. Thus, the observed increase in the level of 2-LTR circles after raltegravir intensification suggests the presence of ongoing, low-level viral replication in these individuals. We also observed a decrease in D-dimer levels with raltegravir intensification, suggesting that residual

Figure 2. Raltegravir intensification leads to a decrease in D-dimer levels. Raltegravir intensification led to a significant decrease in D-dimer levels from week 0 to week 24 (change in D-dimer levels, −0.26 log$_2$ µg/mL in the raltegravir group and +0.49 log$_2$ µg/mL in the placebo group; P= .045). Data for 3 subjects (1 in the raltegravir group and 2 placebo group) are missing.

replication contributes to persistent alterations in the coagulation pathway that have been well-documented in HIV-infected adults [20, 21, 27].

Our data are consistent with results from Buzon et al, who previously found that raltegravir intensification led to an early increase in the level of 2-LTR circles [8]. In their study, 69 subjects with ART-suppressed HIV infection were randomized to receive raltegravir intensification or to continue ART without intensification in an open-label, 48-week study. The level of 2-LTR circles was measured at weeks 0, 2, 4, 12, 24, and 48. The raltegravir group had a transient and significant increase in the level of 2-LTR circles at weeks 2 and 4, compared with baseline. In contrast, an increase in the level of 2-LTR circles was not observed in other raltegravir intensification studies in which 2-LTR circles were measured at later time points (4 weeks [28] and 12 weeks [29] after initiation of intensification), suggesting that most newly formed 2-LTR circles are short-lived and that this surrogate method of detecting ongoing viral replication must be assayed early [23]. Buzon et al also observed in their study that the raltegravir recipients who had an increase in the level of 2-LTR circles had higher levels of immune activation at baseline, which then decreased with intensification [8, 13]. In our current study, we observed that, among the 12 of 31 subjects who had an early increase in the level of 2-LTR circles, the 9 subjects in the raltegravir group had a lower mean level of immune activation (defined as the percentage of CD38$^+$HLA-DR$^+$ CD8$^+$ T cells) at week 24, compared to the 3 subjects in the placebo group (18.4% in the raltegravir group vs 34.4% in the placebo group; $P = .043$).

Although many subjects in our study displayed a transient increase in the level of 2-LTR circles with raltegravir intensification, we observed no substantial change in ultrasensitive plasma HIV RNA levels with intensification. This is consistent with previous intensification studies [7–12] and suggests that (1) most HIV RNA in plasma is due to transient production of virus from a latent reservoir, rather than a reflection of ongoing replication; and (2) there may be distinct viral reservoirs within an ART-suppressed individual, one that is more active and produces low-level rounds of de novo infection and another that is more quiescent and from which there is only stochastic release of viral RNA [30].

As noted above, nearly half (48%) of our study subjects were receiving a PI as part of their ART regimen (Table 1). We observed that the rise in the level of 2-LTR circles in the raltegravir group tended to occur in subjects receiving a PI-based ART regimen. This was also observed in the study by Buzon et al, in which 22% of subjects were receiving a PI-based ART regimen (atazanavir or lopinavir) [8]. Moreover, in our previous raltegravir intensification study in "immunologic nonresponders" (defined as individuals with a CD4$^+$ T-cell count of <350 cells/mm^3 despite at least 1 year of ART-mediated viral suppression), we noted that, although intensification did not lead to a decrease

in plasma HIV RNA levels (as detected by an ultrasensitive single-copy assay), there was a significant decrease in plasma HIV RNA levels in subjects receiving a PI-based ART regimen [11]. Collectively, these data suggest that some PIs may allow low-level viral replication to occur. One reason for this may be because of inadequate tissue penetration at critical lymphoid sites [31]. An alternative (or additional) explanation may be related to the dose-response curves of specific PIs. Siliciano et al have shown that, as a drug class, PIs have a much higher inhibitory potential at peak plasma concentrations than other antiretroviral drug classes [32]. This is due in part to their very steep dose-response curves (as defined by the Hill coefficient); small increases in drug concentration lead to dramatic increases in viral inhibition. However, the converse is also true in that drugs with a high Hill coefficient are vulnerable to small decreases in drug concentration, such that small decreases in drug concentration lead to significant reductions in the degree of viral inhibition [33]. Thus, for PIs with a shorter half-life and a high Hill coefficient, the inhibitory potential can decline dramatically during the dosing interval, and this may allow new infection events to occur when drug concentrations are low. These effects are likely to differ across the various drugs within the PI drug class; future studies are necessary to further elucidate this issue.

Our secondary objective was to assess the potential clinical consequences of low-level viral replication. We measured the effect of raltegravir intensification on levels of IL-6 (a measure of systemic inflammation status) and D-dimer (an integrated measure of clot formation and degradation and thus, an indication of coagulant status). Both biomarkers have been shown to be strong, consistent, and independent predictors of increased morbidity and mortality in HIV infection [20, 21, 27, 34]. Although we observed no effect on IL-6, we found that raltegravir intensification led to a significant decrease in D-dimer levels, despite our cohort having baseline D-dimer levels that were comparable to those of HIV-infected individuals from other cohorts [6, 19]. If elevated D-dimer levels reflect excess clot formation and breakdown, our data support a previously proposed model in which HIV replication either directly or indirectly activates the coagulation cascade and contributes to thrombus formation and cardiovascular morbidity [35]. Of note, in a recent study of simian immunodeficiency virus–infected non-human primates, diffuse clotting in tissues was found at autopsy, supporting this model [36]. As reported previously, in our current study raltegravir intensification did not lead to an improvement in cardiovascular function, as measured by flow-mediated vasodilatation (a marker of macrovascular dysfunction) or hyperemic velocity (a marker of microvascular dysfunction) [12]. It may be that D-dimer levels represent a more sensitive or more proximal measure of cardiovascular function. However, because our sample size was small (and the resulting CI around

the treatment difference was very wide), we cannot assess with certainty whether the observed treatment difference in D-dimer levels has any clinical relevance. It would be important to pursue this finding in future, larger studies.

In summary, we have shown for the first time in a randomized, double-blind, placebo-controlled study that the addition of raltegravir to a stable ART regimen resulted in a rapid and transient increase in the level of 2-LTR circles in a proportion of effectively treated HIV-infected patients, indicating that residual replication persists in some individuals even after long-term suppressive ART. This appeared to be particularly true in patients receiving a PI-based ART regimen, which suggests that with "antiretroviral optimization" (ie, the use of effective, highly bioavailable antiretroviral drugs that have robust lymphoid tissue penetration), ongoing viral replication could be further suppressed to negligible levels. We also found that raltegravir intensification led to a significant decrease in levels of D-dimer, a coagulation biomarker that has previously been shown to be predictive of increased morbidity and mortality in treated HIV disease. The potential of ongoing, low-level viral replication should be considered as a modifiable factor in future cure and treatment strategies.

Notes

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