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# Impact of intensification with raltegravir on HIV-1-infected individuals receiving monotherapy with boosted PIs

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**Background:** Monotherapy with ritonavir-boosted PIs (PI/r) has been used to simplify treatment of HIV-1-infected patients. In previous studies raltegravir intensification evidenced ongoing viral replication and reduced T cell activation, preferentially in subjects receiving PI-based triple ART. However, data about low-level viral replication and its consequences in patients receiving PI/r monotherapy are scarce.

**Methods:** We evaluated the impact of 24 weeks of intensification with raltegravir on markers of viral persistence, cellular immune activation and inflammation biomarkers in 33 patients receiving maintenance PI/r monotherapy with darunavir or lopinavir boosted with ritonavir. ClinicalTrials.gov identifier: NCT01480713.

**Results:** The addition of raltegravir to PI/r monotherapy resulted in a transient increase in 2-LTR (long-terminal repeat) circles in a significant proportion of participants, along with decreases in CD8+ T cell activation levels and a temporary increase in the expression of the exhaustion marker CTLA-4 in peripheral T lymphocytes. Intensification with raltegravir also reduced the number of samples with intermediate levels of residual viraemia (10–60 HIV-1 RNA copies/mL) compared with samples taken during PI/r monotherapy. However, there were no changes in cell-associated HIV-1 DNA in peripheral CD4+ T cells or soluble inflammatory biomarkers (CD14, IP-10, IL-6, C-reactive protein and D-dimer).

**Conclusions:** Intensification of PI/r monotherapy with raltegravir revealed persistent low-level viral replication and reduced residual viraemia in some patients during long-term PI/r monotherapy. The concomitant change in T cell phenotype suggests an association between active viral production and T cell activation. These results contribute to understanding the lower efficacy rates of PI/r monotherapies compared with triple therapies in clinical trials.

## Introduction

Combined ART has significantly improved the life expectancy of HIV-1-infected individuals. Current international guidelines recommend first-line therapy consisting of three drugs from at least two different classes, which generally includes two NRTIs plus a third agent. This combination decreases plasma viral load to undetectable levels by standard assays (<50 HIV-1 RNA copies/mL) and suppresses viral replication for as long as treatment is maintained. Thus, life-long ART is needed to prevent the development of drug resistance and viral recrudescence. However, extended use of ART is expensive and may lead to long-term complications such as hyperlipidaemia, decreased bone mineral density, renal toxicity and

cardiovascular diseases.[1–4] Hence, interest in ART simplification strategies [e.g. monotherapy with ritonavir-boosted PIs (PI/r)] as maintenance therapy for individuals who have already achieved viral suppression with triple ART has grown in recent years.[5]

Current ART cannot eradicate HIV-1 infection, because viral reservoirs remain in the form of silent HIV-1 DNA integrated in the host genome of long-lived resting memory CD4+ T cells. Indeed, the use of novel ultrasensitive procedures has shown that residual plasma viraemia (<50 copies HIV-1 RNA/mL) is present in a significant proportion of individuals on ART in whom viral replication has been well-suppressed for years.[6] However, the origin of this residual viral production during ART remains unknown.

Intensification of ART with raltegravir has been assayed in several clinical trials in an attempt to completely suppress residual viraemia. Although this primary endpoint was not achieved, some studies detected a transient increase in episomes containing two copies of the viral long-terminal repeat (2-LTR circles).[7,8] Given that integrase inhibitors block viral linear DNA from integration into genomic host cell DNA, leading to the accumulation of circular viral DNA molecules (including 2-LTR circles) in recently infected cells, the increase in viral episomes observed after intensification with raltegravir in some individuals suggests the persistence of low-level viral replication during ART. Interestingly, in both studies, the increase in the level of 2-LTR circles after intensification with raltegravir was more frequent in patients receiving a PI-containing ART regimen. Although active replication may occur in compartments that are less accessible to PIs,[9] regimens consisting of three reverse transcriptase inhibitors might also reduce the probability of formation of the linear DNA precursor to episomes.[7] In addition, studies evaluating intensification with raltegravir have also shown a significant decrease in levels of CD8+ T cell activation.[7,10,11] Taken together, these observations suggest that PI/r monotherapy might be even less effective at completely suppressing low-level viral replication than PI-containing triple ART.

Here, we report the results of a pilot trial in which individuals on maintenance monotherapy with PI/r had their treatment temporarily intensified with raltegravir for 24 weeks. We evaluated the impact of intensification on low-level viral replication, viral reservoirs, cellular immune activation and soluble markers of inflammation.

## Methods

### Ethics

We performed a proof-of-concept, single-arm, pilot clinical trial (ClinicalTrials.gov identifier: NCT01480713; EudraCT number 2011-004464-30) at the Hospital Universitari Germans Trias i Pujol (Badalona, Spain). The study was conducted in compliance with the principles of the Declaration of Helsinki and in accordance with the Spanish legislation. The Institutional Ethics Review Committee approved the protocol (approval number AC-11-083) and all participants provided their written informed consent.

### Study design and participants

Sample size was established on the basis of previous data on intensification with raltegravir in participants receiving PI-containing regimens.[7] We recruited 33 HIV-1-infected adults with undetectable plasma viraemia (<50 HIV-1 RNA copies/mL) who had been on fully suppressive PI/r monotherapy (400/100 mg of lopinavir/ritonavir twice daily or 800/100 mg of darunavir/ritonavir once daily) for at least the previous 12 months and had switched from triple ART to PI/r monotherapy while on viral suppression. Other inclusion criteria were an absolute CD4+ T cell count of ≥500 cells/mm$^3$ and absence of virological failure to previous PI-containing regimens. Adherence to treatment was reported by the participants using the adapted SERAD (Self-Reported Adherence) questionnaire.[12] Adherence was further verified by monitoring antiretroviral plasma drug levels during the intensification period and at the end of the follow-up.

Three blood samples were collected during the 2 months preceding initiation of raltegravir (Figure 1a). During the intensification period, samples were collected at weeks 1, 2, 4, 8, 12 and 24. Two additional samples were collected 12 and 24 weeks after withdrawal of raltegravir (i.e. weeks 36 and 48 of the study).

### Quantification of cell-associated HIV DNA

CD4+ T cells were purified from 20×10$^6$ cryopreserved PBMCs by negative immunomagnetic separation (CD4+ T Cell Isolation Kit, Milteny). Total HIV-1 DNA was quantified by Droplet Digital PCR (ddPCR; Bio-Rad) from lysed extracts, as previously described,[13] with a detection limit of 1 copy/million CD4+ T cells. The same methodology was used to measure 2-LTR circles (forward primer, 5′-CTAACTAGGGAACCCACTGCT-3′; reverse primer, 5′-GTAGT TCTGCCAATCAGGGAA-3′; probe, 5′-AGCCTCAATAAAGCTTGCCTTGAGTGC-3′). The RPP30 cellular gene was quantified to normalize sample input. All samples from the same individual were processed in parallel to minimize the effect of experimental variability on the analysis of viral dynamics. FAM/HEX-ZEN/Iowa Black FQ dual-labelled double-quenched probes were purchased from Integrated DNA Technologies.

### Residual viraemia

Throughout the study period, participants' viral load was monitored every 3 months using the Abbott RealTime HIV-1 assay (Abbott Molecular Inc.). In order to quantify plasma viraemia under the detection limit of standard methodologies (50 HIV-1 RNA copies/mL), cryopreserved plasma samples were analysed using an ultrasensitive viral load test.[13] Briefly, up to 7.5 mL of plasma was ultracentrifuged prior to extraction and quantification of viral RNA using the Abbott RealTime HIV-1 assay and the Abbott m2000rt instrument. An in-house calibration curve set (range 10$^1$–10$^3$ copies/mL), which had previously been validated using a standard HIV-1 RNA control from the WHO, was used as a reference.

### Immunophenotyping

Cryopreserved PBMCs (2×10$^6$ cells) were thawed and stained with Fixable Viability Dye eFluor 506 (eBioscience) and the following antibody combination: APC-Cy7-CD3, PerCP-CD4, BV421-CD8, FITC-CD45RA, BV605-CD27, PE-Cy7-CCR7, QB-PE-CD38, APC-HLA-DR and PE-CF594-CTLA-4 (all from Becton Dickinson). Cells were acquired in an LSRFortessa flow cytometer (Becton Dickinson). The analysis was carried out using FlowJo software (v. 9.2.3). First, living T lymphocytes were gated according to morphological parameters and cell viability. Then, CD4+ and CD8+ T cell subsets were identified by the expression of CD45RA, CCR7 and CD27, as described elsewhere.[14] The subsets were naive cells (CD45RA+ CCR7+), central memory cells (CD45RA− CCR7+), transitional memory cells (CD45RA− CCR7− CD27+), effector memory cells (CD45R− CCR7− CD27−) and TEMRA cells (terminally differentiated effector memory cells re-expressing CD45RA) (CD45RA+ CCR7−). Positive gates were delineated based on 'fluorescence minus one' controls. Activation markers (CD38, HLA-DR) and exhaustion markers (CTLA-4 and PD-1) were analysed in CD4+ and CD8+ T cell compartments and within each T cell subset. Absolute numbers of CD38 molecules were assessed using Quantibrite calibration (Becton Dickinson) following the manufacturer's instructions.[15] All timepoints from each participant were acquired and analysed in parallel to minimize the effect of experimental and acquisition variability. All values recorded were compared with baseline values.

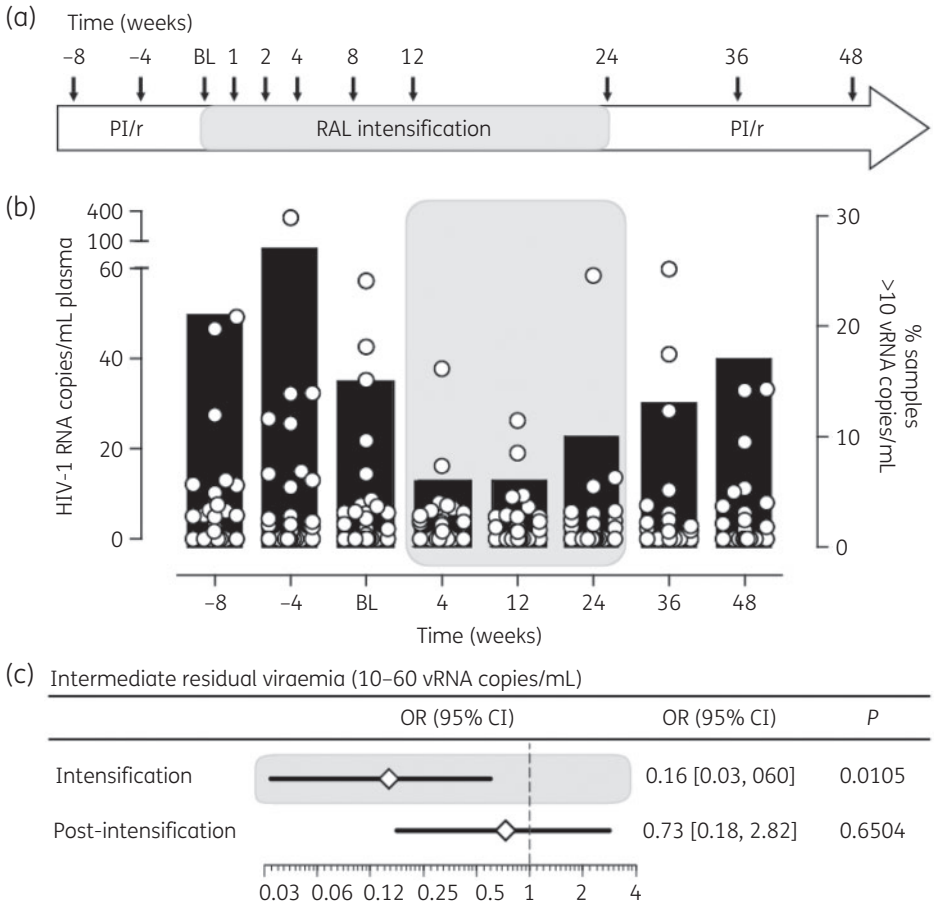


**Figure 1.** Effect of raltegravir intensification on residual viraemia. (a) Blood samples were obtained before intensification (week −8, week −4 and baseline), during intensification (from baseline to 24 weeks) and for up to 24 weeks after withdrawal of raltegravir (week 48). (b) Raw data from ultrasensitive viral load determination (left *y*-axis) and the percentages of samples with >10 HIV-1 RNA copies/mL of plasma (right *y*-axis) are shown for each timepoint. The grey zone represents the intensification period (week 0 to 24). (c) Difference in the probability of detecting intermediate residual viraemia levels (10–60 HIV-1 RNA copies/mL) during raltegravir intensification and after drug withdrawal, compared with the pre-intensification period. A mixed-effects logistic model was fitted for the binary outcome indicative of intermediate residual viraemia, assuming constant probabilities of exceeding 10 copies of residual viraemia for each patient in each period. BL, baseline; RAL, raltegravir; vRNA, HIV-1 RNA.

### *Soluble markers*

Plasma levels of several markers of inflammation and coagulation were measured in frozen plasma samples. D-dimer and C-reactive protein (CRP) were measured using an aggregation assay (Biokit). Soluble CD14 (sCD14), IFN-γ-induced protein 10 (IP-10) and IL-6 were measured using ELISA following the manufacturer's instructions (Diaclone).

### *Statistical analysis*

Longitudinal analysis of residual viraemia, with data below the limit of detection, was first performed by comparing timepoints using the paired Prentice–Wilcoxon test. To evaluate the difference in the frequency of intermediate viraemia levels (>10 HIV-1 RNA copies/mL) during raltegravir intensification and after drug withdrawal, compared with the pre-intensification period, a mixed-effects logistic regression model was fitted for the binary outcome indicative of intermediate residual viraemia, assuming constant probabilities of exceeding 10 copies of residual viraemia for each patient in each period. Longitudinal comparisons between subjects with detectable 2-LTR circles (2-LTR+) and individuals with undetectable 2-LTR circles (2-LTR−) were made using piecewise linear mixed-effects models. Inference on the distribution of the 2-LTR peaks was assessed by calculating an empirical *P* value through a Monte Carlo simulation. Comparisons between timepoints for the levels of cell-associated HIV-1 DNA, T cell immunophenotype and inflammation markers were made using the Wilcoxon signed rank test. The statistical analysis was performed using R and GraphPad Prism 5.

## Results

### *Participant characteristics and disposition*

Thirty-three participants were enrolled in the raltegravir intensification protocol (400 mg twice daily). Of these, 18 individuals (54.5%) were receiving darunavir/ritonavir and 15 (45.5%) lopinavir/ritonavir. The baseline characteristics are summarized in Table 1. Most participants were men (81.8%) and seven were coinfected with HCV. Median time on suppressive ART was 7.4 years and median time on PI/r monotherapy was 3.3 years. To evaluate the reversibility of the potential changes induced by intensification with raltegravir on viral and immune dynamics, 30 individuals

**Table 1.** Demographic characteristics

| | Median | IQR |
|---|---|---|
| Age (years) | 47 | 40–51 |
| Time on PI/r monotherapy (years) | 3.3 | 1.6–4.2 |
| Time on suppressive ART (years) | 7.4 | 4.5–9.2 |
| Zenith viral load ($log_{10}$ HIV-1 RNA copies/mL) | 5.2 | 4.1–5.3 |
| Nadir CD4+ T cells (absolute cells/$mm^3$) | 280 | 181–336 |
| CD4+ T cell counts (absolute cells/$mm^3$) | 751 | 584–907 |
| Percentage of CD4+ T cells | 36 | 30–39 |
| CD8+ T cell counts (absolute cells/$mm^3$) | 770 | 570–915 |
| Percentage of CD8+ T cells | 32 | 28–40 |
| | *n* | % |
| Male | 27 | 81.8 |
| HCV coinfection | 7 | 21.2 |
| Risk factors | | |
| heterosexual | 7 | 21.2 |
| MSM | 19 | 57.6 |
| IVDU | 6 | 18.2 |
| unknown | 1 | 3 |

**Table 2.** Virological data during the study period

| | Time | Median (IQR) | *P* (to baseline) |
|---|---|---|---|
| Residual viraemia (HIV-1 RNA copies/mL) | baseline | 2.2 (0.0–6.6) | |
| | week 12 | 1.7 (0.0–4.9) | 0.2869 |
| | week 24 | 1.15 (0.0–4.4) | 0.2837 |
| | week 48 | 1.0 (0.0–6.9) | 0.2317 |
| | *P* (week 24–week 48) | | 0.3740 |
| 2-LTR (copies/million CD4+ T cells) | baseline | 7.75 (4.25–14.9) | |
| | week 4 | 11 (4.80–16.9) | 0.2121 |
| | week 8 | 13.6 (7.98–19.9) | 0.2681 |
| | week 24 | 7.90 (2.20–13.8) | 0.3028 |
| | week 48 | 8.60 (3.50–13.5) | 0.5563 |
| | *P* (week 24–week 48) | | 0.8926 |
| Total HIV (copies/million CD4+ T cells) | baseline | 486 (299–689) | |
| | week 24 | 448 (251–655) | 0.4646 |
| | week 48 | 441 (227–597) | 0.7621 |
| | *P* (week 24–week 48) | | 0.3188 |

were further followed beyond discontinuation of raltegravir at week 24 (Figure 1a).

Therapeutic drug monitoring indicated optimal plasma drug levels for all participants during the intensification period. Likewise, 97% of the subjects self-reported treatment adherence greater than 96%. During the study, three participants reported mild adverse effects (headache, gastrointestinal disturbances, fatigue) and one moderate event (aggressive anxiety) potentially related to ART, although none of these led to discontinuation of treatment.

Virological failure was not observed during the study. Isolated viraemia blips (preceded and followed by negative results) were detected by routine determination of viral load (Abbott RealTime HIV-1 assay) in four individuals; the blip was during the intensification period in only one case and was not confirmed by ultrasensitive determination of plasma viraemia in the same sample.

### *Residual viraemia*

Residual viraemia was measured using 4.5–7 mL of stored plasma samples collected before intensification (Figure 1a), at weeks 4, 12 and 24 after initiation of raltegravir and also at weeks 12 and 24 after withdrawal of raltegravir [median (IQR) limit of quantification, 0.7 (0.6–0.8) HIV-1 RNA/mL]. Median (IQR) residual viraemia at baseline was 2.2 (0.0–6.6) HIV-1 RNA copies/mL and longitudinal analysis showed no significant changes in median values during intensification or after withdrawal (Figure 1b, left *y*-axis, and Table 2). Indeed, the proportion of participants with detectable residual viraemia (>1 HIV-1 RNA copy/mL) was similar at baseline and at the end of the intensification period (56% versus 53%). However, we observed a higher proportion of samples with intermediate residual viraemia levels (10–60 HIV-1 RNA copies/mL) in the pre-intensification period (21%) than during administration of raltegravir (7%) and a rising tendency was observed after discontinuation of raltegravir (15%) (Figure 1b, right *y*-axis). A mixed-effects logistic regression model revealed a significant decrease in the probability to present intermediate residual viraemia levels during the intensification period (OR = 0.16 and $P = 0.01$; Figure 1c). However, this effect was lost after raltegravir withdrawal (OR = 0.73 and $P = 0.65$). This result reflects the impact of raltegravir on residual viraemia in individuals on maintenance PI/r monotherapy.

### *2-LTR dynamics*

To provide further insights into the efficacy of PI/r monotherapy for suppressing low-level viral replication, we explored the effect of intensifying ART with raltegravir on markers of *de novo* infection. For this purpose, we analysed the dynamics of 2-LTR circles in CD4+ T cells, as episomal HIV-1 DNA is a surrogate marker of recent infection events. 2-LTR circles were systematically detected in 18 participants (54.5%), with a median (IQR) level of 7.75 (4.25–14.9) 2-LTR copies/million CD4+ T cells at baseline. The longitudinal analysis showed a transient increase in the first 2 months following initiation of raltegravir (Figure 2a). However, no significant changes were detected at the end of the intensification period ($P = 0.30$ from baseline to week 24; Table 2). Individual dynamics showed that, in most participants, the highest 2-LTR level was reached between week 1 and week 8 (Figure 2b). The difference in concentrations of peaks was statistically significant ($P = 0.0005$). The results confirm the persistence of low-level viral replication during PI/r monotherapy, which was susceptible to further inhibition by raltegravir.

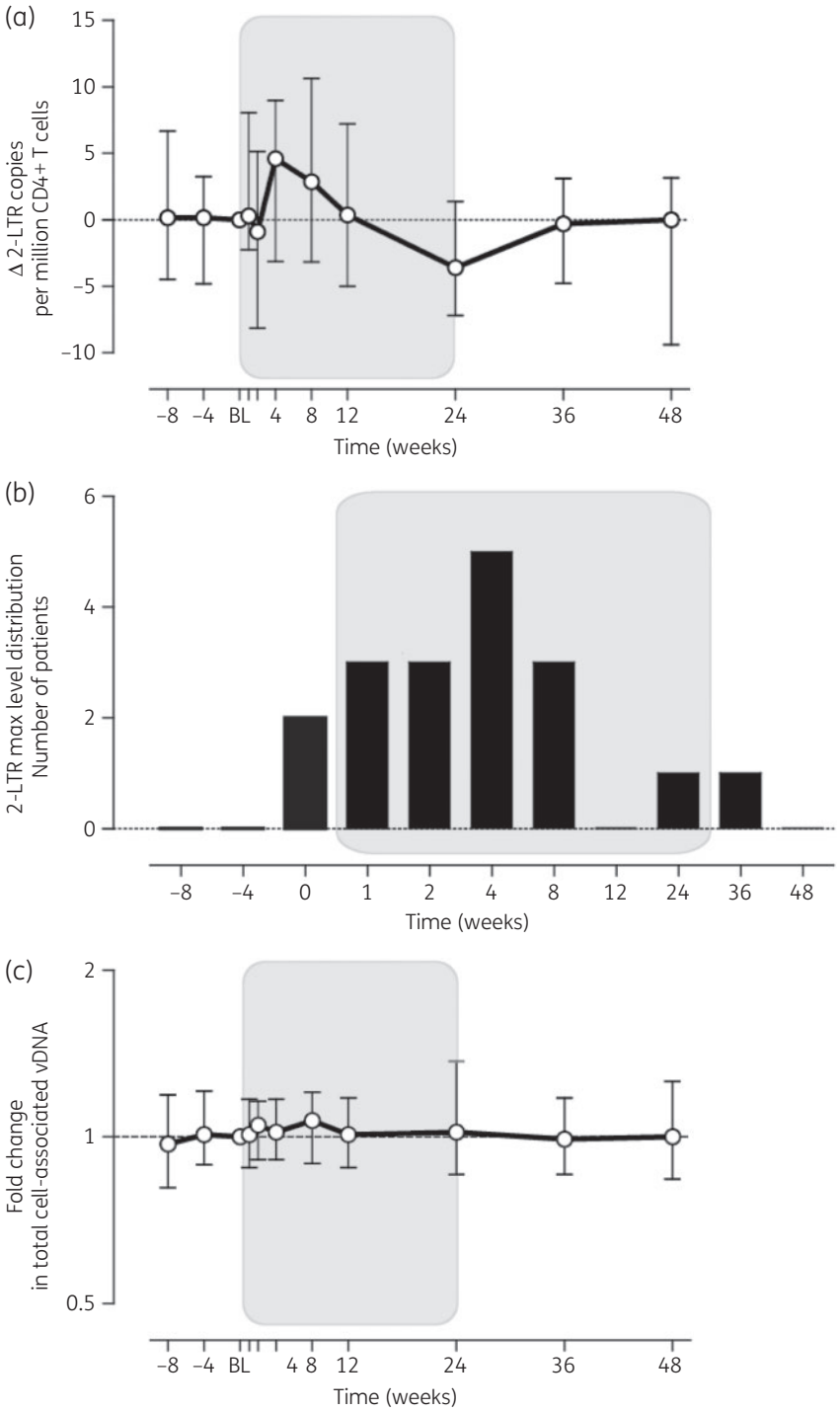


**Figure 2.** Viral reservoir dynamics upon intensification of PI monotherapy with raltegravir. (a) Increases in 2-LTR circles compared with individual baseline levels are shown as the median and IQR. The frequency diagram in (b) summarizes the timepoint at which each individual reached the maximum level of 2-LTR. (c) The dynamics of the proviral reservoir is shown as a fold change in total HIV-1 DNA in CD4+ T cells compared with individual baseline levels. The median and IQR are shown. The grey zone represents the intensification period (week 0 to 24). BL, baseline; vDNA, HIV-1 DNA.

### *Proviral reservoir*

The effect of intensification with raltegravir on the size of the viral reservoir was evaluated by measuring cell-associated viral DNA in purified circulating CD4+ T cells. The median (IQR) level at baseline was 486 (299–689) HIV-1 DNA copies/million CD4+ T cells. Intra-individual longitudinal analysis showed no changes in this parameter during the study period (Figure 2c and Table 2), thus reflecting the steadiness of the proviral DNA reservoir in peripheral CD4+ T cells.

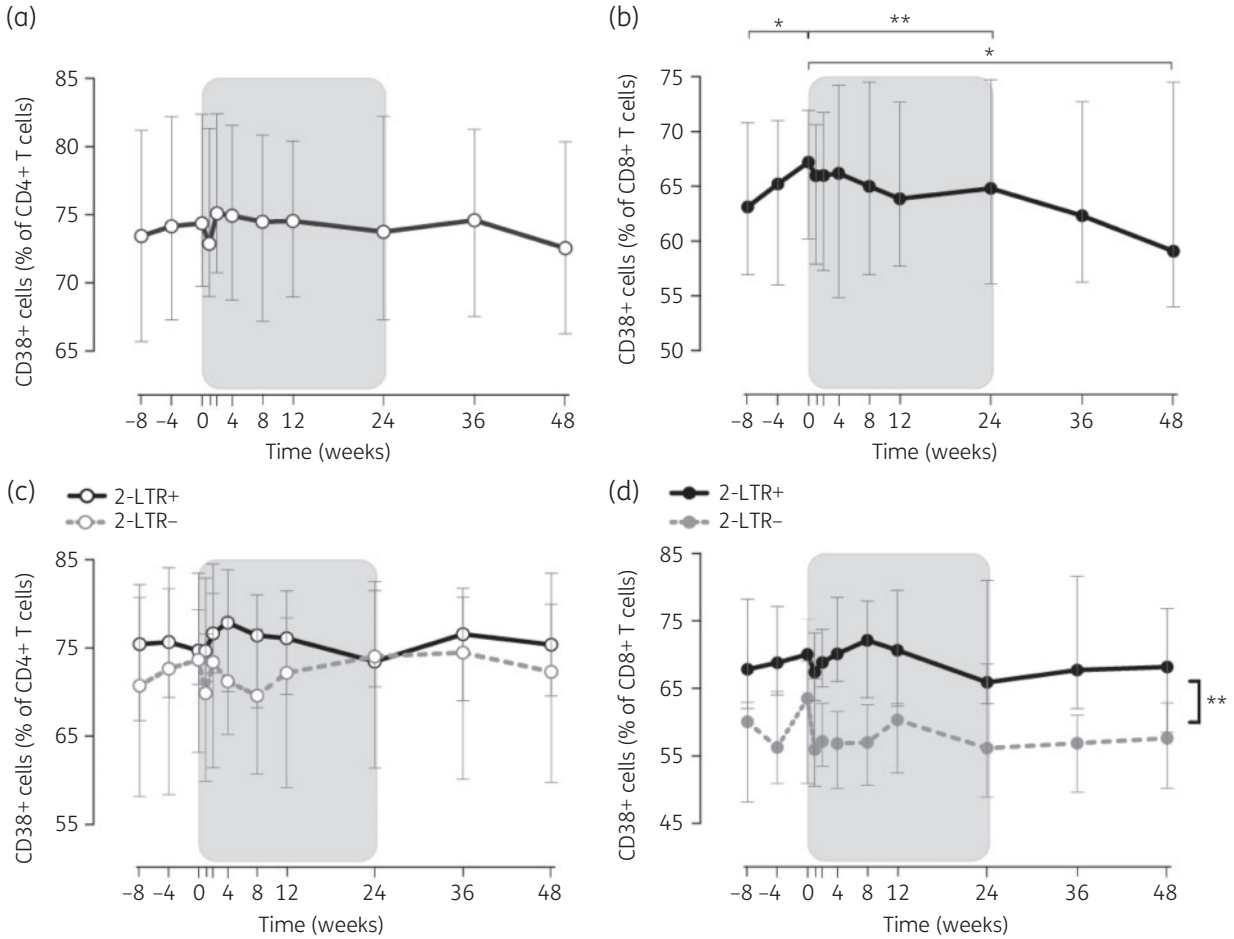


**Figure 3.** Effect of intensification with raltegravir on T cell activation. The graph shows the immune activation levels (determined as a percentage of CD38+ cells) of all HIV-infected individuals analysed both in CD4+ (open symbols) (a) and CD8+ (filled symbols) (b) T cell populations. The median and IQR are represented. Asterisks denote significant differences between timepoints detected using the Wilcoxon signed rank test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). T cell activation was compared between 2-LTR+ (black continuous line) and 2-LTR− (grey broken line) subgroups within CD4+ (c) and CD8+ (d) T cell subsets. The median and IQR are shown. Asterisks denote significant differences between 2-LTR subgroups detected using a linear mixed-effects model (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The grey zone represents the intensification period (week 0 to 24).

### *Analysis of T cell subsets, activation and exhaustion markers*

Intensification with raltegravir reduces CD8+ T cell activation and modestly modulates the maturation profile of CD4+ T cells.[11] Both parameters were also analysed longitudinally. Although the percentage of CD4+ T cells increased between baseline and week 24 ($P = 0.0066$) in parallel with a contraction of the CD8+ T cell compartment ($P = 0.0015$), no significant increases in the CD4+/CD8+ ratio were observed (data not shown). Furthermore, no major longitudinal changes were observed in the frequency of various CD8+ T cell subpopulations (naive cells or central, transitional and effector memory subsets), while changes in CD4+ T cells were associated with an increase in the transitional memory subpopulation (CD4+ CD45RA− CCR7− CD27+, $P = 0.045$, data not shown) from baseline to week 24.

No significant changes in immune activation (percentage of CD38+ cells) were observed within the CD4+ T cell compartment (Figure 3a). In contrast, a general decrease in the expression of this activation marker was observed in the CD8+ T cell compartment during the intensification period ($P = 0.0075$) (Figure 3b). Interestingly, when we compared activation levels according to whether 2-LTR circles had been detected or not (2-LTR+ and 2-LTR−), a slightly higher frequency of CD38+ cells was observed within CD4+ T cells in 2-LTR+ individuals (Figure 3c). This difference reached statistical significance in the CD8+ T cell population ($P = 0.006$) (Figure 3d). No changes were observed in the frequency of the HLA-DR+ or in double CD38+ HLA-DR+ in CD4+ or CD8+ T cells (data not shown).

The analysis of the exhaustion marker CTLA-4 showed no significant changes during the study period, although a transient increase affecting all memory subsets was observed during intensification between weeks 1 and 12, both in the CD4+ and in the CD8+ T cell populations (Figure 4a and b). In contrast with the immune activation levels, exhaustion was similar in CD8+ T cells when individuals were segregated according to detection of 2-LTRs. However, in 2-LTR+ individuals, there tended to be more

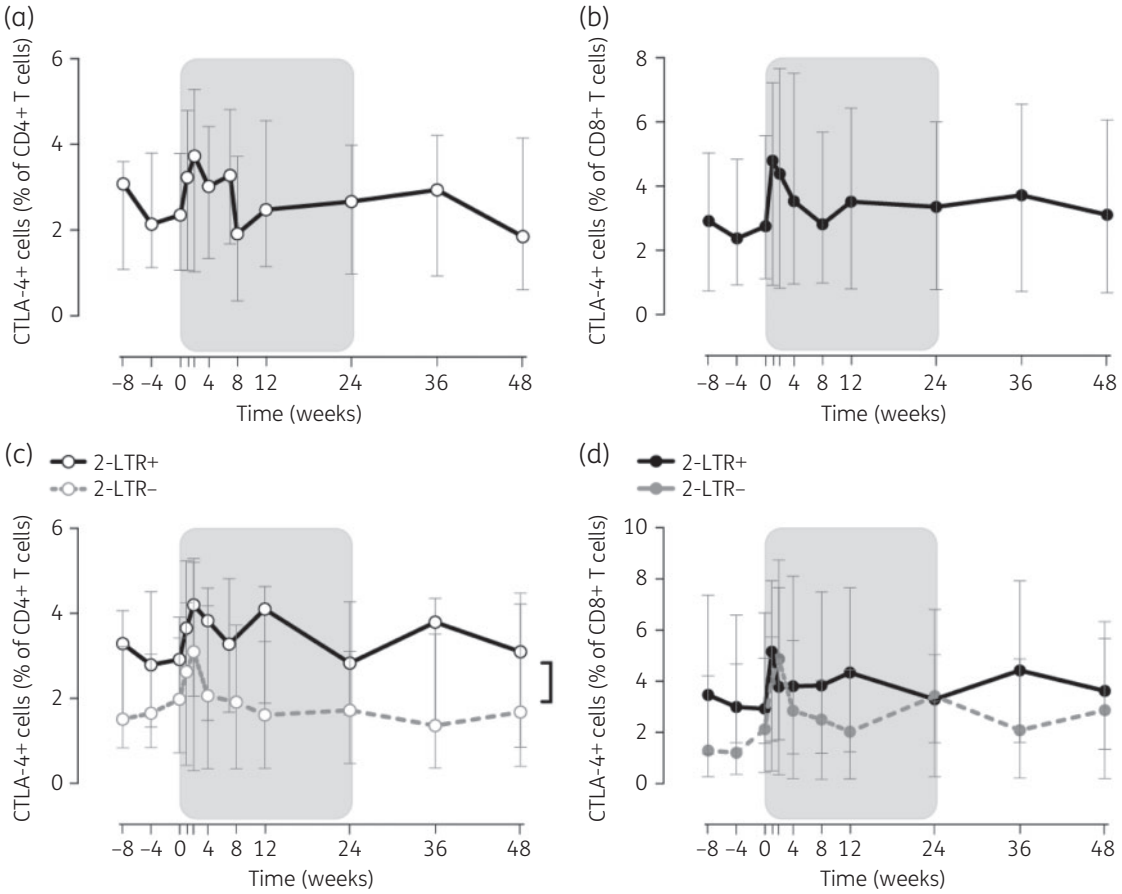


**Figure 4.** Effect of intensification with raltegravir on T cell exhaustion. The graph shows immune exhaustion levels (determined as a percentage of CTLA-4+ cells) of all HIV-infected individuals analysed both in CD4+ (open symbols) (a) and CD8+ (filled symbols) (b) T cell populations. The median and IQR are represented. Asterisks denote significant differences between the timepoints assessed using the Wilcoxon signed rank test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). T cell exhaustion was compared between 2-LTR+ (black continuous line) and 2-LTR− (grey broken line) subgroups within CD4+ (c) and CD8+ (d) T cell subsets. The median and IQR are shown. Asterisks denote significant differences between 2-LTR subgroups assessed using a linear mixed-effects model (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The grey zone represents the intensification period (week 0 to 24).

CTLA-4+ cells in the CD4+ T cell compartment ($P = 0.09$). Intriguingly, the transient elevation in CTLA-4+ cells seemed to be independent of the detection of 2-LTRs (Figure 4c and d).

Finally, the longitudinal analysis of inflammatory and coagulation markers (sCD14, IP-10, IL-6, CRP and D-dimer) showed some fluctuations during the study period (for IL-6, CRP and D-dimer); however, no significant decreases associated with intensification with raltegravir were noticed (Table S1, available as Supplementary data at *JAC* Online).

## Discussion

Randomized clinical trials have shown that maintenance PI/r monotherapy, in HIV-1-infected individuals who are already fully suppressed with standard ART, is able to maintain viral load suppression for months in most individuals. However, viral rebound rates in the long term are higher in patients receiving PI/r monotherapy than triple ART,[16] and thus it is not currently recommended in US and European treatment guidelines.[17,18] Although residual plasma viraemia below the detection limit of standard assays (<50 HIV-1 RNA copies/mL) has been suggested to be the origin of viral failures in this context, there is no clear evidence available.[19,20] In the present single-arm pilot study, we evaluated the effect of intensification with raltegravir on HIV-1-infected individuals on maintenance PI/r monotherapy by measuring changes in residual viraemia, low-level viral replication, HIV-1 DNA reservoirs, T cell immune activation and soluble inflammatory biomarkers.

Although median values of residual viraemia showed no major changes during the study, we found that the probability of having intermediate levels of residual viraemia (10–60 copies/mL) was significantly higher in the pre- and post-raltegravir intensification periods than during the 24-week intensification period, suggesting an impact of treatment intensification on this parameter. This is in contrast with the absence of reductions in residual plasma viraemia during raltegravir intensification in triple therapies.[7,21,22] Thus, our results suggest the lack of complete viral suppression that might explain the lower efficacy rates observed in PI/r monotherapies compared with standard ART.

Previous studies had reported occasional detection of 2-LTR circles upon raltegravir intensification in a proportion of individuals receiving PI-containing HAART.[7,8] Here, 2-LTR circles were also detected in the CD4+ T cells from 18 of the 33 participants (58%).

Of note, we systematically detected 2-LTR circles in those patients even before the addition of raltegravir to the PI/r regimen. As 2-LTRs are surrogate markers of recent infection events,[23] these results indicate the persistence of viral replication. Furthermore, a transient increase was observed after raltegravir initiation, as reflected by the accumulation of 2-LTR peaks between weeks 1 and 8. Taken together, our data demonstrate the persistence of low-level viral replication in a significant proportion of individuals on suppressive PI/r monotherapy. It is also possible that active replication occurs in anatomic compartments that are less accessible to PIs.[9] Still, we did not find any correlation between the detection of 2-LTR circles and the presence of intermediate residual viraemia levels in the patients before raltegravir intensification and neither of those parameters were preferentially associated with darunavir or lopinavir-based regimens.

In accordance with data from studies comparing monotherapy and triple therapy,[24,25] this low-level viral replication observed in PI/r-treated individuals does not seem to drive significant replenishment of the proviral reservoir in the short term, as a 24-week intensification period did not impact on levels of cell-associated viral DNA, at least in circulating peripheral CD4+ T cells.

In contrast with the results from previous intensification studies,[10,11] changes in CD8+ T cell activation were modest in the present study. Indeed, no changes were observed in the frequency of CD38+ HLA-DR+ CD8+ T cells, although a significant reduction in CD38 expression was observed in the whole CD8+ T cell population throughout the intensification period. This change was unrelated to changes in the frequency of naive cells and confirms larger reductions of CD38 expression compared with HLA-DR expression in intensification strategies.[26] However, in this case, CD38 changes after raltegravir intensification were close to the natural variation of CD38 expression during non-intensification periods. When a *post hoc* analysis was performed after classifying participants according to the detection of 2-LTR circles, we observed the previously reported higher CD8+ T cell activation in 2-LTR+ individuals;[7] however, no specific or significant decrease of activation was induced by intensification with raltegravir in those participants. In summary, we found an unanticipated minor effect of raltegravir on CD8+ T cell immune activation.

Similarly, immunological follow-up revealed no major changes in the frequency of CD4+ or CD8+ subsets during the intensification period. However, a striking transient increase in CTLA-4 was noticed in both CD4+ and CD8+ T cells after intensification with raltegravir. The increase peaked between weeks 4 and 8 and was unrelated to the presence of detectable levels of 2-LTRs or any other virological or immunological parameter analysed. CTLA-4 is a negative regulator of T cell receptor-mediated CD4+ T cell activation and function, which is upregulated on HIV-specific T cells;[27,28] therefore, suppression of HIV replication with ART is associated with decreased expression of CTLA-4 and other inhibitory receptors, such as PD-1 and TIM-3, on HIV-specific CD4+ T cells.[27,29–32] Moreover, CTLA-4 is reported to be moderately overexpressed in the total CD4+ population during HIV infection and its expression correlates inversely with CD4+ T cell count and positively with immune dysfunction and exhaustion, which are associated with a progressive loss of effector function and disease progression.[27,28] Consistent with these findings, we observed the highest frequency of CTLA-4+ cells within the effector memory CD4+ T cell subset (data not shown) and a trend toward higher CTLA-4 expression in individuals with detectable levels of 2-LTR circles. The meaning of these transient changes in CTLA-4 expression that were observed in all memory subsets remains unknown and warrants further exploration.

Recent studies have reported that factors associated with virological rebound in individuals receiving PI/r monotherapy include low nadir/baseline CD4+ T cell counts and shorter duration of viral suppression.[33,34] In this sense, the restrictive inclusion criteria applied in the present study ensured that the individuals had a favourable virological and immunological status. Indeed, the participating individuals were able to remain on PI/r monotherapy for a median of 3.3 years, again suggesting a better long-term viral control than those who had to return to ART. However, although the participants' characteristics might have been an unexpected limiting factor in the present study, our data indicate that, at least in long-term suppressed individuals with a good CD4+ T cell count at baseline, PI/r monotherapy can restrain immune activation.

Overall, temporary raltegravir addition uncovered low-level viral replication in a significant proportion of individuals on PI/r monotherapy, as evidenced by the transient increase of viral 2-LTRs circles in peripheral CD4+ T cells. Furthermore, intermediate residual viraemia (10–60 HIV-1 RNA copies/mL) detected during PI/r monotherapy became less frequent during raltegravir intensification. Those results evidenced the incomplete viral suppression occurring in patients on long-term PI/r monotherapy, thus being the potential origin of the lower viral suppression efficacy of this therapeutic simplification strategy, and suggest that dual therapy of PI/r and integrase inhibitors might improve virological parameters.

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### *Author contributions*

All authors made substantial contributions to the conception and design of the work or to the acquisition, analysis and interpretation of the data.

J. R. S., J. M., J. B., B. C. and J. M.-P. contributed to study design and interpretation. J. R. S. and J. M. contributed to patients' recruitment and clinical management. M. C. P., E. G.-M., S. M.-L., A. H.-R., S. M., M. M.-B., L. M. and M. A. M.-F. contributed to data acquisition and V. U. performed the statistical analysis. M. C. P., E. G.-M., V. U., J. B. and J. M.-P. contributed to data analysis and interpretation, and drafted the manuscript including figures and tables; all authors revised it critically for important intellectual content and have approved the final version submitted for publication. All authors fulfil ICMJE criteria for authorship, have had access to all the data in the study and agree to be accountable for the work in ensuring that questions related to its accuracy or integrity are appropriately investigated and resolved.

## Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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