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**Citation:** Massanella M, Ouchi D, Marfil S, Llibre JM, Puertas MC, et al. (2014) Different Plasma Markers of Inflammation Are Influenced by Immune Recovery and cART Composition or Intensification in Treated HIV Infected Individuals. PLoS ONE 9(12): e114142. doi:10.1371/journal.pone.0114142

**Editor:** Shilpa J. Buch, University of Nebraska Medical Center, United States of America

**Received:** May 28, 2014

**Accepted:** November 3, 2014

**Published:** December 2, 2014

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**Data Availability:** The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. Data are part of two clinical trials. All relevant data shown in the manuscript and/or supporting information are available on request to researchers who meet the criteria for access to confidential data. Please contact the Trial Monitor Roser Escrig ([rescrig@flsida.org](mailto:rescrig@flsida.org)) or alternatively the Hospital Germans Trias i Pujol Ethics Committee (<http://www.ceicgermanstrias.cat>) to request data.

**Funding:** This work was supported by HIVACAT program; the RD12/0017/0002 project as part of the Plan Nacional R + D + I cofinanced by Instituto de Salud Carlos III-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER); the European Community's Seventh Framework Program (FP7/2007-2013) under the Project "Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN)" grant agreement n 223131, by the Department of Veterans Affairs, the UCSF Center for AIDS Research (P30 AI36214), the CARE Collaboratory (U19 AI096113) and the James B. Pendleton Charitable Trust and by an unrestricted grant from Merck Sharp & Dohme (MSD 40527). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** BC, JB, JMP, JML, EN, MS and DDR have received fees for consultancy from BMS, GILEAD, ViiV or Jansen outside the submitted work. MSD grant 40527 (Principal Investigator JB) funded the project through the PI's institution (IRSICAIXA). This does not alter the

RESEARCH ARTICLE

# Different Plasma Markers of Inflammation Are Influenced by Immune Recovery and cART Composition or Intensification in Treated HIV Infected Individuals

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## Abstract

**Background:** HIV-1 infection increases plasma levels of inflammatory markers. Combination antiretroviral therapy (cART) does not restore inflammatory markers to normal levels. Since intensification of cART with raltegravir reduced CD8 T-cell activation in the Discor-Ral and IntegRal studies, we have evaluated the effect of raltegravir intensification on several soluble inflammation markers in these studies.

**Methods:** Longitudinal plasma samples (0–48 weeks) from the IntegRal (n=67, 22 control and 45 intensified individuals) and the Discor-Ral studies (44 individuals with CD4 T-cell counts <350 cells/μl, 14 control and 30 intensified) were assayed for 25 markers. Mann-Whitney, Wilcoxon, Spearman test and linear mixed models were used for analysis.

**Results:** At baseline, different inflammatory markers were strongly associated with HCV co-infection, lower CD4 counts and with cART regimens (being higher in PI-treated individuals), but poorly correlated with detection of markers of residual viral replication. Although raltegravir intensification reduced inflammation in individuals with lower CD4 T-cell counts, no effect of intensification was observed on plasma markers of inflammation in a global analysis. An association was found, however, between reductions in immune activation and plasma levels of the coagulation

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marker D-dimer, which exclusively decreased in intensified patients on protease inhibitor (PI)-based cART regimens ( $P=0.040$ ).

**Conclusions:** The inflammatory profile in treated HIV-infected individuals showed a complex association with HCV co-infection, the levels of CD4 T cells and the cART regimen. Raltegravir intensification specifically reduced D-dimer levels in PI-treated patients, highlighting the link between cART composition and residual viral replication; however, raltegravir had little effect on other inflammatory markers.

## Introduction

Human immunodeficiency virus type 1 (HIV-1) infection leads to immune activation that has been related to massive CD4 T-cell depletion occurring mainly in the gut associated lymphoid tissue (GALT), which in turn favors the translocation of bacterial products from the intestinal lumen to the circulation and tissues [1]. In addition, a chronic Type 1 interferon (IFN) response driven by HIV-1 replication, co-infecting pathogens and the homeostatic proliferation, attempting to counterbalance CD4 T-cell depletion have been also reported as potential sources of increased immune activation [2–5]. Cellular markers of immune activation, such as the turnover or the expression of activation and maturation markers in CD4 or CD8 T cells have been commonly used to determine the level of immune damage in HIV-1 infection [6–8]. Soluble markers of inflammation can be also used as biomarkers for immune activation or antiviral responses, and have been widely employed to fully delineate the aberrant immune activation observed in HIV infected individuals [9–11].

Combination antiretroviral therapy (cART) effectively suppresses viral replication and in most cases increases CD4 T-cell counts, but only partly reduces T-cell activation, cell death and soluble inflammatory markers [7, 12–15]. This observation is clinically relevant since many authors have associated increased levels of inflammatory, coagulation or endothelial function markers in cART treated individuals to several clinical outcomes, including death [16], subclinical cardiovascular risk [17, 18], AIDS defining events or non-AIDS defining events [19, 20]. The reasons for this incomplete effect of cART in immune restoration are still elusive. Candidate factors could include an incomplete CD4 T-cell repopulation [12, 21] and HIV-1 persistence, defined by the presence of HIV-1 antigens coming from long-lived reservoirs or from low level HIV-1 replication in sanctuary sites [22, 23].

Intensification of cART regimens has been used as a strategy to contain residual HIV-1 replication in virologically suppressed individuals [24–28]. Although the reported virological and immunological results are controversial [24, 25, 27, 28], a relevant proportion of intensified individuals showed evidence of an effective blockade of new infection events as measured by the presence of episomal viral

DNA (2-long terminal repeat [2-LTR] circles) that was accompanied by a decrease in some markers of immune activation or inflammation [24, 25].

The IntegRal [24, 26, 29] and Discor-Ral [30, 31] studies explored the effect of intensification of cART with the integrase inhibitor raltegravir in virologically suppressed individuals. While the IntegRal study focused on patients with proper immune recovery, the Discor-Ral study investigated patients with unsatisfactory immune recovery (defined by a CD4 T-cell count below 350 cells/ $\mu$ L). Both studies offered an opportunity to address the contribution of incomplete immune recovery and low level HIV-1 replication under cART to the inflammatory status of virologically suppressed HIV-infected individuals.

We analyzed inflammation in baseline samples from these studies to evaluate the association of inflammation and incomplete immune recovery. In addition longitudinal analysis was used to test the effect of the blockade of low level HIV-1 replication under cART in the inflammatory status of virologically suppressed HIV-infected individuals. In order to cover a large number of potential markers, we evaluated a collection of 25 cytokines in baseline and longitudinal samples from both studies. We included pro-inflammatory mediators of innate immunity (i.e. TNF- $\alpha$ , IL-1b, IL-6, IL-10, IL-12, IL-15, type I interferon, such as IFN- $\alpha$ ,  $\alpha$ - and  $\beta$ -chemokines, such as IL-8, MCP-1 and MIP-1 $\beta$ ) or adaptive immunity (i.e. IL-2, IL-4, IL-5, IL-13, IL-17 and IFN- $\gamma$ ), pro-apoptotic inducers (TRAIL), stimulators of hematopoiesis (i.e. GM-CSF and G-CSF), monocyte activation (sCD14), endothelium inflammation (sICAM and sVCAM) and hypercoagulation markers (D-dimer, a fibrin degradation product that can be increased with inflammation). Our data suggest a link between systemic inflammatory markers and CD4 T-cell recovery and also with the composition of cART regimens. Indeed, a specific beneficial effect of raltegravir intensification could be identified in individuals receiving protease inhibitor (PI)-based regimens.

## Materials and Methods

### Ethics statement

The Discor-Ral study is a prospective, open-label, randomized study (ClinicalTrials.gov number NCT00773708) to assess the effect of raltegravir addition to standard antiretroviral therapy for 48 weeks in immunodiscordant individuals (Discor-Ral Study) carried out in the Hospital Universitari Germans Trias i Pujol (Badalona, Spain) [30, 31]. The IntegRal study is a prospective, controlled, open-label study that includes HIV-1-infected subjects on suppressive HAART for at least 1 year (ClinicalTrials.gov number NCT00554398) carried out in three university hospitals in Barcelona [24, 26, 29]. The Ethics Committee of the Hospital Germans Trias i Pujol approved The Discor-Ral Study, while the IntegRal study was approved by the Ethics Committee of the Hospital Germans Trias I Pujol (Badalona, Spain), the Ethics Committee of the Hospital Clinic (Barcelona, Spain) and the Ethics Committee of the Hospital de Sant Pau

(Barcelona, Spain). All participants provided written informed consent to participate in these studies.

## Samples

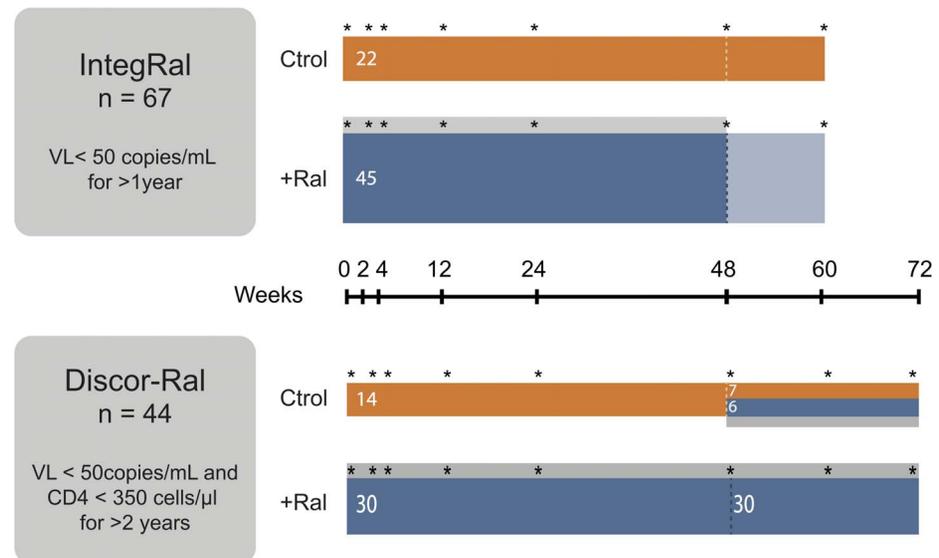
Plasma samples were obtained from EDTA-treated blood, aliquoted and stored at  $-80^{\circ}\text{C}$  at specified time points during the IntegRal and the Discor-Ral studies (Figure 1). Samples from the IntegRal study cover the full intensification period of 48 weeks [26] and an additional 12-week period after raltegravir discontinuation, to assess the stability of the effect of the drug [29]. Samples from the Discor-Ral study included longer intensification periods (up to 60 weeks), but lack additional samples after raltegravir discontinuation [31]. Inclusion criteria were similar for both studies, except that the IntegRal study had no enrollment restriction in terms of CD4 T-cell counts [26], while the Discor-Ral study was restricted to patients with CD4 T-cell counts below 350 cells/ $\mu\text{l}$  during the previous two years [30, 31]. In both studies, HIV-1-infected subjects were eligible if they were 18 years of age or older, received a cART regimen composed of two nucleos(t)ide reverse transcriptase inhibitors (NRTIs), and a protease inhibitor (PI) or a non-NRTI (NNRTI), and were naïve to integrase inhibitors. Their plasma HIV-1 RNA levels had to be below 50 copies/mL at baseline and during at least the previous year for the IntegRal or two years in the Discor-Ral study with more than 4 determinations. In both studies, subjects were randomly assigned 2:1 to add raltegravir (400 mg twice daily) or continue their cART regimen.

## Quantification of soluble markers

Frozen plasma samples were thawed and assayed for the levels of different cytokines and inflammation mediators using Luminex technology. Plasma samples were analyzed (dilution  $\frac{1}{4}$ ) using a Bio-Plex Pro Human Cytokine panel (BioRad, Hercules, CA) that included the determination of the following human cytokines: MIP-1 $\beta$ , IP-10, TRAIL, TNF- $\alpha$ , IFN- $\gamma$ , IFN- $\alpha$ , G-CSF, GM-CSF, MCP-1, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15 and IL-17. For the determination of soluble VCAM-1 (sVCAM-1) and soluble ICAM-1 (sICAM-1) plasma samples were diluted 1/200 and tested using a BioPlex pro human cytokine 2-plex also from BioRad. Plasma levels of D-dimer were analyzed using the HemosIL D-Dimer HS 500 quantitative kit in an ACL TOP equipment (both from Instrumentation Laboratory, Bedford, MA). Plasma levels of soluble CD14 (sCD14) were analyzed as previously described [26, 30] using a commercial ELISA kit (Diaclone, Besançon, France).

## Statistical analysis

Correlation analysis of soluble plasma markers with immune activation, CD4 T-cell counts and other variables at baseline was performed; however, the primary endpoint was the assessment of changes of soluble plasma biomarkers at week 48 in raltegravir intensified patients. Secondary endpoints were the analysis of



**Figure 1. Schematic representation of IntegRal and Discor-Ral studies.** In both studies, enrolled individuals were randomized 1:2 to continue with previous cART (Ctrl, control groups in orange) or intensify their therapy with raltegravir (+Ral, intensified groups in blue). The extent of raltegravir intensification was 48 weeks for the IntegRal study and 72 weeks for the Discor-Ral study (grey areas). The follow up of the IntegRal study was 60 weeks, allowing for the analysis of the effect of raltegravir discontinuation. In the Discor-Ral study six patients from the control arm started raltegravir for 24 weeks (blue area). In all groups, asterisks denote sampling times.

doi:10.1371/journal.pone.0114142.g001

changes at intermediate time points using linear mixed models, and the analysis of changes 12 weeks after raltegravir discontinuation (IntegRal study). We performed separate analyses for IntegRal and Discor-Ral studies and a combined analysis aimed to define common effects of intensification in both studies; corrections for CD4 T-cell counts, HCV status or treatment regimen were performed.

We used the Mann-Whitney U test to compare medians between different arms or different studies and the signed-rank test (paired test) to compare longitudinal differences within groups (baseline-week 48, and week 48-week 60). The relationship between soluble plasma markers and different co-variables was examined using the nonparametric Spearman test. Regression coefficients and *p*-values for each cytokine are reported from a multivariate linear regression model:  $cytokine = intercept + HCV\ status + 2LTR\ detection + age + PI/NNRTI\ based\ cART + nadir + CD4\ counts + gender$ . Specifically, the final multivariate model was derived using a bootstrapped AIC based stepwise selection method. Moreover, the study of longitudinal changes within groups and the mean change comparison between arms (slope coefficient), from baseline to week 48, were evaluated using linear mixed models:  $cytokine = intercept + group + week + group * week$ . A statistically significant interaction term ( $group * week$ ) identified significantly different slopes among treatment groups. All analyses were done using the R package (v 3.0.2) [32, 33].

## Results

### Baseline characteristics of patients enrolled in the IntegRal and Discor-Ral studies

The individuals enrolled in the IntegRal and Discor-Ral studies are described in [Table 1](#). No major differences were observed at baseline among control and intensified groups in each study, except for the previously reported higher prevalence of hepatitis C virus (HCV) infection in the control group of the Discor-Ral study [[30](#), [31](#)]. As expected, participants in the Discor-Ral study had lower nadir and baseline CD4 T-cell counts than participants in the IntegRal study, but also were significantly older, were infected longer and had a higher percentage of PI-based regimens ([Table 1](#)).

All plasma markers examined were detectable in most samples, except for IL-2, IL-15 and IFN- $\alpha$ , which were undetectable in >80% of plasmas tested, hampering a proper analysis of these cytokines. A comparison of detectable soluble plasma markers among control and intensified groups in each study at baseline showed no major differences (data not shown). However, when the IntegRal and Discor-Ral studies were compared at baseline, significantly higher levels of most markers were noticed in the latter study (Figure S1). Since several factors could account for these differences (including lower CD4 T-cell counts and cART composition), we analyzed the association of the levels of soluble plasma markers with demographic, immunological and virological factors ([Figure 2](#)). Univariate analysis showed some relationship of soluble markers with age, the detection of 2-LTR circles during intensification and with HCV co-infection, while inflammatory status appeared to be more related to sex, cART composition (only when PI-based vs NNRTI-based regimens were compared), nadir and current CD4 T-cells values. A multivariate analysis maintained strong associations between HCV infection and levels of IP-10, sICAM-1 and sVCAM-1, between age and sCD14 and an inverse association between CD4 T-cell counts and IL-12 ([Figure 2](#), red boxes). Moreover, the presence of 2-LTR circles, the administration of PI-containing regimens, CD4 T-cell counts, the CD4 nadir, and age were also independently associated with several inflammatory markers ([Figure 2](#), orange boxes). However, while markers of inflammation showed positive and negative associations with the detection of 2-LTR circles, a more consistent scenario was observed for other parameters: higher plasma levels were identified in patients on a PI-based cART (positive correlations) and in individuals with low CD4 T-cell counts or with low nadir CD4 T cell values (negative correlations). Interestingly, sCD14 was the marker with the highest number of significant associations: 2LTR detection, age and nadir CD4 T-cell counts.

### Effect of raltegravir intensification

Longitudinal analyses of soluble plasma markers were performed by comparing baseline and week 48 data and by fitting linear mixed models that included intermediate time points ([Figure 1](#)), as reported for the evolution of CD4 T cells

**Table 1.** Baseline characteristics of the control and raltegravir-intensified individuals.

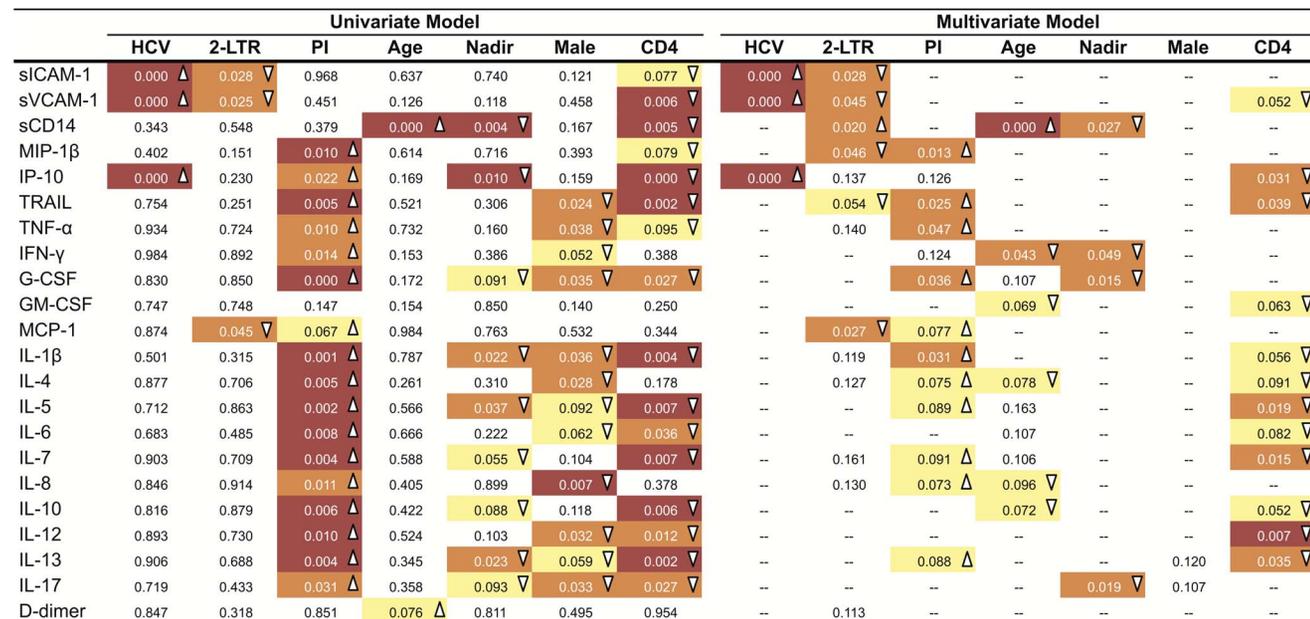
	IntegRal			Discor-Ral			P-value Between Studies <sup>b</sup>
	Control n=22	Intensified n=45	P-value Between Groups <sup>a</sup>	Control n=14	Intensified n=30	P-value Between Groups <sup>a</sup>	
Age, years, Median [IQR]	45 [37–50]	44 [41–49]	0.569	48 [42–51]	45 [44–53]	0.221	<b>0.007</b>
Females n (%)	6 (27.3)	6 (9.0)	0.187	3 (13.3)	4 (14.3)	0.932	1
Hepatitis C co-infection, n (%)	5 (22.7)	13 (28.1)	0.810	7 (50.0)	6 (20.0)	<b>0.005</b>	0.830
PI-based ART at Baseline, n (%)	8 (36.4)	14 (31.1)	0.878	8 (57.1)	18 (60.0)	0.878	<b>0.010</b>
Time from HIV-1 diagnosis, years, Median [IQR]	10 [5–15]	11 [8–17]	0.795	15 [5–20]	14 [7–21]	0.678	<b>0.020</b>
Time with suppressive (VL<50) ART, years, Median [IQR]	5 [3–8]	7 [3–7]	0.311	6 [4–10]	6 [4–9]	0.868	0.070
CD4 T cell at baseline, (cell/μl), Median [IQR]	503 [371–600]	530 [434–678]	0.333	242 [188–292]	253 [208–301]	0.668	< <b>0.0001</b>
Nadir CD4 T-cell count, (cell/μl), Median [IQR]	84 [41–254]	251 [85–405]	0.060	67 [14–108]	90 [24–117]	0.435	< <b>0.0001</b>

PI, protease inhibitors; ART, antiretroviral treatment; VL, plasma viral load (HIV-1 RNA copies/ml);

a p-value between groups: Mann-Whitney U test,

b p-value of the comparison of patients enrolled in the IntegRal (n=67) and Discor-Ral (n=44) studies: Mann-Whitney U test.

doi:10.1371/journal.pone.0114142.t001



**Figure 2. Analysis of baseline levels of soluble plasma markers.** Heat map of the association of baseline levels of the different soluble plasma markers analyzed with the following parameters: HCV co-infection (HCV column), presence of 2-LTR during the study period (2-LTR column), the presence of PIs on the cART regimen (PI column, PI=1, NNRTI=0), age, Nadir CD4 T-cell counts (nadir column), gender (male column, male=1, female=0) and CD4 T-cell counts at study baseline (CD4 columns). Left part shows results from a univariate analysis (spearman test), while right side data correspond to a multivariate analysis carried out using the AIC-based stepwise procedure. In all cases, color codes are as follow: no color means p-value>0.1, yellow 0.1>p-value>0.05; orange 0.05>p-value>0.01 and red p-value<0.01. Upward arrowheads (Δ) indicate positive correlations while downward arrowheads indicate negative correlations.

doi:10.1371/journal.pone.0114142.g002

or immune activation markers [26]. The direct comparison of baseline and week 48 levels of plasma soluble markers showed minimal changes in the IntegRal study. Only D-dimer, G-CSF, IL-6 and TRAIL showed significant decreases in the intensified group that were not observed in control individuals (Figure 3). In contrast, the Discor-Ral study showed a more pronounced effect of raltegravir intensification upon cytokines including IFN- $\gamma$ , IL-13, IL-1b, IL-4, IL-5, IL-7 and IP-10, probably due to higher baseline values. Since changes were not fully consistent in both studies, we explored potential common effects of raltegravir by performing a combined analysis of both studies. This combined analysis showed specific changes induced by raltegravir in D-dimer, IFN- $\gamma$  and TRAIL in intensified, but not in control individuals, with trends for IL-4, IL-5, IL-8 and IL-13. A more powerful analysis using linear mixed models that included all longitudinal data was also applied (Figure S2). This approach detected significant decreases of IP-10, G-CSF, GM-CSF, IFN- $\gamma$ , IL-1b, IL-4, IL-5, IL-7 and IL-8 but failed to show significant differences among slopes calculated in control and intensified groups for these cytokines (Figure 3, right columns). A significant increase of sICAM-1, was seen in both the raltegravir and control groups (Figure 3). Similar results were observed when analyses were performed including corrections for CD4 T-cell counts, HCV status or treatment regimen (data not shown). Analysis of plasma markers 12 weeks after raltegravir discontinuation in the IntegRal study showed no statistically significant changes compared to week 48 values in any marker.

### Association of changes in immune activation and soluble markers

We previously reported that raltegravir intensification of treated subjects with suppressed viremia induced a specific decrease in markers of immunological activation in CD8 T cells, particularly for CD38 [26, 30]. Thus, we explored in the current study whether these changes were associated with soluble markers of inflammation. In this analysis, no significant association between CD8 T-cell activation, as measured by CD38 expression, and inflammatory markers were observed. However, a trend for association with the levels of D-dimer ( $p$ -value=0.09) was seen, which was stronger in the IntegRal study ( $p$ -value=0.07, data not shown).

To better understand this partial association, we have classified individuals undergoing treatment intensification according to the detection of 2-LTR circles with raltegravir intensification or to the presence of a PI in their cART regimen, which has been also related to the detection of 2-LTR circles [24, 25]. No significant changes in plasma levels of D-dimer were observed when these individuals were grouped according to the detection of 2-LTR circles (data not shown). However, a significant decrease was observed in patients treated with a PI-based regimen in the IntegRal study ( $p$ -value=0.045). Although a similar comparison in the Discor-Ral study did not achieve statistical significance, the combined analysis of both studies confirmed a significant effect of raltegravir on the levels of D-dimer in PI-treated patients ( $p$ -value=0.040), that was not

	Comparison baseline - Week 48						Linear Mixed Model		
	IntegRal		Discor-Ral		Both		Both		
	Ctrol	+Ral	Ctrol	+Ral	Ctrol	+Ral	Ctrol Slope	+Ral Slope	Ctrol vs +Ral
sICAM-1	0.240	0.617	0.001 ▲	0.000 ▲	0.001 ▲	0.000 ▲	0.010 ▲	0.000 ▲	0.150
sVCAM-1	1.000	0.929	0.004 ▼	0.008 ▼	0.114	0.076 ▼	0.220	0.610	0.490
sCD14	0.445	0.045 ▲	0.563	0.542	0.307	0.230	0.390	0.700	0.630
MIP-1b	0.761	0.681	0.194	0.178	0.243	0.579	0.190	0.980	0.280
IP-10	0.015 ▲	0.300	0.058 ▼	0.026 ▼	0.002 ▼	0.002 ▼	0.080 ▼	0.010 ▼	0.810
TRAIL	0.394	0.016 ▼	0.076 ▼	0.446	0.487	0.037 ▼	0.180	0.620	0.170
TNF-a	0.486	0.058 ▼	0.068 ▼	0.121	0.385	0.737	0.110	0.360	0.420
IFN-g	0.433	0.218	0.079 ▼	0.001 ▼	0.053 ▼	0.001 ▼	0.000 ▼	0.000 ▼	0.960
G-CSF	0.139	0.004 ▼	0.000 ▼	0.001 ▼	0.094 ▼	0.336	0.060 ▼	0.010 ▼	0.970
GM-CSF	0.449	0.761	0.119	0.094 ▼	0.076 ▼	0.253	0.010 ▼	0.010 ▼	0.450
MCP-1	0.663	0.192	0.217	0.576	0.507	0.549	0.160	0.290	0.570
IL-1b	0.983	0.617	0.194	0.033 ▼	0.313	0.282	0.020 ▼	0.050 ▼	0.420
IL-4	0.338	0.317	0.091 ▼	0.001 ▼	0.519	0.066 ▼	0.130	0.000 ▼	0.700
IL-5	0.931	0.957	0.194	0.021 ▼	0.258	0.077 ▼	0.010 ▼	0.000 ▼	0.890
IL-6	0.276	0.023 ▼	0.135	0.080 ▼	0.647	0.714	0.170	0.390	0.520
IL-7	0.408	0.421	0.135	0.012 ▼	0.472	0.289	0.030 ▼	0.030 ▼	0.600
IL-8	0.360	0.439	0.025 ▼	0.001 ▼	0.282	0.073 ▼	0.030 ▼	0.020 ▼	0.640
IL-10	0.705	0.266	0.049 ▼	0.084 ▼	0.193	0.853	0.020 ▼	0.080 ▼	0.330
IL-12	0.177	0.080 ▼	0.002 ▼	0.009 ▲	0.175	0.539	0.260	0.170	0.890
IL-13	0.296	0.858	0.119	0.009 ▼	0.449	0.055 ▼	0.940	0.140	0.370
IL-17	0.847	1.000	0.063 ▼	0.126	0.042 ▼	0.300	0.110	0.190	0.580
D-dimer	0.862	0.038 ▼	0.086 ▼	0.223	0.822	0.016 ▼	0.960	0.130	0.360

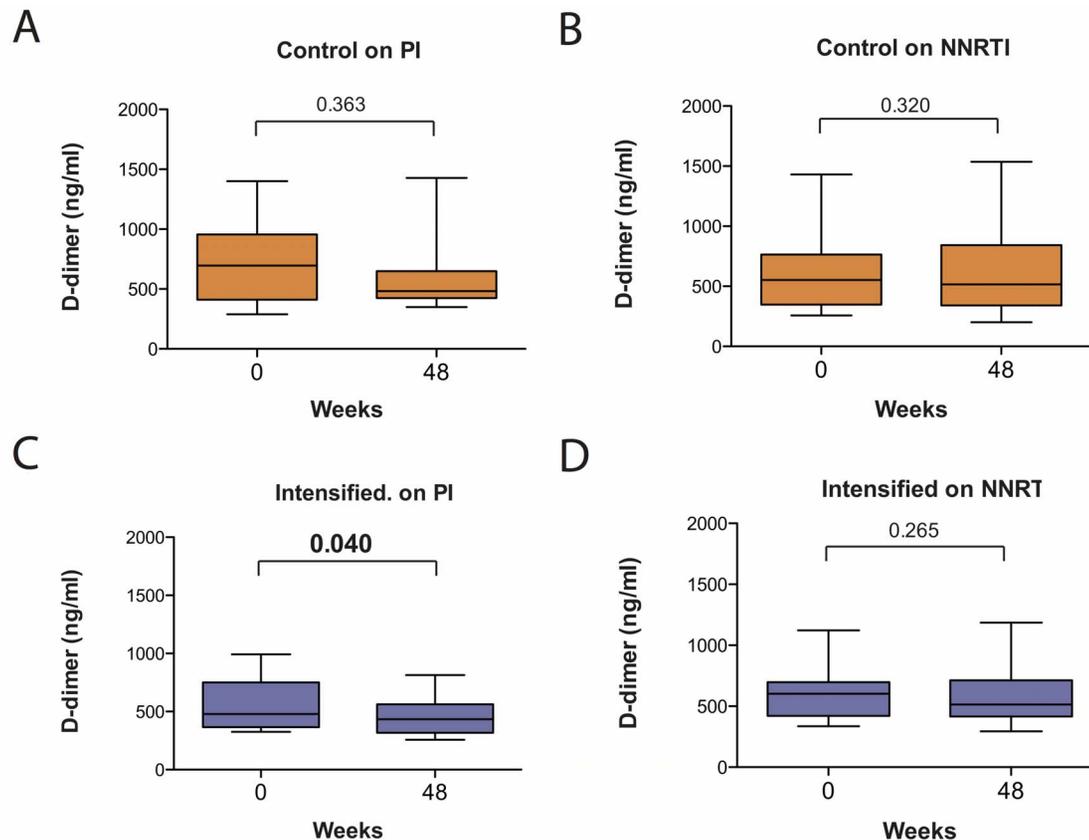
**Figure 3. Longitudinal analysis of soluble plasma markers.** Left part shows the heat map of the *p*-values obtained by comparing the levels of plasma soluble markers at baseline and week 48 in the IntegRal and Discor-Ral studies or in a global analysis of both studies. *P*-values are given for the control (Ctrol) and intensified (+ Ral) groups as indicated. Right part shows the results from a linear mixed model analysis showing *p*-values for slope (change of every marker per week) for control and intensified groups and the interaction slope/group that indicates the existence of significant difference among groups. As in Figure 2, color codes are as follow: no color means *p*-value>0.1, yellow 0.1>*p*-value>0.05; orange 0.05>*p*-value>0.01 and red *p*-value<0.01. Upward arrowheads (▲) indicate positive correlations while downward arrowheads indicate negative correlations.

doi:10.1371/journal.pone.0114142.g003

observed in raltegravir intensified individuals on NNRTI therapy or in control individuals irrespective of their cART regimen (Figure 4). Consistent with the small changes in inflammatory markers induced by raltegravir intensification, no major increase in inflammation was noticed after raltegravir discontinuation (data not shown), as reported for immune activation in the IntegRal study [29].

### Discussion

The impact of raltegravir intensification of suppressive cART regimens on soluble plasma markers might provide new clues regarding the contribution of residual viral replication to the subclinical inflammatory status of treated HIV-1 infected individuals. Our analysis of two separate studies (IntegRal and Discor-Ral) points



**Figure 4. Longitudinal changes in plasma level of D-dimer according to cART regimen.** Individuals from both the IntegRal and Discor-Ral studies were classified according to their cART regimen, PI- or NNRTI-based. The level of D-dimer in plasma at baseline and after 48 weeks of study was compared. The figure shows changes for Control individuals on PI-based regimens (A, n=15), control individuals on NNRTI-based regimens (B, n=17), raltegravir-intensified individuals on PI-based regimens (C, n=27) and raltegravir-intensified individuals on NNRTI based regimens (D, n=33). Comparisons were performed using the signed-rank test (paired test) and p-values are shown for each group.

doi:10.1371/journal.pone.0114142.g004

to a poor association between plasma inflammatory markers and the detection of residual viral replication (assessed by 2-LTR circles) in treated individuals with chronic HIV infection. Instead, we have identified a complex but strong relationship between these markers and other immunological and virological parameters, including CD4 T-cell restoration.

Although we observed a significant association between several cytokines and the number of circulating CD4 T cells, this observation could be influenced by the higher prevalence of PI-based regimens in individuals with low CD4 T-cell counts, a phenomenon previously observed in different cohorts [14]. A multivariate approach performed to identify independent associations confirmed that some plasma markers (including IL-5, IL-7, IL-12, IL-13, TRAIL or IP-10) were independently associated with the number of CD4 T cells. In addition, this analysis also revealed other factors influencing inflammation. Among them, Nadir CD4 T-cell values that showed independent association with sCD14 and IL-17 levels. And the detection of 2-LTR circles and baseline that showed a more

complex association with the levels of inflammation. The interpretation of these data is probably limited by the biased sample selection; namely, a high representation of individuals with low Nadir CD4 T-cell counts (in the Discor-Ral but also in the IntegRal study) and a low number of patients whose mononuclear cells had 2-LTR circles, especially in the Discor-Ral study. New technologies such as droplet digital PCR [34], may help to overcome technical limitations to detect 2-LTR circles and to better identify individuals with residual viral replication [25]. However, the complex relationship of inflammatory markers with several immunological and virological variables may complicate their use as predictors for residual HIV replication under cART.

Indeed, our baseline analysis confirmed the strong association of sCD14 with age [35], the link of HCV co-infection with three soluble markers (IP-10, sICAM-1 and sVCAM-1) [36], and identified a striking association of several cytokines, TRAIL and TNF- $\alpha$  among other, with the cART regimen (PI- or NNRTI-based). In all cases, this latter association revealed higher levels of inflammation in individuals treated with PI-based regimens, an observation that is consistent with previously reported data [11, 18]. However, the mechanisms leading to this increased inflammation are unclear. PI-treated individuals are known to display altered lipid metabolism, resulting in higher levels of plasma lipids and higher cholesterol accumulation in macrophages that may result in higher levels of inflammation and endothelial dysfunction markers in plasma [37–39]. In fact, switching PI-based to raltegravir-based cART results in specific reductions of some of these markers [37]. In addition to metabolic interference of PIs, an alternate explanation for the increased inflammation in these patients can be related to the poor tissue penetration of these compounds that may favor low level viral replication [40, 41]. This hypothesis is supported by the higher prevalence of the detection of 2-LTR circles in PI-treated individuals [24, 25], and may also explain the reduction in inflammation induced by switching PI to raltegravir treatment [37]. However, this could suffer a clinical bias, as treating physicians often include PIs in more advanced or immuno-discordant patients.

In our longitudinal analysis raltegravir was added to PI- or NNRTI-based cART and therefore, no metabolic benefits are expected. Despite this, some significant changes were observed in intensified individuals in the Discor-Ral study, which showed higher baseline levels of inflammatory markers. However, a combined analysis of the Discor-Ral and IntegRal studies failed to identify a general decrease in inflammatory markers associated with raltegravir intensification. In fact, several cytokines showed decreasing trends over time in both control and intensified groups. The observation that most of decreasing cytokines (namely IP-10, TRAIL or IL-7) are also associated with lower CD4 T-cell counts suggests that the observed decrease could be the consequence of slow but significant increases in CD4 T-cell counts reported in both the IntegRal and Discor-Ral studies [26, 31]. However, raltegravir induced a significant reduction of the coagulation marker D-dimer that was exclusively observed in PI-treated individuals. D-dimer levels reflect HIV-induced perturbations of the coagulation cascade, contributing to thrombus formation and cardiovascular morbidity [42]. This observation is

consistent with data reported by Hatano *et al.* [25] and suggests a specific beneficial effect of raltegravir on PI-treated individuals regarding cardiovascular morbidities. Since no drug switch exists in our study, and the effect is not general to all intensified individuals, it seems to be the consequence of a better *in vivo* antiviral profile of raltegravir rather than a non-specific anti-inflammatory activity of this drug. Consistent with this specific effect, no changes in raltegravir-intensified patients were observed for other inflammatory markers such as IL-6, whose levels have been recently shown to predict non-AIDS-defining morbid events during suppressive HAART [43]. Finally, the reduction in D-dimer levels induced by raltegravir was more evident in individuals with high CD4 T-cell counts (IntegRal study) than in patients with CD4 T-cell counts below 350 (Discor-Ral study), although those were more likely on PI-based regimens. This apparent paradox could be related to the low level of 2-LTR circle detection [30]. Alternatively, the profound immunological abnormalities found in patients with low CD4 T-cell counts that are not reversed by raltegravir [12] [31] may sustain D-dimer levels in these patients.

In summary, our data suggest that soluble inflammatory markers show a complex relationship with several factors (age, co-infections, antiretroviral regimen and immune recovery) that may render the use of these markers to identify individuals with residual viral replication under cART poorly reliable. In fact, the detection of 2-LTR circles is poorly related to inflammation, and raltegravir intensification has only minor effects on inflammatory markers. These observations confirm the minimal clinical effect of intensification in terms of standard follow up (CD4 T-cell counts), but highlight the substantial role of cART composition in inflammation and the detection of 2-LTR circles, emphasizing the need for cART regimens with optimal effects on inflammatory markers that may reduce the risk of clinical events in HIV-1 infected individuals.

## Supporting Information

**Figure S1. Baseline plasma levels of soluble inflammatory markers.** The levels of the indicated soluble markers were assessed prior to raltegravir intensification in patients recruited in the IntegRal (n=67, blue symbols) or the Discor-Ral (n=44, red symbols) studies. Control and intensified individuals were grouped for this baseline analysis.

[doi:10.1371/journal.pone.0114142.s001](https://doi.org/10.1371/journal.pone.0114142.s001) (TIFF)

**Figure S2. Linear models for changes in plasma markers.** Data from the Discor-Ral and IntegRal studies were pooled and analyzed as described in methods (linear mixed models). Longitudinal evolution of CD4 T-cell counts and immune activation (CD38<sup>+</sup>HLA-DR<sup>+</sup> CD8 T cells) were included for reference (bottom right graphs).

[doi:10.1371/journal.pone.0114142.s002](https://doi.org/10.1371/journal.pone.0114142.s002) (TIFF)

## Acknowledgments

We thank Rafaela Ayen, Eulàlia Grau, Elisabeth Garcia from IrsiCaixa and Lidia Ruiz from Hematology Department of HUGTiP for technical assistance and sample processing. We are grateful to BLOKIT SA and IZASA SA for support in D-dimer determination. We also thank Adriana Capdevila from Col·legi Sant Miquel dels Sants (Vic, Spain) for preliminary statistical analysis. We finally thank patients and physicians for participating this study.

## Author Contributions

Conceived and designed the experiments: MS JMG JML PD JMP EN JB BC. Performed the experiments: MM MCP MJB SM EO. Analyzed the data: MM DO. Wrote the paper: MM DDR JB.

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