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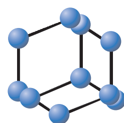

Impact of Different Antiretroviral Strategies on Total HIV-DNA Level in Virologically Suppressed HIV-1 Infected Patients

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Abstract: ***Background***: Total HIV-DNA load in peripheral blood cell (PBMCs) reflects the global viral reservoir that seems not to be affected by antiretroviral treatment. However, some studies reported a different permeability of different drugs in cellular compartments.

Objective: To investigate the relation between the amount of total HIV-1 DNA and different treatment strategies.

Methods: Total HIV-1 DNA was quantified by real time PCR in PBMCs collected from 161 patients with long-term undetectable HIV-RNA receiving different therapy schedules (3-drug regimens or 2-drug regimen containing Raltegravir as integrase inhibitor).

Results: Overall, HIV patients who started therapy with a median pre-ART CD4+ cell count ≥400 cells/mm^3 and HIV viral load of 3 log_{10} copies/ml, achieved a lower amount of HIV total DNA. No significant correlation was found in DNA size when patients were stratified on the basis of different therapeutic protocols. However, HIV DNA load analysis, when only performed in HIV patients with a median pre-ART CD4+ cell count >200 cells/mm^3 and HIV viral load < 3 log_{10} copies/ml, showed a significative DNA decrease in Raltegravir treated group with respect to the NNRTIs-treated group.

Conclusion: The data emphasize that HIV-DNA level represents a predictive factor in long-term suppressive therapy patients. In addition, the diminished reservoir, only observed in patients treated with the NRTI-sparing regimen RAL plus PI/r before immunological and virological derangement, suggests that latest generation drugs, such as integrase inhibitors, might represent an optimal chance in the management of HIV infection.

Keywords: HIV-1 DNA, HIV-RNA, CD4+ cells, antiretroviral therapy, reservoir, NRTI-sparing regimen.

1. INTRODUCTION

Despite the development of more effective antiretroviral protocols, long-lived viral reservoirs represent the main obstacle to eradicating HIV infection [1-3].

Antiretroviral therapy (ART) quickly reduces the pool of activated CD4 cells in peripheral blood and increases the CD4+ T cell count to levels closer to normalcy, but the persistence of competent viral DNA in long-lived CD4 cells seems to be extremely refractory to antiretroviral treatment and immune intervention [4].

HIV-1 DNA, a marker of viral reservoir size, can be found integrated and unintegrated (linear and circular forms) into the host genome in infected cells [3, 5, 6]. Integrated and unintegrated DNA have been extensively studied as different predictive markers of disease progression [7-10]. Independently from RNA level and CD4 counts, a high amount of total HIV-1 DNA seems to be correlated with a rapid progression to AIDS in naïve patients [11, 12] and with viral rebound after treatment interruption in patients receiving therapy [12, 13].

Reservoir dynamics during a suppressive treatment shows a significant initial decay during the first years [14, 15], probably due to the disappearance of non-integrated linear and circular forms [16], but the integrated DNA (also called "provirus") is extremely stable and persists indefinitely in the long life span of CD4 memory [3].

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A critical factor limiting reservoir replenishment remains the timing of the first therapeutical intervention [17, 18]. Whereas the kinetics of plasma viremia is obvious and well-described during an efficient ART, changes in the long-term integrated and episomal HIV-1 DNA are not so clear [19].

In this scenario, the impact of specific ART composition on reservoir size in long-term suppressed antiretroviral therapy patients has been studied to verify a possible correlation between a specific antiretroviral treatment and DNA amount [20-22].

Some therapeutical options seem to influence HIV burden size. In particular, intensification regimens with integrase inhibitors (INI) [23-26] or regimens containing a non-nucleoside reverse transcriptase inhibitor [27] or unconventional simplification regimens like dual or monotherapies with PI [28, 29] have been investigated. Even if different classes of drugs seem to be associated with lower levels of residual viremia [30], NNRTI and Raltegravir-based regimens appear more effective to contain the size of the cellular reservoir [26, 31, 32].

Since unintegrated viral DNA contributes to the total HIV DNA signal, but adds very little to the viral reservoir (due to its limited transcription potential), most attention focused on total proviral DNA [3, 33] correlated to viral rebound in HIV patients on long-term treatment.

On these basis, the quantitative determination of total HIV-l proviral DNA in peripheral blood mononuclear cells (PBMCs) can yield important information on the reservoir and dynamics of HIV infection [34-36] even in the presence of undetectable levels of HIV-RNA viral load in plasma. However, HIV-1 DNA represents a unique biomarker of viral activity, especially when the HIV RNA level drops below detectable limits [20].

PBMCs from HIV-1 patients on long-lasting stable ART have been collected with the dual aim to investigate i) the relationship between the amount of total HIV-1 DNA and immunological/virological parameters, and ii) the possible correlation between the values of HIV-1 DNA load and different antiretroviral regimens (3-drug regimens or 2-drug regimen containing an integrase inhibitor).

2. MATERIALS AND METHODS

2.1. Patients

We performed an observational, cross-sectional study evaluating cellular HIV DNA in PBMCs from HIV-1 infected patients on stable treatment in the last 4 years.

Among all the patients referred to our laboratory or Infectious diseases Unit, we only selected individuals with undetectable HIV RNA load and available PBMC sample at the end of the study and accessible data concerning age, gender, therapy, transmission route, immunological parameters.

The study was authorized by the local ethics committee (Fellowship Study, Prot 104/2013/U/OSS) and written informed consent was obtained from all patients. Our cohort was characterized by long-lasting HIV-1 infection with a median time of 15.0 years (IQR, 11.8-18.6) and a median cART duration of 8.1 years (IQR, 4.4-10.7). Intensive medical evaluation excluded a history of drug abuse and transmission was established to have occurred by sexual contact. Exclusion criteria were acute infectious diseases, inflammatory, cardiovascular or peripheral vascular diseases.

On the basis of therapeutic schedules, patients were divided into 4 groups: patients receiving abacavir/lamivudine (ABC/3TC) or emtricitabine/tenofovir diproxil (FTC/TDF) plus one non-nucleoside reverse trascriptase inhibitor [NNRTI, efavirenz (EFV) or etravirine (ETV) or Rilpivirine RPV)] (group 1) or nevirapine (NVP) (group 2); patients receiving abacavir/lamivudine (ABC/3TC) or emtricitabine/ tenofovir diproxil (FTC/TDF) plus one boosted protease inhibitor (PI/r, darunavir/ritonavir) (group 3) and patients receiving an integrase inhibitor (INI) such Raltegravir (RAL) plus darunavir/ritonavir (group 4).

2.2. CD4+ Cells Count and RNA Viral Load Determination

CD4+T lymphocytes were determined by flow cytometry (FACScan, Becton & Dickinson, Mountain View, CA, USA) using commercially available monoclonal antibodies and plasma HIV-RNA load was detected by standard commercial viral RNA detection assay (COBAS® AMPLICOR, Roche Molecular Systems, Inc., Branchburg, NJ, USA). All samples were tested for CD4+ cell counts and viral RNA at baseline and at time of observation.

2.3. DNA Proviral Load

Total HIV DNA levels were measured in duplicate in peripheral blood mononuclear cells (PBMCs) by a quantitative real time PCR assay, targeting the long terminal repeat region (Biocentric, Bandol, France). Viral and cellular DNA were extracted from PBMCs, separated by density gradient centrifugation, using a QIAamp DNA mini kit (QIAGEN, Courtaboeuf, France) to obtain 100 µl of eluate, according to the manufacturer 's instructions. The DNA concentration was quantified using fluorescence readings at 260 nm (Nanodrop, Labtech, Ringmer, UK) and the samples were eventually diluted in distilled water to reach 1µg of DNA /PCR tube (considered to be equivalent to 150,000 cells) [37]. Results were expressed as number of HIV DNA copies/10^6 PBMCs. For a given sample, an HIV-1 DNA concentration "C" is calculated as reported: C= HIV DNA copies/PCR test (mean value) x 1.000,000/150,000 (copies/10^6 cells). The quantitative amount of total DNA proviral load was only performed at our last observation.

2.4. Statistical Analysis

The continuous variables were expressed as the median [interquartile range (IQR)], and the categorical variables were expressed as percentages. The univariable linear regression model was used to examine the association between HIV-1 DNA and pre-HAART viroimmunological data (nadir CD4 cell count and plasma HIV-RNA zenith) and the HIV-1 DNA and the duration of suppressive ART.

Differences between nominal data were tested for statistical significance by non-parametric test (Mann- Whitney).

A p-value of <0.05 was considered significant.

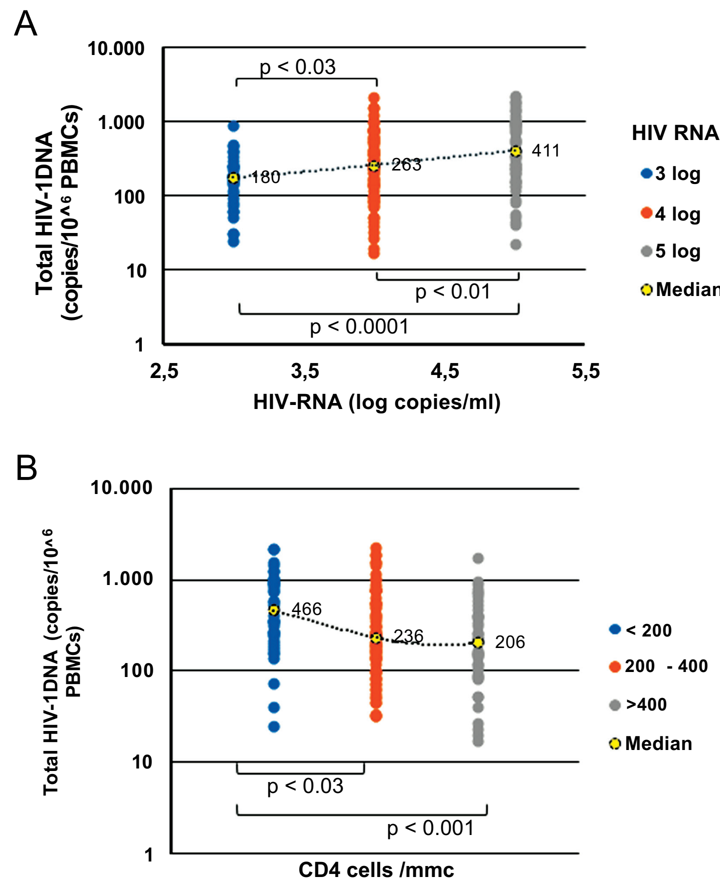

Fig. (1). Correlation between total HIV-DNA and zenith HIV-RNA (**A**) and nadir CD4+ cell count (**B**). Fig. (**1A**). 180, 263 and 411 represent the median copy numbers of HIV DNA load detected in HIV patients regardless of therapy protocols. Fig. (**1B**): 466, 236 and 206 represent the median CD4 cell count/mmc detected in HIV patients regardless of therapy protocols.

3. RESULTS

3.1. Pre-ART HIV-RNA Viral Load and CD4+ Cell Counts are Correlated with HIV DNA Levels at Time of Observation

To evaluate the relation between zenith values of HIV RNA and DNA load levels, HIV-1 patients were stratified on the basis of different pre-ART viremia levels (≤ 3 log_{10} copies/ml, 4 log_{10} copies/ml and $\geq 5log_{10}$ copies/ml of HIV-RNA).

In particular, 38 patients showed pre-ART viral load $\leq 10^3$ log_{10} copies/ml, (median 3.90, IQR, 3.45-3.95), 70 patients had 10^4 log_{10} copies/ml (median 4.50, IQR, 4.25-4.80) and 53 patients 10^5 log_{10} copies/ml (median 5.48, IQR, 5.23-5.65).

Patients who started therapy with lower RNA viral replication (≤ 3 log_{10} copies/ml) showed lower DNA amounts [180 copies/10^6 PBMCs (IQR, 95-255)] in comparison with HIV patients who entered therapy with a higher level of viral replication (4 log_{10} copies/ml and $\geq 5log_{10}$ copies/ml) who reached median DNA levels of 263 copies/10^6 PBMCs (IQR, 114-595) and 411 copies/10^6 PBMCs (IQR, 156-975) respectively [$p<0.03$ and $p<0.0001$] (Fig. **1A**).

Subjects were also stratified on the basis of nadir CD4+ cell counts. In particular, 40 patients showed pre-ART CD4+ cell counts >400 cells/mm^3 (median 476, IQR, 436-520 cells/mm^3), 79 patients had CD4+ cell counts from 200 to 400 cells/mm^3 (median 302, IQR, 263-344 cells/mm^3) and 42 patients CD4+ cell counts <200 cells/mm^3 (median 93, IQR, 61-134 cells/mm^3). Results showed that nadir CD4+ cell values are inversely related to median HIV DNA levels, showing that patients with pre-therapy lower nadir CD4+ counts achieved higher total HIV DNA amount [(466 copies/10^6 PBMCs, (IQR 243-968)] than patients with intermediate or higher CD4+ counts [236 copies/10^6 PBMCs (IQR, 116-544, $p<0.04$) and 206 copies/10^6 PBMCs (IQR, 96-407, $p<0.001$), respectively] (Fig. **1B**).

3.2. Total HIV-1 DNA Level is Associated with Different Therapeutic Protocols

HIV-1 patients were further divided on the basis of specific regimens: *group 1*) 39 patients receiving an NRTI backbone (ABC/3TC or FTC/TDF) plus an NNRTI (EFV or ETV or RPV); *group 2)* 42 patients receiving an NRTI backbone (ABC/3TC or FTC/TDF) plus nevirapine (NNRTIs); *group 3*) 41 patients receiving an NRTI backbone (ABC/3TC or FTC/TDF) plus darunavir/ritonavir (PI/r), and *group 4)* 39 patients receiving a dual therapy of Raltegravir (INI) plus darunavir/ritonavir (Table **1**). No significant differences in median levels of total HIV DNA were observed in these groups (Fig. **2**), even if lower DNA values were reached in patients belonging to groups 2 and 4 (NRTI backbone plus nevirapine and Raltegravir plus darunavir/ritonavir, respectively).

Table 1. Patients' characteristics based on antiretroviral therapy regimens administered in the last four years.

		Therapy		
	Group 1 Backbone plus NNRTI (EFV or ETV or RPV)	**Group 2 Backbone plus NNRTI (NVP)**	**Group 3 Backbone plus PI/r (DRV/r)**	**Group 4 INI (RAL) plus PI/r (DRV/r)**
N° of patients	39	42	41	39
Age	41 (IQR, 38-48)	49 (IQR, 42-55)	45 (IQR, 42-51)	48 (IQR, 42-55)
Male, no (%)	87	76	92	79
Risk group, no (%): Homo/bisexual	62	60	56	52
Risk group, no (%): Heterosexual	34	38	37	43
Risk group, no (%): Drug user	4	2	7	5
Duration of current cART (years)	4.4 (IQR, 3.9-4.8)	4.5 (IQR, 4.1-5.2)	4.2 (IQR, 3.6-5.5)	4.3 (IQR, 3.6-5.1)
Zenith HIV RNA (log copies/ml)	4.96 (IQR, 4.58-5.28)	4.80 (IQR, 4.30-5.38)	4.45 (IQR, 4.00-4.95)	4.00 (IQR, 4.00-5.08)
Current CD4 cell count (cells/mm^3)	706 (IQR, 589-925)	660 (IQR, 521-849)	776 (IQR, 577-943)	789 (IQR, 589-1045)
Nadir CD4 cell count (cells/mm^3)	319 (IQR, 257-400)	296 (IQR, 160-240)	290 (IQR, 176-396)	284 (IQR, 71-390)

Data are median (IQR, interquartile range) and values are expressed as n (%); cART, combination antiretroviral therapy; backbone: abacavir/lamivudine (ABC/3TC) or emtricitabine/tenofovir diproxil (FTC/TDF); NNRTI: non-nucleoside reverse transcriptase inhibitors; EFV: Efavirenz; ETV: Etravirine; RPV: rilpivirine; NVP: Nevirapine; DRV/r: darunavir/ritonavir; INI: integrase inhibitor; RAL: Raltegravir.

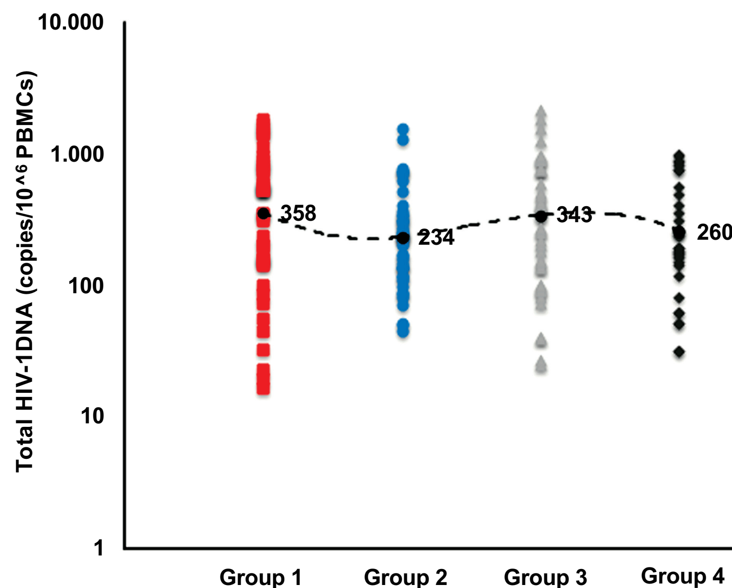

Fig. (2). Median levels of total HIV-DNA in 161 patients on stable ART stratified on the basis of therapy protocols. 358, 234, 343 and 260 represent the median copy numbers of HIV DNA load detected in HIV patients undergoing different therapy protocols. From left to right: patients receiving abacavir/lamivudine (ABC/3TC) or emtricitabine/tenofovir diproxil (FTC/TDF) plus one non-nucleoside reverse trascriptase inhibitor (NNRTI, efavirenz or etravirine or rilpivirine) (group I) or nevirapine (group II); patients receiving abacavir/lamivudine (ABC/3TC) or emtricitabine/tenofovir diproxil (FTC/TDF) plus one boosted protease inhibitor (PI/r, darunavir/ritonavir) (group III), and patients receiving an integrase inhibitor (INI) such Raltegravir (RAL) plus darunavir/ritonavir (group IV).

In particular, the median values of DNA load at the time of observation were 358 (IQR, 148-800), 234 (IQR, 123-344), 343 (IQR, 135-767) and 260 (IQR, 159-787) copies/10^6 PBMCs in groups 1, 2, 3 and 4 respectively, suggesting that only two antiretroviral regimens analyzed seem to have a moderate impact on achieving low level proviral cellular DNA.

Finally, to investigate whether different levels of viral replication and/or CD4+ cell counts could predict therapy success (in terms of smaller reservoir size), we restricted the analysis to HIV-1 patients with CD4+ values > 200 cells/mm^3 divided on the basis of basal viremia levels (3 $\log_{10}$, 4 $\log_{10}$ and 5 $\log_{10}$ HIV-RNA). Results (Fig. **3A**) showed that the lower levels of proviral load were only

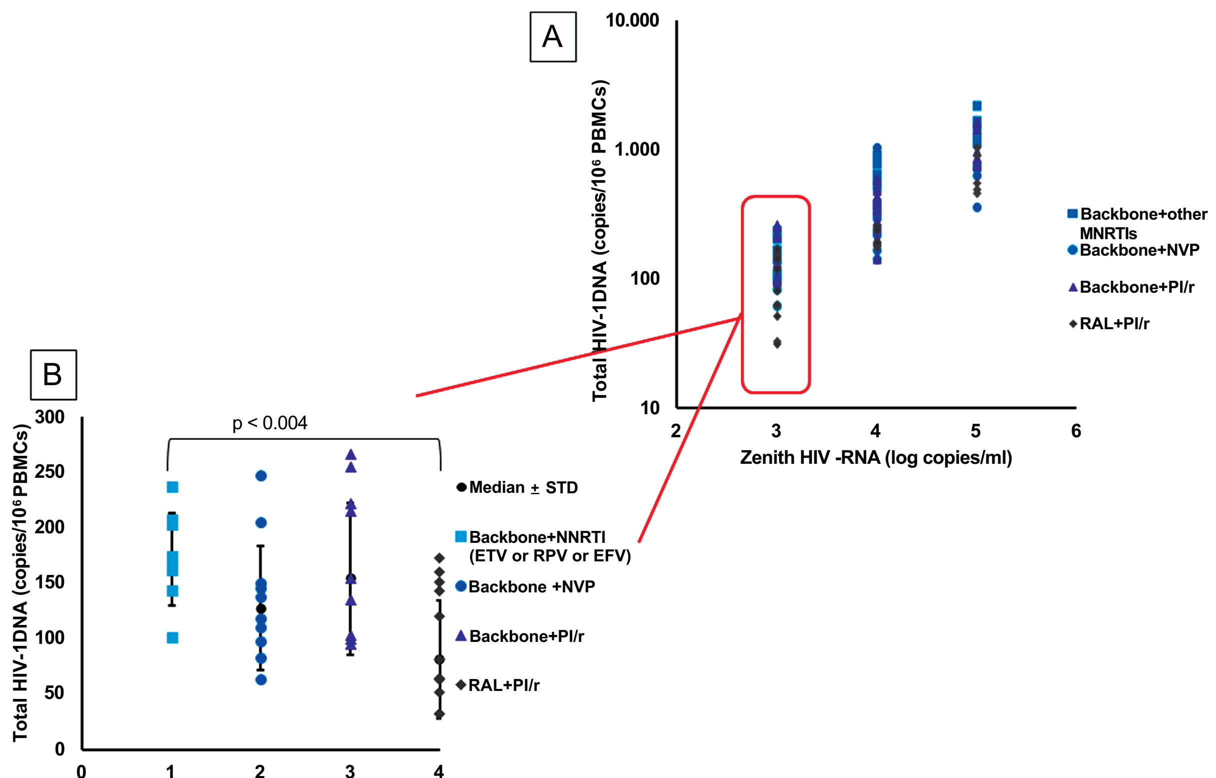

Fig. (3). (**A**). Total HIV-DNA amount in HIV patients treated with different antiretroviral therapy and stratified on zenith HIV-RNA (3 log_{10}, 4 log_{10} and 5 log_{10} HIV-RNA) and CD4+ >200 cells/mm^3. (**B**). Total HIV-DNA amount in HIV patients treated with different antiretroviral therapy protocols, selected on restricted parameters (T CD4 cells > 200 cells/mm^3 and HIV-RNA level <3 log_{10}). Backbone: abacavir/lamivudine (ABC/3TC) or emtricitabine/tenofovir diproxil (FTC/TDF); NNRTI:non-nucleoside reverse transcriptase inhibitors (EFV: Efavirenz; ETV: Etravirine; RPV: Rilpivirine; NVP: nevirapine); PI/r: protease inhibitors (darunavir/ritonavir); RAL: Raltegravir

obtained in subjects who started therapy with CD4+> 200 cells /mm^3 and HIV-RNA viral load <3 Log.

Taking this concomitant condition (T CD4+ > 200 cells/mm^3 and HIV-RNA level <3 log_{10}) as the reference baseline and focusing attention on the amount of HIV DNA in patients treated with specific therapeutical protocols, we did not observe a strong statistical association among the 4 regimens and proviral amount. Neverthelss, HIV DNA load analysis, when only performed in HIV patients with a median pre-ART CD4+ cell count >200 cells/mm^3 and HIV viral load < 3 log_{10} copies/ml, showed a significative DNA decrease in Raltegravir treated group respect to the NNRTIs-treated group (95 vs. 174 copies/10^6PBMCs, $p<0.004$) (Fig. **3B)**.

4. DISCUSSION

HIV persistence in cellular and anatomic reservoirs is a major obstacle to HIV-1 infection eradication. Current antiretroviral therapy successfully suppresses replication in plasma and reduces viral transmission, but is not able to definitively flush out the “hidden virus” established in the first phase of infection [38].

Since the measurement of HIV DNA level, a surrogate marker able to predict viral rebound and disease progression [12, 13], might shed more light on the meaning and dynamics of cellular reservoir [39, 40] our first analysis focused on all patients, regardless of specific therapy. Results demonstrated the close association between HIV-1 DNA and the pre-therapy immune-virological markers, confirming several data on this topic [12, 22]. The HIV-DNA amount is negatively associated with the number of nadir CD4+ cells and directly correlated with the pre-therapy viral load, further confirming that patients who underwent therapy before immune system derangement were more likely to reach more contained proviral DNA levels.

Although available data on the correlation between proviral HIV DNA and different antiretroviral regimens warrant further investigation, much recent research interest has focused on understanding the best therapeutic combinations with better penetration of sanctuaries and lower long-term side-effects in patients on stable virological control [21, 23-29, 39].

HIV DNA is already present after the onset of infection and persists over time, representing a virtually endless reservoir, ready to produce new virus. Hence our study also undertook a quantitative analysis of cellular proviral HIV DNA in patients on stable combination antiretroviral therapy for over 4 years, including four different antiretroviral regimens containing a classic triplice cocktail [two NRTI plus one NNRTI [Efavirenz or rilpivirine or etravirine (group1) or nevirapine (group 2)], two NRTI plus a protease inhibitor with booster of ritonavir (darunavir/r) (group 3)] or a regimen containing two drugs [INI (Raltegravir) plus PI (darunavir/ritonavir) (group 4).

To this aim, we only selected HIV patients on stable treatment with undetectable levels of circulating virus in the last four years with available data concerning age, gender, therapy, transmission route, immunological parameters and PBMC sample available for HIV proviral quantification.

In our experimental conditions, HIV-DNA amount was not significantly altered in PBMCs from patients stratified on the basis of different therapeutic protocols, albeit a slightly smaller reservoir has been documented in nevirapine-treated patients (NNRTI), showing a better penetration score in different anatomical districts [30, 32] and in Raltegravir-treated patients, affecting the viral integration step [41] indicating a partial but not definitive role of nevirapine or Raltegravir regimens in reducing the viral reservoir [17, 26, 31, 42].

In any case, the slight, but not drastic, decrease in PBMC DNA in patients on therapies containing an anti-integrase drug might be due to several reasons, such as the persistence of ongoing replication leading to an transient increase in unintegrated forms (2-LTR) of HIV-1 DNA or the migration of cells with unintegrated DNA from other tissue reservoirs to the peripheral blood [26, 31].

Recently, a decrease in DNA load was demonstrated in HIV patients only after the initiation of the RAL-containing regimen, but not maintained over time, suggesting that most HIV-1 DNA is integrated in the majority of well virologically controlled patients [26, 43-46]. Moreover, we found that the concomitant presence of an HIV RNA value < 3 log and CD4+ cell count nadir > 200 cells/mm^3 at baseline could offer optimistic information on the effectiveness of specific treatment to contain reservoir size. In this connection, the lower HIV-DNA load observed in the RAL-treated group, underlying that a dual therapy containing Raltegravir could have a good efficacy to control the DNA amount in PBMCs and could represent an interesting alternative simplification strategy, especially in patients with comorbidity, due to the good long-term tolerability of Raltegravir and high genetic barrier of protease inhibitors [47].

Our study has some limitations such as: i) the evaluation of HIV-DNA using a PCR-based assay is not able to distinguish replication-competent viruses from viral defective forms, ii) the detection of DNA in PBMCs, representing only a small part of viral reservoir, iii) the lack of analysis of chronic inflammation markers (PCR, D-dimer, Il-6, TNF), iiii) the lack of randomization in the enrollment of patients.

CONCLUSION

In this cohort of virologically suppressed HIV-infected subjects, the amount of HIV DNA was comparable in patients receiving different type of NRTI-based cART including non-nucleoside analogues and PI/r; however, in patients who started therapy with T CD4+ > 200 cells/mm^3 and HIV-RNA level <3 log_{10}, total HIV-DNA was significantly lower Raltegravir plus PI/r treated group respect to NNRTI-group. These data open the way to exploring optimization models of therapy, indicating a potential role of Raltegravir, or other integrase inhibitors, in reducing the size of viral reservoir when switching from a suppressive successful therapy. So, much larger studies will be needed to carefully select patients for NRTI-sparing regimens, to avoid a potentially "reseeding" of reservoir in well-controlled patients.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the local ethics committee [Comitato Etico indipendente dell'Azienda Ospedaliera Universitaria di Bologna, Italia, (Fellowship Study, Prot 104/2013/U/OSS)].

HUMAN AND ANIMAL RIGHTS

No animal were used in this research. All humans research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/) principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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AUTHOR CONTRIBUTION

Isabella Bon acquired, analyzed the data and drafted the manuscript.

Serena Longo, Giuseppina Musumeci and Davide Gibellini contributed to the analysis of results obtained.

Alessia Bertoldi and Vanessa D'Urbano collected and processed plasma samples.

Leonardo Calza and Eleonora Magistrelli attended the patients and helped in data interpretation.

Pier Luigi Viale contributed to the study design.

Maria Carla Re contributed to the study design, supervised the project and wrote the manuscript.

All authors contributed to the revision of the manuscript and approved the final version.

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