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Quantification of HIV-DNA and residual viremia in patients starting ART by droplet digital PCR: Their dynamic decay and correlations with immunological parameters and virological success

Claudia Alteri[a,*,1], Rossana Scutari[b,1], Christof Stingone[c], Gaetano Maffongelli[c], Marta Brugneti[b], Francesca Falasca[d], Salvatore Martini[e], Ada Bertoli[b], Ombretta Turriziani[d], Loredana Sarmati[c], Nicola Coppola[e], Massimo Andreoni[c], Maria Mercedes Santoro[b], Carlo-Federico Perno[a], Francesca Ceccherini-Silberstein[b,1], Valentina Svicher[b,1]

[a] Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy
[b] Department of Experimental Medicine, University of Rome Tor Vergata, Rome, Italy
[c] Infectious Diseases Unit, University Hospital of Rome Tor Vergata, Rome, Italy
[d] Department of Molecular Medicine, University of Rome La Sapienza, Rome, Italy
[e] Infectious Diseases Unit, Second University of Naples, Naples, Italy

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ABSTRACT

Background: Accurate quantification of total HIV-DNA and residual-viremia by sensitive assays is extremely useful to optimize monitoring of ART-treated patients.
Objectives: To evaluate the performances of two ddPCR-based assays for HIV-DNA and residual-viremia quantification, and the correlations of pre-ART HIV-DNA with plasma HIV-RNA, CD4 + T, CD4/CD8 and virological-success (VS) during first-line ART.
Study design: Plasma HIV-RNA, total HIV-DNA, CD4 + T, CD4/CD8 were evaluated at baseline of ART, at VS (viral-load < 50copies/ml), and at 6 months after VS (6moVS) in 57 newly-diagnosed HIV-1 infected patients, receiving first-line modern ART. HIV-DNA (log10 copies/106CD4 + T) and residual-viremia (copies/ml) were measured with in-house ddPCR assays. Correlations were assessed by Spearman and Jonckheere-Terpstra tests.
Results: HIV-DNA and residual-viremia assays showed a good linear trend between the expected and obtained values (R2 = 0.9913 and 0.9945); lower limits of detection were 32 copies/106CD4 + T and 2 copies/ml, respectively. At baseline, median (IQR) plasma HIV-RNA and HIV-DNA were 4.88(4.28–5.36)log10 copies/ml and 4.00(3.36–4.51) log10 copies/106CD4 + T cells. Residual-viremia was 8(2–26) and 4(2–12) copies/ml at VS and 6moVS. Pre-ART HIV-DNA positively correlated with plasma HIV-RNA at BL (Rho = 0.708, $p < 0.001$), and with residual-viremia at VS (Rho:0.383,p = 0.002). Notably, higher HIV-DNA correlated with longer time to achieve VS (median[IQR],weeks: 17.8[12.3–29.0] for HIV-DNA ≥4.5 vs. 7.4[4.1–8.7] for HIV-DNA < 4.5, $p < 0.001$). Furthermore, pre-ART HIV-DNA negatively correlated with CD4 + T and CD4/CD8 at baseline, VS and 6moVS.
Conclusions: Our results support the adoption of ddPCR-based assays for both HIV-DNA and residual-viremia quantifications and corroborate that pre-ART HIV-DNA is an excellent indicator in predicting viroimmunological response and VS in patients starting ART.

1. Background

Human-immunodeficiency virus type 1 (HIV-1) can persist in different cellular reservoirs, despite antiretroviral treatment. Several markers have been proposed to measure the viral reservoir, and among them, total HIV-DNA was considered a good candidate [1].

The clinical relevance of total HIV-DNA has been described both in treated and un-treated patients and for this reason this parameter was defined as a useful and interesting marker in different clinical setting and at different stage of HIV infection [2]. Total HIV-DNA level is

* Corresponding author at: Department of Oncology and Hemato-oncology, University of Milan, 20122, Milan, Italy.
E-mail address: claudia.alteri@unimi.it (C. Alteri).
[1] These authors contributed equally to this work.

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crucial for disease progression since the first time of infection [3,4], and, as shown by few studies, its pre-ART level can predict long-term virological success (VS) in patients starting their first-line ART [5–8].

A close relationship between HIV-DNA and residual viremia has been also reported [9–12]. Even if the full clinical implications of this finding remain unclear, the persistence of residual viremia has been associated with a higher risk of subsequent virological failure in several observational studies [13–17].

2. Objectives

In this study, total HIV-DNA and residual viremia were measured by two *ad hoc* designed assays based on droplet digital PCR (ddPCR). ddPCR is an emerging technique which exhibits improved sensitivity and accuracy to detect low DNA and RNA concentrations compared with conventional quantitative real-time PCR (qPCR) [18]. We used these assays to assess correlations of pre-ART HIV-DNA with plasma HIV-RNA, $CD4^+$T cells and CD4/CD8 ratio at baseline and follow-up as well as with response to first-line ART, in drug naïve patients starting a first line modern ART.

3. Study design

3.1. Study population and design

This longitudinal prospective study includes 57 newly diagnosed HIV-1 infected patients enrolled in three infectious disease units in Italy. All patients started a first line modern ART and were followed until 6 months after VS (defined as 2 consecutive plasma HIV-RNA < 50 copies/ml, measured by COBAS AmpliPrep/COBAS TaqMan HIV-1 v2.0 Test and cobas® 6800 PCR; Roche Molecular System, Inc). Patients that decided to interrupt ART immediately after its initiation were excluded from the study.

For each patient, demographic and clinical information such as age, race, gender, HIV-1 subtype, antiretroviral treatment, plasma HIV-RNA, $CD4^+$T and $CD8^+$T cell count were retrieved and stored in an anonymous database *ad hoc* built for the study.

Peripheral blood mononuclear cells (PBMCs) aliquots, whole blood and plasma samples were collected before the beginning of treatment (baseline), at VS, and at 6moVS. Patients with samples available for total HIV-1 DNA and residual viremia quantification were 57 at baseline and VS and 41 at 6 months after VS (6moVS).

3.2. Ethical committee

All patients signed informed consent forms to participate in this study, for the collection and storage of biological samples and for the anonymous use of their data in clinical research (n. of study protocol: 124/15, prot. 23526/2015).

3.3. Total HIV-DNA quantification

Total HIV-DNA was quantified at baseline, at VS and at 6moVS from a pellet of $\geq 3 \times 10^6$ PBMCs (range of 3×10^6 PBMCs to 6×10^6 PBMCs) by means of the QX200™ Droplet Digital™ PCR System (ddPCR, Biorad) using home-made protocol [19]. Total HIV-DNA (copies/10^6 PBMCs) was then normalized into number copies/10^6 $CD4^+$T cells [20]. Detailed methodology was reported in Supplementary text.

3.4. Residual plasma viremia quantification

Residual viremia was quantified at the VS and at 6moVS in all patients with a plasma HIV-1 viral load < 20 copies/ml, using a home-made protocol. Detailed methodology was reported in Supplementary text.

3.5. Statistical analysis

To verify the correct performance of the HIV-DNA and residual viremia quantification methods, independent experiments using serial dilutions of quantitation standards were performed. Detailed methodology was reported in Supplementary text. Coefficient of determination (R^2) of HIV-DNA and residual viremia quantification methods was assessed by linear regression analysis by plotting the standards' measured copies and comparing them with expected values of serial dilutions. The limit of detection (LOD), defined as the lowest concentration at which 95% of positive samples were detected, was determined by probit regression analysis. Reproducibility of HIV-DNA and residual viremia quantification methods was assessed by intra- and inter-run tests using serial 8e5 and NL4.3 dilutions. The coefficient of variation (CV) was calculated as the standard deviation (SD) of HIV-DNA and HIV-RNA copies/reaction divided by replicates mean.

Patients were stratified in three categories according to the pre-ART total HIV-DNA levels: < 3.5, 3.5–4.5, and ≥4.5 $\log_{10}$ copies/10^6 $CD4^+$T cells.

Spearman correlation and Jonckheere-Terpstra test were used to correlate pre-ART HIV-DNA with i) HIV-DNA decay from baseline to VS and to 6moVS, ii) viral load, $CD4^+$T and CD4/CD8 at baseline, VS and at 6moVS.

Categorical variables were indicated as the absolute number and frequency (%), whereas quantitative variables were expressed as the median and interquartile range (IQR).

All statistical analyses were performed using MedCalc software, version 16.0. (MedCalc, Ostend, Belgium).

4. Results

4.1. Patients' characteristics

The demographic characteristics of the 57 patients are shown in Table 1.

Median year of diagnosis was 2016 (IQR: 2016-2017). Most patients were males (82.5%) with a median age of 37 years (IQR:30-49), and Italians (79.0%). Homosexual and heterosexual contacts were the prevalent infection routes (45.6% and 26.3%, respectively). Most individuals were infected by HIV-1 B subtype (54.4%). The patients enrolled in this study started an antiretroviral treatment after a median (IQR) time of 7.5 (4.5–22.1) weeks from diagnosis. Most patients (77.2%) started a combined antiretroviral regimen including an Integrase Strand Transfer Inhibitor (INSTI) and two Nucleoside Reverse Transcriptase Inhibitors (NRTIs). VS was achieved by all 57 individuals after a median (IQR) time of 7.9 (4.6–14.4) weeks.

4.2. Performance of the assays

Both HIV-DNA and residual viremia assays showed a good linear correlation between expected and observed values (R^2 = 0.9913 and 0.9898, Panel A of Supplementary Figs. 1 and 2). Probit analyses predicted a limit of detection (LOD) of 32 (95% confidence interval: 14–127) copies/$10^6$$CD4^+$T cells for HIV-DNA, and 2 (95% confidence interval: 1–26) copies/ml for residual viremia. Detailed description of the results obtained was reported in Supplementary text.

4.3. Total HIV-DNA

The median HIV-DNA concentration prior to ART was 4.00 (3.36–4.51) $\log_{10}$ copies/10^6 $CD4^+$T cells. By stratifying for pre-ART HIV-DNA, 18 (31.6%) patients had a < 3.5 $\log_{10}$ copies/10^6 $CD4^+$T cells pre-ART HIV-DNA, 25 (43.9%) had a pre-ART HIV-DNA between 3.5 and 4.5 $\log_{10}$, and 14 (24.5%) had a ≥4.5 $\log_{10}$ pre-ART HIV-DNA.

Total HIV-DNA was lower at all subsequent time points with median (IQR) values of 3.23 (2.85–3.73), and 2.90 (2.50–3.5) $\log_{10}$ copies/10^6

Table 1
Patients'characteristics.

Number of patients	57
Male, N (%)	47 (82.5)
Age at HIV-1 diagnosis, median (IQR)	37 (30–49)
Year of diagnosis, median (IQR)	2016 (2016–2017)
Recent infection[a], N (%)	6 (15.8)
Origin, N (%):	
Italy	45 (79.0)
Other	10 (17.5)
Unknown	2 (3.5)
Risk factor, N (%):	
Homosexual	26 (45.6)
Heterosexual	15 (26.3)
Other	6 (10.5)
Unknown	10 (17.6)
Subtype, N (%):	
B	31 (54.4)
CRF02_AG	7 (12.3)
Other (C, F1, G, BF, BC)	19 (33.3)
Time between HIV-1 diagnosis and cART, weeks median (IQR)	7.5 (4.5–22.1)
cART, N (%):	
NRTI + INSTI	44 (77.2)
NRTI + NNRTI	7 (12.2)
NRTI + PI	3 (5.3)
Other	3 (5.3)
At baseline (BL):	
HIV-DNA, log_{10} copies/10^6 $CD4^+$T cells median (IQR)	4.00 (3.36–4.51)
Plasma HIV-RNA, log_{10} copies/ml median (IQR)	4.88 (4.28–5.36)
$CD4^+$T cells count, cells/mm^3 median (IQR)	375 (227–520)
$CD8^+$T cells count, cells/mm^3 median (IQR)	901 (608–1225)
CD4/CD8, median (IQR)	0.38 (0.24–0.53)
At virological success (VS):	
Time between cART and VS, weeks median (IQR)	7.9 (4.6–14.4)
HIV-DNA, log_{10} copies/10^6 $CD4^+$T cells median (IQR)	3.23 (2.85–3.73)
Plasma HIV-RNA, copies/ml median (IQR)	8 (2–26)
$CD4^+$T cells count, cells/mm^3 median (IQR)	524 (329–728)
$CD8^+$T cells count, cells/mm^3 median (IQR)	973 (761–1329)
CD4/CD8, median (IQR)	0.48 (0.35–0.74)
Delta HIV-DNA between VS and BL, log_{10} copies/10^6 $CD4^+$ cells median (IQR)	−0.71 (−1.31; −0.18)
At 6 months after virological success (6moVS):	
HIV-DNA, log copies/10^6 $CD4^+$ cells median (IQR)	2.90 (2.50–3.54)
Plasma HIV-RNA, copies/ml median (IQR)	4 (2–14)
$CD4^+$T cells count, cells/mm^3 median (IQR)	609 (478–825)
$CD8^+$T cells count, cells/mm^3 median (IQR)	850 (673–1085)
CD4/CD8, median (IQR)	0.75 (0.51–1.00)
Delta HIV-DNA between 6moVS and BL, log_{10} copies/10^6 $CD4^+$T cells median (IQR)	−1.02 (−1.36; −0.52)
Delta HIV-DNA between 6moVS and VS, log_{10} copies/10^6 $CD4^+$T cells median (IQR)	−0.40 (−0.76; −0.006)

[a] Information regarding chronic or recent infection was available for 38 patients. cART: Combination Antiretroviral Therapy; INSTI:Integrase Strand Transfer Inhibitor; IQR: Interquartile-range; NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor; NRTI: Nucleoside Reverse Transcriptase Inhibitor; PI: Protease Inhibitor.

$CD4^+$T cells at VS and 6moVS, respectively (Table 1). Total HIV-1 DNA levels decayed rapidly from baseline to VS (median [IQR] delta-HIV-DNA: -0.71[−1.31;−0.18] log_{10} copies/10^6 $CD4^+$T cells), mostly in patients with pre-ART HIV-DNA ≥4.5 log_{10} copies/10^6 $CD4^+$T cells respect to patients with 3.5–4.5 and < 3.5 log_{10} copies/10^6 $CD4^+$T cells pre-HIV-DNA (delta-HIV-DNA: −1.20[−1.58;0.84] vs. -0.71[−1.37;−0.46] vs. −0.06[-0.48;0.53], respectively, P < 0.001) (Fig. 1). A slower decay was observed from VS to 6 months after (−0.40[−0.76; −0.006]). This decay rate did not substantially change after stratifying for pre-ART HIV-DNA (P = 0.961).

4.4. Plasma HIV-RNA and residual viremia

At baseline, median (IQR) plasma viral load was 4.88 (4.28–5.36) log_{10} copies/ml. Plasma HIV-RNA was detectable in 48/57 patients at VS (median [IQR]: 8 [2–26] copies/ml), and in 33/41 patients at 6moVS (median [IQR]: 4 [2–14] copies/ml).

Among the 98 plasma samples available from VS to 6moVS, 34 had a quantifiable HIV-RNA by standard assays (HIV-RNA ranging from 20 to 49 copies/ml), while 64 had an undetectable or < 20 copies/ml HIV-RNA by standard assays. Among these 64 plasma samples, 48 (75.0%) had HIV-1 RNA detected by ddPCR. In particular, 45 (93.7%) samples had residual viremia ranging from 2 to 18 copies/ml, while the remaining three (6.7%) had a residual viremia of 25, 40 and 42 copies/ml, respectively.

4.5. Impact of pre-ART HIV-DNA on HIV-RNA, $CD4^+$T cell count and CD4/CD8 ratio at baseline and during cART

At baseline: Total HIV-DNA prior to ART was strongly correlated with plasma HIV-RNA, $CD4^+$T cells and CD4/CD8 at baseline. In particular, plasma HIV-RNA significantly increased in relation to higher pre-ART HIV-DNA levels (Rho = 0.708, P < 0.001; 4.27 [4.17–4.62] vs. 4.90 [4.62–5.18] vs. 5.47 [5.12–5.88] log_{10} copies/ml in patients

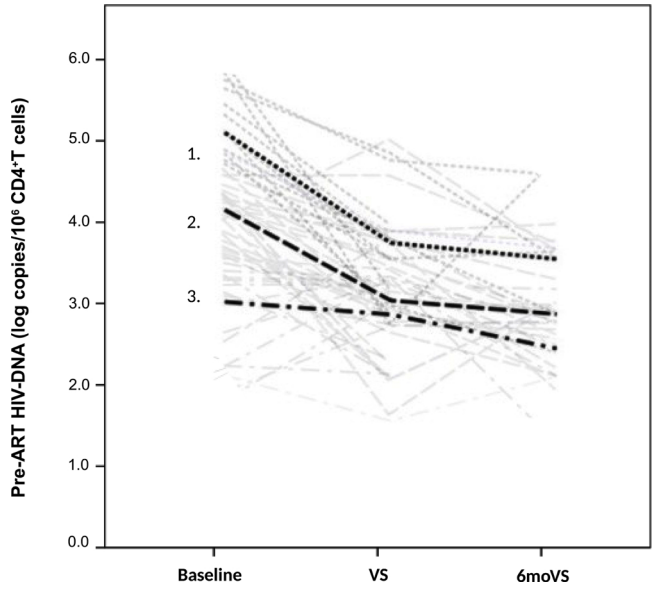

Fig. 1. HIV-DNA decay from baseline to virological success and to 6 months after virological success according to pre-ART HIV-DNA levels. Line 1: median HIV-DNA slope in patients with pre-ART HIV-DNA ≥4.5 log_{10} copies/10^6CD4$^+$T; Line 2: median HIV-DNA slope in patients with pre-ART HIV-DNA 3.5–4.5 log_{10} copies/10^6CD4$^+$T; Line 3: median HIV-DNA slope in patients with pre-ART HIV-DNA < 3.5 log_{10} copies/10^6CD4$^+$T.
Abbreviations: VSvirological success; 6moVS6 months after virological success.

with pre-ART HIV-DNA < 3.5, 3.5–4.5, and ≥4.5 log_{10} copies/10^6 CD4$^+$T cells, respectively, Jonckheere-Terpstra P < 0.001, Fig. 2, Panel A).

Conversely, CD4$^+$T cells significantly decreased in relation to higher pre-ART HIV-DNA levels (Rho = −0.395, P = 0.001; 499 [229–692] vs. 364 [257–457] vs. 289 [62–441], Jonckheere-Terpstra P = 0.017, Fig. 2, Panel B). Accordingly, CD4/CD8 significantly decreased in relation to higher pre-ART HIV-DNA levels (Rho = -0.465, P < 0.001; 0.51 [0.36–0.56] vs. 0.38 [0.29–0.50] vs. 0.20 [0.10–0.45], respectively, Jonckheere-Terpstra P = 0.003, Fig. 2, Panel C).

At virological success: Total HIV-DNA prior to ART was positively correlated with time for VS. In particular, the median (IQR) time to reach VS was 7.7 (4.7–9.0) weeks and 6.7 (4.1–8.4) weeks in patients with pre-ART HIV-DNA < 3.5 and with pre-ART HIV-DNA 3.5–4.5, and sharply increased to 17.8 (12.3–29.0) weeks in patients with pre-ART HIV-DNA ≥4.5 log_{10} copies/10^6 CD4$^+$T cells (Jonckheere-Terpstra P = 0.009).

Moreover, pre-ART HIV-DNA was confirmed to be strongly correlated with residual viremia, CD4$^+$T and CD4/CD8 at VS (Fig. 2, Panels D–F). In detail, a positive correlation was observed between pre-ART HIV-DNA and residual viremia at VS (Rho = 0.361, Spearman P = 0.003, Fig. 2, Panel D), as confirmed by the increase of residual viremia from 2 (0–8), to 6 (2–25) and to 26 (21–39) copies/ml in patients with pre-ART HIV-DNA < 3.5, 3.5–4.5, and ≥4.5 log_{10} copies/10^6 CD4$^+$T cells, respectively (Jonckheere-Terpstra P < 0.001, Fig. 2, Panel D). Conversely, a negative correlation was observed between pre-ART HIV-DNA and CD4$^+$T cells and CD4/CD8 at VS (Rho = -0.298 and -0.234, Spearman P = 0.012 and 0.040, Fig. 2, Panels E and F). Accordingly, CD4$^+$T cells at VS tended to decrease in relation to higher pre-ART HIV-DNA (625 [278–966] vs. 489 [441–631] vs. 435 [184–670], in patients with pre-ART HIV-DNA < 3.5, 3.5–4.5, and ≥4.5 log_{10} copies/10^6 CD4$^+$T cells, respectively, Jonckheere-Terpstra P = 0.084, Fig. 2, Panel E). Of note, CD4$^+$T cells showed a trend of negative correlation with residual viremia at VS (Rho = -0.198, Spearman P = 0.071).

At 6 months after virological success: Pre-ART HIV-DNA concentrations was confirmed to be correlated with CD4$^+$T cell count and CD4/CD8 also at 6moVS (Fig. 2, Panels H and I). In particular, a negative correlation was observed between pre-ART HIV-DNA and CD4$^+$T cells and CD4/CD8 at 6moVS (Rho = −0.409 and −0.341, Spearman P = 0.004 and 0.015, Fig. 2, Panels H and I). Accordingly, CD4$^+$T cells and CD4/CD8 at 6moVS tended to decrease in relation to higher pre-ART HIV-DNA CD4$^+$T cells (727 [539–925] vs. 629 [473–788] vs. 530 [434–596], and 0.78 [0.68–1.40] vs. 0.73 [0.48–0.84] vs. 0.53 [0.29–0.91] in patients with pre-ART HIV-DNA < 3.5, 3.5–4.5, and ≥4.5 log_{10} copies/10^6 CD4$^+$T cells, respectively, Jonckheere-Terpstra P = 0.080 and 0.033, Fig. 2, Panels H and I). Again, CD4$^+$T cells were significantly lower in patients with higher residual viremia at 6moVS (Rho = -0.262, Spearman P = 0.049).

The correlation between pre-ART HIV-DNA and residual viremia observed at VS was not confirmed at 6moVS (Rho = 0.119, Spearman P = 0.229, Jonckheere-Terpstra P = 0.539, Fig. 2, Panel G), although residual viremia was slightly higher in patients with pre-ART HIV-DNA ≥4.5 log_{10} copies/10^6 CD4$^+$T cells respect to patients with pre-ART HIV-DNA < 3.5 and 3.5–4.5 (6 [2–14] copies/ml vs. 4 [2–12] vs. 3 [0–12], respectively). This result can be affected by the limited sample availability at this later time point.

5. Discussion

With this study, we confirm the existence of strong correlations among total HIV-DNA, plasma HIV-RNA, CD4$^+$T cells and CD4/CD8 ratio during ART, thanks to the application of ddPCR-based assays for total HIV-DNA and residual viremia quantification.

Molecular methods characterized by high sensitivity and reproducibility would be crucial for quantifying with accuracy these parameters and consequently assessing the success of antiretroviral therapy and define HIV-1 burden modifications. In this study, we showed that both total HIV-DNA and residual viremia ddPCR-based assays have a good accuracy, reproducibility and sensitivity, as demonstrated by testing a wide range of HIV-DNA and HIV-RNA levels both from standards and patients.

The ddPCR-based assays described in our study showed that HIV-DNA and residual viremia could be accurately quantified above the 32 copies/10^6CD4$^+$T cells and 2 copies/ml, respectively, highlighting the great sensitivity of the methods. The high sensitivity of the residual viremia assay was obtained by testing the entire amount of RNA extract (in quadruplicate). In the setting of very low HIV-RNA copy number, this approach has allowed to reduce false negative results. Indeed, in 31 out of 64 samples, not all the four PCR reactions detected positive HIV-RNA droplets, presumably due to a not homogeneous distribution of HIV-1 RNA through the extract.

Most importantly, the increasing number of non-B subtypes in European countries [21,22] required the adoption of assays appropriate for use in non-B subtypes. When we evaluated the performance of HIV-DNA quantification assay in samples from patients infected by non-B subtypes present in our dataset (N = 26), we found that the median (IQR) value of HIV-DNA quantified at baseline, at VS and at 6moVS was superimposable to that observed in samples from patients infected by B subtype (at baseline: 4.00 [3.32–4.70] vs. 4.00 [3.34–4.26], P = 0.642; at VS: 3.17 [2.86–3.79] vs. 3.28 [2.50–3.72], P = 0.761; at 6moVS: 2.98 [2.46–3.84] vs. 2.89 [2.47–3.16], P = 0.723). Similar results were obtained when non-B subtypes were divided in complex recombinant forms and pure non-B forms. It is important to note that for HIV-DNA quantification we used the primers and probe designed on HIV-1 LTR-5′ reported in Jones et al. [19], supporting with clinical samples the good performance of this ddPCR-based assay, like other recently evaluated HIV-DNA assays [23].

Regarding the quantification of residual viremia, among the 25 plasma samples from participants infected by non-B subtype on suppressive ART with an HIV-RNA < 20 copies/ml by standard assays, 20 (80.0%) had HIV-1 RNA detected by ddPCR, a prevalence superimposable to that observed for B subtype (28 out of 39 [71.8%]

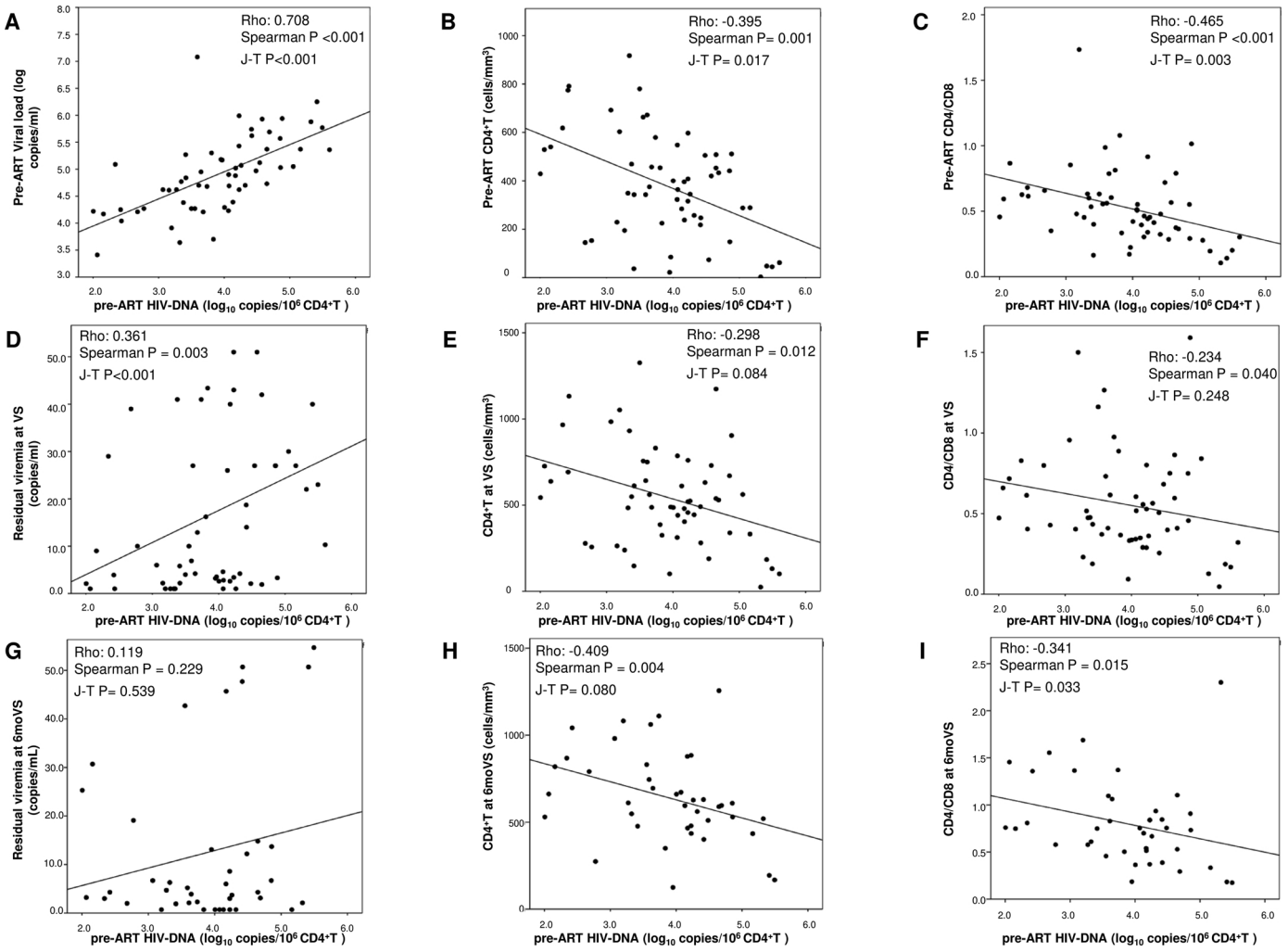

Fig. 2. Correlation of pre-HIV DNA with viral load (a), $CD4^+$T cells (b) and CD4/CD8 (c) at baseline of ART. Correlation of pre-HIV DNA with residual viremia (d), $CD4^+$T cells (e) and CD4/CD8 (f) at virological success. Correlation of pre-HIV DNA with residual viremia (g), $CD4^+$T cells (h) and CD4/CD8 (i) at 6 months after virological success. VS: virological success; 6moVS: 6 months after virological success. Rho and P values were determined by Spearman test and Jonckheere-Terpstra test (J–T).

samples, P = 0.561). Similar results were obtained when non-B subtypes were divided in complex recombinant forms and pure non-B forms.

Since our results support the reproducibility of these assays along different HIV-1 subtypes, the evaluation of their performances in different laboratories will be necessary for the inter-laboratory reproducibility and thus the cross-validation of the methods.

Beyond the performance of the ddPCR-based assays used, this study allowed to support the prognostic significance of HIV-DNA in patients initiating first-line treatment. In particular, our data show that patients initiating antiretroviral treatment with lower pre-ART HIV-DNA load: 1) achieved VS in a shorter time; 2) reached lower levels of HIV-DNA at VS; 3) experienced lower level of residual viremia at VS; 4) maintained higher $CD4^+$T cells and CD4/CD8 at both VS and 6 months after.

By focusing on HIV-DNA slope during antiretroviral treatment, we observed a more pronounced decay of HIV-DNA from baseline to VS in patients characterized by a pre-ART HIV-DNA ≥ 4.5 $\log_{10}$ copies/10^6 $CD4^+$T cells compared to patients with lower pre-ART HIV-DNA levels. These findings are in line with previous studies that defined similar correlations between pre- and post-ART HIV-DNA in infected adults and children [24–26]. Analysing the time needed to reach the VS, we also observed that pre-ART HIV-DNA level was a strong and significant predictor of achieving VS. These data point out that the time to achieve VS strongly depends on the burden of pre-therapy HIV-DNA, in accordance with previous studies [5,6,8], and reinforces the concept that HIV-DNA level before treatment is a good marker for monitoring treatment efficacy [7,8,10,11,27,28]. The increased time to VS and the presence of higher values of residual viremia in patients with higher pre-ART HIV-DNA at VS may be explained by more pronounced ongoing low-level replication or virus production from long-lived cells in these patients respect to those with lower HIV-DNA level [29,30].

Interestingly, our study showed a good correlation between higher levels of pre-ART HIV-DNA and lower $CD4^+$T cells and CD4/CD8 at both VS and 6moVS, supporting the role of pre-ART HIV-DNA as a predictor of immune recovery, and thus clinical progression [10]. Previous studies showed that a lower HIV-DNA load during ART is associated with better immune recovery both in adults and in children [31–34]. In line with these findings, in a recent in vivo model, it was demonstrated a straight correlation between the size of the HIV reservoir in the spleen of mice and the human $CD4^+$T cells [35]. Strikingly, the authors observed a correlation between the human CD4/CD8 in the spleen with integrated as well as total HIV-DNA [35], implying that the $CD8^+$T cells might influence the size of the HIV reservoir, and thus indirectly confirming that pre-ART HIV-DNA load can be related to immune cell activation status during ART [36].

Our results further suggest that residual viremia could have a significant impact on the immunological parameters of patients on sustained effective ART. In particular, our data indicate that there is more

residual HIV-1 RNA in the plasma of patients with low CD4$^+$T cells than in that of patients with higher CD4$^+$T cells at both VS and 6moVS. The clinical implications of residual viremia are still not fully clear, but its occurrence could reflect various clinical scenarios as follows: chronic inflammation, immune system activation, and microbial translocation [37,38]. Thus, understanding the relationship among residual viremia, immune maintenance and reconstitution with further prospective studies, characterised by a higher number of enrolled individuals, is to date mandatory to help guide HIV-1 clinical management in the future.

The small sample size and the inability to measure HIV-DNA and residual viremia among all patients at all study visits, in addition to the missing knowledge of the timing of infection are the main limitations of our study. Moreover, our HIV-DNA assay does not distinguish replication-competent and replication-defective forms of cellular-associated viral genomes, thus not revealing the amount of HIV-DNA that can effectively cause viral rebound. Notwithstanding these limitations, total HIV-DNA is confirmed to be one of the best viral markers to predict the chance of virological suppression and of long-term treatment outcome in patients starting their first-line ART regimen [39].

In conclusion, this study confirms that pre-ART total HIV-DNA, normalized on CD4$^+$T cells, is an excellent indicator of HIV-1 reservoir burden, residual viremia, and immune status. It also appears to be useful to predict the response to antiretroviral treatment since 6 months after VS. Moreover, comparing virological and immunological features of patients, our study arose a potential correlation between poor immunological reconstitution and residual viremia at VS and 6moVS. These data were obtained by using simple, sensitive, and reproducible approach for HIV reservoir measurement that can be performed routinely in clinical practice, and that can be used to better characterize ART response and risk of disease progression.

Author contribution section

C.A., F.C.S. and V.S. conceived the presented idea. C.A. and R.S. developed and performed the analyses, and wrote the manuscript. C.S., G.M., F.F., S.M., O.B., O.T., L.S., N.C., and M.A. provided samples. R.S. and M.B. performed the experiments. M.M.S., C.F.P., V.S. and F.C.S. contributed to the interpretation of the results and revised the manuscript. All authors reviewed and approved the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.06.004.

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