Baseline Cellular HIV DNA Load Predicts HIV DNA Decline and Residual HIV Plasma Levels during Effective Antiretroviral Therapy

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Cellular human immunodeficiency virus type 1 (HIV-1) DNA may be considered a marker of disease progression with significant predictive power, but published data on its correlation with plasma HIV RNA levels and CD4 counts in acute and chronic patients are not conclusive. We evaluated a cohort of 180 patients naïve for antiretroviral therapy before the beginning of treatment and after a virological response in order to define the indicators correlated with HIV DNA load decrease until undetectability. The following variables were evaluated as continuous variables: age, CD4 cell count and log₁₀ HIV DNA level at baseline and follow-up, and baseline log₁₀ HIV RNA level. Primary HIV infection at the start of therapy, an HIV RNA level at follow-up of <2.5 copies/ml, origin, gender, and transmission risk were evaluated as binary variables. The decline of HIV DNA values during effective therapy was directly related to baseline HIV DNA and HIV RNA values, to an increase in the number of CD4 cells, and to the achievement of an HIV RNA load of <2.5 copies/ml. An undetectable cellular HIV DNA load was achieved by 21.6% of patients at the follow-up time point and correlated significantly with lower baseline cellular HIV DNA values and with being in the primary stage of infection when therapy started. In conclusion, early treatment facilitated the achievement of undetectable levels of plasma viremia and cellular HIV DNA and a better recovery of CD4 lymphocytes. HIV DNA levels before and during highly active antiretroviral therapy may be used as a new tool for monitoring treatment efficacy.

The cellular HIV DNA load correlates directly with the number of latently HIV-infected cells that constitute the viral reservoir and is considered an independent marker of disease progression (16) with a strong predictive power for acute and chronic patients (12, 25). Therefore, the cellular HIV DNA load can also be considered a potential indicator for the initiation of highly active antiretroviral therapy (HAART). The HIV DNA load predicts the long-term success of HAART in naïve patients and is able to anticipate virological failure in treated patients. Recently (17), a prospective multicenter study assessed the predictive value of peripheral blood mononuclear cell (PBMC) HIV-1 DNA for determining virological and immunological outcomes in a cohort of 148 patients who were treated (starting from 1998) with a first-line protease inhibitor (PI)-containing regimen, and it demonstrated that a higher baseline HIV-1 DNA level was associated with an increased risk of virological failure after 1 year of HAART. This phenomenon was conserved long-term (7 years) according to univariate analysis but was no longer significant by multivariate analysis. No short-term or long-term effects of HIV-1 DNA level on the immunological outcome were observed. A small follow-up study of 51 patients indicated that the HIV DNA load was the only predictive parameter of virological rebound in the group of non-responders (15). Furthermore, in patients with advanced therapeutic failure, a larger HIV DNA load may be associated with smaller increases in CD4 count. Therefore, HIV DNA levels were recently suggested as a useful tool for the case management of patients in the late stages of disease (3).

HAART-mediated reductions in HIV DNA levels were studied (29) in a 5-year follow-up of 25 patients who were treated with HAART. The study showed that the largest HIV DNA decrease was evident during the 1st year of therapy, followed by milder decreases during the 2nd and 3rd years, without any further diminution, sug-

gesting that any additional benefit of treatment in terms of reduction of the viral reservoir was unlikely. The evolution of HIV DNA was not different for patients with baseline CD4 cell counts that were above or below the median value (127 cells/mm³).

For 236 patients receiving successful therapy for more than 3 years (7), a univariate analysis showed that HIV-1 DNA levels did not correlate with therapy duration, time spent with undetectable HIV-1 RNA, or the occurrence of a viral blip, defined as a viral RNA level of <1,000 HIV RNA copies/ml. The plasma HIV-1 RNA zenith and CD4 cell count nadir remained predictive of HIV-1 DNA levels in the multivariate model. In clinical practice, a significant number of patients reached undetectable HIV DNA levels during HAART.

Given the conflicting reports on the correlation among HIV DNA levels and the virological and immunological responses to treatment, we evaluated a cohort of 180 patients who were studied before the beginning of treatment and after a virological response to HAART to elucidate the indicators that correlated with a decreased or undetectable HIV DNA load.

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MATERIALS AND METHODS

A cohort of 180 HIV-infected subjects who were naïve for antiretroviral therapy were enrolled in five infectious disease units in the Veneto region of Italy. Patients were selected retrospectively among those who achieved virological suppression with first-line therapy within 6 months and maintained plasma HIV RNA levels of <50 copies/ml without virological failures until the follow-up evaluation. A blood sample was collected before the beginning of therapy (T0) and at the time of follow-up (T1), which ranged from 6 months to 6 years after achieving virological suppression.

In the event of a change in HAART, the change was accepted if it was due to intolerance or pill burden convenience. Patients who interrupted treatment or needed treatment modifications for failure were excluded from the study. Patients who had more than one viral blip per year after achieving viral suppression, which was determined by frequent blood sampling (at least 4 times per year), were not considered useful for the aim of the study and were not included.

All of the enrolled subjects gave informed consent to all procedures and the use of their blinded data for a scientific evaluation and publication. The local government, which was represented by the Veneto Regional Health Authority, approved the study and provided funding. This study was conducted in accordance with the Helsinki Declaration and with local legislation.

A primary HIV infection was defined by the presence of the following: (i) a negative or indeterminate HIV antibody enzyme-linked immunosorbent assay associated with HIV RNA-positive plasma or (ii) an initially negative test for HIV antibodies followed by positive serology within 18 months.

HIV genotypic analyses to detect subtypes were performed as previously described (24).

Blood samples collected in EDTA-containing tubes were separated into plasma and cells by Ficoll-Paque Plus density gradient centrifugation. Aliquots of plasma and dried pellets of 2×10^6 PBMCs were stored at -80°C until use. Baseline and follow-up blood samples in EDTA were submitted to the Laboratory of Virology at the University of Padova and stored within 6 h of collection until analysis.

Cellular HIV DNA quantitation. The extraction and purification of DNA from cells were performed using a QIAamp blood kit (Qiagen, Inc., Chatsworth, CA). The real-time TaqMan protocol published by Viard and colleagues (29) was used to quantify the cellular HIV DNA copy number in PBMCs. The cell line 8E5, which contains one copy of integrated HIV DNA in each cell, was used to build a standard curve, with a sensitivity of 5 copies/million PBMCs (21).

Duplicate analysis within the same experiment was conducted for both HIV and beta-globin evaluation, and means for these values were calculated. When delta values were more than 10%, the sample was analyzed again. Baseline and follow-up samples from a patient were always evaluated in the same experiment; this procedure was possible because only complete sample sets (T0 and T1) from the patients, selected retrospectively, were considered. A previously evaluated sample was regularly added as an internal control.

Quantification of residual plasma viremia. Plasma samples were obtained from blood that had been collected, frozen at -80°C within 6 h of collection, and kept at this temperature until tested. Residual viremia was quantified using an ultrasensitive method based on a modified Amplicor HIV-1 Monitor test, version 1.5 (Roche Molecular Systems, Branchburg, NJ), with a limit of detection of 2.5 copies/ml (21). Modifications included pelleting viruses from 2 ml of plasma at $23,600 \times g$ at 4°C for 2 h, adding half of the normal volume of the quantification standard, and resuspending the RNA pellet in 50 μl of diluent. The entire volume of resuspended RNA was assayed using reverse transcription and PCR amplification. These and subsequent detection steps were performed following the manufacturer's protocol.

Patients were subdivided into the following 4 groups at the follow-up time point: (i) subjects with undetectable plasma viremia at the time of evaluation, defined as ultralow (UL; <2.5 HIV RNA copies/ml); (ii) those

with low-level plasma viremia (LL; 2.5 to 20 copies/ml); (iii) those with high-level plasma viremia (HL; 20 to 50 copies/ml); and (iv) those with viral blips (VB; >50 to 1,000 copies/ml). Patients with plasma viremia of >1,000 copies/ml at the follow-up time point were not included.

Statistical analysis. The following variables were analyzed. Age, CD4 cell counts at baseline and follow-up, $\log_{10}$ HIV DNA copies/ 10^6 PBMCs at baseline and follow-up, and $\log_{10}$ HIV RNA copies/ml were evaluated as continuous variables. An HIV RNA level under the lowest threshold level (under 2.5 copies/ml) at follow-up, primary HIV infection, stranger origin, male gender, homosexual risk, and injection drug use were evaluated as binary variables.

All variables were submitted to pairwise correlation analysis in order to outline the underlying linkage pattern in the evaluated population. The correlation was established by linear regression analysis when at least one variable was continuous. When both paired variables were categorical (binary), the chi-square test was used.

Time-to-event procedures were employed as well. In this context, the Kaplan Meier (KM) nonparametric approach (with log rank test) and the semiparametric proportional hazards Cox regression method were used. The latter was performed with an aim toward a multivariate scope.

A time-to-event (survival) analysis was performed by using the lowering of the follow-up HIV DNA load to <5 copies/ 10^6 PBMCs as the relevant event. Therefore, the defined event was a favorable outcome, and the term "hazard" or "risk" meant the probability of this outcome.

The effects of the relevant explanatory variables were investigated by adopting the Cox semiparametric cumulative hazards regression method. Initially, a bivariate analysis was performed. Thereafter, a multivariate approach was exploited.

RESULTS

The median age of the 180 selected patients was 41 years (range, 19 to 79 years). A total of 140 subjects were male (77.8%), and 63/180 subjects (35%) were men who reported having sex with men as a risk factor. A total of 84 patients (46.7%) were heterosexual, 17 patients (9%) were intravenous drug users, and 16 patients had undetermined risk factors. Among 180 subjects, 138 subjects (76.7%) were Italian.

At the beginning of HAART, the median CD4 count was 250 cells/ mm^3 (range, 0 to 1,090 cells/ mm^3), and the median CD4 cell percentage was 15% (range, 0 to 42%). The median HIV RNA level was 5 $\log_{10}$ copies/ml (range, 1.91 to 6.97 $\log_{10}$ copies/ml), and the median HIV DNA level was 3.26 $\log_{10}$ copies/ 10^6 PBMCs (range, 1.34 to 4.84 $\log_{10}$ copies/ 10^6 PBMCs).

In 48 subjects (26.6%), a non-B-subtype strain was detected. Patients with the non-B subtype were more likely to be younger ($P = 0.0007$), have a stranger origin ($P < 0.0001$), be female ($P < 0.0001$), and have lower baseline plasma HIV RNA levels ($P = 0.0075$). A total of 21 patients (11.6%) were enrolled and started therapy during the acute infection period.

Trend of HIV DNA and correlation with other parameters. A reduction of HIV DNA values between T0 and T1 was present in all studied patients.

To assess the relationships between the trend of HIV DNA load (from T0 to T1) and the other parameters, a pairwise correlation analysis was performed. The $\log_{10}$ values for cellular HIV DNA at T1 correlated positively with the baseline values for cellular HIV DNA and plasma HIV RNA ($P < 0.0001$ and $P = 0.0257$, respectively), with achievement of an undetectable value of plasma HIV RNA (<2.5 copies/ml) at T1 ($P < 0.0001$), and with primary HIV infection at the start of therapy ($P < 0.0001$), and they correlated negatively with the baseline value for CD4 count ($P = 0.0236$).

The correlation between the changing values of cellular HIV

FIG 1 Log₁₀-transformed HIV proviral DNA as a function of CD4 count and end (follow-up) plasma viremia (plasma viral load of <2.5 genomic copies/ml or >2.5 genomic copies/ml). Linear predictions are reported, along with 95% confidence intervals. Legend: rnaend=0, partial plasma HIV RNA suppression; rnaend=1, full plasma HIV RNA suppression; time=0, initial observation time (T0); time=1, 1 year of treatment (which is also close to the mean time of observation). Initial observations (time point T0) and final observations (time point T1) are reported as well.

DNA and CD4 cell counts over time was investigated using a multilevel, mixed-effects multivariate regression analysis (Fig. 1). The dependent variable was log₁₀ HIV DNA. After a preliminary inclusion of several explanatory covariates, including CD4 cells, low HIV RNA level (<2.5 copies/ml at T1), primary HIV infection at the start of therapy, non-B HIV subtype, age, nationality other than Italian, intravenous drug use, and men having sex with men, only the CD4 cell count and HIV RNA level (<2.5 copies/ml at T1), along with time, were retained as significant. Time was also included in the random effects section. The observations were grouped by patients. Figure 1 reports the log₁₀ HIV DNA levels as a function of CD4 counts at time points T0 and T1, along with linear predictions.

Examining the coefficients, we noted that if the CD4 cell count increases by 1, the HIV DNA level should be multiplied by 0.998 (i.e., $10^{-0.0017} = 0.998$). If the undetectable HIV RNA level at T1 changes from 0 (partial suppression) to 1 (full suppression), the DNA level should be multiplied by $10^{-0.3273}$ (0.471). If time increases by 1 year, the DNA level should be multiplied by $10^{-0.4539}$ (0.352). For example, with 400 CD4 cells, the HIV DNA level is 477 copies/ml in a patient with partial plasma viral RNA suppression at T1 (time of follow-up = 1 year). Then, if the HIV RNA level at T1 turns from partial to full suppression of plasma viral RNA, the DNA level changes to 224 copies. In general, the HIV DNA level is calculated as follows: $2,652 \times 0.998^{CD4} \times 0.471^{RNA \text{ at T1}} \times 0.352^{time}$, where $10^{3.4237} (10^{intercept}) = 2,652$.

Investigating the possible role of several covariates (CD4 cell count change, age, gender, nationality other than Italian, men who have sex with men or intravenous drug use as a risk factor, achieving <2.5 copies/ml of plasma HIV RNA, primary infection at the start of therapy, and non-B HIV subtype) in predicting a

reduction of the log₁₀ value of the cellular HIV DNA load at T1, a final model showing a significant correlation was obtained with two explanatory covariates that independently exerted significant effects: the CD4 cell count increase from T0 to T1 ($P = 0.004$; 95% confidence interval [95% CI], 0.000 to 0.001) and achieving <2.5 copies/ml of plasma HIV RNA ($P = 0.022$; 95% CI, 0.048 to 0.609).

Undetectable HIV DNA load (<5 copies/10⁶ PBMCs) at T1 and correlation with other parameters. To assess whether some parameters (CD4 cell count, baseline log₁₀ DNA and log₁₀ RNA levels, suppression of plasma viremia to <2.5 genomic copies/ml, primary HIV infection, HIV subtype, ethnicity, gender, and HIV transmission risk) might be related to achieving an undetectable HIV DNA load at T1, a time-to-event (survival) analysis was performed, using the achievement of a cellular HIV DNA load of <5 copies/10⁶ PBMCs as the relevant event. Thirty-nine patients (21.6%) reached this outcome among the 180 patients who were included in the cohort.

The effect of the relevant explanatory variables was investigated by adopting the Cox semiparametric cumulative hazards regression method. Using bivariate analysis, the dependent variable was the successful event, which was defined as a cellular HIV DNA load under the threshold of 5 copies/10⁶ PBMCs. The achievement of <5 copies/10⁶ PBMCs for the cellular HIV DNA load correlated with lower baseline log cellular HIV DNA levels ($P < 0.001$), with achievement of undetectable levels of HIV RNA (<2.5 copies/ml) at T1 ($P = 0.003$), and with having primary infection at the start of therapy ($P = 0.006$). No correlation was found between an HIV DNA load of <5 copies/10⁶ PBMCs at T1 and baseline CD4 cell counts.

This approach failed to obtain a significant result when the

FIG 2 The proportion of patients who were exempt from the relevant event (final log₁₀ cellular HIV DNA level reduced to ≤5 copies) was plotted as a function of time (years). The baseline log₁₀ proviral HIV DNA levels are indicated as the 10th, 50th, and 90th percentiles, corresponding to 269, 1,838, and 6,284 copies, respectively.

explanatory variable was the baseline log₁₀ RNA level. However, a correlation between the dependent variable and the above predictor was demonstrated by two alternative methods: (i) counting the successful proviral DNA suppression events after stratifying the baseline plasma RNA loads into three levels (<10,000, 10,000 to 99,999, and ≥100,000 copies/ml), and using the Pearson chi-square test thereafter ($\chi^2 = 7.4397$; $P = 0.024$); and (ii) performing analysis of variance (ANOVA) on baseline log₁₀ RNA levels versus cellular HIV DNA suppression ($P = 0.0340$).

Using a multivariate analysis, only the log₁₀ baseline cellular HIV DNA value maintained an independent predictive role in the achievement of an undetectable HIV DNA load at T1 ($P < 0.001$; 95% CI, 0.211 to 0.480). In particular, it was demonstrated that an

increase of 1 log in the cellular HIV DNA load at baseline reduced the probability of the favorable event by approximately one-third.

In Fig. 2, the proportion of patients who were exempt from the relevant event (cellular HIV DNA at T1 was reduced to <5 copies) was plotted as a function of time (years). The effects of the baseline log₁₀ HIV DNA values are indicated as 10th, 50th, and 90th percentiles, corresponding to 2.42, 3.26, and 3.79 log₁₀ copies/10⁶ PBMCs, respectively.

Effect of therapy during primary infection. The main characteristics of the population were assessed using a pairwise correlation analysis, which showed that having primary infection at the start of therapy correlated positively with high CD4 counts at baseline ($P < 0.0001$) and at T1 ($P = 0.0076$), achieving <2.5 copies/ml of plasma HIV RNA at T1 ($P = 0.0003$), and low cellular HIV DNA levels at T1 ($P < 0.0001$).

Achieving HIV RNA levels of <2.5 copies/ml at T1 correlated with low baseline values of plasma HIV RNA ($P = 0.0007$), low baseline cellular HIV DNA levels ($P = 0.0004$), low cellular HIV DNA levels at T1 ($P < 0.0001$), and high CD4 cell counts at T0 ($P < 0.0001$).

Effect of HAART on residual plasma viremia. At T1, the subjects were subdivided into the following four groups according to plasma viremia values: (i) UL (<2.5 copies/ml), (ii) LL (2.5 to 20 copies/ml), (iii) HL (>20 to 50 copies/ml), and (iv) VB (>50 to 1,000 copies/ml). Immunovirological values for these patients are shown in Table 1.

The effects of the current treatment on the achievement of an HIV RNA level of <2.5 copies/ml at T1, in terms of using a different third drug that was associated with the same backbone, were evaluated using a semiparametric analysis.

Explanatory treatment variables were efavirenz (71 patients receiving treatment at T1), nevirapine (21 patients), ritonavir-boosted protease inhibitors (83 patients), and a third nucleoside

TABLE 1 Patient characteristics^a

Parameter	Value for patient group			
	HIV RNA at <2.5 copies/ml	HIV RNA at 2.5–20 copies/ml	HIV RNA at 21–49 copies/ml	HIV RNA at 50–1,000 copies/ml
No. (%) of patients				
Total	73 (40.5)	49 (27.2)	25 (13.9)	31 (17.2)
Patients with primary infection	16 (22)	1 (2)	2 (8)	2 (6.4)
CD4 count (cells/mm ³)				
Baseline	296 (0–1,090)	240 (10–390)	181 (6–770)	230 (0–680)
Follow-up	571 (150–1,418)	490 (270–941)	436 (130–1,430)	520 (100–1,170)
% CD4 cells				
Baseline	16 (0–42)	14 (0–29)	11 (2–38)	17.5 (0–33)
Follow-up	30.7 (6–51)	28 (13–44)	22 (5–46)	30 (6–51)
Cellular HIV DNA level (log ₁₀ copies/10 ⁶ PBMCs)				
Baseline	3.09 (1.34–4.84)	3.28 (1.78–3.94)	3.32 (1.96–3.80)	3.51 (2.69–4.82)
Follow-up	1.41 (<0.69–2.89)	2.40 (<0.69–3.52)	1.77 (<0.69–3.15)	2.38 (<0.695–1.91)
No. (%) of subjects reaching DNA level of <5 copies/10 ⁶ PBMCs	27 (37)	7 (14.3)	3 (12)	2 (6.4)
No. (%) of subjects taking PIs	24 (33)	21 (42.8)	15 (60)	17 (54.8)
Length (yr) of follow-up	2 (0.5–5)	1 (0.5–4)	2 (0.5–4)	1 (0.5–5)

^a Patients were divided into groups based on HIV plasma viremia levels according to the results of the ultrasensitive plasma viremia method at follow-up. Two patients (1%) were not included due to a lack of ultrasensitive data. Unless stated otherwise, results are expressed as median values (ranges).

analogue (Trizivir; 4 patients). The variable nonnucleoside analogues included efavirenz and nevirapine.

Using the bivariate Cox regression method, no explanatory treatment variable was significant. Efavirenz was the variable closest to being significant (hazard ratio [HR] = 1.55; $Z = 1.84$).

DISCUSSION

In the study cohort, the decline of HIV DNA values during effective therapy was directly related to HIV DNA and HIV RNA values at the beginning of HAART ($P < 0.0001$ and $P = 0.0257$, respectively), to a gain in the number of CD4 cells ($P = 0.02369$), and to achievement of an HIV RNA load of <2.5 copies/ml ($P < 0.0001$).

A lower HIV DNA load after a defined duration of HAART was significantly related to a higher pre-HAART CD4 cell count and percentage and a prolonged duration of antiretroviral treatment (2, 19, 27, 30). Furthermore, the absence of previous exposure to antiretroviral therapy was a predictive factor for larger decreases in cellular HIV-1 DNA levels (19). Finally, in patients with undetectable levels of plasma viremia after 1 year of HAART, the highest reduction of HIV DNA was obtained in subjects who started treatment in the early asymptomatic phase of infection, with more than 500 CD4 cells/mm³ (2).

In our study, 21.6% of patients achieved a cellular HIV DNA load of <5 copies/10⁶ PBMCs at the follow-up time point. This favorable outcome correlated with lower baseline cellular HIV DNA values ($P < 0.001$) and with being in the primary stage of infection at the start of therapy ($P = 0.006$). A 1-log₁₀ increase in the HIV DNA load at baseline reduced the probability of reaching <5 copies/10⁶ PBMCs by approximately one-third at the follow-up time. Moreover, a strong correlation between cellular HIV DNA decreases and CD4 cell count increases over time was demonstrated.

In agreement with another study (9), a large proportion of patients (40.5%) achieved undetectable levels of plasma HIV RNA (<2.5 copies/ml) at the follow-up time point. This virological endpoint was correlated with low cellular HIV DNA levels at baseline ($P = 0.0004$) and follow-up ($P < 0.0001$), low baseline plasma viremia ($P = 0.0007$), and high CD4 cell counts at baseline ($P < 0.0001$) and follow-up ($P = 0.0329$).

In a cross-sectional analysis after 2 years of effective HAART, only 5 of 84 subjects (6%) had undetectable levels of proviral HIV DNA, and 42 subjects had HIV RNA values of <2.5 copies/ml (23). The use of nonnucleoside reverse transcriptase inhibitor (NNRTI)-based HAART was the only independent predictor of plasma RNA suppression below the cutoff value.

The differences in these results can be explained by the characteristic features of the enrolled patients: different exclusion criteria for virological failure were used. In addition, in the study by Palmisano et al. (23), only 26% of patients were on PI therapy, and $<2\%$ of these patients used boosted PI therapy.

The correlation between the favorable HIV RNA and HIV DNA outcomes was described in a cross-sectional study by Chun et al. (9), who reported the frequency of CD4 T cells carrying HIV proviral DNA. When the data on HIV proviral DNA burden were stratified based on residual plasma viremia, a statistically significant difference was observed between the study subjects with undetectable (0 copies) and detectable (1 to 49 copies) plasma viremia ($P = 0.01$). These results indicate that the frequency of CD4 T cells carrying HIV proviral DNA in infected individuals with undetectable plasma viremia is lower than that for individuals

with detectable plasma viremia. In the current study, our results confirm this observation, using total PBMCs, and provide new insights regarding the predictive value of baseline virological values from archives.

A previous study suggested (22) that low-level persistent viremia was derived from at least two cell compartments: one compartment demonstrated viral production decay over time, whereas a second compartment maintained stable viral production for at least 7 years.

Hatano et al. (14) observed that plasma viremia continued to decline during the first 12 months after viremia was undetectable by conventional methods and remained stable, appearing to achieve a steady-state set point during long-term combination therapy.

This residual viremia was not reduced by treatment intensification (10, 11, 18). However, these results are under debate (8), as it has been suggested that residual viremia does not arise from ongoing cycles of HIV-1 replication and infection of new cells.

Latently infected, resting CD4 T cells and/or unidentified viral reservoirs are capable of producing low levels of virions for prolonged periods in individuals receiving HAART for extended times (4). Previous studies observed that residual plasma viruses and CD4 T cell-associated viruses were separately compartmentalized (6, 26). Nevertheless, in a recent study, clonal sequences recovered from residual HIV-1 viremia in patients receiving intensified HAART were identical to replicating viral RNAs that were recovered from circulating resting CD4⁺ T cells (1).

Our data show that an undetectable level of plasma viremia, low HIV DNA levels, and high CD4 counts are more probably attained by patients who start HAART while having a low level of cellular HIV DNA.

The timing of HAART intervention was suggested to have remarkably different effects on the HIV DNA compartment. Twenty-one of 180 subjects in our population started therapy during the phase of primary infection. A total of 16 subjects had <2.5 copies/ml of HIV RNA at follow-up ($P = 0.0003$). This very early therapy correlated with lower HIV DNA levels at follow-up ($P < 0.0001$) and with achieving a cellular HIV DNA load of <5 copies/10⁶ PBMCs ($P = 0.006$). Using a multivariate analysis, only cellular HIV DNA levels maintained an independent predictive role in the achievement of undetectable cellular HIV DNA in the study population. These data are in agreement with data obtained from patients who were treated in the early, asymptomatic phase of infection (2) and in primary infection (20, 30). Finally, it was reported that patients who were treated before or within 6 months after seroconversion harbored smaller reservoirs of recoverable HIV after 1 year of HAART than patients who were treated for 3 to 6 years during chronic infection (28).

More potent effects on residual viremia have been demonstrated with the use of NNRTIs than with protease inhibitors (5, 21), and a better performance of nevirapine than efavirenz has been suggested (5, 13). In this study, no significant association was demonstrated between HAART composition and undetectable levels of HIV DNA. Efavirenz showed the best performance, approaching statistical significance (HR = 1.55; $Z = 1.84$). It is important that the majority of patients received first-line treatment and that few patients were treated with nevirapine.

A bias in this study needs to be discussed. Only peripheral HIV DNA was suitable for the analysis, and the measure of a clinically useful and easily accessible parameter was planned; not differen-

tiating between integrated and nonintegrated HIV DNA forms makes it difficult to comment on the potential prognostic value of DNA levels, but we were unable to measure the baseline values of nonintegrated virus because we lacked an adequate quantity of PBMCs (about 6×10^7 PBMCs are needed) stored at T0 for this analysis, and the follow-up values without a baseline reference would not be interpretable.

In conclusion, low HIV DNA levels during HAART correlate with CD4 counts and with low-level residual viremia. Treatment during the early phase of HIV infection facilitates the achievement of undetectable levels of plasma viremia and cellular HIV DNA, as well as a better recovery of CD4 lymphocytes. HIV DNA level evaluations before and during HAART may be used as a new tool for monitoring the efficacy of treatment.

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S.G.P. designed and coordinated the study, supervised laboratory experiments, collected the data, interpreted the findings, and wrote the paper; S.A. performed laboratory experiments; C.M. interpreted the data and performed statistical analysis; R.S. helped to design the study, managed the patients, and collected samples; R.F. managed the patients and collected samples; V.M. managed the patients and collected samples; M.C. managed the patients and collected samples; M.G. managed the patients and collected samples; C.B. performed laboratory experiments; M.B. helped in interpretation of the findings and in writing the paper; M.A. designed the study and helped in interpretation of the findings and in writing the paper; G.P. designed the study and helped in interpretation of the findings and in writing the paper; and L.S. designed the study and helped in interpretation of the findings and in writing the paper.

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REFERENCES

- Anderson JA, et al. 2011. Clonal sequences recovered from plasma from patients with residual HIV-1 viremia and on intensified antiretroviral therapy are identical to replicating viral RNAs recovered from circulating resting CD4⁺ T cells. *J. Virol.* 85:5220–5223.
- Andreoni M, et al. 2000. Cellular proviral HIV-DNA decline and viral isolation in naive subjects with <5000 copies/ml of HIV-RNA and >500 CD4 cells treated with highly active antiretroviral therapy. *AIDS* 14:23–29.
- Avettand-Fenoel V, et al. 2010. Higher HIV-1 DNA associated with lower gains in CD4 cell count among patients with advanced therapeutic failure receiving optimized treatment (ANRS 123—ETOILE). *J. Antimicrob. Chemother.* 65:2212–2214.
- Bailey JR, et al. 2006. Residual human immunodeficiency virus type 1 viremia in some patients on antiretroviral therapy is dominated by a small number of invariant clones rarely found in circulating CD4⁺ T cells. *J. Virol.* 80:6441–6457.
- Bonora S, et al. 2009. Ultrasensitive assessment of residual HIV viraemia in HAART-treated patients with persistently undetectable plasma HIV-RNA: a cross-sectional evaluation. *J. Med. Virol.* 81:400–405.
- Brennan TP, et al. 2009. Analysis of human immunodeficiency virus type 1 viremia and provirus in resting CD4⁺ T cells reveals a novel source of residual viremia in patients on antiretroviral therapy. *J. Virol.* 83:8470–8481.
- Burgard M, et al. 2009. Factors influencing peripheral blood mononuclear cell-associated HIV-1 DNA level after long-term suppressive antiretroviral therapy in 236 patients. *AIDS* 23:2165–2171.
- Buzon MJ, et al. 2010. HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. *Nat. Med.* 16:460–465.
- Chun TW, et al. 2011. Relationship between residual plasma viremia and the size of HIV proviral DNA reservoirs in infected individuals receiving effective antiretroviral therapy. *J. Infect. Dis.* 204:135–138.
- Dinso JB, et al. 2009. Treatment intensification does not reduce residual HIV-1 viremia in patients on highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. U. S. A.* 106:9403–9408.
- Gandhi RT, et al. 2010. The effect of raltegravir intensification on low-level residual viremia in HIV-infected patients on antiretroviral therapy: a randomized controlled trial. *PLoS Med.* 7:e1000321.
- Goujard C, et al. 2006. CD4 cell count and HIV DNA level are independent predictors of disease progression after primary HIV type 1 infection in untreated patients. *Clin. Infect. Dis.* 42:709–715.
- Haum-Boukoba S, et al. 2011. Higher efficacy of nevirapine than efavirenz to achieve HIV-1 plasma viral load below 1 copy/ml. *AIDS* 25:341–344.
- Hatano H, et al. 2010. Evidence of persistent low-level viremia in long-term HAART-suppressed, HIV-infected individuals. *AIDS* 24:2535–2539.
- Hatzakis AE, et al. 2004. Cellular HIV-1 DNA load predicts HIV-RNA rebound and the outcome of highly active antiretroviral therapy. *AIDS* 18:2261–2267.
- Kostrakis LG, et al. 2002. Quantitation of human immunodeficiency virus type 1 DNA forms with the second template switch in peripheral blood cells predicts disease progression independently of plasma RNA load. *J. Virol.* 76:10099–10108.
- Masquelier B, et al. 2011. Cellular HIV-1 DNA quantification and short-term and long-term response to antiretroviral therapy. *J. Antimicrob. Chemother.* 66:1582–1589.
- McMahon D, et al. 2010. Short-course raltegravir intensification does not reduce persistent low-level viremia in patients with HIV-1 suppression during receipt of combination antiretroviral therapy. *Clin. Infect. Dis.* 50:912–919.
- Morand-Joubert L, et al. 2005. Contribution of cellular HIV-1 DNA quantification to the efficacy analysis of antiretroviral therapy: a randomized comparison of 2 regimens, including 3 drugs from 2 or 3 classes (TRIANON, ANRS 081). *J. Acquir. Immune Defic. Syndr.* 38:268–276.
- Ngo-Giang-Huong N, et al. 2001. Proviral HIV-1 DNA in subjects followed since primary HIV-1 infection who suppress plasma viral load after one year of highly active antiretroviral therapy. *AIDS* 15:665–673.
- Nicastrì E, et al. 2008. HIV-1 residual viremia and proviral DNA in patients with suppressed plasma viral load (<400 HIV-RNA cp/ml) during different antiretroviral regimens. *Curr. HIV Res.* 6:261–266.
- Palmer S, et al. 2008. Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy. *Proc. Natl. Acad. Sci. U. S. A.* 105:3879–3884.
- Palmisano L, et al. 2005. Residual viraemia in subjects with chronic HIV infection and viral load of <50 copies/ml: the impact of highly active antiretroviral therapy. *AIDS* 19:1843–1847.
- Parisi SG, et al. 2007. Both human immunodeficiency virus cellular DNA sequencing and plasma RNA sequencing are useful for detection of drug resistance mutations in blood samples from antiretroviral drug-naïve patients. *J. Clin. Microbiol.* 45:1783–1788.
- Rouzioux C, et al. 2005. Early levels of HIV-1 DNA in peripheral blood mononuclear cells are predictive of disease progression independently of HIV-1 RNA levels and CD4⁺ T cell counts. *J. Infect. Dis.* 192:46–55.
- Sahu GK, et al. 2009. Low-level plasma HIVs in patients on prolonged suppressive highly active antiretroviral therapy are produced mostly by cells other than CD4 T-cells. *J. Med. Virol.* 81:9–15.
- Sarmati L, et al. 2005. Association between cellular human immunodeficiency virus DNA level and immunological parameters in patients with undetectable plasma viremia level during highly active antiretroviral therapy. *J. Clin. Microbiol.* 43:6183–6185.
- Strain MC, et al. 2005. Effect of treatment, during primary infection, on establishment and clearance of cellular reservoirs of HIV-1. *J. Infect. Dis.* 191:1410–1418.
- Viard JP, et al. 2004. Impact of 5 years of maximally successful highly active antiretroviral therapy on CD4 cell count and HIV-1 DNA level. *AIDS* 18:45–49.
- Watanabe D, et al. 2011. Cellular HIV-1 DNA levels in patients receiving antiretroviral therapy strongly correlate with therapy initiation timing but not with therapy duration. *BMC Infect. Dis.* 11:146.