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Total HIV-1 DNA, a Marker of Viral Reservoir Dynamics with Clinical Implications

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SUMMARY

HIV-1 DNA persists in infected cells despite combined antiretroviral therapy (cART), forming viral reservoirs. Recent trials of strategies targeting latent HIV reservoirs have rekindled hopes of curing HIV infection, and reliable markers are thus needed to evaluate viral reservoirs. Total HIV DNA quantification is simple, standardized, sensitive, and reproducible. Total HIV DNA load influences the course of the infection and is therefore clinically relevant. In particular, it is predictive of progression to AIDS and death, independently of HIV RNA load and the CD4 cell count. Baseline total HIV DNA load is predictive of the response to cART. It declines during cART but remains quantifiable, at a level that reflects both the history of infection (HIV RNA zenith, CD4 cell count nadir) and treatment efficacy (residual viremia, cumulative viremia, immune restoration, immune cell activation). Total HIV DNA load in blood is also predictive of the presence and severity of some HIV-1-associated end-organ disorders. It can be useful to guide individual treatment, notably, therapeutic de-escalation. Although it does not distinguish between replication-competent and -defective latent viruses, the total HIV DNA load in blood, tissues, and cells provides insights into HIV pathogenesis, probably because all viral forms participate in host cell activation and HIV pathogenesis. Total HIV DNA is thus a biomarker of HIV reservoirs, which can be defined as all infected cells and tissues containing all forms of HIV persistence that participate in pathogenesis. This participation may occur through the production of new virions, creating new cycles of infection and disseminating infected cells; maintenance or amplification of reservoirs by homeostatic cell proliferation; and viral transcription and synthesis of viral proteins without new virion production. These proteins can induce immune activation, thus participating in the vicious circle of HIV pathogenesis.

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INTRODUCTION

HIV DNA persists in infected cells during combined antiretroviral therapy (cART), allowing the virus to reemerge from the reservoir if treatment is discontinued (1–3). The virus cannot currently be eradicated from the body, and treatment thus has to be maintained indefinitely. Recent clinical studies have rekindled hopes that HIV infection might be cured, notably by targeting viral reservoirs (4–7). An accurate, clinically relevant marker of HIV reservoirs is therefore needed (8).

Several markers have been proposed to quantify cell-associated HIV reservoirs (6) but there is no consensus method (6, 9–12). Intracellular HIV RNA load indicates the degree of ongoing HIV replication, while coculture assay of resting infected cells indicates their capacity to produce replication-competent virions. In contrast, total cell-associated HIV DNA is a global biomarker that includes integrated and nonintegrated viral genomes coding for both competent and defective viruses.

Total cellular HIV DNA is the focus of this review. It is easy to measure in whole blood, cell pellets, or tissues. Here, we examine the relevance of HIV DNA to HIV pathogenesis and persistence during both the natural and on-treatment course of infection, as well as its implications for tailored therapy and for trials of new approaches targeting HIV reservoirs.

PLACE OF TOTAL HIV DNA AMONG MARKERS USED TO EVALUATE HIV RESERVOIRS

There are many discussions and controversies concerning the best biomarker of HIV reservoirs. Two comprehensive studies have compared a panel of HIV reservoir biomarkers (9, 11), but such studies are limited by the fact that large amounts of blood are needed to test all markers in a given patient. The different approaches may be used differently according to the issue in question, such as the overall level of HIV infection in the body, viral persistence, reservoir activity, or the role of the HIV reservoir in maintaining immune activation. Clearly, no single marker can answer all these questions, but each can provide part of the answer. Markers used to quantify and monitor the HIV reservoir provide complementary information (6, 9).

Blankson et al. proposed to restrict the term "HIV reservoirs" to the cells or tissues where HIV persists in latent form but can reactivate in the form of replication-competent virus (13). This restricts the definition to resting infected cells. An alternative, broader definition of HIV reservoirs can also be proposed: all infected cells and tissues containing all forms of HIV persistence that can participate in HIV pathogenesis. This participation may occur through the production of new virions creating new cycles of infection and disseminating infected cells; maintenance or amplification of reservoirs by homeostatic cell proliferation; and viral transcription and synthesis of viral proteins without new virion production. These proteins can induce immune activation, thus participating in the vicious circle of HIV pathogenesis (14) (Fig. 1). Different biomarkers are relevant to each of these definitions.

Quantitative coculture assays measure the ability of infected cells (purified $CD4^+$ T cells or peripheral blood mononuclear cells [PBMC]) to produce infectious virions upon stimulation (3, 15, 16, 17, 18). It is based on the limiting dilution method, and the results are expressed as infectious units per million cells (IUPM) (6, 9). This viral outgrowth assay requires the purification of PBMC or resting CD4 T cells via use of magnetic beads or flow cytometry, as well as cell activation to induce virus production during several weeks in a secure laboratory and also quantification of cells able to release infectious particles (10, 15). It requires a large volume of blood (120 to 180 ml) or leukapheresis to obtain the necessary large number of viable cells. It is difficult to apply for frequent serial measurements in clinical studies (9, 15) because it is labor-intensive and expensive. Its reproducibility has been estimated in one laboratory in terms of the coefficient of variation (0.95) and the confidence interval (CI) for one measurement ($\pm$0.7 log), indicating it is unreliable for detecting small differences in the size of the HIV reservoir (9, 19, 20). Reproducibility across laboratories has not yet been evaluated. Its limitations are illustrated by the case of the "Mississippi child," a perinatally HIV-1-infected infant who experienced a viremic relapse after entering remission; quantitative coculture remained negative (in a cumulative total of 64 million resting CD4 T cells) throughout the period of virologic remission, whereas HIV DNA was detectable in PBMC and CD4 T cells (positive signal, $<$4 copies/10^6 cells). Her subsequent viral rebound 27.6 months after cART discontinuation proved the persistence of infectious virus somewhere in the "Mississippi child" in blood or unsampled tissues (21). Finally, despite its limits, this assay is certainly useful for some research purposes, such as estimation of blood reservoir productive activity.

Another marker is intracellular HIV RNA load, which measures HIV transcription in infected cells or viral entry. Cell-associated HIV RNA rapidly declined after cART initiation, mimicking cell-free HIV RNA decay, before reaching a plateau (22). This plateau is explained by the fact that cART does not stop HIV transcription. The level of this plateau does not correlate directly with HIV replication, because of insufficient transcription or nuclear retention of viral RNA in resting cells, or transcription of defective viruses (22). Cell-associated HIV RNA might be very useful for cure research, because it quantifies a dynamic process of transcription and reflects the activity of the HIV reservoir (22). It can be helpful for evaluating residual, transcriptionally active infected cells in patients on treatment. Several assays have been developed to quantify this intracellular HIV RNA (22), and their reproducibility across multiple labs needs to be evaluated.

Total HIV DNA, as its name suggests, includes both integrated and unintegrated HIV DNA (that includes episomal 1-long terminal repeat [1-LTR], 2-LTR, and linear HIV DNA) and reflects the global level of the total reservoir (Fig. 1). Each of these separate forms is difficult to quantify in the absence of a standardized, reproducible assay (6). Total HIV DNA assay does not distinguish between replication-competent and -defective viral genomes (9, 17). These forms of HIV DNA can coexist in infected cells. The neosynthesized double-strand HIV DNA is linear. This form is the prevalent form of reverse-transcribed genomes in infected cells and constitutes a preintegrated form of latency (3, 23–25). It is labile if it does not integrate into the host genome (26, 27). HIV DNA can also be circularized in episomal forms with one or two LTRs. Forms with one LTR are due to homologous recombination, whereas forms with two LTRs result from ligation of the viral genome (28). In quiescent CD4 T cells, episomal forms represent less than 10% of unintegrated HIV DNA (3). Two-LTR forms represented 0.1% to 0.6% of integrated forms in cell lines and peripheral blood mononuclear cells infected *in vitro* (29). Data on 2-LTR forms are conflicting. Some authors consider them labile and to be present only during recent infection (30, 31), but *in vitro*

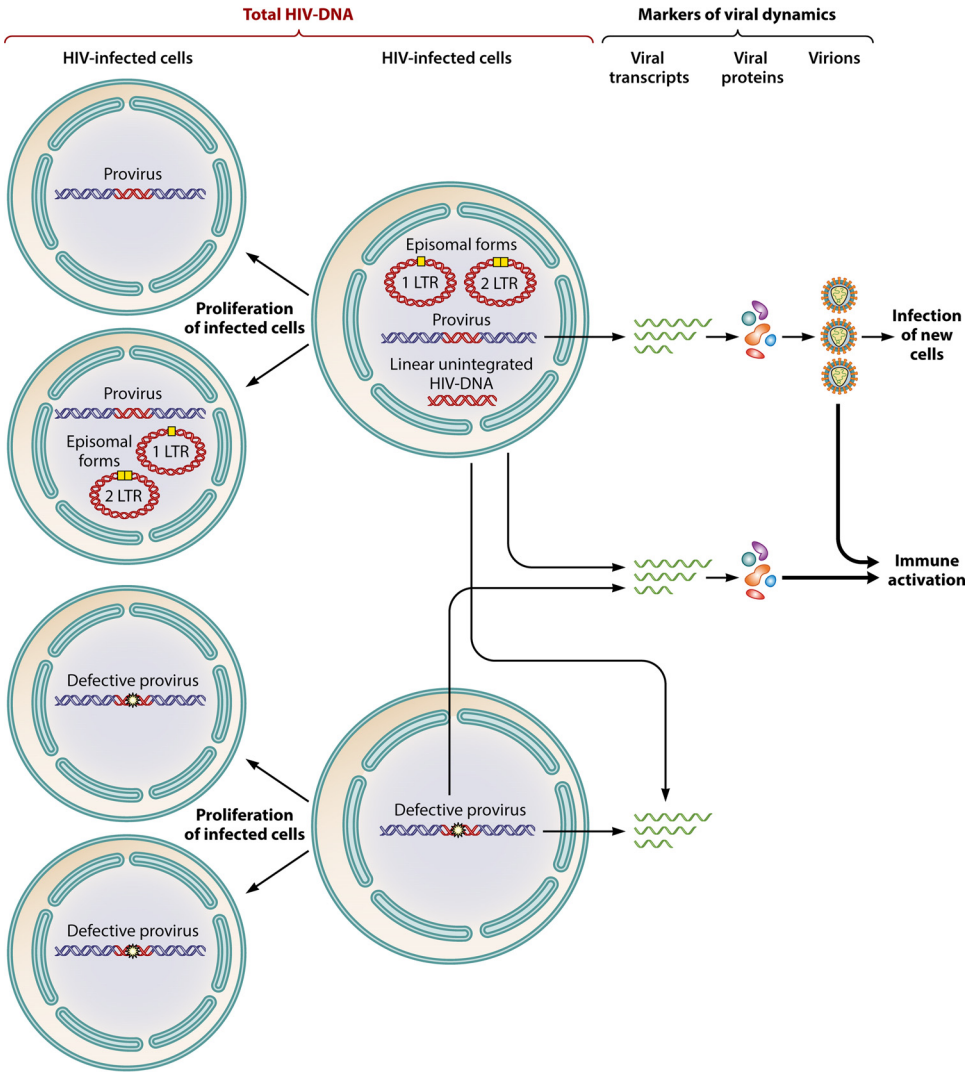

FIG 1 Several forms of HIV DNA compose the total HIV DNA and participate in HIV pathogenesis. The integrated form, the provirus, is the more persistent form and permits production of virions when quiescent infected cells are reactivated. Virions can infect new cells and propagate infection and the HIV reservoir. The provirus form persists in all cells during cell proliferation. Episomal forms with 1-LTR or 2-LTR persist and are diluted in some daughter cells during cell proliferation. Linear unintegrated HIV DNA is the more labile form and is essentially present when the virus is replicating. Defective provirus, with a deletion, nonsense mutation, or hypermutation, cannot produce new virions but can produce transcripts and viral proteins which could activate the immune system and participate in HIV pathogenesis.

studies that eliminated confounding factors, such as infected cell death, and *in vivo* studies have demonstrated that they are stable (32–39). The number of 1-LTR and 2-LTR HIV DNA molecules decreases by dilution during cell division or cell death (32, 34, 40) (Fig. 1). These forms can be renewed by viral replication (30, 41). 2-LTR forms have been quantified during trials evaluating intensification with integrase inhibitors upon the hypothesis that these antiretrovirals enhance 2-LTR HIV DNA if residual replication occurs during cART (42). These unintegrated forms constitute an inducible viral reservoir (43) and can participate in HIV transcription, in the replication cycle (when they are complemented by integrated forms), and in the integration and synthesis of new virions (44–53). Integrated HIV DNA is the most stable form and constitutes the postintegrated form of latency (16, 54). It persists during cell proliferation (Fig. 1). This provirus has an estimated frequency of 1 copy per infected blood cell (55). It contributes to viral rebound after cART interruption. A significant percentage of infected resting CD4 T cells harbors proviruses that are either defective because of hypermutation or deletion or transcriptionally silenced (17, 56). Some studies have suggested that the frequency of resting HIV-infected cells, estimated by total HIV DNA quantification among resting CD4 T cells, is considerably higher than the frequency of infected cells capable of viral outgrowth (differences above 2 logs) (9). Some of these transcriptionally silent cells may be inducible. These data suggest that a significant proportion of the total HIV DNA measured by PCR is not being made into HIV RNA, protein, and viral particles. Cells harboring defective or silent proviruses do not therefore contribute to viremia (17, 56) but could participate in pathogenesis (Fig. 1). Total HIV DNA load correlates with the frequency of cells containing replication-competent virus ($r = 0.73$, $P = 0.0009$) (57). Eriksson et al. also reported a correlation between total HIV

DNA load in resting $CD4^+$ T cells (but not in PBMC) and the quantity of replication-competent virus ($r = 0.45$, $P = 0.08$) (9). Similar results were recently reported by Kiselinova et al. (11). More recently, Noel et al. also reported that the low level of total HIV DNA in cells from long-term nonprogressors (LTNPs) correlated with the low efficiency of virus production after activation *ex vivo* (58).

By comparison with other markers, total HIV DNA has the advantage of easy quantification by standardized, sensitive, real-time PCR (59–68), a method suitable for analyzing large numbers of samples with accuracy. It requires a relatively small amount of blood and can therefore be used for HIV diagnosis in young children, for example. It can be quantified in blood and other body fluids, is unaffected by freeze-thawing, and is the method most widely used to quantify the HIV reservoir in tissue biopsy specimens. It produces reproducible results with small errors (59, 69). The interassay reproducibility of real-time PCR quantification of total HIV DNA has been evaluated in a laboratory by using two controls, one high and one moderate, during more than a 9-month period. Means, coefficients of variation, and 95% confidence intervals (CIs) were, respectively, 3.30 log copies/10^6 leukocytes, 0.03, and ±0.22 log for the high-level sample and 2.40 log copies/10^6 leukocytes, 0.07, and ±0.35 log for the moderate-level sample. Figure 2 shows the high reproducibility of this assay. Reproducibility has also been evaluated for patient samples, with good performance (59). These values are lower than those reported with the viral outgrowth assay (9, 19, 20). A kit is commercially available (Biocentric, Bandol, France) that was developed with the support of the French HIV/AIDS Research Agency (ANRS) to facilitate comparisons of results from different laboratories. For example, HIV DNA load is an inclusion criterion in an ongoing cART de-escalation trial (TRULIGHT; NCT02302547 at ClinicalTrials.gov), in which it is quantified with the same assay after an interlaboratory quality-control procedure. Interlaboratory comparisons have had good results (69). More recently, an assay that dispenses with the extraction step has been proposed (70). Recent technical progress has permitted the development of HIV DNA quantification by digital droplet PCR (ddPCR), which does not require an external quantification standard (71–77). However, some false-positive signals have been observed with ddPCR, which can be a problem when this method is used for diagnosis (76). Moreover, ddPCR cannot help with quantification of low signals, for instance in HIV controllers. In fact, quantification of low signals follows the Poisson distribution, necessitating study of many cells and several replicates, whatever the technology (real-time PCR or ddPCR).

It is very unlikely that a single marker will alone provide sufficient information on viral reservoir status (8). However, the fact that HIV DNA shows a certain degree of correlation with other markers, including viral outgrowth assay results, indicates that HIV DNA may be usefully included in biomarker panels, whatever the question in hand. Total HIV DNA load is the most widely studied marker of the HIV reservoir, having been measured in large patient cohorts and trials at different stages of the infection.

SPECTRUM OF TOTAL HIV DNA LEVELS IN BLOOD

A broad range of total HIV DNA levels has been observed during the natural and therapeutic courses of HIV infection (Fig. 3).

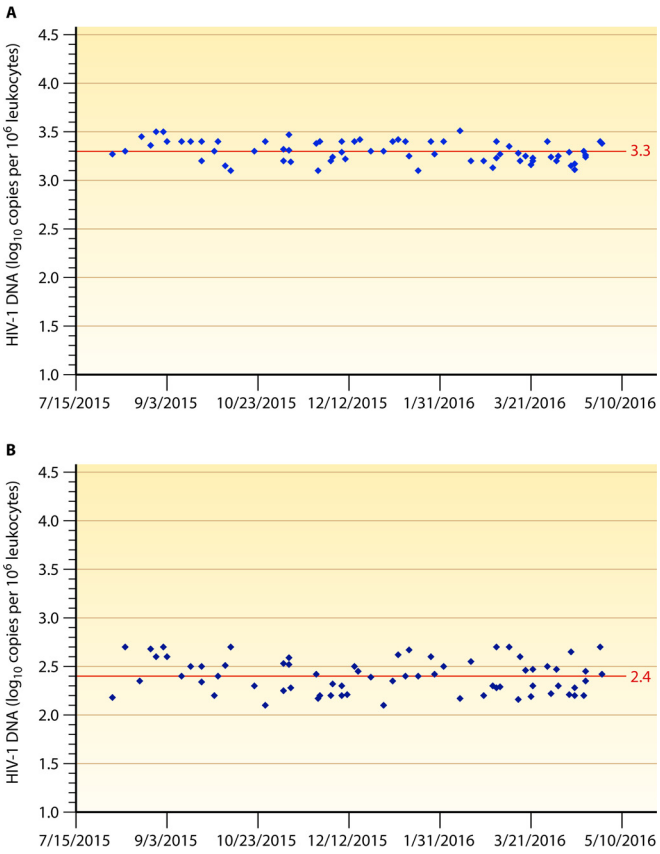

FIG 2 Reproducibility of total HIV DNA quantification in two positive controls by real-time PCR (59). For this experiment, two different pools of blood cells (in EDTA) from HIV-infected patients were prepared. Aliquots were frozen at −80°C. One aliquot of each control (high-level positive control [A] and moderate-level positive control [B]) was tested in all assays performed during a >9-month period to verify the assays reproducibility over time. Results presented here are for 69 quantifications performed by different technicians and with different thermocyclers and with four lots of Biocentric reagents, for the high-level positive control (A) and moderate-level positive control (B). Means (red lines), standard deviations, coefficients of variation, and 95% confidence intervals were, respectively, 3.30 log copies/10^6 leukocytes, 0.11 log, 0.03, and ±0.22 log for the high-level positive-control sample and 2.40 log copies/10^6 leukocytes, 0.18 log, 0.07, and ±0.35 log for the moderate-level positive-control sample.

Total HIV DNA in Blood during Untreated Infection

Cellular HIV DNA can be detected very early after infection. Ananworanich et al. reported that patients studied a few days after infection had a median total blood HIV DNA load of 0.9 log_{10} copies/10^6 PBMC at Fiebig stage I (at which only HIV RNA is detectable; 8 patients) and 2.7 log_{10} copies/10^6 PBMC at Fiebig stage III (detection of HIV RNA and HIV-specific antibodies is possible by enzyme-linked immunosorbent assay [ELISA] but not by Western blotting; 15 patients) ($P = 0.01$) (78). This was recently confirmed in a larger study (79). In the ANRS PRIMO cohort, the median HIV DNA load was 3.30 log_{10} copies/10^6 PBMC (range, <1.84 to 4.93) at an estimated median of 47 days after infection; it was significantly higher in patients with more acute infection (with only 0 or 1 HIV-specific antibody) than in patients included a bit later (with ≥2 antibodies), and also in patients who had a symptomatic primary infection (80). During the 6 months following infection, the median total HIV DNA level

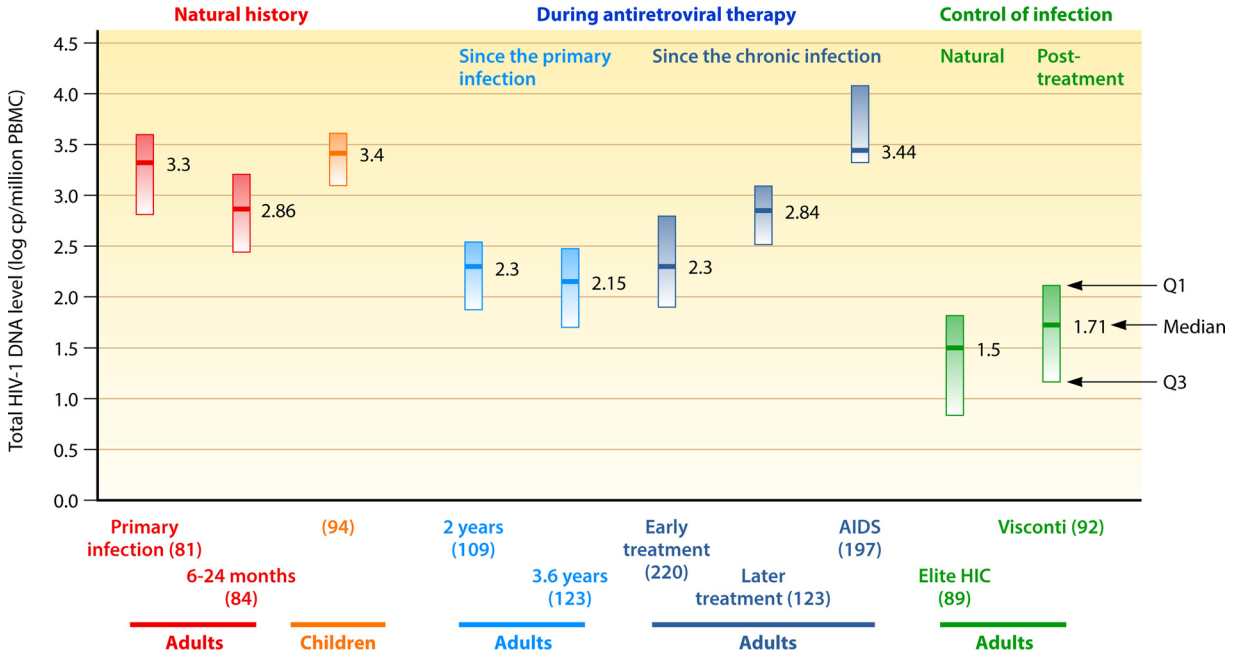

FIG 3 Spectrum of total HIV-1 DNA levels in PBMC during HIV infection. The natural history data are from HIV DNA quantified for 552 adults at the time of the primary infection (PRIMO cohort, ANRS) (81), for 271 patients who had seroconverted 6 to 24 months previously (SEROCO cohort, ANRS) (84), and for 121 perinatally infected children (median age, 6 years), of whom 46.6% and 20.3% were at CDC stage B and C, respectively (ANRS 1244/1278) (94). The data set for "during antiretroviral therapy" is for HIV DNA quantified during antiretroviral therapy initiated during the primary infection and continued for 2 years (90 patients; OPTIPRIM trial, ANRS) (109) or for a median of 3.6 years, with HIV RNA levels of <50 copies/ml for a median of 3.1 years ($n = 35$) (123). HIV DNA was quantified during antiretroviral therapy initiated early during the chronic phase in 116 adults (CD4 cell count, >350/mm^3; plasma HIV RNA, <4.7 log copies/ml), with antiretroviral therapy for a median of 5.3 years (SALTO trial, ANRS) (220) and in 272 adults treated later (median CD4 cell nadir, 228/mm^3; plasma HIV RNA, 5.3 log copies/ml) for a median of 7.3 years, with HIV RNA levels at <50 copies/ml for a median of 3.9 years (123). HIV DNA was quantified in 44 adults with advanced therapeutic failure and AIDS (CD4 count, ≤200/mm^3; HIV RNA level, >4 log; genotypic score showing two or fewer active drugs) (ETOILE trial, ANRS) (197). For the HIV control data set, HIV DNA were quantified for patients who controlled the infection naturally (plasma HIV RNA undetectable for >10 years in the absence of antiretroviral treatment [15 patients]) (HIV controllers cohort, ANRS) (89) or after treatment interruption (14 patients) (VISCONTI study, ANRS) (92). The same standardized assay was used in the same laboratory. A broad range of HIV DNA levels was found. Medians and interquartile ranges are indicated.

in blood was 2.8 to 3.2 $\log_{10}$ copies/10^6 PBMC among patients in the French PRIMO cohort (Fig. 3) and the Ivorian PRIMOCI cohort (674 and 200 patients, respectively) (80–83).

The total blood HIV DNA level was 2.86 $\log_{10}$ copies/10^6 PBMC during the 6 to 24 months following infection in 271 patients included in the ANRS SEROCO cohort (Fig. 3) (84).

During the chronic phase, the median HIV DNA level was 2.65 $\log_{10}$ copies/10^6 PBMC in 130 patients (85) and was quite stable over time (85–87).

Patients who naturally control HIV infection (long-term nonprogressors and elite controllers) have very low and stable total HIV DNA levels, with median values of 2.3 and 1.5 $\log_{10}$ copies/10^6 PBMC reported in, respectively, 66 and 15 patients (88–90). Similarly, posttreatment controllers in the VISCONTI cohort, for whom treatment was initiated early during the primary infection and long-term viral control after treatment interruption was maintained, had low total HIV DNA levels (median, 1.71 $\log_{10}$ copies/10^6 PBMC; 14 patients) (91, 92) (Fig. 3).

Very few data are available for untreated AIDS patients. HIV DNA load expressed per 10^6 CD4 T cells increased in one study during infection, reflecting the increasing proportion of infected cells among the depleted CD4 T cell subset (24 patients) (87).

Infected teenagers included in the ANRS IMMIP study had HIV DNA levels similar to those found in adults (93). Eighty-one Ivorian children, studied mainly at stage B or C of HIV infection, had a median of 3.4 $\log_{10}$ copies/10^6 PBMC (94) (Fig. 3).

Thus, a broad range of total HIV DNA levels is observed among patients. Moreover, HIV DNA levels vary widely during the natural course of infection, especially during the first few weeks.

Decline in Total HIV DNA during cART

The decline in total blood HIV DNA in patients adherent to cART has been extensively studied in both adults and children (57, 77, 78, 82, 95–109). This decline varies among patients. It correlates directly with pretherapeutic HIV DNA levels (97, 102) and HIV RNA levels (97), as well as with the baseline CD4 cell count (102), the CD4 cell increment (97, 100), and the chance of achieving HIV RNA loads of <2.5 copies/ml (107). Pretherapeutic HIV RNA load was also predictive of the decline in HIV DNA load in children, independently of pretherapeutic HIV DNA load (110). HIV DNA load before cART was also predictive of HIV DNA load 2 years after cART initiation and of the time to aviremia in children (111). A sharp decrease (≥0.5 log) in HIV DNA levels was significantly correlated with an improvement in the CD4 T cell count (112). A recent study of children showed lower HIV DNA levels after cART initiation when the plasma HIV RNA level was rapidly controlled (77).

HIV DNA decay is also dependent on the amount of cell-associated HIV RNA in blood and lymph nodes; residual viremia correlates with slower clearance of HIV-1-infected cells, possibly owing to infection of new cells (97). Another, larger study confirmed the association between HIV DNA levels during cART and markers of the dynamics of HIV reservoirs (residual HIV RNA, cell-associated HIV RNA, and 2-LTR HIV DNA) (113). However, another study failed to show a correlation between the HIV DNA slope or level on the one hand and, on the other hand, either residual viremia or the percentage of activated $CD8^+$ T cells (averaged over years 1 to 4 after cART initiation) (108). This point therefore needs further investigation.

The rapid initial HIV DNA decay is mostly due to the decay of linear unintegrated DNA (114). Integrated forms decay slowly (115). 2-LTR forms decline more than integrated forms, and the decay of 2-LTR forms and integrated forms is slower in activated CD38 CD4 T cells than in nonactivated cells (115). In some patients on cART, total HIV DNA can be almost exclusively composed of integrated HIV DNA (104, 114, 116, 117), whereas other studies suggest that some nonintegrated HIV DNA forms can also persist (115, 118) and that unintegrated HIV DNA forms remain more frequent than integrated forms in quiescent CD4 T cells and monocytes after several months of cART (119, 120, 121). Heterogeneity among patients could explain these discrepancies.

Time from HIV-1 Infection to Treatment Initiation Influences Total HIV DNA Decay and Levels during cART

cART reduces the HIV DNA load more markedly when initiated during primary HIV infection (PHI) than during chronic HIV infection (CHI) in adults and children (Fig. 3) (57, 77, 82, 101, 105, 122, 123).

In a longitudinal study of 307 patients, the decline in HIV DNA was analyzed with a nonlinear mixed-effects model that included >1,100 HIV DNA data points. The model showed two phases of decay. During the first 2 years, a rapid decline in HIV DNA was observed both in patients treated since PHI and in patients first treated during CHI, with similar HIV DNA half-lives (113 days and 146 days, respectively). The second decay phase was much slower, and the half-life was significantly shorter in the PHI group (25 years) than in the CHI group (377 years; $P < 0.001$). At the end of the study, after a median duration of viral suppression of 4 years, HIV DNA levels were significantly lower in the PHI group than in the CHI group (median, 2.15 versus 2.84 log copies/10^6 PBMC; $P < 0.0001$) (Fig. 3) (123). Logistic regression analysis showed that starting cART during PHI (odds ratio [OR], 16; 95% CI, 3.5 to 72.3) and a low pretherapeutic HIV DNA level (<3.3 log; OR, 4.8; 95% CI, 1.2 to 19.3) were independent predictors of reaching "optimal viro-immunological responder status" (i.e., HIV DNA of <2.3 log copies/10^6 PBMC, associated with normalization of absolute/relative CD4 T cell counts and the CD4/CD8 ratio) (123). Recently, a mixed-effects model of the ANRS PRIMO cohort data set, using >1,300 HIV DNA values from 327 patients, demonstrated that the timing of cART initiation during PHI could influence the first slope of HIV DNA decline: the earlier cART was initiated after infection, the faster HIV DNA levels fell during the first 8 months on treatment (-0.171, -0.131, and -0.0068 $\log_{10}$ copies/10^6 PBMC per month when cART was initiated 15 days, 1 month, and 3 months after infection, respectively; $P < 0.0001$) (124). Ultimately, the earliness of cART initiation impacts HIV DNA levels observed after 5 years of effective treatment (1.62 and 2.24 $\log_{10}$ copies/10^6 PBMC, respectively, when cART is initiated 15 days and 3 months after infection; $P = 0.0006$) (124).

Buzon et al. reported a statistical correlation between the time from HIV infection to treatment initiation and the total HIV DNA level after 10 years of continuous treatment in a cohort of adults first treated early in the infection (57). The lowest HIV DNA levels were observed in patients who had low baseline levels and who received early treatment (107). In children, the HIV DNA level was markedly lower when viral control was achieved before age 1 year than between the ages of 1 and 5 years or after age 5 years (73). This difference is linked to the duration of uncontrolled viral replication between initial infection and effective treatment. The lower level of HIV DNA in children treated early versus late correlates with a lower level of replication-competent virus (75).

Because of its impact on the HIV DNA level, antiretroviral treatment of newborns within the first hours after infection has consequences for diagnosis during the first months of life, a period when maternal antibodies interfere with serological tests. Antiretroviral prophylaxis of mother-to-child HIV transmission both during pregnancy and in newborns can mask the primary infection in newborns. In the French perinatal cohort, HIV DNA levels, like viremia, were lower at age 1 month among infected infants receiving multidrug prophylaxis (levels were sometimes near the limit of quantification), stressing the need for highly sensitive assays to diagnose neonates born to HIV-infected mothers (59, 125). Under these conditions, total HIV DNA can represent an early diagnostic marker in infants (59, 126). HIV DNA can also be quantified in dried blood spots by real-time PCR, which can be helpful for this diagnosis in resource-limited countries (127).

Thus, the time from HIV-1 infection to treatment initiation strongly impacts the initial rate of decay of total HIV DNA and the level achieved after several years of cART.

Total HIV DNA levels show a broad range of values (Fig. 3) during the natural course of infection, during antiretroviral therapy (initiated during either PHI or CHI), and also during therapeutic failures or AIDS. Patients who control the infection naturally or after treatment interruption have very low HIV DNA levels (37, 61, 88, 89, 91, 92, 128).

TOTAL HIV DNA LEVELS IN TISSUES AND FLUIDS REFLECT THE SPREAD OF INFECTION THROUGH THE BODY

Measurement of total HIV DNA in tissues and fluids provides interesting information on the pathogenesis of HIV infection, as it indicates the spread of the virus through the body (Fig. 4).

Lymph Nodes

The lymph nodes are a major reservoir site, with a large pool of target cells, a high level of activation, and a high level of replication, inducing infection of new cells (Fig. 4). Lymph nodes thus play an important role in the dynamics of HIV reservoirs, in an intricate relation with CD4 T cell dynamics. Follicular helper CD4 T cells (T_{FH}) play an important role in the lymph node HIV reservoir and are infected during their differentiation (129). A recent study compared viral DNA levels in different cell subsets from lymph nodes of simian controllers and progressors. It was found that T_{FH}, one of the most numerous subsets of effector T cells, harbored high levels of viral DNA, similar to those observed in non-T_{FH} from progressors but significantly higher than those observed in non-T_{FH} from controllers (130). This was linked to preferential production of replication-competent virus in B cell folli-

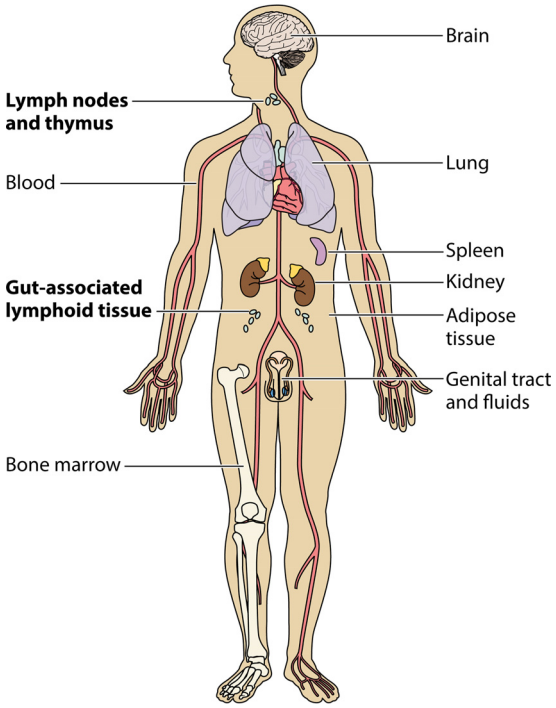

FIG 4 Anatomical HIV reservoirs. Reservoir cells are highly disseminated.

cles of lymph nodes and spleens from controllers, where T_{FH} are localized (130). These B cell follicles were anatomically protected from specific CD8 T cell responses in controllers, explaining the difference in viral DNA levels between T_{FH} and non-T_{FH} cells (130). Moreover, in monkeys receiving cART, infected T_{FH} were more strongly involved in residual replication than other T cells: more simian immunodeficiency virus (SIV) RNA was produced by T_{FH} for a given SIV DNA level (130). Lymph nodes thus constitute an obstacle to HIV remission, as they are not readily accessible by specific immune responses (130) or antiretrovirals (131). Phylogenetic analyses have shown viral evolution in lymphoid tissue during cART; this reflects ongoing replication that replenishes the HIV reservoir (132). When cART is interrupted, many cells expressing different viral RNA variants are found, contributing to viral rebound (133). These new data demonstrate that lymph node cells harboring viral DNA permit productive infection even in the presence of suppressive cART. The very rare T_{FH} in blood also contribute to the HIV reservoir in patients receiving cART (134).

Gut-Associated Lymphoid Tissue

Gut-associated lymphoid tissue (GALT), which harbors 60% of the body's lymphocytes, plays an essential role in the pathogenesis of HIV infection, through Th17 cell depletion, bacterial translocation, and local host cell activation (facilitating HIV replication); this has been reported both in humans and in simian models (135–157) (Fig. 4). Gastrointestinal CD4 T cells have been shown to harbor, on average, 13 times more HIV DNA than blood CD4 T cells during acute and early-stage HIV infection (151, 158–160). Non-CD4 T cells harbored less HIV DNA than CD4 T cells in the gut, but the infection level of non-T leukocytes was higher in GALT than in blood (161). Myeloid cells also harbor HIV DNA in GALT (162). A recent study of patients with acute infection showed that HIV DNA in the gut was significantly lower at Fiebig stage I than at Fiebig stages II to IV (79). The HIV DNA load in GALT declines after cART initiation (78) but does not disappear (150, 158, 163, 164), and HIV DNA levels vary at different gut sites (151, 158–161). Memory effector cells harbored the most HIV DNA in the ileum and rectum (161). HIV DNA in the rectum remained higher in cART-treated patients than in HIV controllers (165). Total HIV DNA in GALT correlates with total HIV DNA load in blood at different stages of the infection, both without and during treatment (139, 158). This finding could partly explain the relevance of HIV DNA to HIV pathogenesis, even when measured only in blood, like the CD4 T cell count and HIV RNA load, two routine follow-up markers.

Other Tissues and Fluids

The central nervous system constitutes a viral reservoir, and circulating monocytes play a particular role in transferring the infection to the brain (166). HIV DNA is also detected in astrocytes (167). cART can often control local replication, but cells harboring viral DNA persist (168, 169).

HIV DNA is quantifiable in semen in nonsperm cells (170, 171). It can persist in the genital tract of women on long-term effective cART (HIV RNA, <50 copies/ml at >6 months) and has been linked to current residual viremia (OR, 3.4; 95% CI, 1.1 to 10.9) and to a history of AIDS-defining illness (OR, 11; 95% CI, 2 to 61) (172). The presence of HIV in genital secretions should be taken into account when estimating the residual risk of transmission.

HIV DNA is also useful for detecting infection of kidney grafts in HIV-infected recipients who had undetectable plasma HIV RNA at the time of transplantation (173). HIV was found to infect the kidney allograft in 68% of cases and might influence graft survival. Detection of HIV DNA and HIV RNA in a patient's urine is a noninvasive way of monitoring kidney graft infection (173, 174).

In the context of stem cell transplantation for AIDS-related lymphoma, total HIV DNA load in the autograft predicts the post-transplant HIV peripheral reservoir size in patients on continuous highly active antiretroviral treatment (175).

Recently, adipose tissue, and particularly memory CD4 T cells in this tissue, has been identified as a potentially important HIV reservoir, with the detection of total viral DNA in untreated macaques but also in patients on effective treatment (176, 177). This reservoir is inducible by *ex vivo* activation.

In the future, measurement of total HIV DNA in different tissues and fluids may help to evaluate therapeutic strategies designed to eradicate viral reservoirs.

TOTAL HIV DNA: A CLINICALLY RELEVANT MARKER

Total HIV DNA Load Is Predictive of the Natural Disease Course, Independently of HIV RNA Load and the CD4 T Cell Count

Total HIV DNA load in PBMC varies widely among untreated HIV-infected adults and children (82, 85, 102, 110, 178, 179), even during PHI, with an interquartile range of 2.7 to 3.5 $\log_{10}$ copies/10^6 PBMC (Fig. 3) (81). The range of HIV DNA levels was smaller than the range of HIV RNA levels, but nonetheless identified several patient subgroups in large cohorts; in particular, LTNPs and elite controllers had significantly lower HIV DNA levels than pro-

gressors (61, 88–90) (Fig. 3). Likewise, symptomatic patients had significantly higher HIV DNA loads than asymptomatic patients during CHI (179). This heterogeneity of HIV DNA loads has noteworthy implications for the course of infection.

Total HIV DNA load in the first 6 months following seroconversion is predictive of immunologic progression, independently of HIV RNA load and the CD4 T cell count (81, 106, 180). In the ANRS PRIMO cohort, patients who progressed rapidly had a median total HIV DNA load of 3.3 log, compared to 3.0 log in other patients (81). Similar results were obtained in the first 19 or 24 months after seroconversion (83, 84), with higher HIV DNA levels having major independent prognostic value for progression to clinical AIDS, <200 CD4 cells/mm^3, and death. Patients who had not progressed more than 5 years after seroconversion had significantly lower HIV DNA load in the months following seroconversion than patients who did not control the infection (2.02 log versus 2.90 log) (181). LTNPs with HIV DNA loads of >1.85 log at seroconversion and a large increase in HIV DNA over time had an increased risk of losing their LTNP status (182). These data show that even small differences in HIV DNA levels influence the course of HIV infection.

The predictive value of HIV DNA load for progression to AIDS and death was confirmed during CHI, independently of age at seroconversion, the CD4 cell count, and HIV RNA load (85).

A meta-analysis of six studies with a total of 1,074 participants indicated that total HIV DNA was a strong predictive marker of AIDS (relative risk [RR], 3.01, 95% CI, 1.88 to 4.82) and of all-cause mortality (RR, 3.49; 95% CI, 2.06 to 5.89) (183). It was a significantly better predictor for progression to AIDS than was HIV RNA (ratio of RRs, 1.47; 95% CI, 1.05 to 2.07) and for a combined endpoint of AIDS and death (ratio of RRs, 1.51; 95% CI, 1.11 to 2.05) (183).

In summary, HIV DNA levels vary among patients during the course of the infection and have high predictive value for disease progression (Fig. 3).

Pretherapeutic Total HIV DNA Levels in PBMC Are Predictive of Virologic, Immunologic, and Clinical Responses to Antiretroviral Therapy

HIV DNA load is predictive of the long-term success of cART. Indeed, patients with lower blood HIV DNA loads at cART initiation have better virologic and immunologic responses to treatment and longer survival (179).

Lower baseline HIV DNA levels were predictive of achieving an undetectable HIV RNA load on cART (103, 178, 184–186), while high HIV DNA load was associated with persistent residual HIV RNA below 50 copies/ml during cART, independent of baseline HIV RNA load (185). This was confirmed by Parisi et al., who showed that baseline HIV DNA load predicted the residual HIV RNA plasma level during effective cART (107).

Pretherapeutic HIV DNA load has been linked to immune cell activation status during cART, correlating positively with the number of Ki-67$^+$ CD8 T cells after 6 months of treatment (187).

Baseline HIV DNA load was predictive of overall survival among patients with relapsing or refractory HIV-related lymphoma treated with high-dose chemotherapy followed by autologous stem cell transplantation (188).

Total HIV DNA Load during cART Is Informative of Patient Pretherapeutic and Therapeutic History

HIV DNA load during treatment correlated with blood HIV DNA load at cART initiation in adults and children (82, 103, 110). In adults, HIV DNA load at years 4, 7, and 10 of antiretroviral treatment correlated positively with the pretherapeutic HIV DNA level (108). Likewise, Ananworanich et al. showed that total blood HIV DNA at cART initiation predicted the HIV reservoir size at week 24 of treatment that was started during PHI ($P < 0.001$) (78). The plasma HIV RNA zenith and the CD4 cell nadir were predictive of HIV DNA load during cART in patients whose plasma HIV RNA remained below the detection limit for more than 3 years; HIV DNA load during cART correlated positively with pretherapeutic HIV RNA load and negatively with the CD4 cell nadir (189, 190, 191, 192). Total HIV DNA load, measured 6 weeks after cART initiation, was the strongest independent predictor of the pretherapeutic HIV RNA level (193). Thus, total HIV DNA load during cART reflects pretherapeutic characteristics of HIV infection, including HIV DNA load, the plasma HIV RNA zenith, and the CD4 cell nadir.

Multivariate analysis indicated that low HIV DNA load in patients with cART for a median of 25 months was significantly associated with prolonged HIV RNA suppression on cART (194). HIV DNA load during cART was independently associated with cumulative HIV RNA viremia over the previous 5 years (93, 192). HIV DNA load could therefore reflect therapeutic adherence over time. Furthermore, in patients on effective cART, total HIV DNA correlated with residual plasma viremia, measured with an ultrasensitive assay (115, 190, 191, 195). Total HIV DNA load, measured 6 weeks after cART initiation, was predictive of virologic outcome in asymptomatic, chronically HIV-1-infected persons (193). Median HIV DNA load was 2.20 log copies/10^6 cells (range, 0.70 to 2.80) in patients treated for at least 10 years without ART interruptions or immunomodulatory therapy and selected on the basis of undetectable HIV RNA throughout follow-up, with at least one yearly HIV RNA measurement, and a total of more than 20 measurements (196). Conversely, in patients with advanced therapeutic failure and AIDS, median HIV DNA load was 3.44 log copies/10^6 PBMC (197) (Fig. 3). cART-treated patients with X4-tropic viruses in HIV DNA, reflecting more advanced disease, had higher levels of HIV DNA than patients with R5-tropic viruses (198).

High HIV DNA load in PBMC is associated with intermittent HIV shedding in the semen of men who have sex with men and who have been on successful antiretroviral therapy for >6 months: after multivariable adjustments, total HIV DNA (OR of 2.6 and 95% confidence interval [CI] of 1.2 to 6.0 for >2.5 $\log_{10}$ copies/10^6 PBMC; $P = 0.02$) and cannabis use accompanying sexual intercourse (OR of 2.8, CI of 1.2 to 6.7; $P = 0.02$) were the two factors significantly associated with HIV RNA detection in seminal plasma (199).

Moreover, multivariate analysis showed that low total HIV DNA loads in patients on long-term suppressive antiretroviral treatment were clearly associated with concomitant residual plasma viremia of <1 copy/ml, a high CD4/CD8 cell ratio, and a high CD4 T cell percentage ($P < 0.0001$) (191). Several studies have shown that a lower HIV DNA load during cART is associated with better immune recovery (194, 200, 201). Similarly, a low HIV DNA load was found to be predictive of better immune restora-

tion in children on cART (202) and was associated with CD4 cell count dynamics during cART combined with interleukin-2 (203). In patients with advanced therapeutic failure and AIDS, a larger viral reservoir was associated with poorer CD4 cell recovery during optimized background therapy (197). This was confirmed by Hatano et al., who showed that high HIV DNA levels during cART were associated with low CD4 cell counts (204).

Overall, total HIV DNA loads in blood, which are influenced by the timing of cART initiation, have been linked to multiple outcomes during cART: cumulative HIV RNA viremia, which reflects therapeutic failure; residual viremia; immune recovery.

Although it is beyond the scope of this review, several studies have investigated the value of analyzing genotypic resistance on HIV DNA, which could detect archived resistant quasispecies (205–207).

In conclusion, total HIV DNA in PBMC or blood can be used as a virologic marker in patients on long-term effective cART. As observed during the natural history of HIV infection, total HIV DNA reflects the dynamics of HIV infection and the HIV reservoir during cART. It is the most easily quantifiable virologic marker in patients on cART, and several studies have shown that it is related to the history of infection and to therapeutic efficacy.

Total HIV DNA in PBMC Is Predictive of the Presence and Severity of Some HIV-Associated Disorders

HIV DNA has an important role in the pathogenesis of neurologic disorders. Indeed, total HIV DNA load in PBMC is predictive of HIV-associated dementia and correlates with the severity of HIV-associated neurocognitive disorders (208, 209). Monocyte HIV DNA load at treatment initiation can predict cognitive performance at 48 weeks (210).

TOTAL HIV DNA CAN HELP GUIDE THERAPEUTIC STRATEGIES

Biological markers are needed to evaluate strategies such as de-escalation (to reduce drug exposure and toxicity) and structured treatment interruption. Several studies have shown that HIV DNA load can be useful in this setting.

De-escalation Therapy

Baseline HIV DNA was found to be predictive of the virologic response to treatment induction with (211) or a treatment switch to (212) ritonavir-boosted protease inhibitor monotherapy. The MONARK study showed that nonresponders to first-line ritonavir-boosted lopinavir (LPV/r) monotherapy had significantly higher baseline HIV DNA levels (3.16 $\log_{10}$ copies/10^6 PBMC) than responders (2.86 $\log_{10}$ copies/10^6 PBMC) (211). Multivariate analysis of the MONOI study data set reported that the HIV DNA level at the time of a switch to ritonavir-boosted darunavir (DRV/r) monotherapy predicted the risk of viral rebound at week 96 (odds ratio, 2.66; $P = 0.04$) in patients with plasma HIV RNA levels of <400 copies/ml for the previous 18 months and <50 copies/ml at screening, with no history of virologic failure on a protease inhibitor-containing regimen, a CD4 lymphocyte nadir of >50 cells/mm^3, no history of HIV-related neurological disease, and no hepatitis B virus coinfection (212). This was confirmed in the MONET randomized study of the switch to DRV/r monotherapy in patients on stable cART for at least 6 months with plasma HIV RNA levels of <50 copies/ml and no history of virologic failure (213). Geretti et al. showed that baseline HIV-1 DNA levels were higher in patients who had at least one HIV RNA result of >50 copies/ml during 144 weeks of follow-up ($P < 0.05$) (213). With the risk of virologic failure being higher during DRV/r or LPV/r monotherapy than during triple therapy (214), boosted PI monotherapy should only be proposed to carefully selected patients, and baseline HIV DNA levels might prove helpful for choosing the best candidates (213). Recent French HIV/AIDS management guidelines state that HIV DNA levels of <2.3 log copies/10^6 PBMC are associated with the success of such strategies (215).

Trials of de-escalation strategies with protease inhibitor-sparing regimens have led to similar results. Low baseline HIV DNA levels (below 2.35 log copies/10^6 PBMC) were independently associated with a lower risk of virologic failure or viral blips when a protease inhibitor was replaced by a nucleoside or nonnucleoside reverse transcriptase inhibitor (216). Another study showed that patients undergoing de-escalation with a nucleoside reverse transcriptase inhibitor dual combination had sustained an HIV RNA load of <50 copies/ml if they had received early treatment (median CD4 nadir, 340/mm^3), had a low HIV RNA zenith (median, 3.9 log/ml), and had a low HIV DNA level (median, 2.5 log copies/10^6 PBMC) (217).

De-escalation strategies should be avoided in patients with high HIV DNA levels, as mentioned in the recent French guidelines (215). The HIV DNA level is being used for inclusion in an ongoing randomized trial of a reductive antiretroviral strategy based on nucleoside reverse transcriptase inhibitor dual combination after triple therapy (TRULIGHT) (study NCT02302547 at ClinicalTrials.gov) for instance. This study is enrolling patients with HIV DNA levels below 2.7 log copies/10^6 PBMC. More work is needed to identify clinically relevant total HIV DNA cutoffs for use in de-escalation strategies.

Structured Treatment Interruption

The HIV DNA load in PBMC at structured treatment interruption was the only biomarker predictive of the time to plasma HIV RNA rebound after treatment interruption, notably in the SPARTAC trial (106, 218), and also of the viral setpoint in patients first treated during PHI (218) or during CHI in the SALTO and other trials (219, 220, 221). HIV DNA load was lower after cART cessation if patients were treated within 60 days after infection, and the viral setpoint was lower in patients who were treated early (222). HIV DNA was recently reported to be the only predictor of progression following treatment interruption (106). A high HIV DNA load in PBMC at cART interruption predicted a shorter time to antiretroviral resumption, independently of the CD4 nadir (220, 221).

The total HIV DNA level in blood at the time of structured treatment interruption also predicted CD8 and CD4 T cell activation status after 12 months off treatment (187). HIV DNA levels 12 months after interruption correlated strongly with the proportion of CD38-expressing CD8 and CD4 T cells (187).

Other Therapeutic Strategies

In a phase III trial of alpha interferon administration during structured cART interruption, the probability of resuming treatment was higher among patients in the interferon arm who had a low CD4 nadir and high baseline HIV-DNA load, suggesting that interferon is less beneficial in patients with a large HIV reservoir or

that the effect of treatment differs because the initial pool of HIV-infected cells is larger (223).

The impacts of therapeutic vaccine strategies can be evaluated by studying viral reservoirs in terms of total HIV DNA. In the ACTG5197 trial, Li et al. reported that a therapeutic rAd5 HIV gag vaccine had no impact on HIV DNA levels and that a higher HIV DNA load was associated with viral rebound after treatment interruption (224).

HIV DNA load could therefore be useful for evaluating therapeutic strategies.

TOTAL HIV DNA CAN BE USED TO EVALUATE TREATMENT STRATEGIES, INCLUDING THOSE TARGETING HIV RESERVOIRS

Total HIV DNA measurement could help to evaluate innovative therapeutic strategies such as intensification, stem cell transplantation, chemotherapy, immune modulation, and antilatency agents.

Different strategies have been compared for their ability to reduce total HIV DNA levels. The OPTIPRIM randomized trial, in which total HIV DNA decay was the primary endpoint, recently showed that standard triple-drug therapy reduced total HIV DNA load in PBMC as effectively as a five-drug regimen when initiated during PHI (109). No decrease in total HIV DNA levels has been observed with intensification strategies using the integrase inhibitor raltegravir, for example, in patients treated during CHI with controlled plasma viremia (42, 225). Similar declines in total HIV DNA in blood were observed in patients for whom standard triple-drug therapy or LPV/r monotherapy were initiated in the MONARK trial (211).

Stem cell transplantation for hematologic disorders can impact HIV reservoirs. The "Berlin patient," suffering from acute myeloid leukemia, received myeloablative conditioning, two sessions of total body irradiation, and two allogeneic stem cell transplants from a donor who was homozygous for the CCR5 Delta32 deletion. His long-term control of HIV was evaluated more than 8 years after treatment interruption. HIV DNA and cell-associated HIV RNA remained undetectable in PBMC, ileum, lymph nodes, and spinal fluid, but HIV DNA was detected at low levels in the rectum and low HIV RNA signals were detected in plasma (4, 226). These signals could correspond to false positives or to defective viruses; their functional significance is unclear, as the patient had no viral rebound (226). Unfortunately, this result has not yet been reproduced.

The impact of chemotherapy on HIV DNA load in lymphoma patients receiving cART has been evaluated in a small study of 9 patients and showed no beneficial effect (227). Some types of anticancer chemotherapy can have antilatency effects (228, 229). Further studies are needed to evaluate the effects of chemotherapy on HIV reservoirs.

The effects of immune modulators such as cytokines can also be appreciated through their impact on total HIV DNA levels. The effect of interleukin-7, which enhances T cell recovery in HIV-infected patients, was evaluated for the HIV reservoir: there were no changes in the frequency of infected cells among target cells (when total HIV DNA levels were expressed as log copies per 10^6 PBMC or 10^6 CD4 T cells) at week 12. In contrast, when expressed as the $\log_{10}$ copies per milliliter of blood, the HIV DNA load increased significantly in individuals treated with 30 μg/kg of body weight interleukin-7 (median change, +0.51 $\log_{10}$ copies/μl; $P = 0.006$, compared with day 0 level), reflecting the augmentation of the absolute number of infected cells in the body (230, 231). Interleukin-7 induced proliferation of CD4 T cells, including latently infected cells (232). These results indicate that interleukin-7 does not modify the proportion of infected cells but increases the total number of infected cells.

Regarding antilatency agents, no decrease in total HIV DNA was observed with valproic acid, a histone deacetylase inhibitor that activates viral transcription, when added to a cART regimen (233). Similar results were recently obtained with vorinostat, another histone deacetylase inhibitor (234). Those authors concluded that, even if vorinostat triggered a significant and sustained increase in HIV transcription from latent cells in most patients, additional interventions would be needed to obtain quantitative virus production and eventually clear latently infected cells (234).

STUDIES OF HIV DNA HELP TO UNDERSTAND HIV PATHOGENESIS AND PERSISTENCE

Total HIV DNA load correlates with immune responses. In LTNPs, total HIV DNA load correlates negatively with p24-specific CD4 Th1 cell proliferation, gamma interferon (IFN-γ) production, and IFN-γ-producing cell frequencies and positively with Gag-specific IFN-γ-producing CD8 T cell frequencies (88). In untreated children, lower HIV DNA levels were associated with higher HIV-specific CD8 T lymphocyte frequencies (235) and with less abundant T cell receptor excision circles (TREC), which reflect the circulating reserve of naive T lymphocytes (236). After cART initiation, a more rapid HIV DNA decline was observed in children with higher baseline TREC levels, indicating that the pool of naive T lymphocytes influences changes in the reservoir size in patients on cART (236).

HIV DNA load has also been linked to systemic immune activation. High HIV DNA levels in patients on cART were associated with higher frequencies of CD4 T cells expressing CD38, HLA-DR, CCR5, and/or PD-1, reflecting immune activation (204). This correlation between HIV burden and cell activation was also found in the sigmoid colon of patients on cART (237). The HIV DNA level in the sigmoid colon was also positively associated with bacterial translocation (quantified in a lipopolysaccharide assay) and with poor Th17 reconstitution, reflecting mucosal barrier damage (156), both of which are key elements in HIV pathogenesis (147, 154, 157). Cytomegalovirus replication in blood and semen, participating in immune activation, was correlated with higher levels of HIV DNA in PBMC of antiretroviral-naive patients (238). This was recently confirmed; in a multivariate analysis, shedding of cytomegalovirus DNA in semen, which is associated with increased activation and proliferation of T cells in blood, was predictive of higher blood HIV DNA load (239).

Quantification of total HIV DNA in blood CD4 T lymphocyte subsets sorted by flow cytometry according to their differentiation status provided information on the viral reservoir distribution. Central memory and transitional memory CD4 T cells have been identified as the main contributors to the HIV blood reservoir in chronic patients first treated during CHI (104). These long-lived memory cells with a high proliferative capacity contribute to the stability of the HIV reservoir. On the contrary, in patients with primary HIV infection, the contributions of T cell subsets to the reservoir are different. In the context of high-level activation, effector memory T cells are the main contributors to the blood reservoir, with central memory T cells being relatively protected

(240, 241). Similarly, when treatment is started during PHI, long-lived central memory cells contribute little to the HIV reservoir in comparison to more highly differentiated and shorter-lived T cells (242). cART initiated during PHI diminished the level of infection of each CD4 T cell subset but did not affect the contribution of each subset; the pattern observed at the time of PHI was similar to that seen after 2 years of early cART (240, 242). It was also similar to the pattern observed in LTNPs harboring the protective human leukocyte antigen allele B27 or B57 (243) and in posttreatment controllers (92).

The observation that the HIV reservoir resides mainly in short-lived CD4 T cells when treatment is initiated during PHI could explain the sharper reduction in total HIV DNA in patients treated during primary infection than in patients treated during CHI. Short-lived CD4 T cells have a low proliferative capacity but are able to give rise to infected daughter cells or to produce replication-competent virus that infects new cells and contributes to HIV persistence. Detection of viral clusters in effector memory CD4 T cells in blood and lymph nodes suggests that the proliferation of these cells helps to maintain the viral reservoir (244). The small fraction of central memory CD4 T cells participating in the HIV reservoir during the primary infection, or after cART initiated in PHI, have a long half-life and high proliferative capacity. They also contribute to the stability of the small reservoir in patients treated during the acute phase of infection. Moreover, central memory T cells play a major role in immune responses, and their protection against HIV infection by very early cART initiation could partly explain the clinical, immunologic, and virologic benefits of early treatment.

To conclude, early cART reduces the contribution of long-lived central memory CD4 T cells to the total HIV reservoir (240, 242), at similar levels to those observed in patients who naturally control HIV without treatment (243). Early cART has a greater impact on HIV reservoirs than later cART. However, a certain amount of total HIV DNA persists despite cART (16), even if treatment is initiated within the first 6 months after infection and is continued for >10 years. Latently infected long-lived memory CD4 T cells (central memory and T memory stem cells) persist in most early-treated individuals (57). Additional interventions will thus be required to eliminate all cells capable of producing replication-competent virus, but treatment initiation during primary infection may be the critical first step to contain HIV reservoirs (92, 245).

DISCUSSION AND CONCLUSIONS

Many studies have shown the clinical relevance of total HIV DNA load. This marker has been used most extensively in studies of HIV reservoir dynamics in both treated and untreated patients. It provides complementary information to standard markers (CD4 T cell count and HIV RNA level) and has predictive value in many settings and at different stages of HIV infection. When measured during cART, it can provide information on the pretherapeutic history (pretherapeutic HIV DNA load, plasma HIV RNA zenith, CD4 T cell nadir) and can predict several treatment outcomes, such as residual viremia, immune recovery, and cell activation status. Finally, it can help to evaluate HIV infection in tissues and body fluids and the impact of new antiretrovirals on tissue reservoirs.

Total HIV DNA load can also help with the choice of therapeutic strategies such as de-escalation, as recently acknowledged in the French guidelines (215).

Total HIV DNA levels, together with other markers, can help to identify candidates for intervention studies aimed at inducing drug-free remission (246). HIV DNA load will not be the only marker used for this purpose, as some individuals with low HIV DNA levels are unable to control the infection after treatment interruption. As each marker of HIV reservoirs provides different information, HIV DNA could be used in combination with other markers. It would also be informative to study immunologic markers of activation, inflammation, and/or T cell exhaustion. Total HIV DNA load could thus be useful for identifying patients who might respond to such cure strategies, among patients with a relatively competent immune system. Changes in reservoir size can be easily monitored by total HIV DNA assay, for example, in large clinical trials of strategies designed to eradicate HIV or to induce a functional cure. Other methods to measure changes in HIV reservoirs during such trials should be included, such as the Tat/Rev-induced limiting dilution assay (TILDA) and/or measurement of cell-associated HIV RNA, in addition to HIV DNA measurements, as some of the observed changes in HIV DNA levels could be due partly to depletion of HIV DNA forms not capable of reinitiating infection. The total HIV DNA level also helps increase understanding the pathogenesis of HIV infection, as shown by studies of CD4 T cell subsets.

One limitation of total HIV DNA as a reservoir marker is that it includes defective and more labile, unintegrated forms. However, unintegrated genomes or defective viruses might also contribute to HIV pathogenesis, by increasing the antigenic load responsible for T cell activation and exhaustion (247, 248). Several studies have shown that unintegrated HIV DNA can participate in HIV transcription and in the synthesis of viral proteins and infectious virus (44-53, 249). A recent study pointed out the role of 1-LTR forms among these unintegrated HIV DNA species (250). These synthesis activities of viral RNA and proteins are at lower levels than those produced by integrated HIV DNA forms, but they are sufficient to induce T cell activation (46). Recently, Siliciano's group showed that defective HIV proviruses can be transcribed during latency reversal (251). Replication can be blocked after this transcription, without production of viral proteins, because of insufficient transcription levels and/or mislocalization of viral RNA (22). Blockade can also occur later. O'Doherty's group recently found that infected resting CD4 cells express low levels of viral protein due to nascent LTR-driven transcription, without releasing infectious viruses, raising the possibility that reservoirs, including defective proviruses, may express HIV proteins and thus be visible to the immune system (252, 253). In untreated patients with abundant unintegrated HIV DNA, total HIV DNA loads in blood cells correlate strongly with the level of CD8 and CD4 T cell activation (187). In antiretroviral-treated adults, total HIV DNA in resting CD4 T cells is strongly associated with CD4 and CD8 T cell activation, whereas there is no association between cell activation and integrated DNA or IUPM coculture results (254). Cell-associated HIV RNA load, which reflects the level of viral transcription, including abortive transcription, also correlates with immune activation in untreated patients, patients on cART, and natural controllers (204, 255, 256, 257). In patients on cART with undetectable viremia, HIV transcript loads correlate negatively with the CD4 T cell count (257) and positively with lymphoproliferative responses to HIV p24 antigen (258). Activa-

tion stimulates transcription of persistent virus (257). These data suggest that defective proviruses might also produce antigens even if they do not produce replication-competent viruses. They could thereby play a role in HIV pathogenesis. Overall, the activity of the viral reservoir contributes to antigenic stimulation of the immune system (22). Several studies have examined the mechanisms of correlation between HIV DNA or cell-associated HIV RNA and activation and HIV pathogenesis. These mechanisms involve pattern recognition receptors: membrane-bound Toll-like receptor (TLR), cytosolic sensors like DNA-dependent activators of IFN regulatory factors, or triggered intrinsic cell defenses. HIV DNA would be particularly more likely to contribute to immune activation if it was transcribed, and especially if it was translated into viral proteins that could then be processed into peptides and presented. For example, cell-associated viral RNA interacts with TLR-7 to stimulate plasmacytoid dendritic cells, leading to α-IFN production that enhances antigen presentation (259). More recently, it was reported that incomplete HIV DNA accumulates in the cytosol before integration and activates apoptotic and inflammatory mechanisms (260, 261). These mechanisms contribute to HIV pathogenesis in infected cells unable to produce new virions (260, 261). Interferon gamma-inducible protein 16 (IFI16) serves as a link to unintegrated double-strand HIV DNA and acts as a sensor, resulting in pyroptosis and CD4 T cell depletion (261, 262). IFI16 expression correlates with CD4 T cell activation (263). HIV DNA can also interact with other sensors prior to integration (264, 265). Links between HIV DNA and activation during effective cART are less clear, but the higher the HIV DNA load, the more likely it is to partially reactivate and produce viral RNA that could contribute to inflammation. It could also induce cell proliferation by integration in specific genes (266), and these genes are likely to produce cytokines and increase activation. Finally, HIV proteins like the capsid can interact with sensors, inducing maturation of dendritic cells and innate and T immune activation, for example (267, 268). HIV DNA, HIV RNA, and proteins constitute pathogen-associated molecular patterns (PAMP) recognized by innate immune sensors and could impact activation, inflammation, and pathogenesis. This supports the utility of quantifying all forms of HIV DNA, including defective and silent forms that can be transcribed or translated without producing infectious virus. This may explain the clinical relevance of total HIV DNA.

In conclusion, the results discussed in this review show that total HIV DNA has clinical relevance as a marker of HIV reservoirs and that its level influences the course of the infection, even if it does not distinguish between replication-competent and -defective viral forms. Total HIV DNA measurement is the simplest and most sensitive, reproducible, and standardized approach for HIV reservoir measurement and can be performed routinely in clinical practice. It can be useful, together with HIV RNA load and CD4 T cell count, for adapting treatments to specific patient subgroups. It is also a promising additional biomarker for monitoring the efficacy of antiretroviral treatments and novel strategies aiming at reducing or eliminating HIV reservoirs.

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