Review

Quantification of Total HIV DNA as a Marker to Measure Viral Reservoir: Methods and Potential Implications for Clinical Practice

Simone Belmonti [1], Simona Di Giambenedetto [1,2] and Francesca Lombardi [2,*]

[1] Dipartimento di Sicurezza e Bioetica Sezione Malattie Infettive, Università Cattolica del Sacro Cuore, 00168 Rome, Italy; simone.belmonti@policlinicogemelli.it (S.B.); simona.digiambenedetto@policlinicogemelli.it (S.D.G.)

[2] UOC Malattie Infettive, Fondazione Policlinico Universitario A. Gemelli IRCCS, 00168 Rome, Italy

[*] Correspondence: francesca.lombardi@gmail.com; Tel.: +39-06-3015-5366

Abstract: The focus of this review is to examine the importance of quantifying total HIV DNA to target the HIV reservoir and the clinical implications and challenges involved in its future application in clinical practice. Despite intrinsic limitations, the quantification of total HIV DNA is currently the most widely used marker for exploring the HIV reservoir. As it allows estimating all forms of HIV DNA in the infected cells, total HIV DNA load is the biomarker of the HIV reservoir that provides most of the insights into HIV pathogenesis. The clinical role of total HIV-DNA in both untreated and treated patients is extensively supported by important lines of evidence. Thus, predictive models that include total HIV DNA load together with other variables could constitute a prognostic tool for use in clinical practice. To date, however, this marker has been primarily used in experimental evaluations. The main challenge is technical. Although the implementation of droplet digital PCR could improve analytical performance over real-time PCR, the lack of standardization has made cross-comparisons of the data difficult. An effort by investigators to compare protocols is needed. Furthermore, the main effort now should be to involve the biomedical industry in the development of certified assays for in vitro diagnostics use.

Keywords: HIV; HIV DNA; total HIV-1 DNA; reservoirs; real-time PCR; digital droplet PCR

1. Introduction

In most patients infected with HIV, the use of combined antiretroviral therapy (ART) results in sustained control of viral replication [1]. However, the virus persists in some specific, latently infected cell-lineages that carry HIV-integrated DNA in their host genome, thus forming the viral reservoir [2,3].

If ART is interrupted, the virus reemerges from the reservoir, leading to a rebound in viremia [4].

Therefore, at the moment HIV cannot be eradicated from the body, and treatment must be maintained indefinitely. Since the reservoir constitutes the major obstacle to eradication of the HIV infection, an accurate and precise measurement of the HIV reservoir is needed to assess strategies for a cure, i.e., a functional or a sterilizing cure [5], and for pilot eradication studies [6]. Quantifying the HIV reservoir is crucial to assess the efficacy of treatments aimed at reducing it and monitoring its changes following specific treatment strategies in studies of intensification, interruption or switching of ART [7]. In particular, measuring the HIV reservoir could be crucial for evaluating patients who are candidates for a simplification of ART, because reducing the drug pressure might be safer in patients who have a small sample-size reservoir. This is noteworthy given the growing importance of less-drug regimens (such as dual regimens) in the management of HIV infection [8].

Thus, a reliable and practical marker is needed to analyze the reservoir persistence and dynamics in HIV-infected subjects. Several markers have been proposed, but there is no

consensus regarding the marker that can best measure the reservoir [9–11]. Indeed, there are several main questions and difficulties that need to be addressed to define and measure the HIV reservoir. Primarily, according to one definition that has been proposed, the HIV reservoir can be considered "the cells where HIV persists in latent form but can reactivate in the form of a replication-competent virus" [12]. However, it should be considered that only some of these latently infected cells contain replication-competent HIV DNA and can be induced ex vivo to produce the virus; in fact, most of these cells harbor defective viral genome [11,13–16]. Memory CD4+ T cells are those most infected, even though different T-cell subsets have been found to harbor latent infection and residual virus has also been detected in different cells during ART [17,18].

Furthermore, the different species of HIV DNA include integrated HIV DNA and unintegrated forms such as the linear pre-integration complex and the 1-long terminal repeat (LTR) and 2-LTR circles [19,20].

Moreover, the latently infected cells are disseminated throughout the body but are more concentrated in sites such as the so-called "anatomical reservoirs" (or "sanctuaries") than in the blood, which is the most accessible organ for sampling [21,22].

Considering these main questions and difficulties concerning the different markers of the HIV reservoir, to date total HIV DNA is the marker that is most widely used and studied. Total HIV DNA is considered an imperfect marker of the HIV reservoir as it is a global biomarker that includes integrated and non-integrated viral genomes, without differentiating the defective forms from the latent ones that can produce infectious viruses. In any case, the clinical role of total HIV DNA has been evaluated in important studies and is supported by the most evidence.

The focus of this review is total HIV-1 DNA—here we examine the importance of quantifying total HIV-1 DNA to target the HIV reservoir, and the clinical implications and challenges involved in its future application in clinical practice.

2. Why Is Total HIV DNA the Most Widely Used Marker of the HIV-Reservoir?

The assay that has been considered to represent the gold standard for measuring the latent, but replication competent, virus is the quantitative viral outgrowth assay (QVOA); it quantifies the number of resting CD4+ T cells releasing infectious virus after in vitro stimulation and subsequent co-culture with feeder cells [23–26].

The great value of this assay is that it can detect individual latently infected cells that release the replication-competent virus, thus providing a valuable estimation of blood reservoir productive activity. However, the major limitations of using this technique are that it is expensive and very labor intensive, even with shortened protocol [23–25].

It also involves large amounts of blood (120–180 mL), often requiring leukapheresis. Moreover, it cannot be performed with tissue biopsies and has been demonstrated to have a wide coefficient of variation, indicating that it is unreliable for detecting small differences in the size of the HIV reservoir [27]. Finally, this technique tends to measure only the "tip of the iceberg", thus underestimating the reservoir, because not all replication-competent latently infected cells can be induced by stimulation [15].

Given these limitations, more reliable, less complex methods are certainly needed for serial measurement of the HIV reservoir in patient management and in clinical studies.

Intracellular HIV RNA load, or cell-associated HIV RNA, is another marker which has been proposed as a marker of HIV transcription in latently infected cells [28]. Indeed, its expression has been used to demonstrate that several drugs could induce latently infected cells from patients under suppressive ART to make HIV transcripts [29]. Therefore, quantification of multiple spliced, singly spliced and unspliced HIV RNA has also been proposed as a marker of the "active HIV reservoir", i.e., of a subset of the total viral reservoir containing cells in which HIV sequences are actively transcribed, and as a marker of residual virus replication [28].

However, whether some transcription is consistent with the latent status or represents ongoing low-level replication must still be determined. Several assays have also been

developed to quantify intracellular HIV RNA [28], but their reliability and reproducibility must still be fully evaluated.

The quantification of total HIV DNA permits estimating all forms of HIV DNA in the infected cells (i.e., resting or activated) that are present in blood as well as in biopsies and tissues. One of the major criticisms regarding the quantification of total HIV DNA is that it does not distinguish between latent defective and replication competent forms.

It has been hypothesized that defective proviruses are probably generated during the initial rounds of replication after transmission, because defective proviruses are generated at a high frequency from the process of reverse transcription. Similarly, it has been hypothesized that defects such as deletions probably occur during minus strand synthesis before the second strand transfer event of reverse transcription [11,15,16]. The most common defects are internal deletions, which varied in size and in location in the genome. The very large deletions (>6Kb) can encompass most of the HIV genome. Some proviruses show 3′ deletions affecting the *env*, *tat*, *rev* and *nef* genes, other proviruses show 5′ deletions affecting *gag* and *pol* genes. A small percentage of proviruses can have small deletions at the packaging signal and the major splice donor site represented. These latter proviruses are probably replication defective owing to a failure to correctly make spliced HIV-RNAs or to package genomes into virions [15,16]. Some clones can contain multiple deletions. A percentage of proviruses harbor guanine-to-adenine (G-to-A) hypermutations. An additional percentage of proviruses can contain both deletion and hypermutation indicating that these processes can occur contextually during the reverse transcription. Hypermutation in turn can alter start codons and/or introduce stop codons in the larger open-reading frames (ORFs) (*gag, gag–pol, env* and *nef*). All these defects and deletions affecting key viral ORFs probably prevent many defective proviruses from being eliminated either by viral cytopathic effects or by eradication strategies that depend on viral protein expression [15,16].

However, if quantifying all forms of HIV-DNA in the infected cells can be considered a structural limit on one hand, on the other it can be considered an advantage. In fact, each of the various forms that are quantified in total HIV DNA play a different role in HIV pathogenesis [30]. This participation can occur through the creation of new cycles of infection and infected cells, with the maintenance /amplification of the reservoir by homeostatic proliferation and through viral transcription, thus producing viral antigens and incomplete viruses without any new production of virions. These proteins can thus induce immune activation/inflammation, thereby participating in maintaining the cycle of HIV pathogenesis and in facilitating the persistence of HIV reservoirs [30].

From this point of view, the key question is how to consider the reservoir. If the HIV reservoir can be defined as all infected cells and tissues containing all forms of HIV persistence, the quantification of total HIV DNA is the biomarker of the HIV reservoir that provides most of the insights into HIV pathogenesis.

On the other hand, several studies have shown that the total HIV DNA load seems to correlate well with the frequency of cells containing the replication-competent virus. Studies by Eriksson et al. [11] and Kiselinova et al. [31] pointed out a correlation between total HIV DNA load in resting cells and quantification of the replication-competent virus. In a different study, Noel et al. reported that a low level of total HIV DNA correlated with low efficiency of virus production after activation [32]. The second main criticism is that the quantification of total HIV DNA does not discriminate between integrated and unintegrated forms. Although the integrated DNA (provirus) is transcribed and leads to synthesis of viral proteins and completion of the viral replication cycle [33], the unintegrated forms cannot lead to a productive infection by themselves [34]. These unintegrated species include linear DNA, which is a component of a pre-integration complex [33], and circular forms, which derive from the previous by means of several mechanisms. The two main circular forms are 1-LTR and 2-LTR, which harbor one and two LTR extremities of cDNA, respectively [34]. Although the proportion between integrated and unintegrated forms can vary in vitro [35], in vivo during ART it appears to be very similar, given the clearance of non-integrated HIV DNA when ART is initiated [35–37]. On the other hand, an accumulation of unintegrated

forms has been observed in ART-naïve subjects [38]. Although the unintegrated forms cannot be copied during cell division, transcription activity has also been shown from linear DNA and 1-LTR and 2-LTR circles [34,39].

Circular forms of DNA are more stable than linear forms, even though unintegrated forms are generally much more labile compared to integrated DNA and have a short half-life [35]. Thus, the quantification of integrated DNA, which is the most stable form of HIV-DNA and serves as the main template for gene transcription and viral replication and is important for viral latency is thought to be the best indicator of the HIV reservoir size. However, it presents several main concerns. The first is technical. Indeed, assays specifically targeting integrated HIV DNA are complex to set up and require extensive replicate testing [11,40]. Furthermore, it has been demonstrated that extrachromosomal forms can be associated with transcription and can contribute to HIV pathogenesis [34,39,41], thus making total HIV DNA a better indicator of HIV persistence. Moreover, due to the clearance of unintegrated forms during ART [35–37], it should be considered that total and integrated HIV DNA levels became generally equal in effective ART [37].

Therefore, even though the quantification of integrated and unintegrated forms can provide information in select studies, it does not appear reliable for routine use as an HIV reservoir marker. Instead, the quantification of unintegrated forms has been proposed as indicative of ongoing viral replication [42,43]; in any case, controversies remain.

Through comparison with these markers, total HIV DNA has the advantage of easier quantification by the quantitative real-time (qPCR) or digital droplet PCR (ddPCR) assays. The measure of total HIV DNA load by the PCR-based assays is quick, easy to perform and has a large dynamic range of quantification. Compared to other assays, the quantification of total HIV DNA can be carried out in blood, other body fluids and in tissue biopsy specimens. It requires a small amount of the sample and is unaffected by freeze-thawing; it also seems to be more cost-effective, less time consuming and easy to implement in the laboratory [44–55].

Therefore, total HIV DNA cannot be considered a perfect maker as it is neither a marker of the replication-competent reservoir, such as the QVOA, a marker of the latent reservoir, such as the integrated HIV DNA, nor a marker of the active reservoir, as is the cell-associated HIV RNA. However, for the above-mentioned reasons, the quantification of total HIV DNA is currently the most widely used marker for exploring the HIV reservoir.

Novel approaches such as IPDA [56] Q4PCR [57] and TILDA [58] represent major advances in quantifying and characterizing intact/replication-competent HIV reservoir. The implementation of these assays might constitute innovative methods for the analysis of the viral reservoir in the future.

Emerging PCR-based techniques used to quantify HIV DNA such as the intact proviral DNA assay (IPDA) and quadruplex quantitative PCR (Q4PCR), distinguish intact proviruses from defective ones, therefore providing a better resolution for studying the dynamics of defective and intact HIV-proviral DNA and requiring a relatively small number of cells.

IPDA is a high-throughput assay that uses two probes to exclude the majority of defective proviruses and to determine the frequency of intact proviruses, albeit without sequence confirmation.

The IPDA utilizes ddPCR to measure proviruses with probes targeting conserved regions. This duplexed ddPCR assay simultaneously targets two HIV-1 regions, the Packaging Signal (Ψ) near the 5′ end of the viral genome and the Rev Responsive Element (RRE) within Envelope (*env*), which when amplified together in the same provirus, includes only genomically intact provirus and excludes the majority of defective proviruses. Input cells are directly measured by a simultaneous ddPCR reaction, enabling the IPDA to report the total frequency of intact and defective proviruses per million input cells. This assay enables much more specific quantification of intact proviral DNA than single-probe assays. However, the Ψ and *env* probe combination may not be entirely predictive of a fully intact provirus [56,59].

Recent studies have described instances of assay failure, attributable to natural HIV polymorphism in primer and/or probe binding regions. [60].

The Q4PCR assay employs long distance PCR at limiting dilution to amplify proviruses, followed by interrogation with four qPCR probes for Ψ, *env*, *pol*, and *gag* in a multiplex reaction to detect amplified HIV genomes. Cell inputs are estimated by quantification of input DNA, and the frequency of intact proviruses is reported after sequence verification through near-full-length genome sequencing (nFGS). Because the Q4PCR employs limiting dilution near-full-length proviral amplification, these nFGS results can be used to provide insight into the clonal composition of intact proviruses [57]. However, inefficiencies in long-distance PCR amplification of proviruses in Q4PCR may lead to underestimates of intact proviral frequencies [57,59].

Notably, proviral intactness does not guarantee virion production and the fraction of intact proviruses that can be induced to produce virions cannot be determined by these assays, IPDA and Q4PCR, which is a critical limitation given that many intact proviruses exhibit low inducibility [15,61].

The transcription-competent (inducible) HIV reservoir can be quantified by several methods including a well-established inducible reservoir assay, *tat/rev* induced limiting dilution assay (TILDA). Data indicate that there are circuits enabling control of viral transcription without cellular activation and showing specifically that Tat positive feedback is sufficient to regulate latency independent of cellular activation [62]. TILDA measures the frequency of cells that harbor viral genomes, and which produce tat/rev multiply-spliced HIV RNA upon maximal stimulation, reducing the likelihood of quantifying defective genome. This method allows to measure the frequency of cells capable of being induced to produce HIV RNA transcripts by combining ultra-sensitive detection of msRNA and maximal activation of CD4+ T cells in a limiting dilution format [58].

However, due to post-transcription blocks in RNA processing not all cells producing tat/rev msRNA transcripts will yield infectious virus; therefore, quantifying these cells can result in overestimating the replication-competent HIV reservoir size [63,64].

TILDA holds great promise for application in clinical trials because it requires less than 1 million viable target cells per condition and can be completed within two days.

Figure 1 summarizes and compares pros/cons of total HIV DNA quantification *vs* pros/cons of other approaches for quantifying the HIV reservoir.
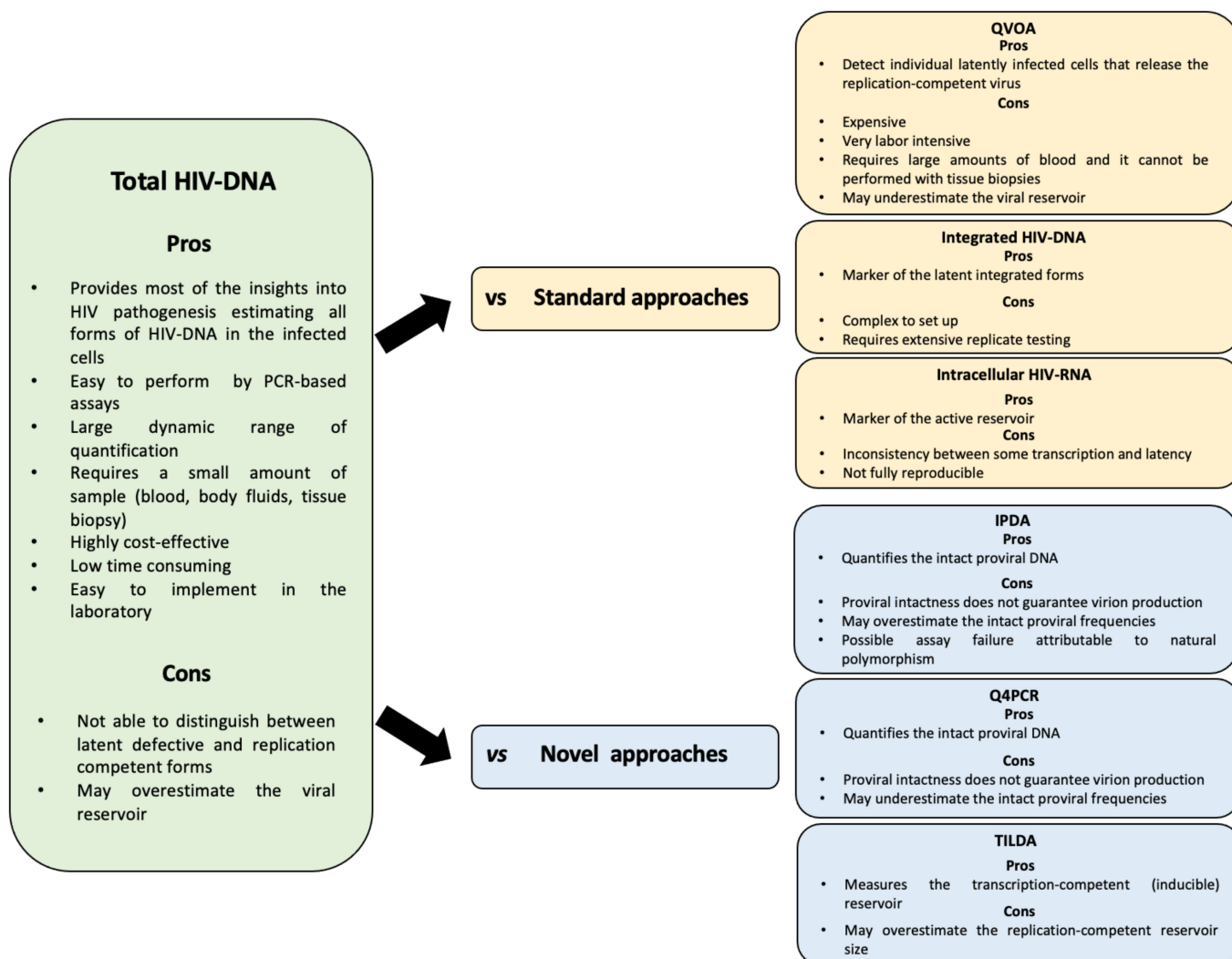
Total HIV-DNA

Pros

- Provides most of the insights into HIV pathogenesis estimating all forms of HIV-DNA in the infected cells
- Easy to perform by PCR-based assays
- Large dynamic range of quantification
- Requires a small amount of sample (blood, body fluids, tissue biopsy)
- Highly cost-effective
- Low time consuming
- Easy to implement in the laboratory

Cons

- Not able to distinguish between latent defective and replication competent forms
- May overestimate the viral reservoir

vs Standard approaches

vs Novel approaches

QVOA
Pros
- Detect individual latently infected cells that release the replication-competent virus
Cons
- Expensive
- Very labor intensive
- Requires large amounts of blood and it cannot be performed with tissue biopsies
- May underestimate the viral reservoir

Integrated HIV-DNA
Pros
- Marker of the latent integrated forms
Cons
- Complex to set up
- Requires extensive replicate testing

Intracellular HIV-RNA
Pros
- Marker of the active reservoir
Cons
- Inconsistency between some transcription and latency
- Not fully reproducible

IPDA
Pros
- Quantifies the intact proviral DNA
Cons
- Proviral intactness does not guarantee virion production
- May overestimate the intact proviral frequencies
- Possible assay failure attributable to natural polymorphism

Q4PCR
Pros
- Quantifies the intact proviral DNA
Cons
- Proviral intactness does not guarantee virion production
- May underestimate the intact proviral frequencies

TILDA
Pros
- Measures the transcription-competent (inducible) reservoir
Cons
- May overestimate the replication-competent reservoir size

Figure 1. Total HIV DNA vs other approaches for quantifying the HIV-reservoir.

3. Technical aspects

Technical concerns regarding measurement of total HIV DNA include the specimens and the assays.

3.1. The Specimens

One of the major criticisms concerning measurement of the HIV reservoir is the distribution of latently infected cells in the different organs and tissues. Blood is the most accessible organ as it can easily be obtained by venipuncture; other tissues are not as easily sampled. Therefore, most studies have explored blood-associated total HIV DNA despite concerns as to whether it is representative of the latent reservoir size in the whole body. In fact, it has been shown that most infected cells can be found in the lymph nodes and the gastrointestinal tract [65–68], and the activation status of the latently infected CD4+ is higher in these sites than in the blood [67]. However, a phylogenetic analysis carried out by Josefsson L. et al. revealed genetic similarity of HIV DNA in CD4+ T-cells from lymph nodes and peripheral blood, which indicates an interchange of infected cells among these compartments during infection [69]. Furthermore, in a different study it was noted that total HIV DNA load in rectal cells correlated with the blood-associated total HIV DNA levels [70]. However, this finding was not confirmed in other reports [66,67]. On the other hand, some authors considered how the blood HIV DNA could be generally accepted as a

reflection of the entire HIV reservoir in the same way that blood HIV RNA or the CD4+ T cell count are generally accepted as a reflection of virus activity in the whole body [71].

Quantification of the total HIV-DNA levels in the blood can be performed directly on whole blood cells or can require a more laborious step by separation of PBMCs or by purification of CD4+ T cells. Depending on the method used the results are expressed as copies/leukocytes, copies/PBMCs or copies/ CD4+ T cells, respectively. Some studies found a good correlation between the results of the whole blood sample tests for total HIV DNA and those of PBMC samples [49,72]. Another study found that HIV DNA load could predict disease progression regardless of the mode of results expression [73].

3.2. The Assays

Historically, the method most used to measure total HIV DNA is qPCR. In this assay, amplification is carried out with primers and probe targeting of a conserved region of the HIV genome; quantification is assessed to calculate the frequency of infected cells based on a standard curve prepared by serial dilution of a standard (i.e., 8E5 cell line) [44–53].

However, there are no certified methods for in vitro diagnostic use and several in-house protocols have been developed. These homebrew HIV DNA quantification protocols may differ in the different genomic regions analyzed and in the different standards, probes and control genes used.

Total HIV-DNA quantification protocols have been developed using both Taqman probes and SYBR green-based PCR, targeting LTR region, gag-pol junction, gag gene or pol gene [44–53]. An alternative method uses two sets of primers that target the genomic regions of LTR and gag by molecular beacon probes [74].

The methods that target the conserved region of the long terminal repeat have been reported to have more sensitivity, specificity, and reproducibility than the others [75,76]. Other protocols that have been proposed to quantify total HIV DNA using technologies such as Nested- PCR or semi-nested PCR [53,77] are used less frequently. Thus, in the last two decades the qPCR has become the tool most used for the quantification of total HIV-DNA. Despite its success, qPCR remains an indirect measurement tool. It requires a validated standard curve and is susceptible to small changes in the efficiency of the reaction, exponentially amplifying the noise and making it difficult to differentiate target low levels from assay noise. dPCR is a more recent alternative to real-time PCR for the measurement of total HIV DNA load. This technology allows for absolute quantification of the target nucleic acid without reliance on the use of an external standard. Indeed, in this technique each sample is divided into thousands of independent microscopic "end-point" PCR reactions so that only one or no nucleic acid molecule will be found in each partition [78]. In such a this sufficiently diluted sample, the template follows the POISSON distribution, which can be used to derive the target concentration based on the frequency of the positive and negative distributions. The high costs and labor-intensive procedure underlying the micro partitioning have historically limited the use of dPCR. Developments in the field of microfluidics have made digital PCR platforms commercially available in a low-cost and practical format.

Micro partitioning can be achieved by emulsification of the aqueous PCR reaction mixture in a thermostable oil, as in droplet digital PCR (ddPCR) [79]. This technique was applied to total HIV DNA quantification and showed high accuracy and precision [54,55].

When qPCR and ddPCR techniques were compared in a study conducted by Heinrich et al. [80], they showed a similar level of analytical sensitivity. In a different study, ddPCR provided a significant increase in precision compared to qPCR; moreover, additional benefits of the ddPCR over the qPCR assay included relative insensitivity to mismatches in primer and probe sequences [54]. Accordingly, Bosman et al. compared ddPCR platforms with a semi-nested qPCR and showed a higher reproducibility for ddPCR than qPCR; however, some false-positive signals were observed with ddPCR [81]. The sensitivity of the ddPCR assay makes it advantageous for analyzing molecules with a rare frequency, i.e., in

the presence of a high background of non-target molecules. Theoretically, this makes it an ideal model for studying the HIV reservoir.

4. The Clinical Relevance of Total HIV-DNA Load

Multiple studies have demonstrated the clinical relevance of quantifying total HIV-DNA load. This marker has been used extensively in studies of HIV reservoir dynamics in both treated and untreated subjects.

4.1. During Infection

Results of several relevant studies support the predictive value of the measure of total HIV DNA level during the natural history of HIV infection. In fact, total HIV DNA load has been shown to be predictive of disease progression independently of HIV RNA load and the CD4+ T cell count in untreated HIV-positive subjects [82–87].

Rouzioux et al. showed that total HIV DNA level was a major predictor of progression to AIDS independently of HIV RNA level and CD4+ T cell count from 6 to 24 months after seroconversion [82].

A meta-analysis of six studies of untreated patients reported that total HIV-DNA was a better predictor of progression to AIDS and of all-cause mortality than plasma HIV-RNA load in untreated patients [86].

In another more recent study, N'takpe et al. analyzed the association between blood-associated total HIV DNA load and long-term mortality in African adults who started ART early. The authors found that patients with a high HIV DNA level at baseline had a higher 6-year mortality risk than other patients, irrespective of plasma viral load, CD4+ T cell count and ART [87].

A different study showed that total HIV DNA in blood can be detected very early after infection, with high levels seeded in primary infection at the peak and with the HIV- DNA set point rapidly established [88,89]. Furthermore, total HIV DNA load has been shown to be significantly lower in patients with primary infection, i.e., at stage Fiebig I versus Fiebig II–IV [90]. In the ANRS PRIMO cohort, the median HIV DNA load was significantly higher in patients who had a symptomatic primary infection [91].

Elite controllers and long-term non progressors are characterized by a very low and stable cell-associated HIV-DNA level [32,92,93]. Total HIV DNA levels in the blood correlated positively with plasma viremia and negatively with CD4+ T cell count [82]. Moreover, a link has been observed between HIV DNA levels and T-cell activation [94] and in primary infection early levels of HIV DNA predict the extent of T-cell proliferation [95]. Furthermore, the HIV DNA load plays an important role in the pathogenesis of HIV-associated neurological disorders. In fact, some studies reported that the blood-associated total HIV DNA level is associated with the occurrence and severity of HIV-associated neurological disorders [96,97].

Finally, of note, the quantification of total HIV DNA load in the peripheral reservoir of monocytes was shown to be predictive of cognitive performance in an elegant study by Valcour VG et al. [98].

4.2. During ART

There is evidence that the earlier ART begins, more prominent is the decrease in total HIV DNA load [88,89]. Furthermore, unlike the slow decrease that is observed in patients treated at the chronic stage, this decay is faster in acutely infected patients receiving ART. Indeed, several studies showed that total HIV DNA levels dropped rapidly during the first year of ART and then showed a subsequent slower decline from years 1 to 4 until they reached a plateau phase where no further decay was observed [99,100]. However, several more recent studies reported that after several years of suppressive ART, HIV DNA levels could continue to slowly decline [101–103]. The authors suggest that this decay of HIV DNA during effective ART could reflect the ongoing loss of HIV-infected cells partially

counterbalanced by clonal proliferation of infected cells [101,102]. In any case, this point needs further investigation.

It has been shown that total HIV- DNA load during ART reflects pre-ART characteristics of infection, such as baseline HIV- DNA load, pre-ART HIV-RNA load and the CD4+ cell count nadir [104–106]. Furthermore, total blood-associated HIV- DNA levels correlated with the levels of residual plasma viremia in patients under suppressive ART [107,108].

It has also been reported that the presence of X4-tropic viral DNA variants is related to a larger size of the HIV DNA blood reservoir compared to the presence of R5-tropic viruses in patients fully responding to ART [109]. In a different setting, total HIV-DNA load was not associated with soluble biomarkers of inflammation in treated patients [110].

Lower baseline HIV-DNA levels were predictive of achieving a sustained virological response on ART [108,111], whereas several reports showed that baseline HIV DNA load predicted the level of residual plasma viremia during effective ART [112,113].

In a recent study baseline total HIV-DNA and CD4+ T cell count resulted to be independent predictors of total HIV-DNA after treatment [114].

Of note, it has been demonstrated that the measurement of total HIV DNA load is informative when measured at the time of treatment interruption, as it is the only biomarker predictive of the time to plasma HIV RNA rebound after treatment interruption, both in patients treated early during primary infection and in patients treated late during chronic infection; low levels predict a higher probability of maintaining viral control [84,115–117].

In particular, the study by Williams JP. et al. measured total HIV DNA load in a highly characterized randomized population of individuals with primary HIV infection. Results showed that those with higher levels of HIV DNA when the treatment was stopped experienced faster viral rebound than those with lower levels of HIV DNA [84].

Instead, the study by Assoumou L. et al. evaluated whether HIV replication could be controlled following interruption of treatment started early in the course of infection, but not during the primary infection. In this study, patients who had low HIV DNA levels at the time of treatment interruption maintained viral control for long periods [116]. In addition, the study by Piketti C. et al. predicted a shorter time to treatment resumption, independently of the CD4 nadir in patients treated during chronic infection [117].

A specific topic deserves discussion here, i.e., the strategies of de-escalation therapies and the role of HIV DNA load in this setting. The use of less-drug regimens seems to indicate a new era of significant changes in the management of ART for people living with HIV. These therapies aim to combine virological efficacy with the limited long-term toxicity associated with use of the nucleos(t)ide reverse transcriptase inhibitors (NRTIs), limited adverse effects and reduced costs when compared to standard three-drug ART [8,118]. Within this context, the quantification of total HIV DNA levels could prove helpful for choosing the best candidates, in whom it may be safer to reduce drug pressure. Furthermore, it could be useful for evaluating these new strategies in terms of effective action on the reservoir.

Of note, several studies have demonstrated the predictive value of total HIV DNA load on the risk of virological failure following treatment de-escalation. Indeed, the total HIV DNA levels at the time of a switch to ritonavir-boosted darunavir (DRV/r) monotherapy predicted the risk of viral rebound at week 96 in the MONOI trial [119].

Accordingly, in the MONET randomized study Geretti et al. showed that baseline HIV DNA levels were higher in patients who switched to DRV/r monotherapy and who had at least a viral rebound during the 144 weeks of follow-up [120].

In addition, higher blood-associated HIV DNA copy number at the time of a dolutegravir (DTG)-based monotherapy initiation, was associated with virological failure in the DOMONO trial [121].

In the setting of virological suppression, some two-drug regimens (2DRs) have demonstrated comparable effectiveness to standard triple ART for maintenance therapy. Thus, several studies evaluated the impact of 2DRs as maintenance/switch treatment on the cellular reservoir size [8,118]. A sub study of the ATLAS-m trial compared the impact of 48 weeks of atazanavir/ritonavir plus lamivudine dual therapy versus maintaining the

atazanavir/ritonavir plus two NRTIs regimen on total HIV DNA levels and found a similar decline in the two study groups [122].

When compared to other virologically effective 2DRs, the combination of DTG plus lamivudine (3TC) had several advantages in terms of lower risk of drug interactions and metabolic complications. Furthermore, it has shown excellent viro-immunological properties in both naïve and ART-experienced HIV-infected patients [8–118]. Thus, this strategy currently constitutes the most widely prescribed two-drug regimen. An observation from the single arm ANRS167 LAMIDOL trial suggests that, in virally suppressed patients, the treatment switch to DTG plus 3TC from a standard triple regimen could have a favorable effect on the cellular reservoir in terms of control of total HIV DNA levels in the blood after 48 weeks [123]. A different longitudinal, matched, controlled study by Lombardi F. et al. showed that after 48 weeks of treatment this simplification strategy had the same impact on the cellular viral reservoir as the triple regimen [103]. In fact, similar changes in total blood-associated HIV DNA levels were observed in the dual therapy group and the triple therapy group.

The same group conducted a different study on the HIV DNA decay in ART-naïve patients starting treatment with DTG plus 3TC versus a standard DTG-based triple regimen. The preliminary study findings seem to suggest that, at least in the short term, using DTG plus 3TC is not detrimental for controlling the HIV reservoir [124].

Figure 2 summarizes the main studies supporting the clinical role of total HIV DNA.
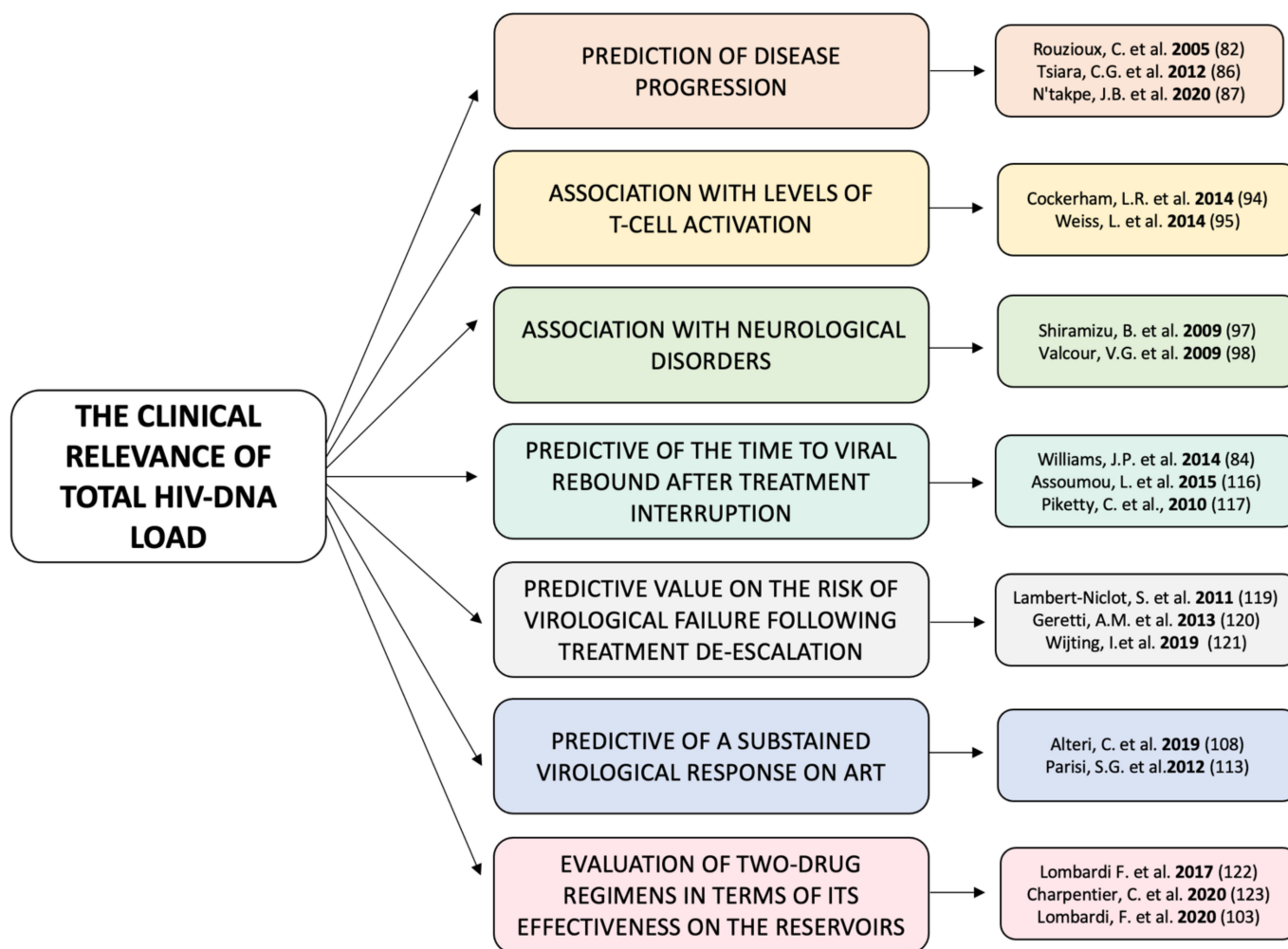

Figure 2. Main studies supporting the clinical role of total HIV-DNA.

5. What Are the Challenges Now?

Despite intrinsic limitations, the quantification of total HIV DNA load is broadly accepted as a measure of the viral reservoir as well as a promising marker for monitoring its changes following specific treatment strategies.

Since it has been demonstrated that measuring the levels of total HIV-DNA makes it possible to estimate the spread of the infection and reflects the ability of antiretroviral therapy to purge reservoirs, it could be an important factor in establishing disease progression and predicting the outcome of ART. It could also be a useful biomarker for monitoring the efficacy of ART, particularly for such novel treatment as long-acting injectable treatments [125,126], as well as novel strategies aimed at reducing or eliminating HIV reservoirs. Furthermore, measuring total HIV DNA load could help identify candidates for therapeutic strategies such as simplification and structured interruption of therapy.

However, data suggest that HIV DNA should not be the only marker used for these clinical purposes. For example, it has been observed that subjects with low HIV DNA levels are not always able to control the infection after interruption of therapy or after simplification.

Therefore, even though HIV DNA should be considered a marker that provides information about the size of the viral reservoir that is not available with the standard markers of HIV RNA load and CD4+ T cell count, this information must necessarily be integrated with that provided by traditional markers and other parameters that are now available (i.e., ultrasensitive plasmatic viremia), as well as clinical data, to better evaluate the disease and to optimize the management of ART.

Predictive statistical models that include HIV DNA together with the other variables might constitute a useful and truly prognostic tool in clinical practice. For example, in this regard some teams derived a predictive score for virological failure to define an appropriate selection of patients for treatment simplification to a dual ART [127]. The inclusion of an important variable, such as total HIV DNA load, could improve the predictive value of the derived score as well as allow validation for clinical purposes.

Although HIV DNA load might be part of clinical practice in the future, to date it is a marker that is mainly used in experimental evaluations. There are no established clinical cut-offs or referencing intervals with a clinical relevance. The main challenge seems to be technical. In fact, to date no certified assay to measure total HIV DNA load has been developed. Few commercial assays have been developed, but they have not been certified for in vitro diagnostic use.

Several "in-house" PCR methods have been used to quantify total HIV DNA worldwide. The assays typically differ as to the standard used, the genomic regions analyzed and/or technique performed, such as real time PCR or droplet digital PCR.

The implementation of droplet digital PCR could improve the accuracy and precision of the total HIV DNA quantification over real-time PCR. However, the heterogeneity of methods has made it difficult to interpret the data generated by different labs because of different levels of sensitivity, accuracy, and precision. Thus, the lack of standardization has made it difficult to carry out cross-comparisons of the data.

There is a need for investigators to compare protocols to permit the validation and standardization of the different methods. In this regard, an effort was made by some teams through multicenter quality controls evaluating the inter-laboratory reproducibility of total HIV DNA quantification [128–130].

In any case, the main effort now should be to involve the biomedical industry. The development of certified systems could be the key for implementing the use of total HIV DNA load in routine diagnostic settings.

Author Contributions: Conceptualization, S.B., S.D.G. and F.L.; methodology, S.B. and F.L.; data curation, S.B. and F.L.; writing—original draft preparation, S.B.; writing—review and editing, S.D.G. and F.L.; visualization, S.B. and F.L.; supervision, F.L. All authors have read and agreed to the published version of the manuscript.

References

1. Phanuphak, N.; Gulick, R.M. HIV treatment and prevention 2019: Current standards of care. *Curr. Opin. HIV AIDS* **2020**, *15*, 4–12. [CrossRef]
2. Finzi, D.; Blankson, J.; Siliciano, J.D.; Margolick, J.B.; Chadwick, K.; Pierso, T.; Smith, K.; Lisziewicz, J.; Lori, F.; Flexner, C.; et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat. Med.* **1999**, *5*, 512–517. [CrossRef] [PubMed]
3. Siliciano, J.D.; Kajdas, J.; Finzi, D.; Quinn, T.C.; Chadwick, K.; Margolick, J.B.; Kovacs, C.; Gange, S.J.; Siliciano, R.F. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat. Med.* **2003**, *9*, 727–728. [CrossRef]
4. Chun, T.W.; Justement, J.S.; Murray, D.; Hallahan, C.W.; Maenza, J.; Collier, A.C.; Sheth, P.M.; Kaul, R.; Ostrowski, M.; Moir, S.; et al. Rebound of plasma viremia following cessation of antiretroviral therapy despite profoundly low levels of HIV reservoir: Implications for eradication. *AIDS* **2010**, *24*, 2803–2808. [CrossRef]
5. Bruner, K.M.; Hosmane, N.N.; Siliciano, R.F. Towards an HIV-1 cure: Measuring the latent reservoir. *Trends Microbiol.* **2015**, *23*, 192–203. [CrossRef] [PubMed]
6. Delagrèverie, H.M.; Delaugerre, C.; Lewin, S.R.; Deeks, S.G.; Li, J.Z. Ongoing clinical trials of human immunodeficiency virus latency-reversing and immunomodulatory agents. *Open Forum Infect. Dis.* **2016**, *3*, ofw189. [CrossRef]
7. Avettand-Fenoel, V.; Hocqueloux, L.; Ghosn, J.; Cheret, A.; Frange, P.; Melard, A.; Viard, J.P.; Rouzioux, C. Total HIV-1 DNA, a marker of viral reservoir dynamics with clinical implications. *Clin. Microbiol. Rev.* **2016**, *29*, 859–880. [CrossRef] [PubMed]
8. Badowski, M.; Pérez, S.E.; Silva, D.; Lee, A. Two's a company, three's a crowd: A review of initiating or switching to a two-drug antiretroviral regimen in treatment-naïve and treatment-experienced patients living with HIV-1. *Infect. Dis. Ther.* **2020**, *9*, 185–208. [CrossRef]
9. Falcinelli, S.D.; Ceriani, C.; Margolis, D.M.; Archin, N.M. New frontiers in measuring and characterizing the HIV reservoir. *Front. Microbiol.* **2019**, *18*, 2878. [CrossRef]
10. Rouzioux, C.; Richman, D. How to best measure HIV reservoirs? *Curr. Opin. HIV AIDS* **2013**, *8*, 170–175. [CrossRef]
11. Eriksson, S.; Graf, E.H.; Dahl, V.; Strain, M.C.; Yukl, S.A.; Lysenko, E.S.; Bosch, R.J.; Lai, J.; Chioma, S.; Emad, F.; et al. Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. *PLoS Pathog.* **2013**, *9*, e1003174. [CrossRef] [PubMed]
12. Blankson, J.N.; Persaud, D.; Siliciano, R.F. The challenge of viral reservoirs in HIV-1 infection. *Annu. Rev. Med.* **2002**, *53*, 557–593. [CrossRef]
13. Reuse, S.; Calao, M.; Kabeya, K.; Guiguen, A.; Gatot, J.S.; Quivy, V.; Vanhulle, C.; Lamine, A.; Vaira, D.; Demonte, D.; et al. Synergistic activation of HIV-1 expression by deacetylase inhibitors and prostratin: Implications for treatment of latent infection. *PLoS ONE* **2009**, *4*, e6093. [CrossRef]
14. Bouchat, S.; Gatot, J.S.; Kabeya, K.; Cardona, C.; Colin, L.; Herbein, G.; de Wit, S.; Clumeck, N.; Lambotte, O.; Rouzioux, C.; et al. Histone methyltransferase inhibitors induce HIV-1 recovery in resting CD4(+) T cells from HIV-1-infected HAART-treated patients. *AIDS* **2012**, *26*, 1473–1482. [CrossRef]
15. Ho, Y.C.; Shan, L.; Hosmane, N.N.; Wang, J.; Laskey, S.B.; Rosenbloom, D.I.; Lai, J.; Blankson, J.N.; Siliciano, J.D.; Siliciano, R.F. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* **2013**, *155*, 540–551. [CrossRef]
16. Bruner, K.M.; Murray, A.J.; Pollack, R.A.; Soliman, M.G.; Laskey, S.B.; Capoferri, A.A.; Lai, J.; Strain, M.C.; Lada, S.M.; Hoh, R.; et al. Defective proviruses rapidly accumulate during acute HIV-1 infection. *Nat. Med.* **2016**, *22*, 1043–1049. [CrossRef] [PubMed]
17. Descours, B.; Avettand-Fenoel, V.; Blanc, C.; Samri, A.; Mélard, A.; Supervie, V.; Theodorou, I.; Carcelain, G.; Rouzioux, C.; Autran, B. Immune responses driven by protective human leukocyte antigen alleles from long-term nonprogressors are associated with low HIV reservoir in central memory CD4 T cells. *Clin. Infect. Dis.* **2012**, *54*, 1495–1503. [CrossRef] [PubMed]
18. Cobos-Jiménez, V.; Booiman, T.; Hamann, J.; Kootstra, N.A. Macrophages and HIV-1. *Curr. Opin. HIV AIDS* **2011**, *6*, 385–390. [CrossRef]
19. Bushman, F. Measuring covert HIV replication during HAART: The abundance of 2-LTR circles is not a reliable marker. *AIDS* **2003**, *17*, 749–750. [CrossRef]
20. Sharkey, M.E.; Teo, I.; Greenough, T.; Sharova, N.; Luzuriaga, K.; Sullivan, J.L.; Bucy, R.P.; Kostrikis, L.G.; Haase, A.; Veryard, C.; et al. Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. *Nat. Med.* **2000**, *6*, 76–81. [CrossRef]
21. Brenchley, J.M.; Schacker, T.W.; Ruff, L.E.; Price, D.A.; Taylor, J.H.; Beilman, G.; Nguyen, P.L.; Khoruts, A.; Larson, M.; Haase, A.T.; et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* **2004**, *200*, 749–759. [CrossRef]

22. Zeng, M.; Southern, P.J.; Reilly, C.S.; Beilman, G.J.; Chipman, J.G.; Schacker, T.W.; Haase, A.T. Lymphoid tissue damage in HIV-1 infection depletes naïve T cells and limits T cell reconstitution after antiretroviral therapy. *PLoS Pathog.* **2012**, *8*, e1002437. [CrossRef] [PubMed]

23. Laird, G.M.; Eisele, E.E.; Rabi, S.A.; Lai, J.; Chioma, S.; Blankson, J.N.; Siliciano, J.D.; Siliciano, R.F. Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay. *PLoS Pathog.* **2013**, *9*, e1003398. [CrossRef]

24. Sanyal, A.; Mailliard, R.B.; Rinaldo, C.R.; Ratner, D.; Ding, M.; Chen, Y.; Zerbato, J.M.; Giacobbi, N.S.; Venkatachari, N.J.; Patterson, B.K. Novel assay reveals a large, inducible, replication-competent HIV-1 reservoir in resting CD4+ T cells. *Nat. Med.* **2017**, *23*, 885–889. [CrossRef]

25. Wonderlich, E.R.; Subramanian, K.; Cox, B.; Wiegand, A.; Lackman-Smith, C.; Bale, M.J.; Stone, M.; Hoh, R.; Kearney, M.F.; Maldarelli, F. Effector memory differentiation increases detection of replication-competent HIV-l in resting CD4+ T cells from virally suppressed individuals. *PLoS Pathog.* **2019**, *15*, e1008074. [CrossRef] [PubMed]

26. Lewin, S.R.; Rouzioux, C. HIV cure and eradication: How will we get from the laboratory to effective clinical trials? *AIDS* **2011**, *25*, 885–897. [CrossRef]

27. Crooks, A.M.; Bateson, R.; Cope, A.B.; Dahl, N.P.; Griggs, M.K.; Kuruc, J.D.; Gay, C.L.; Eron, J.J.; Margolis, D.M.; Bosch, R.J.; et al. Precise quantitation of the latent HIV-1 reservoir: Implications for eradication strategies. *J. Infect. Dis.* **2015**, *212*, 1361–1365. [CrossRef] [PubMed]

28. Pasternak, A.O.; Lukashov, V.V.; Berkhout, B. Cell-associated HIVRNA: A dynamic biomarker of viral persistence. *Retrovirology* **2013**, *10*, 41. [CrossRef] [PubMed]

29. Archin, N.M.; Liberty, A.L.; Kashuba, A.D.; Choudhary, S.K.; Kuruc, J.D.; Crooks, A.M.; Parke, D.C.; Anderson, E.M.; Kearney, M.F.; Strain, M.C.; et al. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature* **2012**, *487*, 482–485, Erratum in *Nature* **2012**, *489*, 460. [CrossRef]

30. Imamichi, H.; Dewar, R.L.; Adelsberger, J.W.; Rehm, C.A.; O'Doherty, U.; Paxinos, E.E.; Fauci, A.S.; Lane, H.C. Defective HIV-1 proviruses produce novel protein-coding RNA species in HIV-infected patients on combination antiretroviral therapy. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 8783–8788. [CrossRef] [PubMed]

31. Kiselinova, M.; de Spiegelaere, W.; Buzon, M.J.; Malatinkova, E.; Lichterfeld, M.; Vandekerckhove, L. Integrated and total HIV-1 DNA predict ex vivo viral outgrowth. *PLoS Pathog.* **2016**, *12*, e1005472.

32. Noel, N.; Peña, R.; David, A.; Avettand-Fenoel, V.; Erkizia, I.; Jimenez, E.; Lecuroux, C.; Rouzioux, C.; Boufassa, F.; Pancino, G.; et al. Long-term spontaneous control of HIV-1 is related to low frequency of infected cells and inefficient viral reactivation. *J. Virol.* **2016**, *90*, 6148–6158. [CrossRef]

33. Sherman, M.P.; Greene, W.C. Slipping through the door: HIV entry into the nucleus. *Microbes Infect.* **2002**, *4*, 67–73. [CrossRef]

34. Sloan, R.D.; Wainberg, M.A. The role of unintegrated DNA in HIV infection. *Retrovirology* **2011**, *8*, 52. [CrossRef]

35. Koelsch, K.K.; Liu, L.; Haubrich, R.; May, S.; Havlir, D.; Günthard, H.F.; Ignacio, C.C.; Campos-Soto, P.; Little, S.J.; Shafer, R.; et al. Dynamics of total, linear nonintegrated, and integrated HIV-1 DNA in vivo and in vitro. *J. Infect. Dis.* **2008**, *197*, 411–419. [CrossRef] [PubMed]

36. Suspène, R.; Meyerhans, A. Quantification of unintegrated HIV-1 DNA at the single cell level in vivo. *PLoS ONE* **2012**, *7*, e36246. [CrossRef]

37. Mexas, A.M.; Graf, E.H.; Pace, M.J.; Yu, J.J.; Papasavvas, E.; Azzoni, L.; Busch, M.P.; di Mascio, M.; Foulkes, A.S.; Migueles, S.A.; et al. Concurrent measures of total and integrated HIV DNA monitor reservoirs and ongoing replication in eradication trials. *AIDS* **2012**, *26*, 2295–2306. [CrossRef] [PubMed]

38. Agosto, L.M.; Liszewski, M.K.; Mexas, A.; Graf, E.; Pace, M.; Yu, J.J.; Bhandoola, A.; O'Doherty, U. Patients on HAART often have an excess of unintegrated HIV DNA: Implications for monitoring reservoirs. *Virology* **2011**, *409*, 46–53. [CrossRef]

39. Munir, S.; Thierry, S.; Subra, F.; Deprez, E.; Delelis, O. Quantitative analysis of the time-course of viral DNA forms during the HIV-1 life cycle. *Retrovirology* **2013**, *10*, 87. [CrossRef] [PubMed]

40. De Spiegelaere, W.; Malatinkova, E.; Lynch, L.; van Nieuwerburgh, F.; Messiaen, P.; O'Doherty, U.; Vandekerckhove, L. Quantification of integrated HIV DNA by repetitive-sampling Alu-HIV PCR on the basis of poisson statistics. *Clin. Chem.* **2014**, *60*, 886–895. [CrossRef] [PubMed]

41. Chan, C.N.; Trinité, B.; Lee, C.S.; Mahajan, S.; Anand, A.; Wodarz, D.; Sabbaj, S.; Bansal, A.; Goepfert, P.A.; Levy, D.N. HIV-1 latency and virus production from unintegrated genomes following direct infection of resting CD4 T cells. *Retrovirology* **2016**, *13*, 1. [CrossRef] [PubMed]

42. Orlandi, C.; Canovari, B.; Bozzano, F.; Marras, F.; Pasquini, Z.; Barchiesi, F.; de Maria, A.; Magnani, M.; Casabianca, A. A comparative analysis of unintegrated HIV-1 DNA measurement as a potential biomarker of the cellular reservoir in the blood of patients controlling and non-controlling viral replication. *J. Transl. Med.* **2020**, *18*, 204. [CrossRef] [PubMed]

43. Buzón, M.J.; Massanella, M.; Llibre, J.M.; Esteve, A.; Dahl, V.; Puertas, M.C.; Gatell, J.M.; Domingo, P.; Paredes, R.; Sharkey, M.; et al. HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. *Nat. Med.* **2010**, *16*, 460–465. [CrossRef] [PubMed]

44. Vicenti, I.; Meini, G.; Saladini, F.; Giannini, A.; Boccuto, A.; Schiaroli, E.; Zazzi, M. Development of an internally controlled quantitative PCR to measure total cell-associated HIV-1 DNA in blood. *Clin. Chem. Lab. Med.* **2018**, *56*, e75–e77. [CrossRef] [PubMed]

45. Zhao, Y.; Yu, M.; Miller, J.W.; Chen, M.; Bremer, E.G.; Kabat, W.; Yogev, R. Quantification of human immunodeficiency virus type 1 proviral DNA by using TaqMan technology. *J. Clin. Microbiol.* **2002**, *40*, 675–678. [CrossRef] [PubMed]

46. Christopherson, C.; Kidane, Y.; Conway, B.; Krowka, J.; Sheppard, H.; Kwok, S. PCR-Based assay to quantify human immunodeficiency virus type 1 DNA in peripheral blood mononuclear cells. *J. Clin. Microbiol.* **2000**, *38*, 630–634. [CrossRef]

47. Désiré, N.; Dehée, A.; Schneider, V.; Jacomet, C.; Goujon, C.; Girard, P.M.; Rozenbaum, W.; Nicolas, J.C. Quantification of human immunodeficiency virus type 1 proviral load by a TaqMan real-time PCR assay. *J. Clin. Microbiol.* **2001**, *39*, 1303–1310. [CrossRef]

48. Lillo, F.B.; Grasso, M.A.; Lodini, S.; Bellotti, M.G.; Colucci, G. Few modifications of the Cobas Amplicor HIV Monitor 1.5 test allow reliable quantitation of HIV-1 proviral load in peripheral blood mononuclear cells. *J. Virol. Methods.* **2004**, *120*, 201–205. [CrossRef] [PubMed]

49. Casabianca, A.; Gori, C.; Orlandi, C.; Forbici, F.; Federico Perno, C.; Magnani, M. Fast and sensitive quantitative detection of HIV DNA in whole blood leucocytes by SYBR green I real-time PCR assay. *Mol. Cell. Probes.* **2007**, *21*, 368–378. [CrossRef] [PubMed]

50. Casabianca, A.; Orlandi, C.; Canovari, B.; Scotti, M.; Acetoso, M.; Valentini, M.; Petrelli, E.; Magnani, M. A real time PCR platform for the simultaneous quantification of total and extrachromosomal HIV DNA forms in blood of HIV-1 infected patients. *PLoS ONE* **2014**, *9*, e111919.

51. Izopet, J.; Tamalet, C.; Pasquier, C.; Sandres, K.; Marchou, B.; Massip, P.; Puel, J. Quantification of HIV-1 proviral DNA by a standardized colorimetric PCR-based assay. *J. Med. Virol.* **1998**, *54*, 54–59. [CrossRef]

52. Hong, F.; Aga, E.; Cillo, A.R.; Yates, A.L.; Besson, G.; Fyne, E.; Koontz, D.L.; Jennings, C.; Zheng, L.; Mellors, J.W. Novel assays for measurement of total cell-associated HIV-1 DNA and RNA. *J. Clin. Microbiol.* **2016**, *54*, 902–911. [CrossRef]

53. Vandergeeten, C.; Fromentin, R.; Merlini, E.; Lawani, M.B.; DaFonseca, S.; Bakeman, W.; McNulty, A.; Ramgopal, M.; Michael, N.; Kim, J.H.; et al. Cross-clade ultrasensitive PCR-based assays to measure HIV persistence in large-cohort studies. *J. Virol.* **2014**, *88*, 12385–12396. [CrossRef]

54. Strain, M.C.; Lada, S.M.; Luong, T.; Rought, S.E.; Gianella, S.; Terry, V.H.; Spina, C.A.; Woelk, C.H.; Richman, D.D. Highly precise measurement of HIV DNA by droplet digital PCR. *PLoS ONE* **2013**, *8*, e55943.

55. Kojabad, A.A.; Farzanehpour, M.; Galeh, H.E.G.; Dorostkar, R.; Jafarpour, A.; Bolandian, M.; Nodooshan, M.M. Droplet digital PCR of viral DNA/RNA, current progress, challenges, and future perspectives. *J. Med. Virol.* **2021**, *93*, 4182–4197. [CrossRef]

56. Bruner, K.M.; Wang, Z.; Simonetti, F.R.; Bender, A.M.; Kwon, K.J.; Sengupta, S.; Fray, E.J.; Beg, S.A.; Antar, A.A.R.; Jenike, K.M.; et al. A quantitative approach for measuring the reservoir of latent HIV-1 proviruses. *Nature* **2019**, *566*, 120–125. [CrossRef] [PubMed]

57. Gaebler, C.; Lorenzi, J.C.C.; Oliveira, T.Y.; Nogueira, L.; Ramos, V.; Lu, C.L.; Pai, J.A.; Mendoza, P.; Jankovic, M.; Caskey, M.; et al. Combination of quadruplex qPCR and next-generation sequencing for qualitative and quantitative analysis of the HIV-1 latent reservoir. *J. Exp. Med.* **2019**, *216*, 2253–2264. [CrossRef] [PubMed]

58. Lungu, C.; Banga, R.; Gruters, R.A.; Procopio, F.A. Inducible HIV-1 reservoir quantification: Clinical relevance, applications and advancements of TILDA. *Front. Microbiol.* **2021**, *12*, 686690. [CrossRef] [PubMed]

59. Gaebler, C.; Falcinelli, S.D.; Stoffel, E.; Read, J.; Murtagh, R.; Oliveira, T.Y.; Ramos, V.; Lorenzi, J.C.C.; Kirchherr, J.; James, K.S.; et al. Sequence evaluation and comparative analysis of novel assays for intact proviral HIV-1 DNA. *J. Virol.* **2021**, *95*, e01986–e02020. [CrossRef]

60. Kinloch, N.N.; Ren, Y.; Conce Alberto, W.D.; Dong, W.; Khadka, P.; Huang, S.H.; Mota, T.M.; Wilson, A.; Shahid, A.; Kirkby, D.; et al. HIV-1 diversity considerations in the application of the Intact Proviral DNA Assay (IPDA). *Nat. Commun.* **2021**, *12*, 165, Erratum in *Nat. Commun.* **2021**, *12*, 2958. [CrossRef] [PubMed]

61. Siliciano, J.D.; Siliciano, R.F. Low inducibility of latent human immunodeficiency virus type 1 proviruses as a major barrier to cure. *J. Infect. Dis.* **2021**, *223*, 13–21. [CrossRef] [PubMed]

62. Razooky, B.S.; Pai, A.; Aull, K.; Rouzine, I.M.; Weinberger, L.S. A hardwired HIV latency program. *Cell* **2015**, *160*, 990–1001. [CrossRef]

63. Yukl, S.A.; Kaiser, P.; Kim, P.; Telwatte, S.; Joshi, S.K.; Vu, M.; Lampiris, H.; Wong, J.K. HIV latency in isolated patient CD4+ T cells may be due to blocks in HIV transcriptional elongation, completion, and splicing. *Sci. Transl. Med.* **2018**, *10*, eaap9927. [CrossRef]

64. Hong, F.; Jacobs, J.L.; Aga, E.; Cillo, A.R.; Fyne, E.; Koontz, D.L.; Zheng, L.; Mellors, J.W. Associations between HIV-1 DNA copy number, proviral transcriptional activity, and plasma viremia in individuals off or on suppressive antiretroviral therapy. *Virology* **2018**, *521*, 51–57. [CrossRef]

65. Belmonte, L.; Olmos, M.; Fanin, A.; Parodi, C.; Baré, P.; Concetti, H.; Pérez, H.; de Bracco, M.M.; Cahn, P. The intestinal mucosa as a reservoir of HIV-1 infection after successful HAART. *AIDS* **2007**, *21*, 2106–2108. [CrossRef] [PubMed]

66. Chun, T.W.; Nickle, D.C.; Justement, J.S.; Meyers, J.H.; Roby, G.; Hallahan, C.W.; Kottilil, S.; Moir, S.; Mican, J.M.; Mullins, J.I.; et al. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. *J. Infect. Dis.* **2008**, *197*, 714–720. [CrossRef] [PubMed]

67. Yukl, S.A.; Gianella, S.; Sinclair, E.; Epling, L.; Li, Q.; Duan, L.; Choi, A.L.; Girling, V.; Ho, T.; Li, P.; et al. Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy. *J. Infect. Dis.* **2010**, *202*, 1553–1561. [CrossRef]

68. Yukl, S.A.; Shergill, A.K.; McQuaid, K.; Gianella, S.; Lampiris, H.; Hare, C.B.; Pandori, M.; Sinclair, E.; Günthard, H.F.; Fischer, M.; et al. Effect of raltegravir-containing intensification on HIV burden and T-cell activation in multiple gut sites of HIV-positive adults on suppressive antiretroviral therapy. *AIDS* **2010**, *24*, 2451–2460. [CrossRef]

69. Josefsson, L.; Palmer, S.; Faria, N.R.; Lemey, P.; Casazza, J.; Ambrozak, D.; Kearney, M.; Shao, W.; Kottilil, S.; Sneller, M. Single cell analysis of lymph node tissue from HIV-1 infected patients reveals that the majority of CD4+ T-cells contain one HIV-1 DNA molecule. *PLoS Pathog.* **2013**, *9*, e1003432. [CrossRef]

70. Avettand-Fenoel, V.; Prazuck, T.; Hocqueloux, L.; Melard, A.; Michau, C.; Kerdraon, R.; Agoute, E.; Rouzioux, C. HIV-DNA in rectal cells is well correlated with HIV-DNA in blood in different groups of patients, including long-term non-progressors. *AIDS* **2008**, *22*, 1880–1882. [CrossRef] [PubMed]

71. Rouzioux, C.; Avettand-Fenoël, V. Total HIV DNA: A global marker of HIV persistence. *Retrovirology* **2018**, *15*, 30. [CrossRef] [PubMed]

72. Lin, L.; Yue, Y.S.; Wang, N.D.; Wei, L.Y.; Han, Y.; Song, X.J.; Qiu, Z.F.; Cao, W.; Li, T.S. Whole blood as an alternative to peripheral blood mononuclear cell for detection of total HIV-1 DNA. *BMC Infect. Dis.* **2020**, *20*, 941. [CrossRef] [PubMed]

73. Avettand-Fènoël, V.; Boufassa, F.; Galimand, J.; Meyer, L.; Rouzioux, C. HIV-1 DNA for the measurement of the HIV reservoir is predictive of disease progression in seroconverters whatever the mode of result expression is. *J. Clin. Virol.* **2008**, *42*, 399–404. [CrossRef]

74. Beloukas, A.; Paraskevis, D.; Haida, C.; Sypsa, V.; Hatzakis, A. Development and assessment of a multiplex real-time PCR assay for quantification of human immunodeficiency virus type 1 DNA. *J. Clin. Microbiol.* **2009**, *47*, 2194–2199. [CrossRef]

75. Rozera, G.; Abbate, I.; Bruselles, A.; Bartolini, B.; D'Offizi, G.; Nicastri, E.; Tommasi, C.; Capobianchi, M.R. Comparison of real-time PCR methods for measurement of HIV-1 proviral DNA. *J. Virol. Methods.* **2010**, *164*, 135–138. [CrossRef]

76. Avettand-Fènoël, V.; Chaix, M.L.; Blanche, S.; Burgard, M.; Floch, C.; Toure, K.; Allemon, M.C.; Warszawski, J.; Rouzioux, C.; French Pediatric Cohort Study ANRS-CO 01 Group. LTR real-time PCR for HIV-1 DNA quantitation in blood cells for early diagnosis in infants born to seropositive mothers treated in HAART area (ANRS CO 01). *J. Med. Virol.* **2009**, *81*, 217–223. [PubMed]

77. Pasternak, A.O.; Adema, K.W.; Bakker, M.; Jurriaans, S.; Berkhout, B.; Cornelissen, M.; Lukashov, V.V. Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA. *J. Clin. Microbiol.* **2008**, *46*, 2206–2211. [CrossRef]

78. Vogelstein, B.; Kinzler, K.W. Digital PCR. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9236–9241. [CrossRef]

79. Hindson, B.J.; Ness, K.D.; Masquelier, D.A.; Belgrader, P.; Heredia, N.J.; Makarewicz, A.J.; Bright, I.J.; Lucero, M.Y.; Hiddessen, A.L.; Legler, T.C.; et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal. Chem.* **2011**, *83*, 8604–8610. [CrossRef]

80. Henrich, T.J.; Gallien, S.; Li, J.Z.; Pereyra, F.; Kuritzkes, D.R. Low-level detection and quantitation of cellular HIV-1 DNA and 2-LTR circles using droplet digital PCR. *J. Virol. Methods.* **2012**, *186*, 68–72. [CrossRef]

81. Bosman, K.J.; Nijhuis, M.; van Ham, P.M.; Wensing, A.M.; Vervisch, K.; Vandekerckhove, L.; de Spiegelaere, W. Comparison of digital PCR platforms and semi-nested qPCR as a tool to determine the size of the HIV reservoir. *Sci. Rep.* **2015**, *5*, 13811. [CrossRef] [PubMed]

82. Rouzioux, C.; Hubert, J.B.; Burgard, M.; Deveau, C.; Goujard, C.; Bary, M.; Séréni, D.; Viard, J.P.; Delfraissy, J.F.; Meyer, L. 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.* **2005**, *192*, 46–55. [CrossRef] [PubMed]

83. Goujard, C.; Bonarek, M.; Meyer, L.; Bonnet, F.; Chaix, M.L.; Deveau, C.; Sinet, M.; Galimand, J.; Delfraissy, J.F.; Venet, A.; et al. 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.* **2006**, *42*, 709–715. [CrossRef]

84. Williams, J.P.; Hurst, J.; Stöhr, W.; Robinson, N.; Brown, H.; Fisher, M.; Kinloch, S.; Cooper, D.; Schechter, M.; Tambussi, G.; et al. HIV-1 DNA predicts disease progression and post-treatment virological control. *Elife* **2014**, *3*, e03821. [CrossRef] [PubMed]

85. Minga, A.K.; Anglaret, X.; d' Aquin Toni, T.; Chaix, M.L.; Dohoun, L.; Abo, Y.; Coulibaly, A.; Duvignac, J.; Gabillard, D.; Rouet, F.; et al. HIV-1 DNA in peripheral blood mononuclear cells is strongly associated with HIV-1 disease progression in recently infected West African adults. *J. Acquir. Immune. Defic. Syndr.* **2008**, *48*, 350–354. [CrossRef]

86. Tsiara, C.G.; Nikolopoulos, G.K.; Bagos, P.G.; Goujard, C.; Katzenstein, T.L.; Minga, A.K.; Rouzioux, C.; Hatzakis, A. Impact of HIV type 1 DNA levels on spontaneous disease progression: A meta-analysis. *AIDS Res. Hum. Retrovir.* **2012**, *28*, 366–373. [CrossRef] [PubMed]

87. N'takpe, J.B.; Gabillard, D.; Moh, R.; Gardiennet, E.; Emieme, A.; Badje, A.; Kouame, G.M.; Toni, T.D.; Karcher, S.; Carrou, J.L.; et al. Association between cellular HIV-1 DNA level and mortality in HIV-1 infected African adults starting ART with high CD4 counts. *EBioMedicine* **2020**, *6*, 102815. [CrossRef]

88. Laanani, M.; Ghosn, J.; Essat, A.; Melard, A.; Seng, R.; Gousset, M.; Panjo, H.; Mortier, E.; Girard, P.M.; Goujard, C.; et al. Impact of the timing of initiation of antiretroviral therapy during primary HIV-1 infection on the decay of cell-associated HIV-DNA. *Clin. Infect. Dis.* **2015**, *60*, 1715–1721. [CrossRef]

89. Ananworanich, J.; Chomont, N.; Eller, L.A.; Kroon, E.; Tovanabutra, S.; Bose, M.; Nau, M.; Fletcher, J.L.K.; Tipsuk, S.; Vandergeeten, C.; et al. HIV DNA set point is rapidly established in acute HIV infection and dramatically reduced by early ART. *EBioMedicine* **2016**, *11*, 68–72. [CrossRef] [PubMed]

90. Ananworanich, J.; Sacdalan, C.P.; Pinyakorn, S.; Chomont, N.; de Souza, M.; Luekasemsuk, T.; Schuetz, A.; Krebs, S.J.; Dewar, R.; Jagodzinski, L.; et al. Virological and immunological characteristics of HIV-infected individuals at the earliest stage of infection. *J. Virus. Erad.* **2016**, *2*, 43–48. [CrossRef]

91. Ghosn, J.; Deveau, C.; Chaix, M.L.; Goujard, C.; Galimand, J.; Zitoun, Y.; Allègre, T.; Delfraissy, J.F.; Meyer, L.; Rouzioux, C. Despite being highly diverse, immunovirological status strongly correlates with clinical symptoms during primary HIV-1 infection: A cross-sectional study based on 674 patients enrolled in the ANRS CO 06 PRIMO cohort. *J. Antimicrob. Chemother.* **2010**, *65*, 741–748. [CrossRef] [PubMed]

92. Martinez, V.; Costagliola, D.; Bonduelle, O.; N'go, N.; Schnuriger, A.; Théodorou, I.; Clauvel, J.P.; Sicard, D.; Agut, H.; Debré, P.; et al. Combination of HIV-1-specific CD4 Th1 cell responses and IgG2 antibodies is the best predictor for persistence of long-term nonprogression. *J. Infect. Dis.* **2005**, *191*, 2053–2063. [CrossRef] [PubMed]

93. Lambotte, O.; Boufassa, F.; Madec, Y.; Nguyen, A.; Goujard, C.; Meyer, L.; Rouzioux, C.; Venet, A.; Delfraissy, J.F. HIV controllers: A homogeneous group of HIV-1-infected patients with spontaneous control of viral replication. *Clin. Infect. Dis.* **2005**, *41*, 1053–1056, Erratum in *Clin. Infect. Dis.* **2005**, *41*, 1694. [CrossRef] [PubMed]

94. Cockerham, L.R.; Siliciano, J.D.; Sinclair, E.; O'Doherty, U.; Palmer, S.; Yukl, S.A.; Strain, M.C.; Chomont, N.; Hecht, F.M.; Siliciano, R.F.; et al. CD4+ and CD8+ T cell activation are associated with HIV DNA in resting CD4+ T cells. *PLoS ONE* **2014**, *9*, e110731. [CrossRef] [PubMed]

95. Weiss, L.; Chevalier, M.F.; Assoumou, L.; Didier, C.; Girard, P.M.; Piketty, C.; Costagliola, D.; Rouzioux, C. T-cell activation positively correlates with cell-associated HIV-DNA level in viremic patients with primary or chronic HIV-1 infection. *AIDS* **2014**, *28*, 1683–1687. [CrossRef] [PubMed]

96. Shiramizu, B.; Paul, R.; Williams, A.; Shikuma, C.; Watters, M.; Grove, J.; Valcour, V. HIV proviral DNA associated with decreased neuropsychological function. *J. Neuropsychiatry Clin. Neurosci.* **2007**, *19*, 157–163. [CrossRef] [PubMed]

97. Shiramizu, B.; Williams, A.E.; Shikuma, C.; Valcour, V. Amount of HIV DNA in peripheral blood mononuclear cells is proportional to the severity of HIV-1-associated neurocognitive disorders. *J. Neuropsychiatry Clin. Neurosci.* **2009**, *21*, 68–74. [CrossRef]

98. Valcour, V.G.; Shiramizu, B.T.; Sithinamsuwan, P.; Nidhinandana, S.; Ratto-Kim, S.; Ananworanich, J.; Siangphoe, U.; Kim, J.H.; de Souza, M.; Degruttola, V.; et al. HIV DNA and cognition in a Thai longitudinal HAART initiation cohort: The SEARCH 001 Cohort Study. *Neurology* **2009**, *72*, 992–998. [CrossRef]

99. Viard, J.P.; Burgard, M.; Hubert, J.B.; Aaron, L.; Rabian, C.; Pertuiset, N.; Lourenço, M.; Rothschild, C.; Rouzioux, C. Impact of 5 years of maximally successful highly active antiretroviral therapy on CD4 cell count and HIV-1 DNA level. *AIDS* **2004**, *18*, 45–49. [CrossRef]

100. Besson, G.J.; Lalama, C.M.; Bosch, R.J.; Gandhi, R.T.; Bedison, M.A.; Aga, E.; Riddler, S.A.; McMahon, D.K.; Hong, F.; Mellors, J.W. HIV-1 DNA decay dynamics in blood during more than a decade of suppressive antiretroviral therapy. *Clin. Infect. Dis.* **2014**, *59*, 1312–1321. [CrossRef]

101. Gandhi, R.T.; McMahon, D.K.; Bosch, R.J.; Lalama, C.M.; Cyktor, J.C.; Macatangay, B.J.; Rinaldo, C.R.; Riddler, S.A.; Hogg, E.; Godfrey, C.; et al. Levels of HIV-1 persistence on antiretroviral therapy are not associated with markers of inflammation or activation. *PLoS Pathog.* **2017**, *13*, e1006285. [CrossRef] [PubMed]

102. Golob, J.L.; Stern, J.; Holte, S.; Kitahata, M.M.; Crane, H.M.; Coombs, R.W.; Goecker, E.; Woolfrey, A.E.; Harrington, R.D. HIV DNA levels and decay in a cohort of 111 long-term virally suppressed patients. *AIDS* **2018**, *32*, 2113–2118. [CrossRef] [PubMed]

103. Lombardi, F.; Belmonti, S.; Borghetti, A.; Fabbiani, M.; Marchetti, S.; Tamburrini, E.; Cauda, R.; di Giambenedetto, S. Evolution of cellular HIV DNA levels in virologically suppressed patients switching to dolutegravir/lamivudine versus maintaining a triple regimen: A prospective, longitudinal, matched, controlled study. *J. Antimicrob. Chemother.* **2020**, *75*, 1599–1603. [CrossRef]

104. Burgard, M.; Boufassa, F.; Viard, J.P.; Garrigue, I.; Ruffault, A.; Izopet, J.; Vabret, A.; Descamps, D.; Colson, P.; Seigneurin, J.M.; et al. Factors influencing peripheral blood mononuclear cell-associated HIV-1 DNA level after long-term suppressive antiretroviral therapy in 236 patients. *AIDS* **2009**, *23*, 2165–2171. [CrossRef] [PubMed]

105. Fourati, S.; Flandre, P.; Calin, R.; Carcelain, G.; Soulie, C.; Lambert-Niclot, S.; Maiga, A.; Ait-Arkoub, Z.; Tubiana, R.; Valantin, M.A.; et al. Factors associated with a low HIV reservoir in patients with prolonged suppressive antiretroviral therapy. *J. Antimicrob. Chemother.* **2014**, *69*, 753–756. [CrossRef]

106. Cuzin, L.; Pugliese, P.; Sauné, K.; Allavena, C.; Ghosn, J.; Cottalorda, J.; Rodallec, A.; Chaix, M.L.; Fafi-Kremer, S.; Soulié, C.; et al. Levels of intracellular HIV-DNA in patients with suppressive antiretroviral therapy. *AIDS* **2015**, *29*, 1665–1671. [CrossRef] [PubMed]

107. Chun, T.W.; Murray, D.; Justement, J.S.; Hallahan, C.W.; Moir, S.; Kovacs, C.; Fauci, A.S. Relationship between residual plasma viremia and the size of HIV proviral DNA reservoirs in infected individuals receiving effective antiretroviral therapy. *J. Infect. Dis.* **2011**, *204*, 135–138. [CrossRef]

108. Alteri, C.; Scutari, R.; Stingone, C.; Maffongelli, G.; Brugneti, M.; Falasca, F.; Martini, S.; Bertoli, A.; Turriziani, O.; Sarmati, L.; et al. Quantification of HIV-DNA and residual viremia in patients starting ART by droplet digital PCR: Their dynamic decay and correlations with immunological parameters and virological success. *J. Clin. Virol.* **2019**, *117*, 61–67. [CrossRef]

109. Lombardi, F.; Belmonti, S.; Rapone, L.; Borghetti, A.; Ciccullo, A.; Gagliardini, R.; Baldin, G.; Montagnani, F.; Moschese, D.; Emiliozzi, A.; et al. HIV-1 non-R5 tropism correlates with a larger size of the cellular viral reservoir and a detectable residual viremia in patients under suppressive ART. *J. Clin. Virol.* **2018**, *103*, 57–62. [CrossRef]

110. Falasca, F.; di Carlo, D.; de Vito, C.; Bon, I.; d'Ettorre, G.; Fantauzzi, A.; Mezzaroma, I.; Fimiani, C.; Re, M.C.; Vullo, V.; et al. Evaluation of HIV-DNA and inflammatory markers in HIV-infected individuals with different viral load patterns. *BMC Infect. Dis.* **2017**, *17*, 581. [CrossRef]

111. Hatzakis, A.E.; Touloumi, G.; Pantazis, N.; Anastassopoulou, C.G.; Katsarou, O.; Karafoulidou, A.; Goedert, J.J.; Kostrikis, L.G. Cellular HIV-1 DNA load predicts HIV-RNA rebound and the outcome of highly active antiretroviral therapy. *AIDS* **2004**, *18*, 2261–2267. [CrossRef] [PubMed]

112. Havlir, D.V.; Koelsch, K.K.; Strain, M.C.; Margot, N.; Lu, B.; Ignacio, C.C.; Miller, M.D.; Wong, J.K. Predictors of residual viremia in HIV-infected patients successfully treated with efavirenz and lamivudine plus either tenofovir or stavudine. *J. Infect. Dis.* **2005**, *191*, 1164–1168. [CrossRef]

113. Parisi, S.G.; Andreis, S.; Mengoli, C.; Scaggiante, R.; Ferretto, R.; Manfrin, V.; Cruciani, M.; Giobbia, M.; Boldrin, C.; Basso, M.; et al. Baseline cellular HIV DNA load predicts HIV DNA decline and residual HIV plasma levels during effective antiretroviral therapy. *J. Clin. Microbiol.* **2012**, *50*, 258–263. [CrossRef] [PubMed]

114. Yue, Y.; Li, Y.; Cui, Y.; Wang, N.; Huang, Y.; Cao, W.; Han, Y.; Zhu, T.; Lyu, W.; Xie, J.; et al. Therapeutic prediction of HIV-1 DNA decay: A multicenter longitudinal cohort study. *BMC Infect. Dis.* **2021**, *21*, 592. [CrossRef] [PubMed]

115. Lafeuillade, A.; Poggi, C.; Hittinger, G.; Counillon, E.; Emilie, D. Predictors of plasma human immunodeficiency virus type 1 RNA control after discontinuation of highly active antiretroviral therapy initiated at acute infection combined with structured treatment interruptions and immune-based therapies. *J. Infect. Dis.* **2003**, *188*, 1426–1432. [CrossRef]

116. Assoumou, L.; Weiss, L.; Piketty, C.; Burgard, M.; Melard, A.; Girard, P.M.; Rouzioux, C.; Costagliola, D. A low HIV-DNA level in peripheral blood mononuclear cells at antiretroviral treatment interruption predicts a higher probability of maintaining viral control. *AIDS* **2015**, *29*, 2003–2007. [CrossRef]

117. Piketty, C.; Weiss, L.; Assoumou, L.; Burgard, M.; Mélard, A.; Ragnaud, J.M.; Bentata, M.; Girard, P.M.; Rouzioux, C.; Costagliola, D. A high HIV DNA level in PBMCs at antiretroviral treatment interruption predicts a shorter time to treatment resumption, independently of the CD4 nadir. *J. Med. Virol.* **2010**, *82*, 1819–1828. [CrossRef] [PubMed]

118. Rossetti, B.; Montagnani, F.; de Luca, A. Current and emerging two-drug approaches for HIV-1 therapy in ART-naïve and ART-experienced, virologically suppressed patients. *Expert. Opin. Pharmacother.* **2018**, *19*, 13–738. [CrossRef]

119. Lambert-Niclot, S.; Flandre, P.; Valantin, M.A.; Peytavin, G.; Duvivier, C.; Haim-Boukobza, S.; Algarte-Genin, M.; Yazdanpanah, Y.; Girard, P.M.; Katlama, C.; et al. Factors associated with virological failure in HIV-1-infected patients receiving darunavir/ritonavir monotherapy. *J. Infect. Dis.* **2011**, *204*, 1211–1216. [CrossRef]

120. Geretti, A.M.; Arribas, J.R.; Lathouwers, E.; Foster, G.M.; Yakoob, R.; Kinloch, S.; Hill, A.; van Delft, Y.; Moecklinghoff, C. Dynamics of cellular HIV-1 DNA levels over 144 weeks of darunavir/ritonavir monotherapy versus triple therapy in the MONET trial. *HIV Clin. Trials.* **2013**, *14*, 45–50. [CrossRef]

121. Wijting, I.; Rutsaert, S.L.; Rokx, C.; Burger, D.M.; Verbon, A.; van Kampen, J.; Boucher, C.; Rijnders, B.; Vandekerckhove, L. Predictors of virological failure in HIV-1-infected patients switching to dolutegravir maintenance monotherapy. *HIV Med.* **2019**, *20*, 63–68. [CrossRef]

122. Lombardi, F.; Belmonti, S.; Quiros-Roldan, E.; Latini, A.; Castagna, A.; D'Ettorre, G.; Gagliardini, R.; Fabbiani, M.; Cauda, R.; de Luca, A.; et al. Evolution of blood-associated HIV-1 DNA levels after 48 weeks of switching to atazanavir/ritonavir + lamivudine dual therapy versus continuing triple therapy in the randomized AtLaS-M trial. *J. Antimicrob. Chemother.* **2017**, *72*, 2055–2059. [CrossRef]

123. Charpentier, C.; Peytavin, G.; Raffi, F.; Burdet, C.; Landman, R.; Lê, M.P.; Katlama, C.; Collin, G.; Benalycherif, A.; Cabie, A.; et al. Pharmacovirological analyses of blood and male genital compartment in patients receiving dolutegravir + lamivudine dual therapy as a switch strategy (ANRS 167 LAMIDOL trial). *J. Antimicrob. Chemother.* **2020**, *75*, 1611–1617. [CrossRef] [PubMed]

124. Lombardi, F.; Belmonti, S.; Borghetti, A.; Ciccullo, A.; Fabbiani, M.; di Giambenedetto, S. Comparison of HIV-DNA decay in naive patients starting dolutegravir plus lamivudine or dolutegravir-based triple therapy. *Infez. Med.* **2020**, *28*, 461–463. [PubMed]

125. Czarnogorski, M.; Garris, C.; D'Amico, R.; Flamm, J.; Sinclair, G.; Wohlfeiler, M.; Mena, L.; Dalessandro, M.; McHorney, C.; Mansukhani, S.; et al. CUSTOMIZE: Overall results from a hybrid III implementation-effectiveness study examining implementation of cabotegravir and rilpivirine long-acting injectable for HIV treatment in US healthcare settings; final patient and provider data. In Proceedings of the IAS Conference on HIV Science, Berlin, Germany, 18–21 July 2021; Available online: https://theprogramme.ias2021.org/Abstract/Abstract/899 (accessed on 23 July 2021).

126. Czarnogorski, M.; Garris, C.; Stassek, L.; Mansukhani, S.; D'Amico, R.; Dalessandro, M.; Williams, M.; Wu, S.; Wohlfeiler, M.; Flamm, J.; et al. CAB+RPV LA implementation outcomes and acceptability of monthly clinic visits improved during COVID-19 pandemic across US healthcare clinics (CUSTOMIZE: Hybrid III implementation-effectiveness study). In Proceedings of the IAS Conference on HIV Science, Berlin, Germany, 18–21 July 2021; Available online: https://theprogramme.ias2021.org/Abstract/Abstract/881 (accessed on 23 July 2021).

127. Borghetti, A.; Moschese, D.; Cingolani, A.; Baldin, G.; Speziale, D.; Ciccullo, A.; Lombardi, F.; Emiliozzi, A.; Belmonti, S.; Antinori, A.; et al. Lamivudine-based maintenance antiretroviral therapies in patients living with HIV-1 with suppressed HIV RNA: Derivation of a predictive score for virological failure. *HIV Med.* **2019**, *20*, 624–627. [CrossRef]

128. De Rossi, A.; Zanchetta, M.; Vitone, F.; Antonelli, G.; Bagnarelli, P.; Buonaguro, L.; Capobianchi, M.R.; Clementi, M.; Abbate, I.; Canducci, F.; et al. Quantitative HIV-1 proviral DNA detection: A multicentre analysis. *New Microbiol.* **2010**, *33*, 293–302. [PubMed]

129. Gantner, P.; Mélard, A.; Damond, F.; Delaugerre, C.; Dina, J.; Gueudin, M.; Maillard, A.; Sauné, K.; Rodallec, A.; Tuaillon, E.; et al. Interlaboratory quality control of total HIV-1 DNA load measurement for multicenter reservoir studies. *J. Med. Virol.* **2017**, *89*, 2047–2050. [CrossRef] [PubMed]

130. Vicenti, I.; Dragoni, F.; Giannini, A.; Callegaro, A.; Napolitano, G.; Turriziani, O.; di Carlo, D.; Giambenedetto, S.; Lombardi, F.; Belmonti, S.; et al. Creation of an Italian HIV DNA network for the validation and clinical use of HIV-1 DNA quantification assays. In Proceedings of the 12th Italian Conference on AIDS and Antiviral Research (ICAR), Digital Edition. Riccione, Italy, 12–16 October 2020.