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Novelties in Evaluation and Monitoring of Human Immunodeficiency Virus-1 Infection: Is Standard Virological Suppression Enough for Measuring Antiretroviral Treatment Success?

*Valentina Svicher[1], Giulia Marchetti[2], Adrianna Ammassari[3], Francesca Ceccherini-Silberstein[1], Loredana Sarmati[4], and Impact Study Group**

[1]Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, Rome, Italy; [2]Department of Health Sciences, Clinic of Infectious Diseases - University of Milan, ASST Santi Paolo e Carlo, Milan, Italy; [3]HIV/AIDS Department, I.N.M.I."L. Spallanzani", Rome, Italy; [4]Department of System Medicine, Clinic of Infectious Diseases, University Hospital "Tor Vergata", Rome, Italy
**Members are listed in the Acknowledgments section.*

Abstract

The high potency and tolerability of the currently available antiretroviral drugs has modified HIV-1 infection from a life-threatening disease to a chronic illness. Nevertheless, some issues still remain open to optimize the management of HIV-1 infected patients in term of maintenance of virological suppression over time, identifying patients that could benefit from simplification therapy, and reducing co-mordibities driven by chronic inflammation. The availability of robust and affordable virological and immunological markers can help in solving these issues by providing information on the burden of HIV-1 reservoir in all the anatomical compartments in which the virus replicates as well as on persistent inflammation, immune activation and senescence despite successful virological suppression. In this light, this review is aimed at providing new insights (arising from a two-day Italian expert meeting hold in Rome in March 2016) in evaluation and monitoring of HIV-1 infection from a virological, immunological and clinical perspective. Particular attention has been focused on role of novel parameters (such as total HIV-1 DNA, residual viremia, and immunological markers) in optimizing treatment strategies, enhancing medical adherence, and individualizing monitoring. **(AIDS Rev. 2017;19:119-133)**

Corresponding author: Francesca Ceccherini-Silberstein, ceccherini@med.uniroma2.it

Key words

HIV-DNA. Residual viremia. Low level viremia. Genotypic resistance testing. Inflammation. Immune-activation.

Correspondence to:
Francesca Ceccherini-Silberstein
Department of Experimental Medicine and Surgery
University of Rome Tor Vergata
Via Montpellier, 1
00133 Rome, Italy
E-mail: ceccherini@med.uniroma2.it

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Introduction

In recent years, human immunodeficiency virus (HIV) infection has become a chronic illness with a life-long treatment, and hence, control and monitoring of the infection play a major role in assessing the durability of therapeutic effectiveness, and the appropriate use of antiretroviral drugs may reduce the risk of virological failure. According to the current guidelines, a number of laboratory tests are recommended for monitoring treatment response during combined antiretroviral therapy (cART) and the evaluation of treatment failure (http://aidsinfo.nih.gov/guidelines). In clinical routine, HIV-1 RNA and CD4 cell count are commonly used to verify virological suppression and immune restoration. Because of high rates of therapeutic efficacy obtained with newer cART regimens, frequency of monitoring in effectively treated HIV-positive patients on stable cART has been lowered to every 6 months for HIV RNA and once a year for CD4 cell count (http://aidsinfo.nih.gov/guidelines). The above current monitoring schedule appears, however, mainly oriented to exclude treatment failure while it does not provide any information on virological purging and chronic inflammation status. This latter, in fact, is currently considered sustaining the appearance of the so named "non-AIDS comorbidities" typically now present in long-treated HIV patients. With the purpose of treatment optimization in chronic patients with long-life expectancy, it would appear relevant to better evaluate not only the classical cART efficacy parameters but also the condition of persistent inflammation assessing the existing relationships between HIV-1 infection, immune system/host factors, and cART (Figs. 1 and 2). So far, accurate and highly sensitive assays to measure low-level or residual HIV-1 replication and reservoir during virological suppression have been investigated. In a similar fashion, novel immunological markers can provide information on the level of persistent inflammation, immune activation, and senescence in patients despite successful virological suppression. The availability of such virological and immunological markers could provide valuable information to the provider and patient, by eventually enhancing medication adherence, optimizing cART, and individualizing monitoring.

With regards to virological failure, guidelines recommend the execution of resistance testing for identification of mutations in the reverse transcriptase and protease genes to select the most appropriate antiretrovirals for cART switch. To date, guidelines recommend the search for integrase strand transfer inhibitors (INSTI) mutations only if the provider is concerned about this possibility, based on drug exposure of the patient and local mutation transmission rates. However, because of widespread use of this drug class in first-line treatment as well as in rescue cART regimens, mutation patterns at treatment failure may profoundly change in the near future with a clear increase of INSTI-associated resistance mutations in naïve and cART-experienced patients. The aim of this paper is to summarize novelties and considerations arising from a two-day Italian expert meeting hold in Rome in March 2016 in evaluation and monitoring of HIV-1 infection from a virological, immunological, and clinical perspective. In particular, the following topics will be discussed and summarized in Tables 1 and 2:

HIV-1 RNA: Viral burden, high pre-cART viremia, residual viremia, and low-level viremia.

1. HIV-1 DNA levels quantification
2. Resistance testing for INSTIs
3. Immunological and inflammation markers.

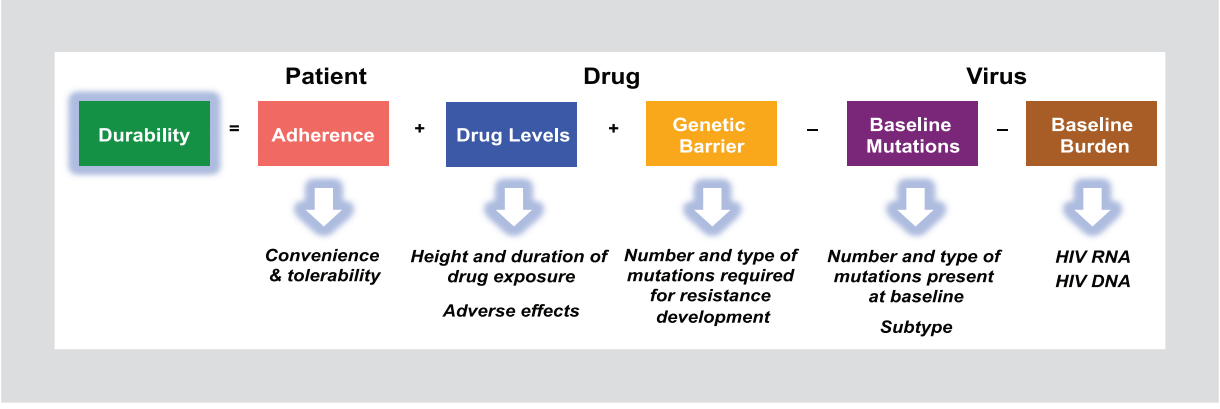

Figure 1. *Factors influencing long-term viral suppression, adapted from Massimo Andreoni personal communicaton.*

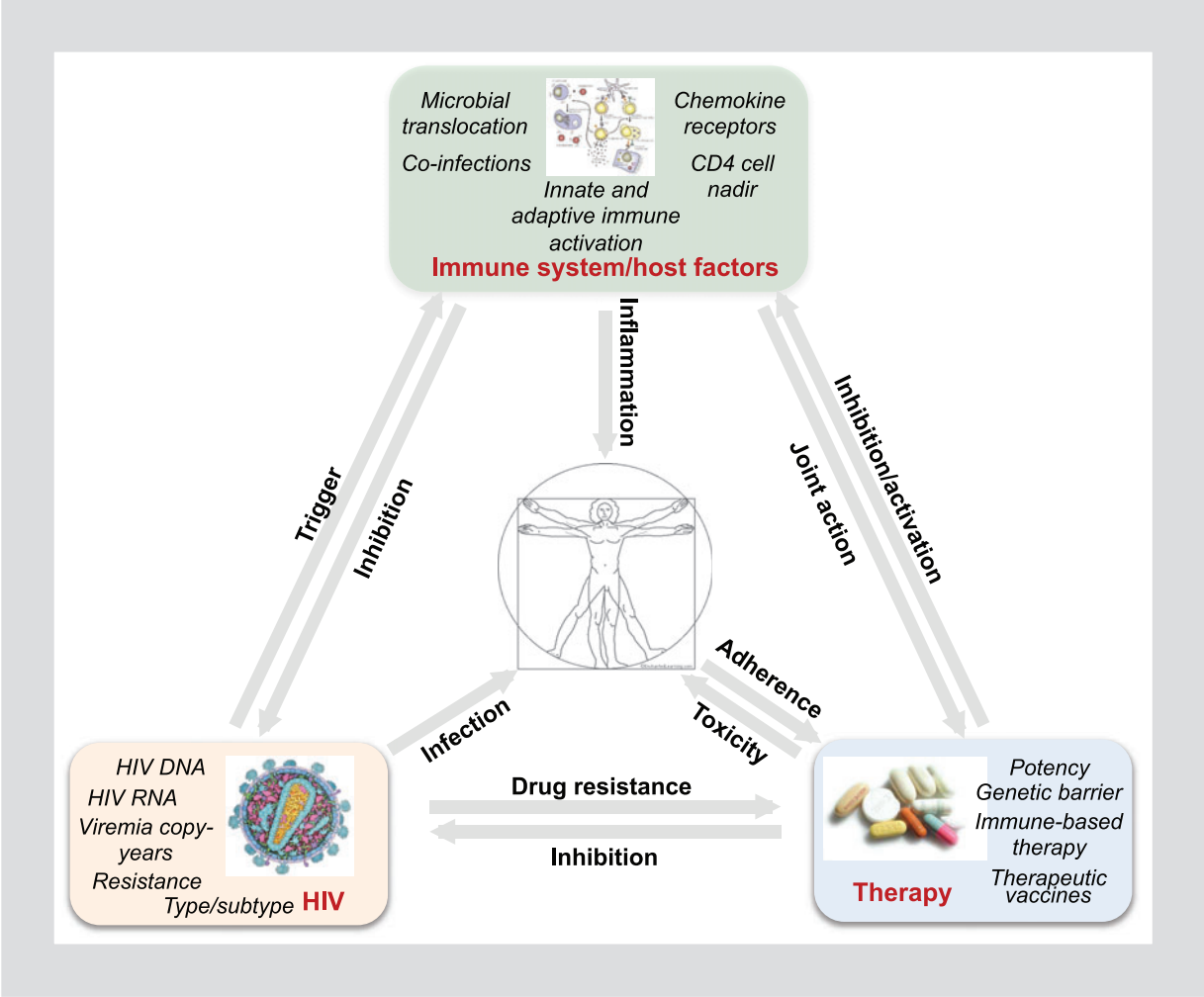

Figure 2. *Factors influencing antiretroviral treatment success.*

HIV-1 RNA: Viral Burden, High Pre-cART Viremia, Residual Viremia, and Low-level Viremia

Viral burden

Quantification of plasma HIV-1 RNA is a measure of HIV-1 replicative potential and can provide crucial information on disease progression (Table 1). The previous important studies have showed that the risk of AIDS and death in HIV-1-infected drug-naïve, and drug-treated patients were directly related to plasma viral load[1]. Likewise, a potential association between high viral load and an increased rate of non-AIDS-related events was recently highlighted even in patients with high CD4 cell count[2]. Overall findings support a direct role of HIV-1 replication (beyond immune-suppression) in mechanisms underlying HIV-1 pathogenesis and the need of a proper therapeutic approach in patients with high viremia at baseline. To reinforce this concept, recently, the attention has been focused on the so-called viremia copy-years (defined as the number of copies of HIV-1 RNA per mL per year circulating in plasma over the number of years from seroconversion) that provide a time-updated measure of cumulative HIV exposure[3-6]. It has been shown that a high viremia copy-year after initiating antiretroviral therapy is associated with increased risk of all-cause mortality independently from CD4 cell count, suggesting that cumulative HIV replication causes harm independent of its effect on the degree of immunodeficiency[3].

Overall findings highlight the importance of measuring plasma HIV-1 RNA to retrieve information on disease progression and to set up an adequate antiretroviral therapy in term of potency and genetic barrier (Table 1).

Table 1. Virological markers and their association with clinical outcome

Laboratory test	Clinical outcome	References
Viral burden • HIV-1 RNA at baseline • High pre-HAART HIV RNA • Viremia copy-years after initiating ART	Faster disease progression in chronically infected patients Increased rate of non-AIDS related events Impact on virological response to first-line therapy Increased risk of all-cause mortality	Mellors et al., Science 1996 Reekie et al., AIDS 2011 Santoro et al., Antiv Therapy 2013; Di Biagio et al., J Medical Virol 2014 Khatchatourian et al. EACS 2015 Mugavero et al., CID 2011; Wright et al., JAIDS 2014; Chirouze et al., JAIDS 2015; Olson et al., JAIDS 2016
Residual viremia	Higher burden of HIV-1 cellular reservoir (measured as cellular HIV-1 DNA Higher transcriptional activity of HIV cellular reservoir Risk of virological failure Shorter time under virological success in PI/b monotherapy Promote persistent immune activation despite virological suppression	Parisi et al., CMI 2012; Parisi et al., CMI 2015; Falasca et al., JAIDS 2015 Chillo et al., CROI 2016 Doyle et al., CID 2012; Maggiolo et al. JAIDS 2012; Henrich et al., Plos Pathogens 2012; Gianotti et al., CMI 2015 Lambert-Niclot et al., JID 2011; Arribas, et al., JIAS 2013 Imamichi et al., CROI 2016
Low-level viremia	Increased risk of virological failure Detection of drug-resistance mutations Progressive enrichment of drug resistance mutations Higher risk of clinical progression and development of non-Hodgkin lymphoma	Laprise et al., CID 2013; Hofstra et al., Plos One 2014; Swenson et al., AIDS 2014; Gonzalez-Serna et al., CID 2014; Young et al., BMC Infect Dis 2015 Santoro et al., CID 2014; Gonzalez-Serna et al., CID 2014; Swenson et al., AIDS 2014; Delaugerre et al., Plos One 2012; Vardahanabhuti et al., AVT 2015 Antinori et al., 15th European AIDS Conference 2015; Zheng et al., JAIDS 2014; Achenbach et al. CID 2014
HIV-1 DNA - Total HIV-1 DNA at baseline	Predictor for disease progression Predicts time and magnitude of viral rebound in treatment experienced patients after therapy interruption Positively correlated with the risk of virological rebound Predicts virological success in the setting of simplification therapy	Tsiara, et al., AIDS Res Hum Retr 2012 Williams eLife 2014 Parisi et al., CMI 2012; Torres-Cornejo et al., AIDS 2014; Parisi et al., CMI 2015; Ceccherini-Silberstein et al., CROI 2016 Marcellin et al. CROI 2016 Geretti et al. HIV Clin Trials. 2013

HIV: human immunodeficiency virus; ART: antiretroviral therapy.

High pre-cART viremia

Different studies have shown that the level of viremia at baseline of cART can influence the time of achieving virological success and the risk of virological failure[7-9].

To shed more light on high viremic patients, the study has evaluated for the first time virological response to first-line cART by stratifying patients into more stringent viremia ranges (< 30,000 copies/ml, 30,000-100,000 copies/ml, 100,000-300,000 copies/ml, 300,000-500,000 copies/ml, and > 500,000 copies/ml)[7]. The authors showed that the prevalence of patients reaching virological success at week 48 of treatment was > 90% in all viremia ranges, with the only exception of range > 500,000 copies/ml (virological success = 83%; $p < 0.001$)[7]. Higher pre-cART viremia was tightly correlated with longer median time to achieve virological success, with a lower probability to achieve virological undetectability and with a higher risk of virological faluire[7].

Another recent study, led on > 8,000 patients starting a first-line cART, has shown that time to achieve virological suppression was significantly faster in patients with pre-treatment viremia < 100,000 copies/ml and in patients receiving an integrase inhibitor[8]. Patients with pre-treatment viremia > 100,000 copies/ml had also a higher risk of virological rebound[8]. These results were confirmed in another study also showing that in the

subset with HIV-1 RNA >100,000 copies/ml, virologic success was only associated with the use of integrase inhibitors[9]. Although further studies are necessary to unravel this issue, these findings support a potential role of integrase inhibitors in controlling massive HIV-1 replication.

These results support the need to better define and characterize the threshold of viremia that properly defines a patient as high viremic. Furthermore, although there is no evidence that quadruple-class therapy confers an advantage in patients with high pre-treatment viral load compared to standard cART[10], overall findings also highlight the need to optimize the management and therapeutic strategies in this setting of patients.

So far, most research efforts were focused on identifying key parameters that could help clinicians in simplifying antiretroviral therapy. In this light, the concept of viral burden cannot be limited only to the quantification of plasma HIV-1 RNA. There is strong evidence that viral burden can be better appreciated by integrating multiple virological parameters that take also in account the level of residual replication under antiretroviral therapy and the extent of HIV-1 cellular reservoir in peripheral and also in other anatomical compartments where HIV replicates. At this regard, a recent study has shown that the level of residual viremia, the burden and transcriptional activity of HIV-1 cellular reservoir (measured as intracellular HIV-1 DNA and HIV-1 RNA, respectively) can help identifying patients maintaining for longer time virological suppression despite interrupting antiretroviral therapy[11]. The role of these parameters will be discussed in the following paragraphs (Tables 1 and 2, Figs. 1 and 2).

Residual viremia

Despite the potency of the currently available antiretroviral regimens, a residual viremia can persist in most patients. Using a single-copy HIV-1 RNA assay, Maldarelli et al. observed that >80% of individuals had stable viremia after 60 weeks of antiretroviral therapy, with a median viral load of 3.1 copies/ml (range, 1-49 copies/ml)[12].

Residual viremia is mainly due to the ongoing or intermittent release of viral particles from HIV-1 long-lived reservoirs. It has been shown a tight correlation between the level of residual viremia and the burden of HIV-1 cellular reservoir (measured as cellular HIV-1 DNA)[13] (Table 1). In another study, the level of residual viremia under suppressive cART has been positively associated with the transcriptional activity of HIV cellular reservoir[14] (Table 1). This suggests that the extent of residual viremia depends not only on the burden of HIV-1 cellular reservoir but also on its capability to express viral genes and produce new viral particles.

In the setting of a fully active cART, viral particles released from the cellular reservoir cannot establish new rounds of infection giving rise to a stable set point of residual viremia that persists despite several years of suppressive cART[12]. However, a decreased adherence (even modest) can promote ongoing cycles of viral replication thus posing the basis for virological failure and the generation of drug- and immune-escape mutations[15]. This strongly supports the need to strictly monitor patient's adherence to treatment to maximally restrict HIV-1 replication and maintain long-term virological success.

Other factors can contribute to residual viremia. Among them, HIV-1 replication in anatomical reservoirs (such as the central nervous system, lymph nodes, and gut) plays an important role[16]. Ongoing viral replication in anatomical reservoirs can be related to suboptimal penetration of antiretroviral drugs and can be exacerbated by HIV-1 cell-to-cell transmission. At this regard, Fletcher et al. showed that in lymph-node samples, the concentrations of some frequently used antiretroviral drugs are much lower than in peripheral blood and correlated with persistent virus replication[17]. In this light, new formulations of antiretroviral drugs with enhanced penetration in HIV anatomical sanctuaries are expected to maximally reduce HIV replication in tissues.

The extent of residual viremia can have important clinical implications. Several studies have highlighted a direct correlation between the extent of residual viremia, and the subsequent risk of virological failure in patients receiving a first- or subsequent-line cART[18-21] (Tables 1 and 2). Using a commercial assay, the previous study showed that the rate of viral rebound was 34.2% in patients with viremia ranging from 40 to 49 copies/ml, and decreased to 11.3% in patients with detectable viremia < 50 copies/ml, and to 4.0% in patients with undetectable viremia[18]. Despite these results, further studies are necessary to investigate whether the "no-signal" information (undetectable HIV-1 RNA) provided by commercially available assays for HIV-1 RNA quantification, may be used in clinical practice. For this reason, ultra-sensitive assays for HIV-1 RNA quantification were also evaluated. For the research purpose, Maggiolo et al. showed that the rate of virological rebound was 0.4% in patients with residual viremia < 3 copies/ml compared to 3.2% in

patients with residual viremia ranging from 3 to 50 copies/ml[19]. Similar results have been obtained in the setting of simplification strategy (Tables 1 and 2). Indeed, a previous study has shown that factors associated with virological failure in patients receiving darunavir/ritonavir monotherapy were a residual viremia > 1 copy/ml at the time of starting simplification therapy, shorter time of antiretroviral treatment before monotherapy, and a level of adherence < 100% during monotherapy[22].

From a pathogenetic point of view, residual viremia could contribute to persistent immune activation and inflammation thus favoring disease progression in cART-treated patients (Tables 1 and 2). A recent study has highlighted a link between residual viremia and atherosclerosis[23], thus suggesting a potential contribution of residual viremia on the development of non-AIDS defining events. Besides, it has been published that not only the entire viral particles but also the proteins produced by cells infected with defective provirus (defined as "zombie" proviruses) can promote persistent immune activation despite virological suppression[24]. This suggests that HIV, even when not viable, can continue to exert its properties in inflammation pathogenesis.

Despite all these findings, it should be noted that ultrasensitive assays to measure residual viremia have been so far used only for research purposes, require a large amount of plasma samples, and are highly costly and time-consuming. This limits the use of residual viremia in routine daily clinical practice, and the potential to propose strong clinical indications according to this virological parameter (Table 2). Thus, further methodological improvements are necessary to position residual viremia in the armamentarium of virological parameters used to optimize the management of HIV-1-infected patients.

Low-level viremia (LLV)

LLV is defined as a persistent viral load ranging from 50 and 400 copies/ml while on cART, and should be distinguished from viral blip defined as a single increase in plasma HIV-1 RNA above 50 copies/ml in cART-treated patients. So far, there is vivid discussion regarding the source and clinical relevance of LLV. By a phylogenetic approach, a previous study has shown that LLV during effective antiretroviral therapy can originate from two distinct (but not mutually exclusive) processes: (i) A clonal outgrowth from long-lived HIV-1-infected cells without new cycles of viral replication, (ii) ongoing viral replication that may contribute to the selection of new drug-resistant mutations[25]. The role of ongoing replication in the genesis of LLV has been highlighted also in other two studies. The former has shown that LLV can be preceded by persistently detectable residual viremia despite achieving virological success[26]. The latter highlighted a relationship between a decreased adherence during antiretroviral treatment and subsequent development of LLV[27]. These findings are important since they can provide plausible explanations for the divergent clinical outcomes associated with LLV and may pose the basis for an individualized therapeutic approach of patients with LLV.

So far, there is an extensive debate on clinical consequences and optimal management of patients with LLV (particularly for those with viremia < 200 copies/ml). Different studies have highlighted a correlation between LLV and an increased risk of virological failure[26,28,29] (Tables 1 and 2). In particular, a recent study has shown that the cumulative incidence of virological failure was 6.6% for patients with undetectable viremia, and raised to 22.7% and 24.2% for patients with viremia range 50-199 copies/ml, and 200-499 copies/ml, respectively[28]. These results may shed new light for the management of patients with LLV, especially for those with LLV < 200 copies/ml.

This is even more critical considering that LLV can be associated with the detection of drug-resistance mutations[30-34] (Table 1). In a recent study, ≥ 1 drug-resistance mutation was detected in 52.8% of patients with plasma HIV-1 RNA ranging from 50 and 200 copies/ml, and in 70% of patients for plasma HIV RNA ranging from 201 to 500 copies/ml[30]. Other studies have shown a progressive enrichment of drug-resistant mutations (and in the extent of genetic variability in pol gene) during persistent LLV[31,32]. Importantly, the detection of drug-resistance during LLV has been associated with an increased risk of virological failure[33,34] (Table 1). Overall findings strongly support the use of drug-resistance testing to inform on the development of drug-resistance mutations and to optimize antiretroviral therapy in patients with LLV even in those with LLV < 200 copies/ml.

Finally, a new study has shown that LLV was an independent risk of clinical progression[35] (Tables 1 and 2). LLV ranging from 51 and 200 copies/ml on antiretroviral therapy was also associated with greater CD8 T-cell activation and increased risk of developing non-Hodgkin lymphomas than full suppression to < 50 copies/ml[36,37]. Although this topic needs further

investigation, there is the evidence for a potential pathogenetic role of LLV in favoring the developing of non-AIDS comorbidities.

HIV-1 DNA level quantification

After reverse transcription and entry into the nucleus, HIV-1 DNA can be integrated into human genome or circularized giving origin to 1-LTR and 2-LTR forms. Circular forms predominate during primary infection and have been detected in at least 50% of drug-naïve patients[38], and thus are considered a surrogate marker of ongoing viral replication. Integrated HIV-1 DNA allows HIV-1 to persist throughout the entire lifespan of both productively and latently infected cells, thus representing the major obstacle for HIV-1 eradication. So far, different assays have been developed for the quantification of integrated, circular, or total cell-associated DNA (hereafter defined as total HIV-DNA)[39-41]. However, currently, there is a solid consensus on the use of total HIV-1 DNA as an accurate parameter to measure the size of HIV cellular reservoir in clinical practice. Indeed, during last years, different studies have investigated the prognostic role of total HIV-1 DNA for monitoring treatment efficacy. In the setting of triple antiretroviral therapy, a recent study (led on 433 patients receiving a first-line antiretroviral regimen) has shown that the amount of total HIV-1 DNA at baseline was positively correlated with the risk of virological rebound (with a prognostic value higher than baseline plasma HIV-1 RNA)[39]. Total HIV-1 DNA has been also shown to predict time to achieving virological success even if less accurately than plasma HIV-1 RNA[39]. These results are in agreement with other studies showing that patients with higher baseline HIV-1 DNA are exposed to an increased risk of early virological failure[40-42].

Similarly, the amount of HIV-1 DNA can be useful to predict virological success in the setting of simplification therapy (Table 1). In the MONOI trial, a higher amount of total HIV-1 DNA was correlated with an increased risk of virological rebound in patients receiving a darunavir/ritonavir monotherapy[43]. Similarly, in the MONET trial, baseline HIV-1 DNA level was higher in patients who experienced (over 144 weeks) at least 1 HIV-1 RNA measurement > 50 copies/ml in both triple- and mono-therapy arms[44]. These findings support the role of total HIV-1 DNA in identifying patients that could benefit from simplification strategy.

The dynamics of total HIV-1 DNA under antiretroviral therapy have been investigated also in the setting of primary infection. It is so far well established that antiretroviral therapy during acute HIV-1 infection can remarkably reduce the burden of HIV-1 cellular reservoir (measured as total or integrated HIV-1 DNA) in periphery and in HIV-1 anatomical reservoirs[12]. These findings strongly support the critical role of early treatment in limiting the establishment of HIV-1 cellular reservoir and viral dissemination. Early treatment may also imbalance virus/host relationship to the advantage of the host, thus favoring host-driven HIV-1 control in the absence of therapy. Again, the quantification of total HIV-1 DNA can be useful in this setting. At this regard, a recent study has shown that, in HIV-1 acutely infected patients receiving antiretroviral therapy, the amount of total HIV-1 DNA at the time of treatment interruption was negatively correlated with the duration of off-therapy virological remission in both adults and pediatric infection[12]. This suggests the use of HIV-1 DNA in identifying patients that might interrupt antiretroviral therapy in clinical trials aimed at achieving HIV-1 cure.

The role of total HIV-1 DNA in disease progression is also under investigation (Table 1 and Fig. 3). It has been highlighted a negative correlation between the amount total HIV-1 DNA and CD4 cell count in drug-naïve[39,45]. In the setting of primary infection, it has been shown a strong predictive value of total HIV-DNA levels for progression to low CD4 cell count, to AIDS and to death, independently of HIV-RNA levels and CD4+ T cell counts. This finding was confirmed in the Spartac cohort showing that a higher total HIV-1 DNA was correlated with faster disease progression (with a prognostic value higher than plasma HIV-RNA)[46].

The amount of HIV-1 DNA may also play a role in modulating immune activation and inflammation. Indeed, a recent study has shown a correlation between the amount of total HIV-1 DNA at baseline and immunological parameters (such as interleukin-6 [IL-6], CD14, CD4, and CD8 cell count) in patients receiving a first-line antiretroviral therapy[39]. In a similar fashion, a positive association between integrated HIV-1 DNA load and frequency of CD8+DR/DP/DQ+ cells has been recently observed[47], suggesting a close correlation between HIV persistence and immune activation despite consistently suppressive therapy. In the gut, an increased level of HIV-1 DNA has been correlated with higher microbial translocation and in turn higher immune activation[48]. In the cerebrospinal fluid, HIV-1 DNA was detected in most virological suppressed individuals, at a level comparable to that observed in drug-naïve patients[49]. These findings highlight that the burden of HIV-1 cellular reservoir plays a critical role

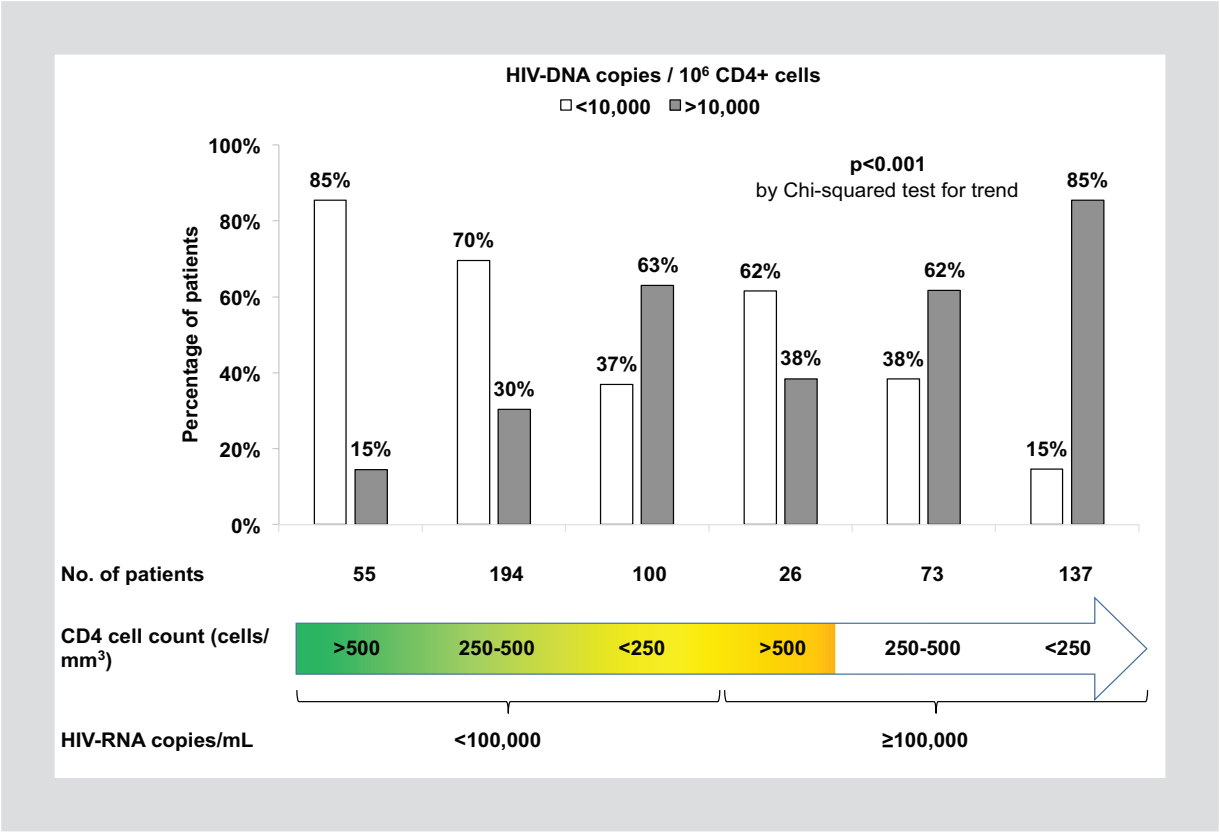

Figure 3. *Figure reports the percentage of patients with total human immunodeficiency virus (HIV)-1 DNA < 10,000 copies/106 CD4+ cells (white bars) and with total HIV-1 DNA > 10,000 copies/106 CD4+ cells (grey bars) stratified according to CD4+ cell count (> 500 cells/mm^3, 250-500 cells/mm^3, and < 250 cells/mm^3) and to levels of plasma HIV-1 RNA (< 100,000 copies/ml and > 100,000 copies/ml), adapted from Ceccherini-Silberstein et al.[39].*

in modulating HIV-1 pathogenetic potential in peripheral blood and in all anatomical compartments where HIV-1 replicates.

So far, the assays for total HIV-1 DNA quantification have not been standardized yet (Table 2). This gap should be rapidly filled in the light of recent findings strongly supporting the role of total HIV-1 DNA as a virological parameter that could help in treatment decision and in optimizing the management of HIV-1-infected individuals.

Genotypic resistance test to INSTIs

Nowadays, a number of international guidelines for ART strongly suggest the use of drug-resistant testing for the choice of the first-line therapy and of alternative therapy in the case of virologic failure.

The use of drug-resistance test in drug-naïve patients is based on the evidence of the possible transmission of drug-resistance mutations. A number of countries periodically report data on HIV drug-resistant prevalence with the ultimate aim to oversee transmitted drug resistance (TDR). In Europe, the surveillance SPREAD program has been monitoring TDR since 2001. In the last report[50], Hofstra et al. documented a value of TDR of 8.3% in 2008-2010, which is substantially unchanged compared to that observed in 2002-2005. Mutations associated with resistance to nucleoside reverse transcriptase inhibitors (NRTIs) were the most frequently observed (prevalence, 4.5%) followed by those associated with resistance to non-NRTIs and protease inhibitors (2.9% and 2%, respectively). INSTIs were excluded from this study due to the unavailability of integrase sequence data at that time.

INSTI is a new class of antiretroviral drugs designed to block HIV integrase enzyme activity. To date, raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG) have been approved, and numerous international guidelines have now introduced INSTIs in the first-line antiretroviral therapy due to their potent antiviral activity and good tolerability. RAL and EVG have a low/moderate genetic barrier to resistance and shared extensive cross-resistance. DTG, the most recent INSTI,

has a high potency, long half-life, and high genetic barrier. These characteristics permitted its use in once daily administration for treatment-naive or treatment-experienced patients (naïve to INSTIs). Drug resistance against DTG is rare, however, a variable cross-resistance with other drugs of the class is possible.

Till date, data on INSTIs TDR in drug-naive patients in Europe are limited (Table 2). A recent study[51] that analyzed 278 samples collected within the SPREAD study, with the aim to evaluate the INSTI-resistant variants circulating in Europe, did not document any resistance transmission in the HIV-1 integrase gene across Europe before the introduction of INSTIs, even if the authors alerted that polymorphisms, contributing to INSTIs resistance, were not so rare. Therefore, the surveillance on INSTI TDR emergence, which could increase with the use of these type of drugs, should be maintained (Table 2).

The first few cases of TDR for INSTIs in drug-naive patients were published in 2010-2011[52,53] but, to date, testing for INSTIs mutations has not been routinely recommended in patients starting first-line ARV. Recent studies in Europe[54] and USA[55] did not detect major INSTIs resistance in drug-naïve patients. The single study, which explored the possibility of TDR using ultra-deep sequencing analysis, confirmed the absence of any primary ISTIs mutations in different groups of naïve patients, even if high percentage of polymorphisms associated with ISTIs resistance was documented in the group of men having sex with men[56].

The possible emergence of drug-resistance to RAL, EVG, and DGT used in first-line therapy or in drug-experienced patients was shown in a number of studies and N155H, Q148H/K/R, and Y143R were the main drug-resistance mutations identified for RAL and EVG. RAL and EVG show extensive cross-resistance, thus performing the resistance test on the integrase at failure, also in cases of LLV is strongly encouraged to evaluate the possibility of consecutive reuse or exclusion of these INSTIs in patients failing an INSTI-containing regimen (Table 2).

Differently, from RAL and EVG, DGT has a high genetic barrier, therefore, it has the indication to be used even in patients that failed a previous INSTI-containing regimen. However, recently, it has been demonstrated that some mutations selected by the first-generation INSTIs were associated with a decreased DGT susceptibility[57,58]. Although rare, DTG-resistance is often associated with the emergence of R263K, known *in vitro* to impair viral replication capacity, DNA integration, and integrase strand-transfer activity. Mutations impairing DGV activity can emerge during DGT monotherapy in patients with a previous failure to INSTIs[59]. For these patients, a twice administration of DGV is recommended and a careful evaluation of the composition of cART regimen is necessary.

Finally, INSTIs resistance mutations can also occur in patients failing ARV with LLV (Table 2). A recent study, analyzing 120 patients failing antiretroviral therapy, showed a prevalence of RAV primary mutations in around 30% of sequences with a proportion of detected mutations, respectively, of 18.2% and 37.5% at viremia levels of 51-500 and 501-1000 copies/mL, respectively. Cross-resistance to EVG was found in 28.3% of overall sequences with the highest prevalence at the 1,001-10,000 copies/mL[60].

HIV: A Tale of (Hyper) Immune Activation/ Inflammation

HIV-driven inflammation/immune activation infection persists indefinitely on cART, at levels significantly higher than HIV-uninfected individuals[61] and has been associated to hampered CD4 reconstitution and disease progression[62].

Numerous factors have been investigated as possible causes of chronic immune activation/inflammation and hypercoagulability in treated HIV, and include viral co-infections[63], ongoing low-level viral replication[64], gastrointestinal impairment, and subsequent microbial translocation[65-67] (Table 2).

Evidence for the association between immune activation/inflammation and morbidity/mortality in the course of virologically effective cART

Successfully-treated HIV-infected patients present an increased risk of non-AIDS-related morbidity/mortality[68], such as cardiovascular disease, non-AIDS-defining cancers, osteopenia/osteoporosis, liver and kidney failure, neurocognitive impairment, in all establishing a condition of increased frailty. Together, the findings of heightened prevalence of clinical conditions known to feature an inflammatory pathogenesis and the hyper-inflamed/activated status of treated HIV, prompted the demonstration of an association between elevations in inflammatory, immune activation and coagulation biomarkers, and increased risk of non-AIDS morbidity within observational and cohort studies (most of which are reviewed in Deeks et al.[69]).

It is worth noting that, contrarily to untreated infection, in treated HIV, monocyte-/macrophage-related inflammation has proven a stronger clinical prognostic power versus T-cell activation[62], consistent with the unique maturation/functional properties of activated T-lymphocytes in treated versus untreated patients[61] and with the crucial involvement of innate immunity in the pathogenesis of non-AIDS clinical conditions, above all cardiovascular disease.

Interestingly, recent literature findings have also provided evidence of an association between markers of gut damage and disease progression: Prolonged mucosal IL-17 deficiency have indeed been associated to disruption of the intestinal microbiome with increased IDO1 activity, in turn contributing to overall mortality rates in virologically suppressed individuals[70].

While such ever-growing body of findings collectively lends support to the hypothesis of inflammation as cause of non-AIDS comorbidity in cART patients, comorbidities themselves are known to drive inflammation (Table 2). Therefore, a definite cause-effect nexus between inflammation and non-AIDS comorbidity is still undemonstrated, and should be assessed through *ad hoc* designed, randomized studies.

Theoretically, some evidence of causality could be gathered from small pilot interventional studies on anti-inflammatory agents in cART-treated patients. However, despite a large number of such studies have been performed to date (most of which are reviewed in Deeks et al.[69]), they fail to provide broad indications due to some intrinsic limitations: (i) Different endpoints (some of which weakly validated) used; (ii) contradictory results, at least partly attributable to biological differences, that include the differential role exerted by specific immune activation/inflammatory pathways in different stages of disease, and in the presence/absence of therapy, and the possibility that intervening on a single pathway through a given agent will lead to the activation of compensatory pathway(s). Finally, surrogate markers validated to detect inflammation and/or immune activation are still lacking.

How do inflammation, immune activation, and coagulation cause disease in the setting of HIV infection?

An important and yet unanswered question is how the state of (hyper) immune activation/inflammation and coagulability causes disease during cART.

HIV-positive cirrhotic patients presented high levels of lipolysaccharide (LPS)-dependent macrophage activation markers (sCD14)[71], thus suggesting that activation of innate immune cells with the subsequent up-regulation of pro-inflammatory, pro-fibrogenic cytokines may indeed promote liver fibrosis in the setting of HIV-hepatitis C virus coinfection.

Circulating LPS has been proven as predictor of atherosclerosis in the general HIV-uninfected population[72]. In the animal model, intravenous LPS challenge resulted in heightened D-dimer levels and cardiovascular lesions, thus providing evidence that hypercoagulation and cardiovascular pathology are a consequence of excessive microbial translocation in pathogenic simian immunodeficiency virus infection[73]. Of note, treated HIV-infected patients with early atherosclerosis feature higher sCD14 levels compared to individuals with no vascular damage[74]. Taken together, these data identify translocating bacteria as a selective stimulus to macrophage activation during cART and possibly explain why parameters of innate immune activation proved much stronger predictor of disease progression in treated HIV infection than markers of adaptive immunity.

Immune cell defects have been identified as a contributing factor for the pathogenesis of other non-AIDS-related comorbidities in treated HIV: T-lymphocyte activation has been shown in osteopenia/osteoporosis (most of which are reviewed in Ofotokun et al.[75]), and skewed/activated T-cell homeostasis in both peripheral blood and cerebrospinal fluid in the setting of neurocognitive impairment (most of which are reviewed in Saylor et al.[76]).

Finally, physiological aging of HIV-infected patients on cART has also to be acknowledged as an adding feature of chronic activation/inflammation. Experimental evidence in mice has shown a selective increase in mortality and pro-inflammatory cytokine release in older animals following LPS administration; further, levels of pro-inflammatory cytokines correlated with age in human volunteers, underscoring age-related differences in the immune response to antigenic stimuli[77].

Immune monitoring of HIV infection

Routine CD4+ T-cell count monitoring: Enough or too much?

Since 1980s, the clinical monitoring of HIV-infected patients has relied on the regular CD4+ measurement. However, several data are accumulating that highlight

the limitations of CD4+ monitoring in patients on stable and successful cART.

In particular, a recent large study involving 1820 patients on virologically suppressive cART, with more than 20000 CD4+ T-cell counts collected from 1998 to 2011, demonstrated: (i) Above 99% likelihood for CD4+ to remain > 200/μL; (ii) no CD4+ loss in patients on cART for more than 2 years[78]. Most interestingly, the utility of CD4+ monitoring is uncertain also in patients with inefficient immunologic recovery of cART: Despite these individuals do present an increased risk of clinical progression[79], no consensus has been established on possible alternative therapeutic approaches.

Furthermore, recent data investigating the cost-effectiveness of CD4+ T-cell monitoring *vis-à-vis* clinical usefulness in both resource-limited settings and in richer countries, found that patients' follow-up by regular CD4+ measurement might be less cost-effective than HIV RNA alone[80,81].

While these findings seem to suggest that routine CD4+ T-cell might be redundant, on the other hand, CD4+ T-cell count alone fails to fully capture immune competence and residual immune activation/inflammation during cART, as witnessed by the onset clinical events at high CD4+ count.

How can we estimate (and monitor) immune activation?

Given the crucial pathogenetic role of immune activation/inflammation, the issue now arises on how it can (and should) be estimated and monitored in virologically suppressed patients. Numerous markers have been associated with the outcome of HIV disease in either settings, yet issues exist regarding the clinical validation of such parameters; in addition, clinicians require a tool able to capture the complex immunologic abnormalities known to pose patients at risk of increased morbidity and mortality. In this respect, the CD4/CD8 T-cell ratio has gained much attention given its association with immune activation and clinical risk in treated individuals[82,83] (Table 2). Indeed, CD4/CD8 T-cell ratio may indeed represent a novel biomarker for the clinical management of HIV disease given its ability to provide simultaneous information on both the clinical and immune status of HIV-infected individuals on cART.

For all the above considerations, clinician (and patients) are nowadays experiencing a paradox, whereby in the face of routine (a perhaps somehow addictive) assessment, CD4+ count is rarely trusted as proxy of "health" status in treated HIV, and therefore increasingly neglected in clinical decision-making (as finely scrutinized in Sax[84]).

Taken together, results from these data advocate the need to thoroughly rethink the frequency of CD4+ monitoring in patients on long-term, whereas on the same time, prompting research to identify and validate surrogate markers of immune competence/activation that might be exploited in the clinic to hopefully couple clinical efficacy and cost-effectiveness (Table 2).

Table 2. Key-points

HIV-1 RNA and DNA
Viral burden
• The effects of HIV are complex and usually occur in a time frame not observed in clinical practice
• Current guidelines underestimate the importance of viral burden. To consider viral burden as a mono-dimensional variable based solely on one quantity is unrealistic and usefulness
• Viral burden (defined as the number of copies of HIV-RNA/ml/year) should be considered in a new and more sophisticated way as a possible prognostic marker for functional cure
High viremia and risk of virological failure
• Further studies are needed to evaluate if HIV-RNA > 100,000 copies/ml is the level of viral load that defines the high viremic patient
• The use of more than 3 drugs for the beginning of treatment in drug-naive patients with high viremia is not supported by data from the literature and therefore it is not recommended in clinical practice

(Continue)

Table 2. Key-points (*Continued*)

Low-level viremia

- Although a direct association between immune activation and development of LLV was not recognized, there is an evidence of a correlation between LLV and the increase/persistence of immune activation and reduction in the number of CD4 lymphocytes
- The presence of LLV requires a more stringent virological and laboratory monitoring

Residual viremia

- Some studies showed that residual viremia is associated with a higher risk of virological failure in patients receiving cART
- Further studies are necessary to support the role of residual viremia in selecting patients candidate to LDR therapy
- So far, single-copy assays to measure residual viremia are costly and time consuming, thus limiting their use in clinical practice. Thus, further methodological improvements should be set up to introduce this parameter in routine clinical practice

HIV-1 DNA

- Before treatment: The measurement of total HIV-DNA in drug naïve patients is a marker of the size of HIV cellular burden, therefore it could be included as a virological parameter at HIV diagnosis (and/or before therapy) to optimize the management of HIV-1 infected patients. Pre-ART HIV-DNA content should be measured/normalized in CD4+ cells
- During treatment: Accurate quantification of HIV-1 DNA in peripheral blood cells (CD4+ cells), can be used for monitoring disease progression in patients receiving antiretroviral therapy
- Since total HIV DNA pre-ART is an independent marker for virological rebound, after obtaining a virological success, patients with pre-ART HIV Total DNA > 10,000 copies/million CD4 cells require closer virological monitoring (e.g. 3-4 months instead of 6-12 months)
- To create networks to obtain a well-standardized method for HIV DNA detection, with high sensitivity and reproducibility establishing a cutoff for total DNA, and secondarily for integrated DNA and non- integrated DNA
- HIV DNA test for diagnosis in newborns from HIV infected mothers should be maintained

Genotypic resistance test to INSTIs

- In ART naive patients who start a INSTI-based therapy, the need for the execution of the INSTI genotypic resistance testing is not supported by the existing epidemiological evidence. However, performing this assay is recommended, for the purpose of clinical and epidemiological surveillance. This recommendation takes in account several factors, among which: The increased usage of INSTI; the different genetic barrier of these antiretrovirals; the lack of epidemiological data in clinical practice.
- In patients failing INSTI-based regimens, the emergence of mutations for INSTI may also occur with low-level viremia values; therefore, performing a genotypic resistance testing for INSTI is strongly recommended also in patients with low-level viremia during INI treatment

Immune activation/inflammation in cART-treated patients

- Clinical and observational studies strongly suggest (but do not definitely prove) that persistent low-level inflammation/ hypercoagulation causes non-AIDS clinical events (mainly cardiovascular and cognitive impairment) and a "frail" clinical phenotype
- The use of immune-activation and inflammation markers in the clinical monitoring and in therapeutic decisions of HIV + patients is not supported by existing scientific evidence. Further studies are advocated to investigate:
  The clinical significance of changes in single biomarkers;
  The possible use of biomarkers to identify patients at higher risk of non-AIDS events (mainly cardiovascular and cognitive impairment);
  The safety and efficacy of specific anti-inflammatory/anticoagulant interventions in reducing inflammation and the risk of non-AIDS events and clinical progression.
- CD4+ T-cell count provides important information in the initial assessment of HIV+ patients (before treatment) or in the presence of clinical events and virologic failure of therapy
- In patients with consistently suppressed HIV viremia after at least 2 years of cART, and high CD4+ T-cell count, the frequency of CD4+ testing can be safely reduced. This approach can result in a substantial saving and possibly allocate resources to offer larger access to treatment
- The CD4+/CD8+ T-lymphocyte ratio should always be assessed and can be considered a good indicator of the immune function and activation. Further studies are advocated that assess the threshold CD4+/CD8+ value predictive of increased risk of clinical progression

HIV: human immunodeficiency virus; LLV: low-level viremia; cART: combined antiretroviral therapy, LDR: low dose rate; INSTIs: integrase strand transfer inhibitors.

Conclusions

Despite the high rates of virological success under treatment, some issues still remain open for an optimal management of HIV-1 infected patients. Current research is showing that the availability of advanced virological and immunological markers to measure HIV-1 cellular reservoir, residual replication, and persistent inflammation can provide an added value and can help in optimizing treatment strategies in term of mainte-

nance of virological success, simplification therapy, and reducing the burden of comorbidity. These markers can also be useful in better understanding factors assessing off-therapy virological remission, and thus could be useful in therapeutic strategies aimed at achieving HIV cure. Thus, further joint efforts between virologists, immunologists, and clinicians are necessary to position these parameters into clinical practice and current guidelines.

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