Advances in detection and monitoring of plasma viremia in HIV-infected individuals receiving antiretroviral therapy

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**Purpose of review**
This review will describe advances in detection and results of monitoring persistent viremia in patients on long-term suppressive therapy. In addition, the review explores the usefulness of these methods in determining the effectiveness of new HIV-1 eradication strategies in purging persistent HIV-1 reservoirs.

**Recent findings**
Quantification of plasma HIV-1 RNA levels remains essential for determining the success of combination antiretroviral therapy (cART) in treated patients. Recently, several new platforms with improved sensitivity for quantifying HIV-1 RNA have been developed and the application of these assays has revealed that low-level viremia persists in patients on suppressive therapy. In addition, new technological advances such as digital PCR have been proposed to increase the sensitivity of measuring and characterizing persistent HIV-1 viremia. The application of these assays will be important in determining the effectiveness of future HIV-1 eradication strategies.

**Summary**
The level of HIV-1 RNA in patient plasma remains an important marker for determining the success of cART. New sensitive assays have found that HIV-1 persists in the plasma of patients on suppressive therapy that may have implications for the clinical management of this disease and strategies for eliminating HIV-1 infection.

**Keywords**
eradication strategies, HIV-1 monitoring, persistent viremia, plasma HIV-1 RNA

**INTRODUCTION**
Recent observational studies have shown that combination antiretroviral therapy (cART) reduces the probability of HIV-1 transmission and provides clinical benefits to those on therapy [1]. As a result, new treatment guidelines for patient care recommend offering cART to patients immediately upon diagnosis of HIV infection regardless of their CD4\(^+\) cell count [2]. In early initiation of cART has been associated with lower levels of cell-associated HIV-1 DNA and lower transient viral set point after treatment interruption [3,4,5]. With these new guidelines, monitoring the levels of HIV-1 RNA in patient plasma after initiation of cART has become essential and more prevalent [2,6–8]. When treatment is initiated, HIV-1 RNA levels should be monitored every 3 months and plasma HIV-1 RNA should decrease to less than 50 copies/ml by 24 weeks regardless of prior treatment experience [9]. New third-generation real-time HIV-1 RNA assays have a lower limit of quantification to 40 or 20 copies/ml and report qualitative detection below these levels in patients receiving effective therapy [10,11]. Current antiretroviral therapy effectively suppresses but does not eradicate HIV-1 infection. During cART, reduction of HIV-1 RNA levels to less than 50 copies/ml is frequently achieved; however, persistent low-level viremia has been detected in plasma and cells of HIV-infected patients using these third-generation HIV-1 RNA assays and other research-based...
New advances in immunological and virological monitoring

**KEY POINTS**
- Quantification of plasma HIV-1 RNA levels is essential during cART.
- HIV-1 RNA persists in the plasma of patients on long-term suppressive therapy albeit at low levels.
- The source of persistent viremia is currently unknown.
- Reactivation of latently infected CD4+ T cells contributes to persistent viremia during suppressive therapy.
- Eradication strategies should include reactivation of latent HIV-1 reservoirs and monitoring of persistent viremia.

ultrasensitive assays [12–17]. The source and significance of this persistent virus is currently unknown but recent studies show that detectable low-level HIV-1 RNA of less than 50 copies/ml can result in viral rebound albeit the lower the level of persistent viremia the less likely a viral rebound will occur [18,19]. Therefore, monitoring persistent virus is not only crucial for determining the effectiveness of cART but will be essential for monitoring the success of future eradication strategies for curing HIV-1 infection. This review will discuss the sensitivity and accuracy of new third-generation HIV-1 RNA assays for detecting and monitoring persistent viremia in patients initiating and maintaining cART. The findings of research-based ultrasensitive assays that measure HIV RNA down to a single copy will be described. Because of the fact that new agents that reduce HIV latency are currently in clinical trials, this review will describe new technologies and methods being developed for quantifying persistent viremia in patients on long-term therapy, which may ultimately be used for assessing the effectiveness of these new HIV-1 eradication strategies.

**THERAPEUTIC MONITORING OF PLASMA HIV-1 RNA LEVELS**

Three general methods are currently used for monitoring the success of cART in HIV-1 infected patients: standard endpoint PCR assays with a detection limit of 400 copies/ml; ultrasensitive endpoint PCR assays with a detection limit of 50 copies/ml; and real-time PCR assays with lower limits of detection ranging from 20 to 48 copies/ml. Cross-platform comparisons have found good correlation between the endpoint PCR assays and the real-time PCR assays, but the new third-generation real-time methodologies, Roche Taqman v2.0 and Abbott RealTime 0.6 ml, have broader reportable ranges and are the most sensitive with lower detection limits of 20 and 40 copies/ml, respectively [20–26]. Additional comparative studies have shown better detection rates of citrated versus EDTA plasma control samples containing 25–50 copies/ml by these new real-time assays [27]. This may be due to EDTA chelating the manganese in the PCR mix after RNA extraction that can affect the efficiency of the PCR reaction. However, these third-generation real-time PCR assays were very specific and no evidence of carryover contamination leading to false positives was detected [27]. Overall, the lower detection limits of the new real-time assays may result in increased measurements of viral blips or transient detectable viral RNA and the impact of these measurements on clinical studies will have to be evaluated [28]. The increased occurrence of measurable low-level HIV-1 RNA by these more sensitive real-time assays should be carefully interpreted. HIV-1 treatment is not curative; rather, the virus persists and is merely suppressed during therapy. Although low-level viral RNA has been found to be a predictor of subsequent suboptimal virological control, it may not lead to virological failure or require a regimen change [29,30].

**LEVELS OF PERSISTENT VIREMIA IN PATIENTS ON LONG-TERM SUPPRESSIVE THERAPY AS DETERMINED BY THE SINGLE-COPY ASSAY**

Application of experimental assays with sensitivities in the range of 10 copies/ml has demonstrated that many patients with viremia suppressed to less than 50 copies/ml have persistently detectable HIV-1 RNA in plasma [31,32]. To investigate the source of residual viremia and to quantify plasma HIV-1 RNA below 50 copies/ml, a more sensitive and precise assay than had previously existed was developed. This assay uses larger plasma sample volumes (7 ml), improved nucleic acid isolation and purification techniques, and real-time RT-PCR to accurately quantify HIV-1 in plasma samples to 1 copy/ml with a limit of detection of one copy in 3.8 ml of plasma, making it 20–50 times more sensitive than currently approved commercial assays [15]. Because the assay has a detection limit of 1 RNA copy it is known as the single copy assay (SCA). To control for recovery of HIV-1, each plasma sample is spiked with 30,000 copies of an internal virion standard derived from an unrelated retrovirus, the replication competent avian sarcoma-leukosis retrovirus vector RCAS BP(A) [15].

SCA gave virtually identical results when compared with a standard (bDNA) assay on samples from
patients initiating triple therapy, but revealed the presence of low-level viremia that persisted long after viremia became undetectable by the standard bDNA assay [33]. Additional studies using this assay have shown that 77% of samples from patients with plasma HIV-1 RNA consistently below 50 copies/ml had quantifiable viremia [15,33]. Notably, in one study of 40 patients, persistent viremia was evident even after 7 years of therapy and all the patients had at least one sample with detectable viremia over the course of those 7 years [34]. The level of viremia correlated with pretherapy plasma HIV-1 RNA but not with the specific treatment regimen. Furthermore, a nonlinear mixed effects model revealed a biphasic decline in plasma RNA levels occurring over weeks 60–384: an initial phase of decay with a half-life of 39 weeks and a subsequent phase with no perceptible decay where levels of plasma HIV RNA remained stable ranging from 1–5 copies/ml or less with an overall median viral load of 3 copies/ml [34]. The level of pretherapy viremia extrapolated for each phase of decay was significantly correlated with total baseline viremia for each patient ($R^2=0.27, P=0.001$ and $R^2=0.19, P<0.005$, respectively). These findings support a biological link between the extent of overall baseline viral infection and the infection of long-lived reservoirs. The clinical significance of low-level viremia, especially between 1 and 10 copies/ml, in patients on long-term suppressive therapy is currently not established. However, these data suggest that there is a constant source of viremia in patients on long-term suppressive therapy.

The source and dynamics of persistent viremia in treated patients is currently under investigation. Recent studies of treatment intensification with raltegravir have produced conflicting results: no reductions in low-level plasma viremia indicating the source of persistent viremia is long-lived HIV-infected cells and increases in episomal HIV DNA indicating active replication persists in some infected individuals on suppressive therapy [35,36]. These results indicate that low-level viremia could arise from several different sources. These sources may include ongoing replication cycles in cells located in sanctuary sites wherein drug levels are suboptimal, long-lived HIV-infected cells that replicate and produce virus, and/or activation of viral expression from latently infected cell reservoirs [13,33,37–39]. Evidence for the view that long-lived cells are the probable source of persistent viremia includes four studies using a number of different treatment protocols that show no perceptible change in persistent viremia in patients receiving intensified treatment [36,40,41*,42]. Moreover, genetically homogeneous sub-populations can often be observed in patients under long-term treatment, as well as in the viral population that rebounds during treatment interruption further indicating that persistent viremia arises from long-lived latently infected cells rather than ongoing cycles of replicating virus [43,44]. The role of ongoing HIV-1 replication in tissue compartments and cellular reservoirs, however, remains to be defined as a number of findings suggest that the reservoir is largely established and maintained in tissues, and that the infected cells circulating in blood may not necessarily be representative of the much larger population of infected cells in tissue [45**]. For example, sequences of persistent HIV populations in plasma are often not found in peripheral blood resting memory CD4$^+$ T cells [43,46]. On the contrary, a recent study of two patients found that HIV-1 sequences isolated from virus outgrowth assays of resting memory CD4$^+$ T cells matched the predominant plasma clones [47*]. These results indicate that persistent viremia is derived in part from reactivation of latent proviruses from resting memory CD4$^+$ T cells.

**PLASMA VIREMIA DURING DISRUPTION OF HIV-1 LATENCY IN PATIENTS ON LONG-TERM SUPPRESSIVE THERAPY**

One well defined latent HIV-1 reservoir is infected resting memory CD4$^+$ T cells carrying an integrated form of the viral genome [48]. The precise molecular mechanisms contributing to the generation and maintenance of this reservoir, however, remain to be elucidated. The scarcity of such cells, even among those carrying integrated HIV DNA, makes these cells extremely difficult to identify, isolate, and study. However, this latent reservoir is now widely recognized as the major obstacle to HIV-1 eradication [49].

Approaches for the reactivation of HIV-1 from resting memory T cells and the subsequent elimination of these cells include T-cell activating cytokines [50–52], T-cell receptor and T-cell receptor signaling pathway agonists [53–57], histone deacetylase inhibitors [58–60], histone methyltransferase inhibitors [61], DNA methylase inhibitors [62,63], and compounds such as 5-hydroxynaphthalene-1,4-dione [64], and disulfiram [65] that reverse latency through mechanisms that are not yet completely understood. Clinical trials of disulfiram and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) have been initiated [66**,67**]. In conducting these two different HIV-1 reactivation trials, HIV-infected patients on long-term suppressive therapy with undetectable plasma viremia (by conventional methods) for 6 months to 1 year have been given: 14 daily doses of disulfiram (500mg) or two separate doses of SAHA (400mg) given 4–5 weeks apart. To evaluate the effectiveness of these two interventions in reactivating latent
HIV-1, many different analyses were conducted including measuring plasma HIV-1 RNA levels by SCA. An interim analysis of persistent viremia was measured for 14 patients prior, during, and after treatment with disulfiram (15 separate measurements) and an increase in viremia was measured during disulfiram therapy and after therapy was discontinued (53 and 88% increase, respectively) compared with baseline but these increases in plasma viremia were found to be statistically insignificant [67**]. For the eight patients enrolled in the SAHA study, no significant change in plasma HIV-1 RNA levels were detected 8–24 h after exposure to SAHA compared with baseline, although increased levels of intracellular HIV-1 RNA were measured in resting memory CD4⁺ T cells [66**]. To date, reactivation of latent HIV-1 RNA from resting memory cells does not translate into statistically significant increases in plasma HIV-1 RNA. These initial results may be due to the very low levels of persistent plasma viremia in patients on long-term therapy thereby limiting the detection of the brief effects of these new reactivation therapies. Another explanation could be that resting memory CD4⁺ T cells are not the only source of persistent viremia in treated patients.

**QUANTIFICATION OF PERSISTENT VIREMIA USING DIGITAL DROPLET PCR METHODOLOGY**

In conducting SCA only a limited number of patient samples can be analyzed because each assay run requires two standard curves: one for HIV-1 and one for RCAS⁺ [15]. Although the limit of detection of SCA is the most sensitive of all assays to date (0.3 copies/ml in 7 ml plasma), due to the extremely low levels of persistent viremia in the plasma of patients on long-term therapy, new eradication treatment strategies that evoke more than an one log reduction in the size of viral reservoirs contributing to residual viremia may become unmeasurable even by SCA. Digital droplet PCR (ddPCR) is a new technology that quantifies DNA copy number by dividing a 20 μl mixture of sample and PCR reagents into 20,000 droplets, each containing a single molecule and fluorescent-labeled probe. PCR amplification of these droplets to end-point amplification is performed in a conventional thermal cycler [68*,69]. For droplets that contain template, specific cleavage of TaqMan probes generates a strong fluorescence signal. Positive droplets containing the DNA target are measured by flowing one droplet at a time through a fluorescence reader. This technology provides an absolute measurement of the DNA target without the use of a standard curve. Recent use of ddPCR for the measurement of HIV-1 RNA, wherein HIV RNA transcripts were serially diluted, reverse transcribed and then quantified using ddPCR, found this method had a broad dynamic range from 0.3 to 1 x 10⁵ copies [70]. Due to saturation of the positive droplets, ddPCR was found to be inaccurate above 1 x 10⁵ copies indicating further dilution would be needed to quantify high copy numbers. In addition, ddPCR detected similar HIV-1 RNA levels when compared with SCA in patients with less than 50 copies/ml (mean 2.0-fold difference). However, viral sequence variation in primer/probe binding sites resulted in varying amplitude of positive droplets causing different signal-to-noise ratios and difficulty in setting a threshold for positive/negative droplets. Although this technology will need further improvement, it holds promise for providing absolute quantification of residual plasma HIV-1 RNA equal to or better than current ultrasensitive assays.

**CONCLUSION**

The level of HIV-1 RNA in patient plasma remains an important marker for determining the success of cART. New sensitive assays have revealed that HIV-1 persists in the plasma of nearly 80% of patients on suppressive therapy. Understanding the source and dynamics of this persistent viremia is essential for developing effective strategies for HIV-1 eradication. In addition, new technologies may improve the quantification of plasma HIV-1 RNA levels during and after latent reservoir reactivation as part of future HIV-1 eradication efforts.

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**Conflicts of interest**

The author has no conflicts of interest in writing this article.

**REFERENCES AND RECOMMENDED READING**

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).


This in-depth clinical study revealed that suppressive antiretroviral therapy reduces the risk of HIV transmission in HIV-discordant couples.


This report is a proof-of-concept study which provided the first evidence that the antilatency activity of vorinostat, an HDAC inhibitor, decreased the levels of HIV RNA in resting CD4⁺ T cells.


This interim report is a proof-of-concept study that provided the first evidence that the antilatency activity of disulfiram demonstrated a 14% decline in the latent reservoir size and average 4.5-fold increase in viremia.


This article described the digital droplet technique for measuring absolute DNA copy numbers in patient samples.
