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Evolution of Experimental Design and Research Techniques in HIV-1 Reservoir Studies: A Systematic Review

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Abstract

Although HIV-1 has evolved from a deadly to a chronic disease over the past 20 years, an HIV-1 cure is still lacking due to the presence of persisting cellular viral reservoirs which are spread throughout the body in different anatomical compartments. Hence, the identification and characterization of these HIV-1 reservoirs were the focus of many studies during the past decades. In this review, a systematic literature screening and text mining approach were implemented to assess the evolution in experimental design of these HIV-1 reservoir studies. For this purpose, the online databases PubMed, Web of Science, and ClinicalTrials.gov were consulted and 1768 articles were identified, of which 106 are included in this review. We observed several evolutions that indicate a more structured approach of recent HIV-1 reservoir studies. This includes the use of well-characterized patient cohorts, tissue sampling at several time points and anatomical compartments, the inclusion of patients with different treatment status (on and off antiretroviral therapy), and the implementation of state-of-the-art research techniques such as single genome sequencing. In addition, there is an increased interest and sampling of lymphoid tissues and cerebrospinal fluid together with methods to investigate cellular subsets and HIV-1 sequences. Overall, this review describes an observed shift from detecting and quantifying HIV-1 toward a qualitative in-depth assessment of anatomical reservoirs and cellular subsets playing a role in HIV-1 persistence/latency. These trends coincide with the evolution in focus from controlling HIV-1 replication by currently available antiretroviral therapy toward HIV-1 curative strategies. **(AIDS Rev. 2020;22:16-24)**

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Key words

HIV-1 reservoir. HIV-1 latency. Tissue compartments. HIV-1 cure. Research methodology.

Introduction

Over the past decades, major advances were made in the field of HIV-1 care, but a curative strategy is still out of reach due to the presence of persistent cellular viral reservoirs which are spread across different anatomical compartments in the human body and cause viral rebound after therapy cessation. Despite the fact that early treatment initiation can limit the size of these HIV-1 reservoirs, it cannot prevent its establishment

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during the early days of infection or revoke its latent state. Hence, an additional intervention targeting these reservoirs will be mandatory to achieve a cure[1].

An HIV-1 reservoir is defined as any infected cell population where HIV-1 persists as integrated provirus despite optimal combined antiretroviral therapy (cART)[2]. These infected cells are present in the peripheral blood and mainly consist of resting memory CD4+ T-cells. Other anatomical compartments are suggested to harbor latently infected cells such as lymphoid tissue spread throughout the body[3], the gastrointestinal tract, and the central nervous system[2,4]. The latent HIV-1 reservoir can be defined as that part of the HIV-1 reservoir that harbors replication-competent proviruses which can produce new infectious particles and infect other cells once treatment is interrupted and the latent state of the cells is reversed[4]. Hence, to achieve an HIV-1 cure, these different reservoirs and compartments need to be studied in depth to identify their respective clinical significance and to unravel the mechanisms by which latency is established or maintained[5,6].

There are different ways to investigate the viral reservoir in HIV-1-infected individuals and although viral load measurements are the gold standard in follow-up of chronically infected HIV-1 patients to assess viral control and therapy compliance, once the patient is virologically suppressed, it cannot longer provide information on the HIV-1 reservoir. Therefore, other, more sensitive assays, are used to quantitatively and qualitatively measure the HIV-1 reservoir such as polymerase chain reaction (PCR)-based methods (e.g., quantitative or digital PCR), cell-culture-based methods (e.g. viral outgrowth assays), immunophenotyping techniques and several sequencing approached developed for HIV-1 full-length and integration site investigation[5].

In this systematic review, we assessed how the study of anatomical compartments has evolved over time in the context of HIV-1 reservoir studies, especially in terms of compartment sampling, study participants, study design and research techniques.

Materials and methods

Systematic literature screening

We performed a systematic literature search on HIV-1 reservoir studies using the online databases PubMed (www.ncbi.nlm.nih.gov/pubmed), Web of Science (www.webofknowledge.com), and clinicaltrials.gov for the time period 1987-2019 (Fig. 1).

The following search terms were used in PubMed and Web of Science to construct the initial dataset of articles: "HIV-1 reservoir lymph node", "HIV-1 reservoir lung", "HIV-1 reservoir gut", "HIV-1 reservoir bone marrow", "HIV-1 reservoir rectal tissue", "HIV-1 reservoir gut-associated lymphoid tissue (GALT)", "HIV-1 reservoir brain", "HIV-1 reservoir central nervous system", and "total body HIV-1 reservoir". In addition, the term HIV-1 reservoir was also searched for in clinical trials related to HIV-1 interventional and observational studies with published articles (on clinicaltrials.gov). Here, advanced search terms included "interventional studies" or "observational studies, completed".

The total set of 1768 identified articles was pooled together, duplicates removed, and the first filtering was performed based on following exclusion criteria: review articles, non-English manuscripts, no full-text available and article publication date before 2000 without recent citation. Recent citation was defined as articles that were cited in the past 10 years (from 2010 onward). The remaining set of articles were manually evaluated based on their abstract with the following exclusion criteria: *in vitro* studies, studies on animals including primates, only peripheral blood mononuclear cells included, no relevant reservoirs, non-HIV-1 and studies focusing on autopsies.

Data processing and visualization

The included articles were represented as a data matrix in Microsoft Excel (2016) (Supplemental Table 1-3). These tables include information on the number of patients included and their main characteristics, the number of compartments that were studied and which compartments and research techniques that were applied to investigate these compartments. In terms of possible HIV-1 compartments, we especially focused on blood, lymph nodes, bone marrow, central nervous system, lungs and the gastrointestinal tract. In addition, the research techniques were grouped in four categories: PCR-based quantification, non-PCR-based quantification, immunophenotyping and qualitative sequencing approaches (Supplemental Table 4).

The graphs summarizing the data from these articles were made in R (v.3.4.0) using RStudio (v1.0.143) and the ggplot2 package (v2.2.1). Trends were made visual by implementing non-parametric moving regression analysis with Locally Weighted Scatterplot Smoothing.

Results

HIV-1 reservoir studies: systematic literature search

The systematic literature screening resulted in the inclusion of 106 HIV-1 reservoir studies over the time

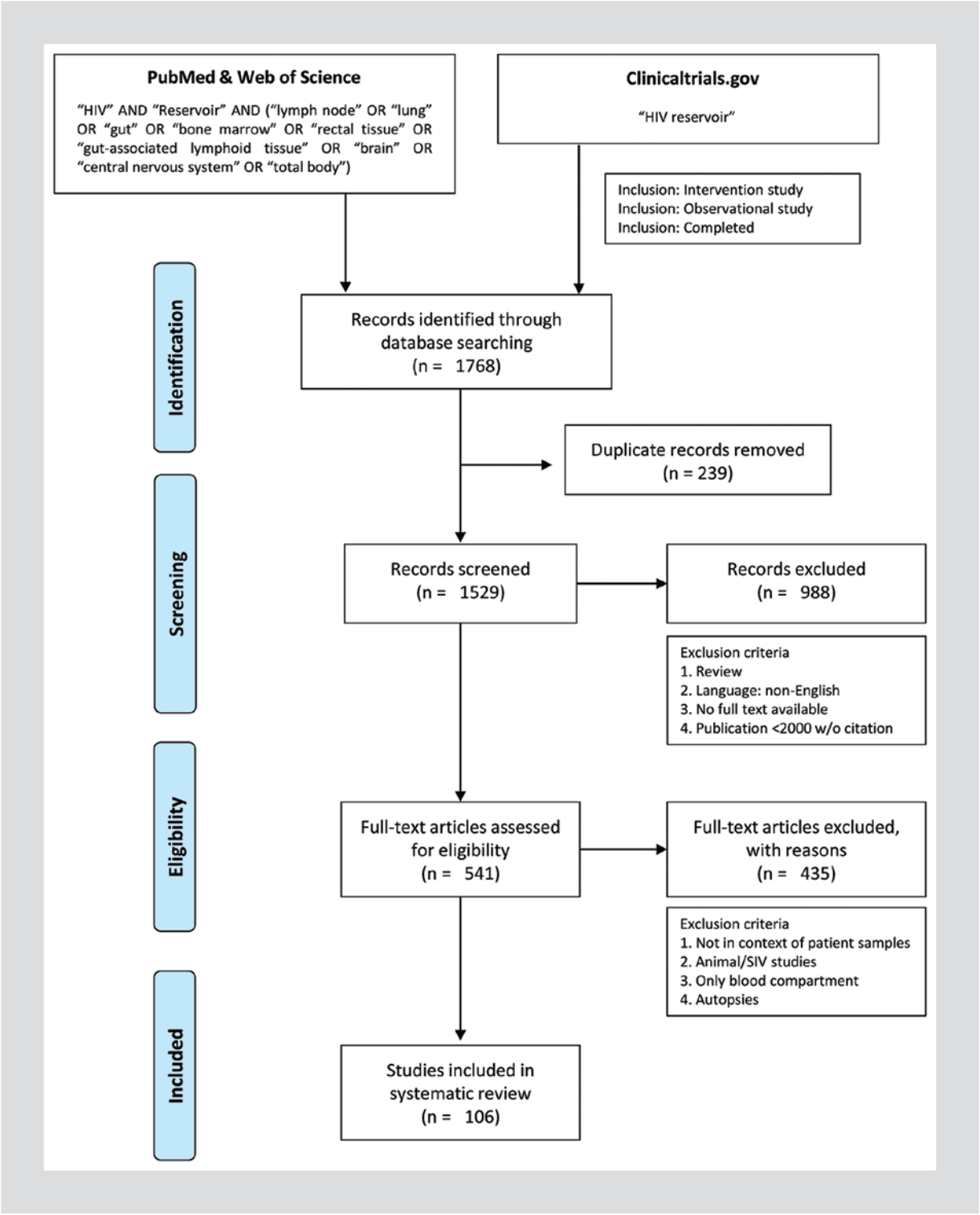

Figure 1. *Systematic literature screening pipeline. Overview of the stepwise approach used to identify the set of 106 HIV-1 reservoir studies in the time period 1987-2019.*

period 1987 to present, with an observed increase in the amount of studies between 2010 and 2018 which underlines the efforts made in HIV-1 reservoir research to better understand the latent reservoir and work toward a cure (Fig. 2a).

Anatomical compartments

In total, 229 samples from five different compartments were reported across 106 reservoir studies with an increasing number of compartments sampled per study

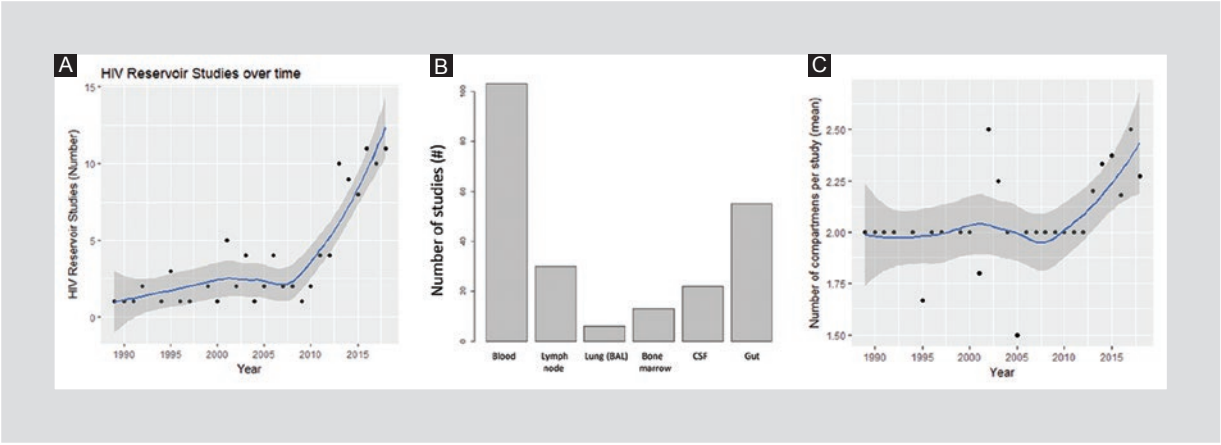

Figure 2. *Evolution of HIV-1 reservoir studies.* **A:** *The absolute number of HIV-1 reservoir studies is plotted per year for the time period 1987-2019. The Locally Weighted Scatterplot Smoothing regression line is added in blue;* **B:** *The number of reservoir studies including one of the following compartments: blood, lymph node, lung bronchoalveolar lavage, bone marrow, cerebrospinal fluid, or gut;* **C:** *Evolution of the average number of compartments included in reservoir studies over the time period 1987-2019. The Lowess regression line is added in blue.*

over time (Fig. 2b and c). Since 2010, reservoir studies include two or more compartments and even sample up to five different compartments in a single study.

Overall, gut (55 studies) and lymph node (30 studies) tissues were sampled the most besides the blood compartment which was sampled in 103 out of 106 studies (Fig. 3a-c). However, when investigating the compartment sampling over time, there is an observed increase in cerebrospinal fluid (CSF) and lymph node sampling together with a decreased sampling of gut tissues in recent years (Fig. 3b-d). Interest in bone marrow (BM) has declined over the years and the amount of studies including lung sampling has been low (Fig. 3e and f).

Longitudinal sampling and patient inclusion

Although the majority of studies still focus on a single cross-sectional time point (66 vs. 40 studies), longitudinal sampling at multiple (> 1) time points increased over the recent years, reaching 40% of total HIV-1 reservoir studies (Fig. 4a and b).

Zooming in on ART status of patients enrolled, 38% of the reservoir studies included patients on and off therapy, 45% of the studies included only patients on ART, and 16% only included patients off ART. In addition, there is a stable inclusion of healthy volunteers over time, reaching on average 20% of reservoir studies published.

When combining longitudinal sampling and ART status, studies that consider one time point focus more on controlled aviremic participants that are on ART (Fig. 4c).

Research methods

When dissecting the research methods used to study the viral reservoir, we observe that in 77% of the studies, PCR-based techniques are used, 48% non-PCR based, 35% immunophenotyping and 25% qualitative analysis. Since 2000, there has been an increase in qualitative analyses as well as a continuous increase in the implementation of immunophenotyping techniques (Fig. 5a-d). Although 65% of studies perform subset sorting, there is an increase in usage of bulk cells around the 2000 (Fig. 5e and f).

Discussion

Where the initial goal of HIV-1 research was to understand the natural course of disease and the effect of cART on virus and host, the focus has now shifted toward identifying the drivers of viral persistence in HIV-1-infected patients and tackle the last hurdle toward a cure, namely, the latent HIV-1 reservoir. By conducting this systematic review, we identified three shifts in reservoir studies that merit attention.

Importance of multiple tissues and compartment sampling

Although immune cells isolated from peripheral blood have been extensively studied due to their easy access, the importance of viral sanctuaries in peripheral tissues has gained research attention over the past years. In this review, we especially focused on lymph nodes,

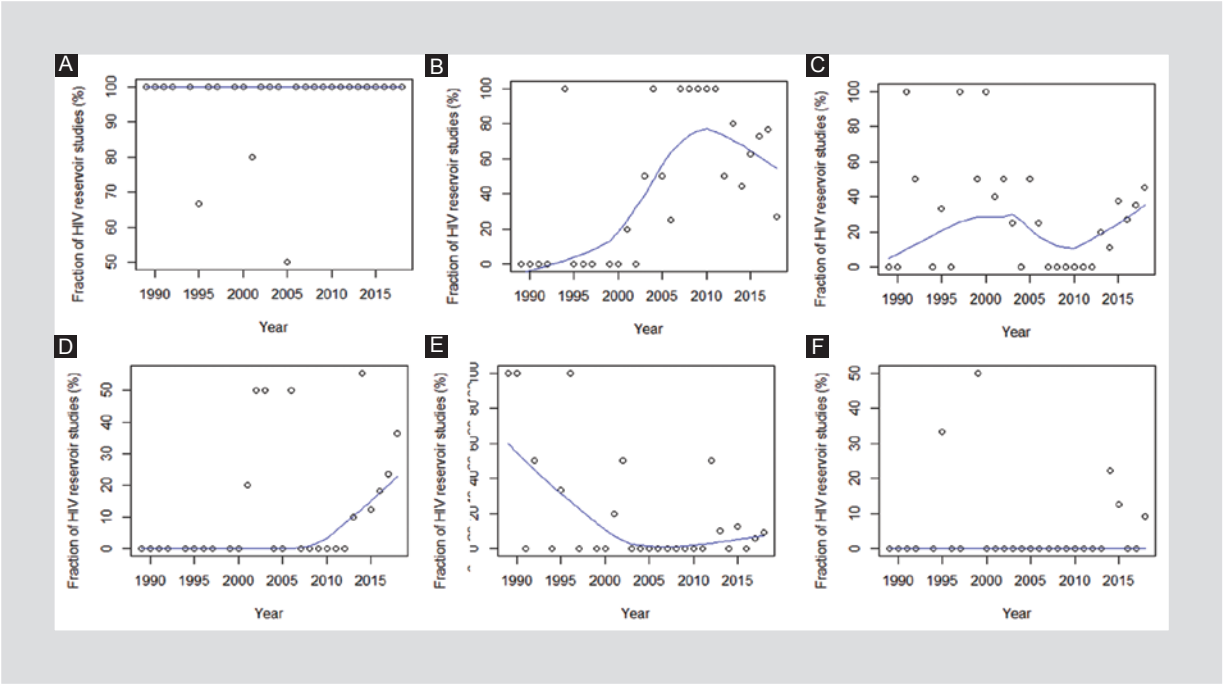

Figure 3. *Evolution in the study of individual anatomic compartments in HIV-1 reservoir studies. For each compartment, the fraction of studies including the compartment of interest is plotted over the time period 1987-2019:* **A:** *blood;* **B:** *gut;* **C***: lymph node;* **D:** *cerebrospinal fluid;* **E:** *bone marrow; and* **F:** *lung (bronchoalveolar lavage). The Locally Weighted Scatterplot Smoothing regression line is added in blue.*

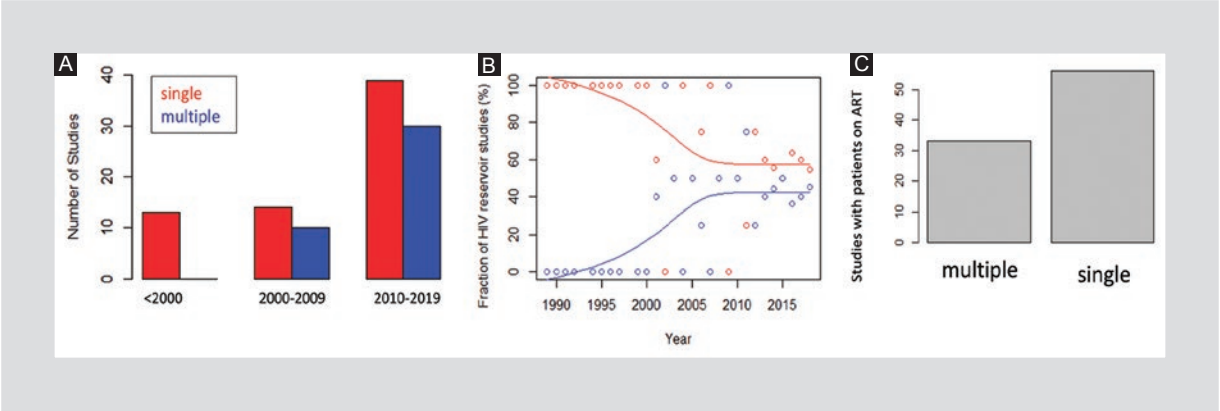

Figure 4. *Longitudinal sampling in HIV-1 reservoir studies.* **A:** *the number of reservoir studies which include single (red) or multiple (blue) time points in three time periods: 1987-1999, 2000-2009, and 2010-2019;* **B:** *fraction of reservoir studies including single (red) or multiple (blue) time points over the time period 1987-2019 with respective Locally Weighted Scatterplot Smoothing regression line;* **C:** *the number of studies with patients on antiretroviral therapy which include multiple or single time points.*

bone marrow, the central nervous system, the lungs, and the gastrointestinal tract as possible HIV-1 compartments.

Lymph nodes and lymphoid tissue are important sites for viral production, storage, and persistence[2,4]. The frequency of infections per cell is mostly higher in here than in immune cells in the blood[7]. Previous studies have shown that the intracellular drug concentration is lower in here, compared to in the blood, which can explain why it is a site of viral persistence[2,8]. Furthermore, lymph nodes play a key role as primary defense organ after infection[9]. Cells from the site of infection migrate to the lymph node and cause a very early site of HIV-1 infection[10,11]. Therefore, this reservoir remains of very high interest, although sampling is challenging.

The gastrointestinal tract is known to sequester the majority of lymphocytes and the GALT is a major viral reservoir in HIV-1 patients[3,12]. These lymphocytes are depleted early after primary infection[2] and do not return to their normal levels despite long-term cART[13]. A possible mechanism underlying HIV-1 persistence in this compartment may be the continuous

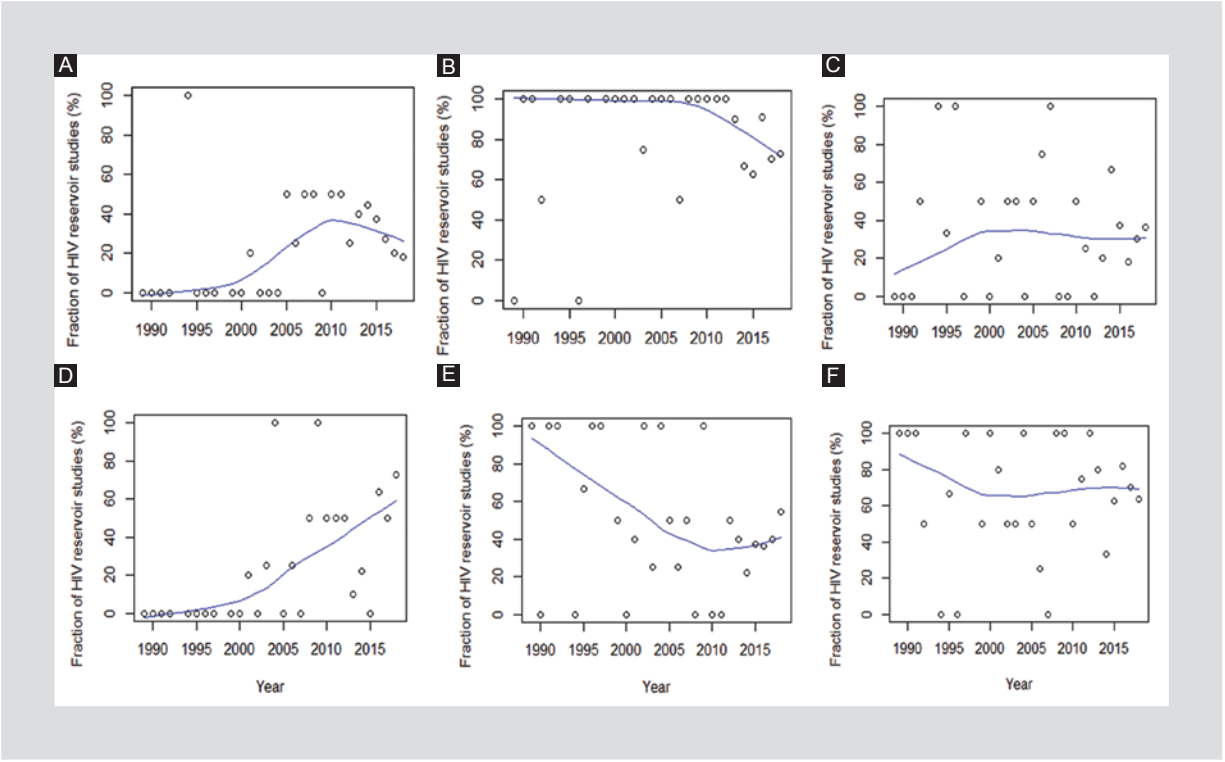

Figure 5. *The evolution of research methods and techniques used in HIV-1 reservoir studies. The fraction of reservoir studies including one of the following research method categories* **A:** *qualitative techniques;* **B:** *polymerase chain reaction (PCR)-based quantification;* **C:** *immunophenotyping or* **D:** *non-PCR-based quantification. The fraction of reservoir studies including bulk cells (***E***) or subset sorting (***F***) in the time period 1987-2019.*

immune activation caused by antigenic stimulation of resting B- and T-cells, increasing their turnover providing a constant source of susceptible cells[13]. When comparing compartments, the GALT is the most studied compartment besides the blood in absolute numbers. Indeed, the gut harbors possibly the biggest HIV-1 reservoir in the body, this in combination with being accessible for biopsies explains the broad interest for this reservoir[2,3,12]. However, we see a decline in the fraction of studies including the GALT in the past few years, potentially due to the low amount of immune cells recovered, limiting the types of analysis that can be conducted. Moreover, it is important to acknowledge differences between immune cells and gene expression between different regions of the colon, requiring a total colonoscopy for ileal biopsy compared to the left colon (sigmoid) biopsies[14].

Various cells can be infected within the brain, mostly from the monocyte lineage[2]. This reservoir is clinically relevant because patients can develop neurological complications such as HIV-1-associated dementia when the control of HIV-1 is not sufficient[15]. Although HIV-1-associated dementia is rare when patients are treated with cART[2], mild neurocognitive impairment is commonly observed despite suppressive cART. In this light, the CSF has been proposed as a viral sanctuary; however, the relevance in HIV-1 latency is unclear and the role in HIV-1 viral rebound after treatment interruption is doubtful[16,17]. It is, therefore, mostly studied as a sanctuary site, evolving separately from the other compartments[11,16].

In the lungs, it has been shown that HIV-1 infection worsens alveolar macrophage immune functions and in this way, it makes patients more susceptible to bacterial and viral infections[18]. These macrophages are the major viral reservoir in the lungs[13]. However, their relevance in latency is unclear and seems to be minor[4]. Finally, although studies have been investigating the role of $CD34^+$ hematopoietic precursor cells, there is no clear answer to date whether these cells contain latent virus[19,20]. With caution, we can suggest that the major source of virus in the bone marrow is likely to be found in migratory $CD4^+$ T-cells from the peripheral blood[4]. Lung and bone marrow biopsies lost interest over the years, as they appear to be less relevant, with a poor access to biopsies and low cell yields of the samples, not allowing for thorough downstream analysis[4,19,21].

We can conclude that there is a clear consensus about the relevance of the lymphoid tissue as an important HIV-1 reservoir. Overall, we see that the study setup and techniques have been adapted to answer to these reservoir challenges and mostly to have a better study of the involved reservoirs.

Increased uniformity in patient inclusion and longitudinal sampling

Over time, there is an increased uniformity in the study population and time points of sampling due to the urge to better understand the kinetics and dynamics of the HIV-1 reservoir. This is most evident in the studies sampling one time point with almost all of these studies including virologically suppressed patients on cART. The reason for this is mainly to limit bias caused by comorbidities, coinfection, ongoing disease, and AIDS-related illnesses[2,13,22]. Recent studies further limit heterogeneity by focusing, for example, on very early treated patients[4,6,23]. The same evolution is seen in studies that sample on multiple time points, they mostly compare patients on and off cART. In this way, they can study the effect of treatment initiation or evaluate an intervention by analytical treatment interruption (ATI)[24-26].

Although sampling at different time points offers the opportunity that the patient is his own control, this multiple sampling is also very demanding for the patients and, therefore, not always feasible.

Importance of multiple research methods to assess size and quality of HIV-1 reservoirs

With the shift toward the assessment of the quality of the HIV-1 reservoir in the context of disease relapse after ATI and the role in immune activation during cART, we observe a trend toward a more combined approach including quantitative and qualitative measurements. PCR-based techniques allow us to quantify total HIV-1 DNA, integrated HIV-1 DNA, 2 LTR circles, spliced RNA, and cell-associated (CA) RNA and provide very important information on the size of the reservoir in patients, informing on the viral burden, and total reservoir size[4,27]. PCR-based methods remain the most frequently used through the years, due to their high availability, relative low cost, and high throughput[6,23,28]. However, these assays greatly overestimate the latent reservoir since they cannot distinguish between defective and intact virus[23,28]. The quantitative viral outgrowth assay is an example of a non-PCR based methods and is currently the gold standard to detect replication-competent virus[29]. The main disadvantages are its cost and the resource-intensive approach. Furthermore, this assay is not perfect in detecting all replication-competent virus which can lead to an underestimation[23,28,30]. A non-PCR-based technique more focused toward tissues is next-generation *in situ* hybridization. These assays can detect low copy viral DNA and RNA signals *in situ* using RNA/DNA scope techniques and have been a major advance in the study of these tissues[28,31,32]. Many variations exist both for PCR and non-PCR-based quantitative techniques, all focusing on other demands, being more sensitive, quicker, measuring immune responses, or being less expensive, with the PCR-based assays being the most flexible[23,30]. Recent advances in sequencing have changed the field of HIV research once more. Qualitative measurements rise in use after 2000. This is in line with the shift from measuring the amount of HIV-1 present, to analyzing the HIV-1 reservoirs and their respective relevance in HIV-1 persistence and viral rebound[30]. Single genome and proviral sequencing is a technique which analyses HIV-1 DNA and RNA down to a single copy. A great advantage of single genome sequencing is that the genetic composition of the viral DNA and RNA can be unraveled. In this way, genetic changes between anatomic compartments and cell types can be measured and viral rebound can be linked to these sites. A disadvantage of this technique is that until recently, we were only able to sequence small parts of the virus. A possible solution for this problem is near full-length sequencing assays, able to sequence more than 90% of the virus, allowing for a more in-depth analysis of the viral genome[30]. Another approach is to sequence the integration site of the virus in the human genome, providing us with more information on the interplay between virus and host[33]. Due to this wide variation of results based on over- and underestimation of the functional reservoir, a combination of different techniques, preferentially both quantitative and qualitative, should be proposed to produce a confident estimate of the HIV-1 reservoir[5,23].

Although all these novel techniques are promising, it is important to adjust the compartments, research techniques, and time points of sampling according to the research question, taking into account various limitations due to sampling issues or discordance between the availability and the high input needed for some of the techniques.

Implications for future research

This systematic research clearly shows how advances made in cure research are correlated with a shift in the way clinically oriented research is conducted. The study interest and design have evolved, especially in the past 10 years. This corresponds to a shift in interest toward a complete understanding of the HIV-1 reservoir after viral control with cART became a reality and research moved on toward finding a cure for HIV-1. Indeed, the lessons learned from performing this systematic review were implemented in our recently published HIV-1 reservoir study and included the following key features[34]. We started from a homogeneous study population, employing strict inclusion criteria to avoid as much as possible bias related to interparticipant variability. Therefore, virological, immunological, and treatment inclusion criteria were applied. Participants were sampled on and off cART, to better understand the mechanisms and origin of *in vivo* viral rebound when stopping cART. In-depth sampling was done while on cART and single genome sequencing was applied to analyze the phylogenetic relationships between the viruses from different cell subsets and plasma rebound virus. We focused on blood, lymphoid tissue and gut samples as these reservoirs gave us the highest potential of (a) being able to collect enough sample to apply sequencing techniques and (b) providing answers to our research question as former research was convincing about their important contribution to the HIV-1 reservoir. As expected, further analysis of bronchoalveolar lavage, CSF, BM, and genital samples was not contributive to our analysis due to the poor amount of viral sequences that could be obtained. When we look at the evolution in the current research, we see a relapse in studies focusing on these compartments for the specific reasons mentioned above.

Although extensive sampling studies are very informative and needed to move toward an HIV-1 cure, there are several limitations that need to be considered. As formerly addressed, the availability and input of sample needed to conduct different assays requires a strict selection beforehand in how resources are used. Furthermore, applying different sequencing techniques often comes at a high cost. Another important limitation is the relatively low number of participants that are routinely included in these studies due to their invasive nature and the ethical considerations that come with the extensive sampling and treatment interruption.

Conclusion

Based on these observations, we can conclude that HIV-1 reservoir and cure research are evolving toward a targeted research approach with the use of a vast array of techniques to achieve this goal. Furthermore, the study results underline the need to combine both quantitative and qualitative assays to further identify replication-competent and intact full-length viruses and to define relevant anatomical reservoirs in order to design targeted therapy. In this context, the whole body approach[35] with extensive tissue sampling and analysis,[34,36] clearly demonstrates a trend of integrated research within one study participant to get as much information as possible. This approach should help us to better understand the establishment and dynamics of the viral reservoir and its contribution to viral rebound after ATI.

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Supplementary data

Supplementary data is available at AIDS Reviews journal online (http://www.aidsreviews.com). This data is provided by the author and published online to benefit the reader. The contents of all supplementary data are the sole responsibility of the authors.

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