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38° Cycle

Coordinator: Prof. Lorenza Trabalzini

Multidisciplinary assessment of the HIV-1 reservoir following switch from 3-drug to 2-drug antiretroviral regimens in virologically suppressed patients over an 18 months follow-up period. An observational, prospective pilot study.

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MY PhD ACTIVITIES

During the three years (2022-2025) of my attendance at the Doctoral School in Biochemistry and Molecular Biology, I joined the Laboratory of Microbiology and Virology at the Department of Medical Biotechnologies of the University of Siena. The Department has been hosting the HIV Monitoring Laboratory (HML), started as a public health service since 1990 and has been involved in several Human Immunodeficiency Virus (HIV) related research projects. In the last few years, HML has extended research activity on emerging and re-emerging viruses, including Dengue (DENV), West Nile (WNV) and Zika (ZIKV). Moreover, due to the emergence of the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), part of the research activity has been also directed to this pathogen during the pandemic and thereafter.

Currently, the main strategy for combating viral infections is a combination of large-scale vaccination and the use of antiviral drugs to treat disease cases. However, vaccines are available only for a minority of viral pathogens, thus the demand for new antiviral strategies has significantly increased. Factors contributing to this growing demand include the ever-increasing prevalence of chronic viral infections, the emergence of new viruses and the re-emergence of old viruses. Indeed, due to globalization and climate changes, viruses confined in specific and isolated areas are re-emerging and rapidly spreading to new geographic areas (Pierson & Diamond, 2020).

Related to this issue, the assessment of antiviral effects in vitro is a key approach for the screening of either de novo or repurposed candidate compounds. Among the variety of methods that have been developed, cell-based assays are the most valuable methods to define antiviral activity in vitro (Boldescu et al., 2017).

During my PhD, attending a lab with a strong focus in antiviral drug discovery and a variety of running projects, gave me the opportunity to participate in multiple antiviral drug related activities. In effect, in drug discovery, HML is currently engaged in several projects aimed at investigating the in vitro antiviral activity of promising broad-spectrum libraries of antivirals, which represent an attractive option to treat new emerging viral diseases, such as SARS-CoV-2, WNV, DENV and ZIKV.

During my PhD, I was involved in projects aimed at investigating new candidate antiviral compounds:

- **TUSCAVIR (Tuscany Antiviral Research Network), Tuscany Region Health Call 2018.**

This project aimed to set up an interdisciplinary research consortium providing qualified services for the development and investigation of novel antiviral therapies.

- **Genomic epidemiology and phylogenesis of SARS-CoV-2 in Italy: identification and characterization of circulating variants of clinical and public health relevance, National PRIN 2020 program**. This project was not only about in vitro antiviral activity investigation of novel candidate compounds to treat COVID-19, but also about Next Generation Sequencing (NGS) of SARS-CoV-2 isolates from summer 2020 to winter 2021 to trace the epidemiology of the virus in Italy. Italy has been one of the most and earliest affected countries by the SARS-CoV-2 pandemic. In the context of the PRIN 2020 grant, an Italian network consisting of 14 clinical centers and named SCIRE (SARS-CoV-2 Italian Research Enterprise) was created to trace SARS-CoV-2 evolution.
- **APICE (Antibodies and Peptides Inhibiting Coronavirus Entry), Tuscany COVID19 Call 2020.** Our laboratory collaborated with Professor Bracci's biochemistry group within the Department of Medical Biotechnology of the University of Siena to evaluate the antiviral properties of a heparan sulfate-binding peptide.
- **Blocking Coronavirus infection: developing inhibitors of SARS-CoV-2 protease and multifunctional compounds interfering with virus entry and replication, Intesa San Paolo Bank COVID-19 Call.** Granted research in collaboration with the research group of Prof. Sandra Gemma of the University of Siena, Department of Biotechnology, Chemistry and Pharmacy. The project aimed to contribute to the discovery of new antiviral agents for the treatment of SARS-CoV-2 infection and possible novel coronaviruses that may emerge in the future. The grant was renewed for a second round for the development of new Mpro inhibitors and was based on the use of the PROTAC (Proteolysis Targeting Chimera) technology.

As an original project within the HML laboratory, I investigated the in vitro combinatorial effects of three licensed antivirals to treat COVID-19, namely Remdesivir, Nirmatrelvir, and Molnupiravir. I also investigated the combination of Remdesivir with four monoclonal antibodies against SARS-CoV-2 (sotrovimab, bebtelovimab, cilgavimab and tixagevimab). These analyses were performed in a live virus cell based in vitro assay as a proxy for in vivo combination therapy (Fiaschi et al., 2024).

As opposed to the pressing needs in the area of emerging viruses, antiretroviral therapy (ART) has changed HIV-1 infection from a fatal disease to a chronic condition and continuously

progressed to improved quality of life for people living with HIV. However, ART cannot clear the infection, since HIV-1 is able to persist indefinitely in the so-called latent reservoir. Therefore, strategies to eradicate or control HIV-1 without ART are a high priority.

In this context, I have been involved in the **MARISA** project (Multidisciplinary Assessment of the blood and gut-associated HIV Reservoir and Immunity following Switch from 3-drug to 2-drug Antiretroviral regimens in virologically suppressed patients - MARISA), granted within the PRIN 2022 program, that aims to evaluate the impact of switching from triple to dual antiretroviral therapy in the HIV reservoir using non-routine molecular and cellular techniques. Also, about HIV-1 persistence, I recently started a pilot activity in collaboration with the Infectious Diseases Department of the Siena University Hospital that aims to understand both the underlying mechanisms and the implications of a condition known as “persistent residual viremia” despite antiretroviral treatment. This project is currently being extended to other Italian centers providing clinical samples for this challenging context.

INTRODUCTION

HIV-1

- **Overview**

Human Immunodeficiency Virus (HIV) was first isolated in 1938 (Barré-Sinoussi et al., 1983) and then identified as the causative agent of Acquired Immunodeficiency Syndrome (AIDS). HIV is one of the viruses belonging to the Lentivirus group of the Retroviridae family (Mayer et al., 2025), able to infect CD4+ T lymphocytes and to cause their progressive depletion over time.

Since the beginning of the epidemic, more than 90 million people have been infected and about 44million people have died. At the end of 2024, people living with HIV (PLWH) were estimated to be about 40.8 million, with 1.3 million of newly diagnosed infections in 2024, 25% of which in Southern Africa, and 630,000 people dead from HIV-related causes globally (WHO, HIV data and statistics; www.unaids.org/sites/default/files/media_asset/UNAIDS_FactSheet_en.pdf).

Nowadays, HIV is still a major global public health concern with most of the PLWH located in low/middle- income countries (n 2016, the Joint United Nations Program on HIV/AIDS - UNAIDS), along with 11 United Nations Organizations, planned to end AIDS by reaching the 90–90–90 targets by 2020 (90% of people infected with HIV known about their conditions, 90% of all diagnosed people receiving antiretroviral therapy, 90% of all people receiving antiretroviral therapy having viral suppression). These targets have been further raised to the 2025 target that aims to achieve the 95–95–95 HIV testing, treatment and viral suppression within all demographics and groups and geographic settings.

Remarkable progress has been made and, in 2024, the global HIV response was closer than ever to reaching these goals: currently an estimated 87% of all people living with HIV knows their HIV status, 89% of people who know their HIV-positive status are receiving antiretroviral therapy, and 94% of people on treatment has a suppressed viral load (www.unaids.org, update report 10.07.2025) but, even if some regions are very close to achieving the 95-95-95 target, there is a disparity in these conditions between different geographical areas (Fig. 1) and even before the recent funding losses, the improvements against HIV were uneven.

HIV testing and treatment coverage and viral suppression levels among people living with HIV is still considerably delayed in eastern Europe, central Asia, the Middle East, North Africa, Asia and the Pacific. Notably, in 2024, 9.2 million people living with HIV were not receiving antiretroviral treatment, and half of them lived in sub-Saharan Africa. Despite the above-

described progress, collapse in funding at the beginning of 2025 triggered a crisis in the global AIDS response. The sudden withdrawal of the USA as the single biggest contributor, mainly through the President's Emergency Plan for AIDS Relief program, to the global HIV response disrupted treatment and prevention programs around the world in early 2025. UNAIDS modelling shows that if the funding permanently disappears, there could be an additional 6 million HIV infections and an additional 4 million AIDS-related deaths by 2029.

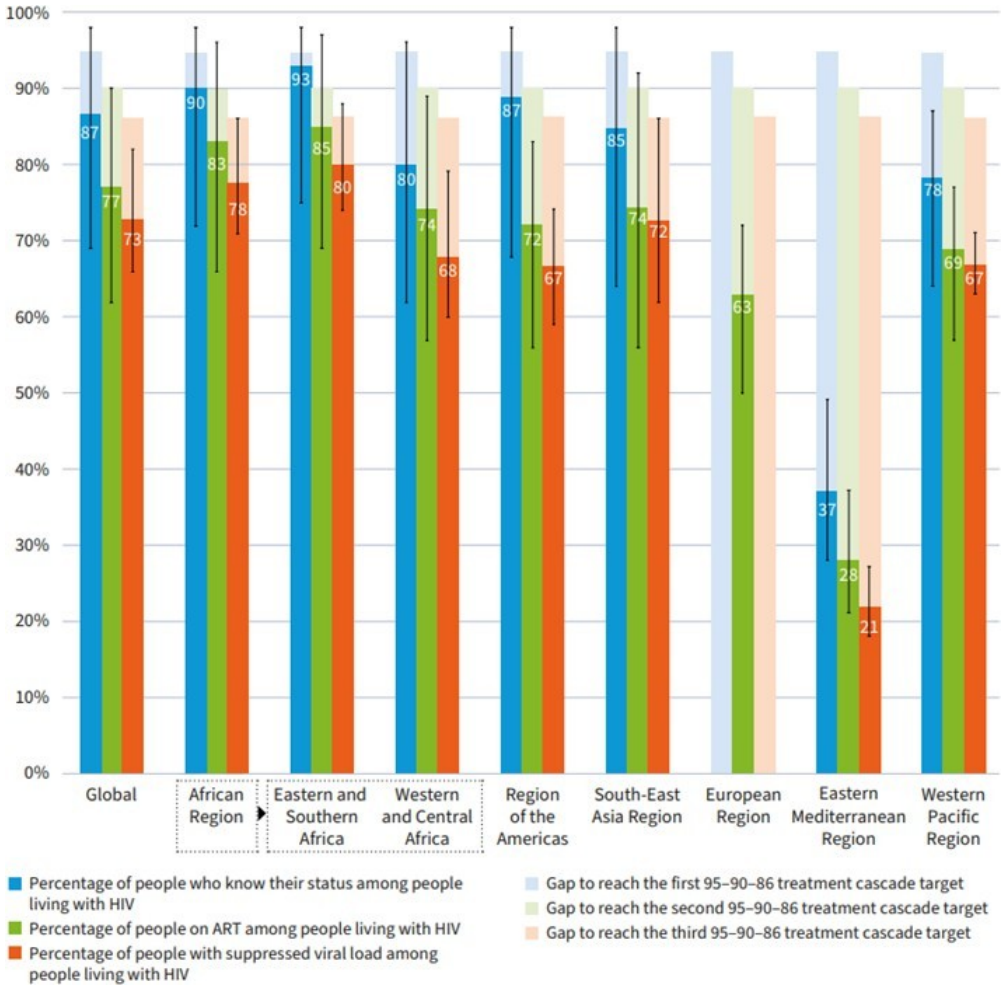


Figure 1. Progress towards the 95-95-95 testing, treatment and viral load suppression target, by region, in 2024. Source: UNAIDS epidemiological estimates 2025.

- **Epidemiology**

Two types of HIV with a different genetic composition, HIV-1 and HIV-2, originated from separate zoonotic transmission events from non-human primate simian immunodeficiency viruses in Central African chimpanzees (HIV-1) and West African sooty mangabey monkeys (HIV-2) (MURPHY, 2018). They are differently distributed worldwide: HIV-1 is responsible for

the global pandemic, while HIV-2 is characterized by a reduced virulence and occurs mainly in west Africa regions with sporadic cases in Europe, India and United States (Campbell-Yesufu & Gandhi, 2011). HIV is characterized by an impressive genetic diversity, due to three main factors: error-prone reverse transcriptase (RT), high replication rate and genetic recombination (Nair et al., 2024; Santoro & Perno, 2013; Suligoi et al., 2010). Based on genetic homology, HIV-1 is divided into four groups: M (main), N (non-M, non-O), O (outlier) and P, with different prevalence and geographic distributions, but all causing similar clinical symptoms (Peng, 2024; Santoro & Perno, 2013).

Group M accounts for the most common circulating subtypes of HIV-1 and it is responsible for the global epidemic. According to the current classification of HIV-1 sequences, 9 subtypes (A, B, C, D, F, G, H, J and K) belong to group M; in addition, some subtypes can be additionally divided into distinct sub-subtypes (e.g. the subtypes A1 through A8, F1 and F2). However, the classification of such sequences is complicated by the HIV-1 high mutation rate and propensity to develop new recombinant forms. Indeed, in addition to pure subtypes, at least 159 circulating recombinant forms (CRFs) have been detected so far (https://www.hiv.lanl.gov/components/sequence/HIV/crfdb/crfs.comp, last accessed 15 August 2025).

CRFs indicate the case in which two or more subtypes of HIV-1 mix their genetic material and create a new hybrid mosaic virus which is identified in three or more epidemiologically unlinked individuals (Fan et al., 2024; Rhee & Shafer, 2018; Santoro & Perno, 2013; Suligoi et al., 2010; Taylor et al., 2008; Tongo et al., 2015). Notably, novel mosaic forms, referred to as unique recombinant forms (URFs), are continuously reported which may in turn spread as CRFs.

Despite most data and research dealing with subtype B as the largely prevailing subtype in western countries, subtype C predominates worldwide with a prevalence of about 50%. It is mainly found in the southern African region, the Indian sub-continent, but also in east African countries, Brazil and the southern provinces of the People's Republic of China, whereas HIV-1 subtype B remains most prevalent in North America, western Europe, Australia and Japan (Jacobs et al., 2014) and it is responsible for 12.1% of infections, followed by subtype A (10.3%) and CRF02_AG (7.7%). HIV-2 is divided into eight lineages (from A to H) and of these, only A and B have infected a relevant number of people (Sharp & Hahn, 2011).

- **Genome Organization**

The HIV virion is about 110-120 nm in diameter and has an icosahedral structure with a streamlined inner electron-dense core, surrounded by an outer structure that forms the envelope (Fig. 2).

The envelope is acquired during the virion budding and is made up of a phospholipid bilayer, where the two major glycoproteins are anchored: gp120 (surface glycoprotein) and gp41 (transmembrane glycoprotein). The surface of the envelope has 72 projections composed of heterodimers of the two surface glycoproteins, further organized as trimeric spike-like structures. The central **core** is composed of four viral proteins: the capsid protein (p24), the matrix protein (p17), the nucleocapsid (p7) and p6 (Pancera et al., 2014).

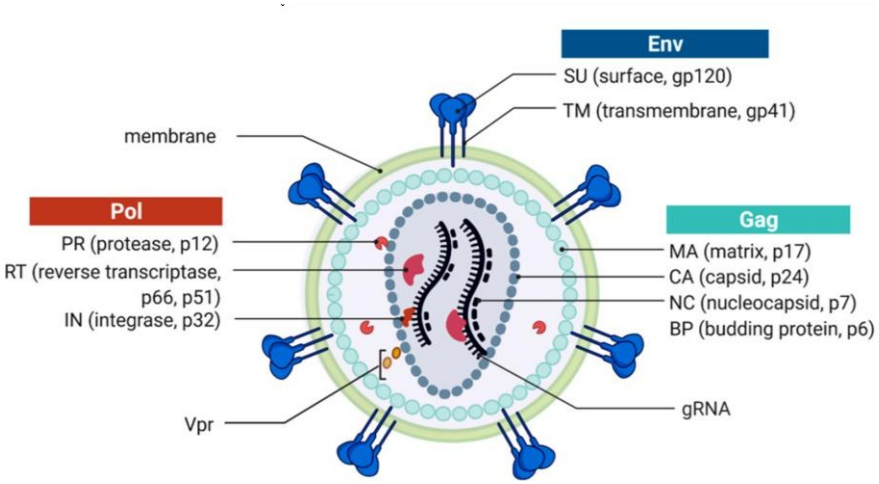


Figure 2. Schematic representation of the HIV-1 mature virion structure (adapted from van Heuvel et al., 2022).

The HIV-1 RNA genome consists of two identical copies of single-stranded +RNA of about 9.4 Kb, which are capped at their 5' end and polyadenylated at their 3' end (Fig. 3). At both the 5' end and 3' end there is a repeated sequence (R, ~ 100 nt) and adjacent there are unique regions called U5 (~ 70 nt) and U3 (~ 450 nt). Upon reverse transcription of the viral RNA genome into proviral DNA, two identical long terminal repeats (LTR) regions are generated both at the 5' and 3' terminus by juxtaposition of U3-R-U5. Only the 5'-LTR (~ 634-bp in length) functions as promoter control for viral transcription, using the host transcription machinery by recruiting many ubiquitously expressed or cell-type specific factors to control proviral transcription, both for its activation and repression (Shukla et al., 2020).

As for the other retroviruses, from 5' to 3' the viral genome contains the gag gene, encoding the proteins of the outer core membrane; the *pol* gene coding for the enzymes protease (PR), RT, RNase H and integrase (IN), and finally the *env* gene coding for the two envelope glycoproteins gp120 and gp41 (King, 1994). All the viral enzymes are produced by proteolytic cleavage from a larger precursor molecule. The synthesis of a **Gag-Pol** precursor protein (p180) occurs during the translation thanks to a ribosomal frameshift between the open reading frames (ORFs) of the two genes. Subsequent cleavages of p180 yields the **Gag** proteins and the PR (p10), the RT/RNase H (p66/p51) and the IN (p32).

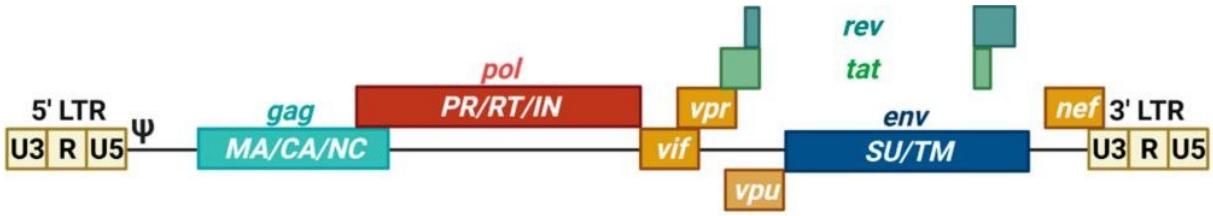


Figure 3. Schematic representation of the HIV-1 genome (adapted from van Heuvel et al., 2022).

- The 55-KDa **Gag** myristylated precursor is cleaved into the matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), and p6 proteins to compose the central core.
- HIV **PR** is an aspartyl protease responsible for the post-translational processing of the viral Gag and Gag-Pol polyproteins and functions as a homodimer composed of two non-covalently associated monomers (Freed, 1998; Kaplan, 1994).
- HIV **RT** is a heterodimer composed of the p66 and the p51 subunits. The p51 subunit is composed of the first 450 amino acids of the RT serving as a scaffold for the p66 subunit. The latter is composed of all 560 amino acids of the entire RT-RNAseH protein, providing both to the RNA- dependent and DNA-dependent DNA-polymerase activity and the RNA degradation in RNA-DNA hybrid duplexes (Beard & Wilson, 1993; Sluis-Cremer et al., 2004).
- The 32-KDa **IN** protein is the C-terminal cleavage product of the pol region and catalyzes the insertion of the viral cDNA into the host cell genome by cleaving the host DNA, then joining the linear double stranded form of the viral DNA creating covalent bonds between the ends of the generated fragments. HIV IN comprises three structural domains: the N-terminal domain, the catalytic core domain and the C-

terminal domain (Engelman & Cherepanov, 2012; Esposito & Craigie, 1999). Recently, it has been suggested that the IN can also influence viral particle maturation by interacting with the viral RNA genome during particle morphogenesis. Loss of IN–RNA binding leads to mislocalization of the viral genome inside the virions and prevents viral replication in target cells (Kessl et al., 2016).

- Finally, the protein product of the ***env*** gene is synthesized in the endoplasmic reticulum (ER) as an 88-KDa polypeptide. This protein undergoes heavy glycosylation through the ER and Golgi network. The resulting molecule, gp160, is cleaved by the cellular serine protease, furin, to generate the transmembrane (gp41) and surface (gp120) subunits; such cleavage is required for viral infectivity (Wiley et al., 1991).

In addition to gag, pol and env, tat and rev have been identified as regulatory proteins required for virus replication.

- The 14-kDa trans-activating protein, **Tat**, is required for HIV-1 transcription and accumulates inside the nucleus. It enhances the rate of transcription through the binding of the transactivation response (TAR) element found at the 5' end of HIV transcripts, and by recruiting cellular factors that improve the processivity of the cellular RNA polymerase II complex (Musinova et al., 2016).
- The 18 kDa **Rev** protein mediates the transport to the cytoplasm of singly spliced and unspliced viral RNAs by the nuclear exportation system (Pollard & Malim, 1998). Rev was also found to stimulate protein expression levels, to enhance encapsidation of the genomic RNA into virions (Blissenbach et al., 2010) and interestingly, to inhibit the integration of the viral genome and thus playing a role in prevention of cellular superinfection (Grewe & Überla, 2010).

The other four small proteins (new, vpr, vif and vpu in HIV-1 or vpx in HIV-2) are referred to as "accessory" since, although they play a significant role in vivo, their expression is usually dispensable for virus growth in many in vitro systems.

- **Vif** is a 193-amino acid protein present in the cytoplasm and incorporated in the virion. It interferes with the activity of cellular protein APOBEC3G, inducing its ubiquitination and degradation by proteasomes. APOBEC3G is involved in the innate immune response by introducing mutations in the viral genome during transcription and causing a reduction in the virus infectivity (Reddy et al., 2016).
- **Vpr** is a 100-amino acid protein that is expressed late during the infection cycle and is

packaged in significant quantities into virus particles through a specific interaction with the P6 domain of the viral Gag precursor. It has two main functions: (i) to prevent the proliferation of the host cells by arresting the cell cycle at the G2 phase, thus favoring viral gene expression; (ii) to allow infection of non-dividing cells by helping the transport of the viral genome into the nucleus of the host cells (Fabryova & Strebel, 2019), as well as inducing proteasomal degradation of the DNA repair enzymes HLTF, UNG2 and MUS81 to prevent the restriction of viral cDNA (Nodder & Gummuluru, 2019).

- **Vpu** is an 81-amino acid single-pass trans-membrane protein that is only present during the late stages of HIV-1 infection. It prevents superinfection by inducing CD4 degradation and promotes virus release by antagonizing the restriction factor tetherin (González, 2015). Moreover, recently Vpu has been found to hijack DNA repair mechanisms to promote degradation of nuclear viral cDNA in cells that are already productively infected (Volcic et al., 2020). Vpu is absent in HIV-2, whereas its genome contains **Vpx,** another small protein facilitating viral replication in certain host cells, particularly myeloid cells like macrophages and dendritic cells, which would otherwise be resistant to infection.
- **Nef** is a 210-amino acid protein located at the inner face of the plasma membrane. It is responsible for several effects, such as the downregulation of CD4 receptor by promoting its endocytosis and lysosomal degradation; the support in virus budding by removing the Env receptor from the cell surface; the decreased expression of major histocompatibility complex class I on the cell's surface, thus limiting the ability of infected cells to be cleared by the immune system. Nef also activates T cells, inducing the translocation of transcription factors to the nucleus, leading to greater HIV transcription (Furler et al., 2019).

• Life Cycle

The entire replication cycle of HIV-1 is completed, in vitro and in vivo, in approximately 24 hours and it can be divided into the following steps: binding and entry, uncoating, reverse transcription, provirus integration, viral protein synthesis and assembly, budding (Fig. 4) (Kim et al., 1989).

The binding and entry pathways consist in a multi-step process that starts with the binding of the viral protein gp120 to the host cell CD4 receptor. The virus mainly targets T helper lymphocytes, but also macrophages, dendritic and microglial cells, and resting T cell subsets.

The interaction between the viral envelope glycoprotein gp120 and the cellular receptor molecule CD4 is the first step of the HIV-1 replication cycle. Although the binding of virions to the CD4 is essential for HIV infectivity, their subsequent interaction with a co-receptor, the seven membrane-spanning chemokine receptor type 5 (CCR5, mainly used by macrophages and dendritic cells) or C-X-C motif chemokine receptor type 4 (CXCR4, mainly localized in memory and naïve T cells, hematopoietic cells and thymocytes), is required for membrane fusion and entry (Suligoi et al., 2010; van Heuvel et al., 2022). Occasional HIV-1 strains may use alternative chemokine receptors or multiple receptors. After the formation of the gp120/CD4/co-receptor complex, the virus exposes the fusion peptide at the N-terminus of gp41, which inserts into the cell membrane leading to the fusions of the membranes (Engelman & Cherepanov, 2012).

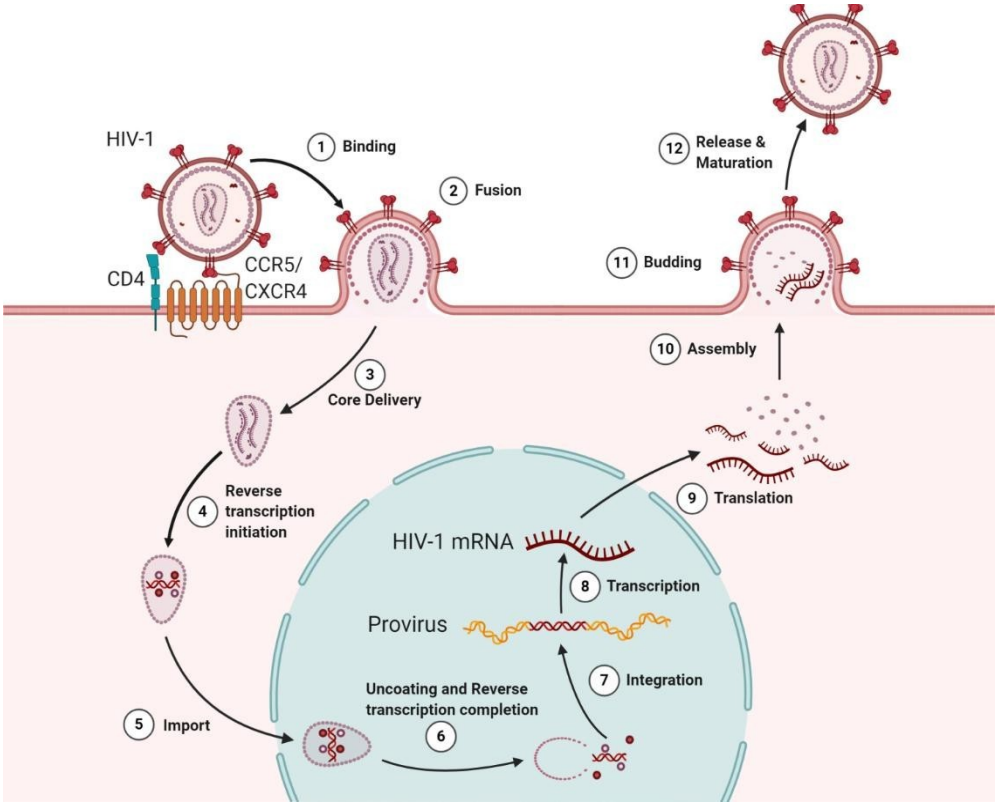


Figure 4. Schematic representation of the HIV-1 life cycle inside the host cell adapted from Ramdas et al., 2020.

Following entry, viral particles are partially uncoated in the cytoplasm. Reverse transcription of the viral RNA into a dsDNA (cDNA) seems to start in the cytoplasm, but the nuclear import

occurs quite rapidly, before the completion of reverse transcription that will be completed inside the nucleus (Dharan et al., 2020). Reverse transcription starts at the primer-binding site of the viral RNA, involving two enzymatic activities of the RT: polymerase and RNase H activities and it is initiated at the 5' end of the primer binding sequence region.

The resulting RNA–DNA duplex is almost completely degraded by RNase H activity. Only the 3' polypurine tract, found to the left of the U3 sequence, is resistant to RNase H degradation allowing its use as the primer for the plus-strand DNA synthesis during the reverse transcription process. The result is the proviral DNA, a linear, dsDNA longer than the RNA genome because of the presence of LTR regions at both ends that are generated by polymerase jumps during the retrotranscription process (Cimarelli & Darlix, 2014; Malet et al., 2019). Following reverse transcription, the IN, together with a pre-integration complex made of both cellular and viral proteins, catalyzes insertion of the viral DNA into the genome of the host cell through two sequential reactions: 3' processing and strand transfer (Malet et al., 2019; Suligoi et al., 2010).

Host enzymes complete the integration process by repairing the single-strand gaps flanking the unjoined viral DNA 5' ends, resulting in the establishment of a stable provirus (Engelman & Cherepanov, 2012).

As IN associates with the ends of the HIV DNA, the internal HIV sequence can be also defective or deleted, making the HIV proviral integration landscape highly diverse in the same individual. The exact sites of viral cDNA integration into the host genome are still to be defined; however, HIV integration site preferences include actively transcribed genes, gene rich regions of chromosomes, often within introns, while promoter regions are mostly excluded (Anderson & Maldarelli, 2018).

Once integrated into the host DNA, the proviral DNA is replicated together with the host DNA. Activation of HIV transcription and gene expression is dependent on the activity of both cellular (Sp1, NFkB, and others) and viral (Tat) factors. The primary transcript is spliced by a finely regulated strategy to generate over 30 species of alternative viral mRNAs. The early proteins Tat, Rev and Nef increase the level of viral transcription, promoting transcription of other genes and exportation of viral mRNA from the nucleus into the cytoplasm. The virion assembles at the plasma membrane when the genomic RNA is associated with the nucleocapsid proteins. The specificity of HIV-1 genome encapsidation results from an interaction between NC and an approximately 120-nt sequence, known as the packaging

signal or ψ-site, located between the 5' LTR and the Gag initiation codon (Berkowitz et al., 1996).

The immature virion acquires its envelope by budding from the cell surface. In this step, in addition to the viral encoded proteins, virions incorporate several cellular proteins, including major histocompatibility antigens, intercellular cell adhesion molecules such as ICAM-1 and cyclophilin A. This strategy is believed to enhance the infectivity of the new viral particles. HIV buds from specialized regions of the plasma membrane which are enriched in cholesterol and glycolipids. Presumably this envelope modified lipid composition facilitates virion morphogenesis and the fusion with target cells (Nguyen & Hildreth, 2000).

The final step of the viral life cycle is mediated by the PR and it occurs concomitant with or soon after budding, converting immature particles to infectious virions via the proteolysis of the precursor peptides Gag and Gag-Pol to yield the structural components MA, CA and NC, and the enzymes PR, RT and IN (Engelman & Cherepanov, 2012).

- **Route of transmission and natural history of infection**

HIV-1 infection is the result of direct inoculation of the virus across a mucosal surface that may occur in case of sexual contact, parenteral or vertical transmission. Nowadays, sexual transmission is the most common route, due to the exchange of semen, genital secretion or blood from an infected individual to the uninfected partner. Transmission from contaminated blood, blood products or transplantation of infected tissues poses the greatest risk, with as much as 95% persons becoming infected, however current molecular screening procedures have virtually eliminated this possibility.

Vertical transmission from mother to child occurs occasionally during pregnancy, during childbirth or during breast-feeding. The risk of mother-to-child transmission is 25 to 60% in untreated women, but it is dramatically reduced by prophylactic treatment of the mother and newborn with antiretroviral drugs and with bottle feeding (Abrams, 2004; Shaw & Hunter, 2012).

The natural history of HIV-1 infection can be divided into three phases: primary or acute infection, asymptomatic period and symptomatic stage including opportunistic disease stage (Fig. 5).

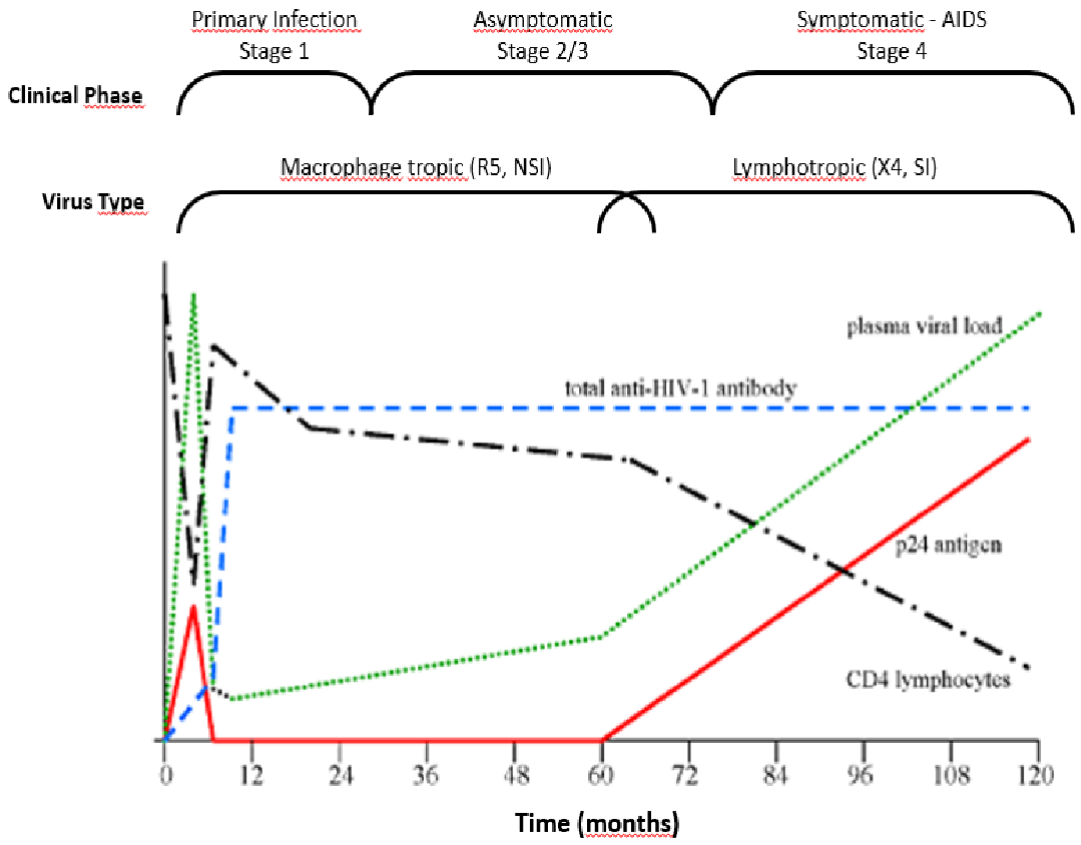


Figure 5. Relative timescale of clinical, virological and immunological events during HIV-1 infection. Adapted from Pinzone et al., 2019.

After inoculation, HIV passes through mucosal, submucosal and replicates in lymphoreticular tissue. During the initial phase, the virus directly infects and kills susceptible cells, eventually producing high viremia that spreads the virus throughout the body (Melhuish & Lewthwaite, 2018).

The time from exposure to the onset of signs and symptoms is approximately 10 to 30 days. A typical presentation may include acute onset of fever, lethargy, maculopapular rash, myalgia, headache, sore throat, cervical lymphadenopathy, arthralgia, oral ulcers, photophobia, oral candida, and rarely meningoencephalitis. At the beginning of the infection, the CD4 T cell count usually diminishes but after the acute illness resolves, CD4 T cell counts generally rise again, although not to pre-infection levels. Typically, viremia decreases to a virus-host specific plateau level defined as the viral “set-point”. Virus specific CD8 T lymphocytes appear early and help to reduce the level of circulating virus through the lysis of infected cells and through the release of chemokines, which inhibit HIV-1 cell entry. At this point, the CD8 T cells count is increased, and there is an inversion in the CD4/CD8 ratio, a hallmark of HIV-1 infection

that will be hardly restored to normal levels even in the presence of successful therapy (Sabin & Lundgren, 2013).

Primary HIV-1 infection is followed by an asymptomatic period, characterized by a virologic quasi-steady state. The clinical latency is caused by a latent reservoir made up of proviruses integrated into the host cell genome and not transcriptionally active. The most important feature of this period is the gradual loss of CD4 T cells, caused by continuous viral replication. The rise in viral load is proportional to CD4 T cells loss, and the CD4 T cells count largely reflects the degree of impairment of the immunological function and the consequent risk of opportunistic infections. The duration of this phase is highly variable, depending on unclear virus and host genetic factors. A very small number of individuals, the so-called long-term non progressor, can remain in the asymptomatic phase for decades even without taking antiretroviral therapy (ART) treatment. Due to some genetic mutations or immune system modifications, they can maintain their CD4 T cells count stable and the level of viremia low, probably due to natural control of HIV replication by the immune system (Suligoi et al., 2010).

The last phase is a clinically symptomatic stage, which is a consequence of the progressive and profound deterioration of the immune system. When the CD4 T cell count drops to AIDS-defining levels (CD4 T cell count less than 200/µl), opportunistic infections, neoplastic diseases, and wasting syndrome can occur. These conditions remain the leading cause of death in untreated individuals as well as in a minority of unsuccessfully treated people (Melhuish & Lewthwaite, 2018).

Antiretroviral Therapy

The primary goals of ART are to block virus replication and restore the immunological function, reducing HIV-associated morbidity and the risk of HIV transmission. Since ART introduction, the death of infected people has decreased 80% in industrialized countries.

Antiretroviral drugs are divided into nine classes (Fig. 6, Tab. 1) and include the nucleoside/nucleotide RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand

transfer inhibitors (INSTIs), fusion inhibitors, CCR5 antagonists, attachment inhibitors, post-attachment inhibitors and capsid inhibitors. In addition, two drugs, ritonavir and cobicistat are used as pharmacokinetic enhancers to improve the pharmacokinetic profiles of PIs and of

elvitegravir (INSTI). All these drug classes have different targets during the viral life cycle (Melhuish & Lewthwaite, 2018; Rathbun et al., 2023).

NRTIs inhibit the synthesis of viral cDNA carried out by the RT. NRTIs are active against both HIV-1 and HIV-2 and they are nucleosides analogues that are incorporated into the elongating proviral DNA chain with high affinity, halting DNA elongation (Cihlar & Ray, 2010). NRTIs were the first drugs to be licensed for clinical use but achieved only partial success until not combined with later classes to build the paradigm of combination therapy which remains a cornerstone in HIV treatment. Mostly for historical reasons, NRTI have been and still are part of the combination regimens preferred over time as indicated by treatment guidelines (Geretti & Easterbrook, 2001; Luber, 2005).

NNRTIs include compounds that are active only against HIV-1, due to the specificity of the substrate. NNRTIs bind to the p66 subunit of the RT, inducing a conformational change in the enzyme that allosterically inhibits its enzymatic activity (Li et al., 2016).

INSTIs block the strand-transfer of viral DNA into the host genome through competitive binding with cellular DNA to the active site of the enzyme. While first generation INSTIs were characterized by a modest genetic barrier, second generation INSTIs (e.g. dolutegravir) have overcome this problem (Zhao et al., 2022). Second-generation INSTIs are indeed recommended in most first choice treatment for ART-naive as well as for ART-experienced people, based on their potency, tolerability and convenience (Li et al., 2016; Malet et al., 2019).

PIs were introduced in late 1995 to build the first combination treatment enabling a virtually complete block of HIV replication. They act against both HIV-1 and HIV-2 (Li et al., 2016) by inhibiting the cleavage of polyproteins resulting in the generation of non-infectious viral particles. First-generation PIs were characterized by a low genetic barrier and suboptimal tolerability. However, later PI generations (e.g. darunavir) have overcome these limitations, showing a better safety profile and a greater antiviral potency, as well as an excellent genetic barrier (Fernández-Montero et al., 2009). However, long-term toxicity remains a limitation for all PIs and the class has been globally superseded by second-generation INSTIs.

Fusion inhibitor enfuvirtide is the only approved fusion inhibitor and can prevent the fusion between cellular and viral membranes (Reeves et al., 2023). Enfuvirtide was a welcome news for people living with multidrug-resistant HIV, but it is no longer used, since it requires twice daily subcutaneous injections and more convenient options are available.

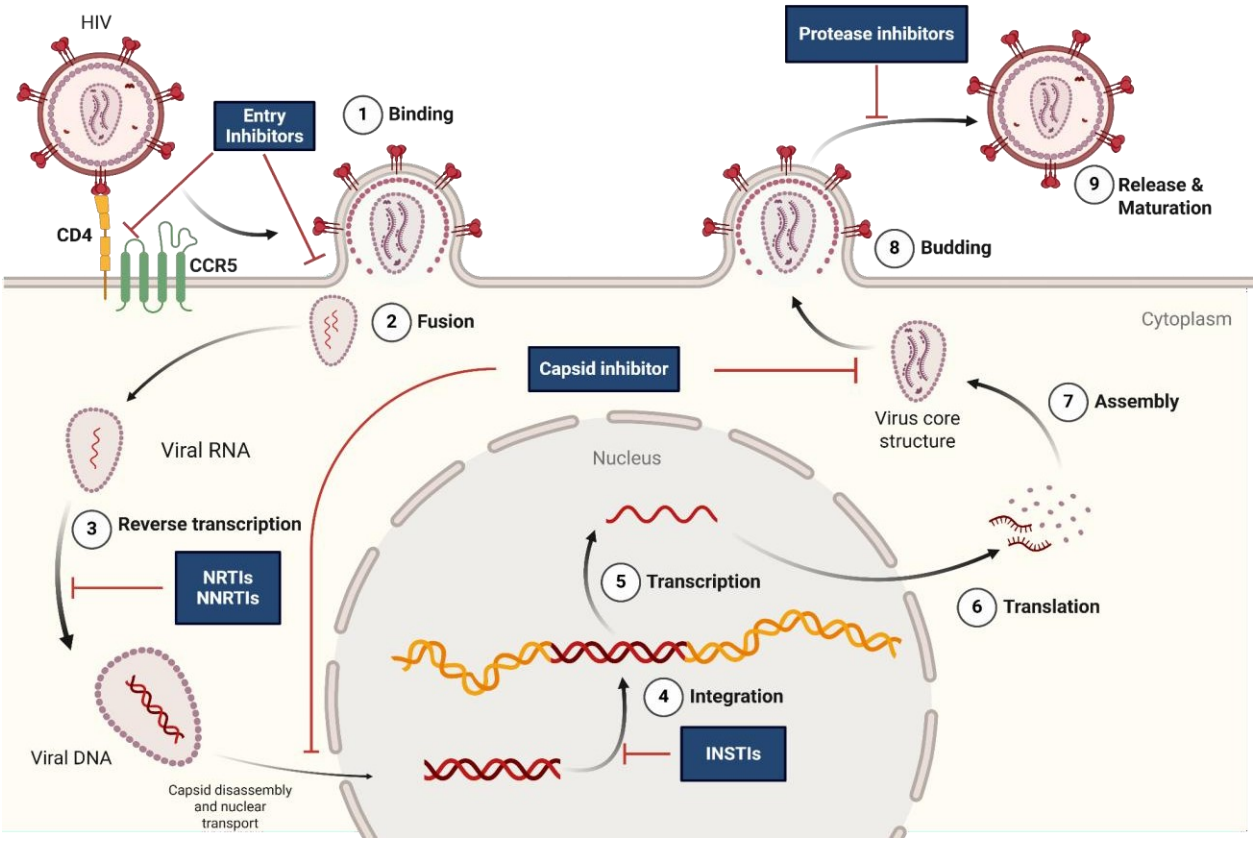


Figure 6. Schematic representation of the mechanism of action of the main classes of antiretroviral drugs (created in https://BioRender.com).

CCR5 antagonist maraviroc (MVC) (Yost et al., 2009) binds to the CCR5 coreceptor, blocking the interaction with the gp120 V3 loop and the subsequent entry events. MVC is effective only in patients harboring CCR5 tropic viral populations, while no efficacy was observed against CXCR4 tropic viruses, hence a virus tropism test is strictly recommended before administration.

Attachment inhibitor fostemsavir is a prodrug of the active compound temsavir that binds directly to the gp120 subunit within the HIV envelope and inhibits the interaction between the virus and the cellular CD4 receptors, preventing attachment. Fostemsavir has been approved for the treatment of HIV-1 people with limited treatment options in 2020 by the FDA (Markham, 2020) and in 2021 by the EMA.

Post-Attachment inhibitor ibalizumab is a monoclonal antibody approved for the treatment of multidrug resistant HIV-1. Ibalizumab blocks viral entry by interfering with the formation of CD4-HIV envelope complex through its binding to CD4 extracellular domain 2; ibalizumab is active against both CCR5 and CXCR4 tropic viruses but remains available only in USA and

Canada due to the choice of the manufacturer not to commercialize the product in EU (Emu et al., 2018; Kufel, 2020).

Capsid inhibitor lenacapavir targets the HIV capsid protein p24, which forms the shell that embeds the genetic material, impairing the release of the viral genome. Additionally, lenacapavir impairs the transport of the pre-integration complex into the nucleus. Thus, it affects both early and late phases of viral replication, which is a novel feature among antiretrovirals (Tailor et al., 2024).

Drug Class	Drug Name	Abbreviation	FDA Approval Year
NRTIs	Zidovudine	ZDV	1987
	Abacavir	ABC	1988
	Didanosine	DDI	1991
	Zalcitabine	DDC	1992
	Stavudine	d4T	1994
	Lamivudine	3TC	1995
	Tenofovir isoproxil fumarate	TDF	2001
	Emtricitabine	FTC	2003
	Tenofovir alafenamide	TAF	2016
NNRTIs	Nevirapine	NVP	1996
	Efavirenz	EFV	1998
	Etravirine	ETR	2008
	Rilpivirine	RPV	2011
	Doravirine	DOR	2018

INSTIs	Elvitegravir	EVG	2012
	Dolutegravir	DTG	2013
	Raltegravir	RAL	2017
	Bictegravir	BIC	2018
	Cabotegravir	CAB	2021
PIs	Ritonavir	RTV	1996
	Indinavir	IDV	1996
	Nelfinavir	NFV	1998
	Lopinavir	LPV	2000
	Atazanavir	ATV	2003
	Fosamprenavir	FPV	2003
	Saquinavir	SQV	2004
	Tipranavir	TPV	2005
	Darunavir	DRV	2006
Fusion Inhibitor	Enfuvirtide	T-20	2003
CCR5 Antagonist	Maraviroc	MVC	2007
Post-Attachment Inhibitor	Ibalizumab	IBA	2018
Attachment Inhibitors	Fostemsavir	FTR	2020
Capsid Inhibitor	Lenacapavir	LEN	2022

Table 1 List of antiretroviral drugs for the treatment of HIV-1 infection.

- **Current ART guidelines**

Current guidelines (https://clinicalinfo.hiv.gov/en/guidelines, last reviewed September 2024; EACS Guidelines version 12.1, November 2024) recommend that ART should be started immediately or as soon as possible after diagnosis, regardless of CD4 count, in order to rapidly suppress virus replication, improve retention in care, and reduce the risk of HIV transmission. Drug resistance testing is recommended at diagnosis to guide treatment selection depending on the presence of transmitted drug resistance mutations. If ART needs to be started before the availability of genotypic testing results, it is recommended to select a first-line regimen with a high barrier to resistance, preferably including a second generation INSTI or a boosted PI.

The initial ART regimen generally includes three HIV medicines from at least two different drug classes, often consisting of two NRTIs plus a second generation INSTI (DTG or BIC), or an NNRTI (DOR), or a boosted PI (DRV/c or DRV/r). As shown in many clinical trials, this strategy has resulted in suppression of HIV replication and in increased CD4 T cells count in most PLWH.

Drugs belonging to other classes are mainly recommended in second line regimens, in patients with insufficient treatment options because of multidrug resistance, or because of adverse effects or costs.

- **Two-drug regimens**

Nowadays, there is wide evidence that supports the use of two-drug regimens (2-DRs) in PLWH, both as first line treatment and as switch option for virologically suppressed PLWH who meet defined characteristics: sustained suppression of HIV viral load to <50 copies/mL, no historical antiretroviral resistance to the 2-DR being considered, and HBV immunity with detectable anti-HBs antibodies (since current 2-DRs lack protection against HBV infection or reactivation). The currently favored 2-DRs are:

- DTG/3TC (first-line treatment and switch)
- DTG/RPV (switch)
- 3TC/DRV (switch)
- CAB/RPV (switch, injectable)

The objectives of treatment simplification include improving tolerability, eliminating or

decreasing adverse events, thus improving the quality of life of PLWH.

Therapeutic switch to dual regimen represents an increasingly established therapeutic option for selected PLWH. Clinical trials, such as the SALSA (Llibre et al., 2023) and the TANGO trials (Osiyemi et al., 2022), demonstrated the non-inferiority of 2-DRs compared to 3-DRs. A recent systematic review and meta- analysis confirmed that the DTG/3TC regimen shows high rates of virological suppression and low rates of virological failure, discontinuation, and drug resistance, both in randomized trials and real-world data (Fraysse et al., 2025). The 48-week data from the DOLCE study also provides strong evidence that the combination of DTG/3TC is as effective as 3-DR (Figueroa et al., 2025). Furthermore, a 2024 retrospective study demonstrated the efficacy of DTG/3TC dual therapy even in treatment-naïve patients with baseline viral loads > 500,000 copies/mL, suggesting its potential as a first-line regimen for naïve patients (Dou et al., 2024). These approved 2-DRs have demonstrated comparable safety, efficacy, and non-inferiority with respect to triple therapy, without being associated with higher rates of virological rebound, while offering reduced pharmacological exposure and an improved tolerability profile (Hidalgo-Tenorio & Martínez- Sanz, 2025). Despite solid data supporting that switching to a 2-DR as a valid strategy to maintain virological suppression comparable to 3-DRs, to date little is known about the long-term impact that treatment simplification has on the HIV-1 reservoir, in terms of total and intact HIV proviral DNA and cell-associated HIV RNA. Only the RUMBA study (De Scheerder et al., 2025), a phase IV, randomized controlled switch trial, has investigated the impact of switching from a 3-DR to the 2-DR (DTG/3TC) with a primary focus on changes in the HIV viral reservoir over time.

- **Social and economic impact of switching from a 3-DR to a 2-DR among PLWH**

The definition of the global effects of 2- and 3-DRs, both used as standard of care on HIV management, would suggest new therapeutic approaches for HIV treatment to be developed by National Health Systems, thus addressing the issue of the economic sustainability of ART in a population of PLWH that increases by 10% every year.

The introduction of 2-DRs, particularly DTG/3TC and DTG/RPV, represents a major step in ART optimization. Economic implications include drug-cost savings and potential reductions in toxicity-related care, leading to a reduction of the resources needed for prevention and monitoring programs. Budget- impact analyses in high-income settings predict significant

savings, although these may be partly offset by the current need for viral load monitoring and genotypic resistance testing before and after switch (Butler et al., 2021).

Additionally, social implications are relevant as well since a lower pill burden and improved tolerability may enhance adherence, quality of life, and reduce transmission and treatment-related stigma, particularly when 2-DRs are available as single-tablet regimens or long-acting injectables.

- **Long-acting regimens**

Some people are eligible to receive newer long-acting medicines that offer a way to reduce the frequency of treatment administration. Currently available long-acting drugs are administered as injections with schedules that range from every two weeks to every six months, depending on the drug (https://hivinfo.nih.gov/understanding-hiv/fact-sheets/long-acting-hiv-medicine).

Currently FDA approved long-acting HIV medicines include the 2-DR CAB/RPV, administered as intramuscular injection every 2 months, LEN and IBA. CAB and LEN can also be administered alone as Pre- exposure prophylaxis (PrEP) to people who do not have HIV to reduce the risk of acquiring infection from sexual intercourses by about 99% and from injection drug use by at least 74% (https://hivinfo.nih.gov/understanding-hiv/fact-sheets/pre-exposure-prophylaxis-prep).

HIV-1 Latency

Viral latency is a reversible condition characterized by a nonproductive infection from infected cells. A key step in the HIV-1 replication cycle is the integration of its DNA into the host genome, enabling the virus to persist in blood cells and in other anatomic compartments for years (Jütte et al., 2023). This distinctive characteristic of HIV-1 makes ART effective in blocking viral replication and halt disease progression towards the development of the AIDS (Tioka et al., 2025) but not in clearing the infection. Indeed, upon ART interruption, viral rebound from the latent reservoir is rapid, leading to detectable viremia usually within weeks of therapy interruption (Chou et al., 2024).

HIV-1 can evade the immune system thanks to its genetic variability, driven by its error-prone RT (10^{-3} to 10^{-5} bp/cycle) and by the high replication rate of the virus (10^{9} to 10^{10} virions/day).

In addition, viral reservoirs support immune evasion, as CTLs cannot easily target latently infected cells (Chvatal-Medina et al., 2023). Although the concept of reservoir being known from the introduction of ART (Finzi et al., 1997), the molecular mechanisms leading to the establishment and persistence of HIV-1 have still to be completely elucidated.

The latent reservoir is established when cells harboring proviral DNA are reverted to a resting phenotype with reduced gene expression, causing the cell to be non-permissive for HIV-1 production but providing a sanctuary to evade the immune response and ART (Chvatal-Medina et al., 2023; Thomas et al., 2020). The 90-95% of the reservoir exists as defective provirus (Tioka et al., 2025), which contain deletions, insertions, or hypermutations introduced during reverse transcription that render the virus unable to support new infections (Bruner et al., 2016; Ho et al., 2013). However, most defective proviruses retain promoter activity and can express viral antigens upon stochastic reactivation, contributing to chronic immune activation.

Many factors contribute to the transcriptional silencing that characterizes latency. Ex vivo studies in PLWH on ART revealed that HIV-1 integration occurs often within actively transcribed host genes in resting CD4+ T cells (Han et al., 2004). However, epigenetic regulation plays an important role as well: methylation of CpG islands in the HIV-1 promoter silences viral gene expression in vitro, as shown in cell lines and primary CD4+ T cells, promoting HIV-1 latency, and is inversely correlated with viremia and proviral reactivation in infected individuals (Blazkova et al., 2010). Long-term non-progressors and elite controllers show increased methylation patterns of LTR and CpG islands, a mechanism that may contribute to more efficient immune control and reduced immune activation (Palacios et al., 2012). Moreover, in cellular models, proviruses integrated into heterochromatic regions remain transcriptionally silent, reinforcing latency (Jordan, 2001).

The persistence of the latent reservoir is sustained by the long half-life (~ 43–44 months) of the cells that harbor it, with a natural decay of the latent HIV-1 reservoir estimated to be about 73 years. Therefore, all HIV-1-infected individuals need to take life-long ART (Anderson & Maldarelli, 2018). The viral reservoir is dynamic and exists in at least in three main states: (i) deep latency, with no viral RNA expression, (ii) low RNA transcription but no protein translation, and (iii) dynamic viral activation, with higher expression of viral RNA, allowing translation of proteins that lead to competent virions assembly and release (Busman- Sahay et al., 2021).

Even if HIV-1 can infect different lymphoid and myeloid lineages in multiple anatomical

compartments, the main cellular reservoir is represented by resting memory CD4 T cells, which harbor 16-fold more integrated HIV DNA than naïve cells (Anderson & Maldarelli, 2018; Chvatal-Medina et al., 2023; Pinzone et al., 2019; Wong & Yukl, 2016).

Memory T cells can be categorized into central memory cells, transitional memory cells, effector memory cells, tissue-resident memory cells, and stem cell memory cells. Each subset is phenotypically defined by the surface expression of specific chemokine and homing receptors. These subsets are infected to different extents and contribute variably to viral persistence (Avettand-Fènoël et al., 2016; Vanhamel et al., 2019; Ventura, 2020). HIV-1 reservoirs are not limited to the blood, rather they are mainly present in the lymphoid tissue, the gastrointestinal tract, the male genital tract, the central nervous system, adipose tissue, liver, skin, bone marrow, and breast tissue (Chvatal-Medina et al., 2023).

The size and stability of the reservoir are mainly maintained by clonal expansion (Yeh et al., 2021). Since its discovery, the stability of the latent reservoir was believed to be due to the natural longevity of different subsets of resting memory CD4 T cells. However, recent works suggest that the HIV-1 latent reservoir is maintained through proliferation of infected cells (Ta et al., 2022). Over time, the proportion of clonally expanded HIV-1-infected cells increases, suggesting that reservoir persistence results not only from the survival of the original infected cells, but also from ongoing cellular proliferation (R. Liu et al., 2020). Additional factors, such as residual viral replication during suppressive ART, may contribute to reservoir stability (Anderson & Maldarelli, 2018; Ventura, 2020).

Taken together, these data highlights how HIV-1 latency results from a combination of viral integration, transcriptional silencing, long-lived cellular reservoirs, and clonal expansion and, in this context, developing strategies aimed at eliminating or functionally controlling HIV-1 infection without ART is a high priority.

HIV-1 Eradication Strategies

Currently, seven people have been functionally cured from HIV-1 infection, as shown by remaining free of viral rebound for a period that spans from months to over 10 years without ART (Xiao et al., 2025). All patients had leukemia, and during pre-transplant therapy, the pool of infected cells was significantly depleted. This was followed by stem cell transplantation, the

majority of which involved donors homozygous for the Δ32 CCR5 deletion, rendering the recipient's cells non-permissive to infection by R5- tropic HIV-1 viruses (X. Liu et al., 1996). However, due to the limited availability of these donor cells and the unique circumstances predetermining these cases, including inherent risks and cost of any bone marrow transplantation, this type of cure is not feasible for widespread use.

The majority of current efforts that aim to find a cure to HIV-1 infection are concentrated on the so-called 'shock and kill' strategy, a method proposed to eliminate latently infected cells, involving the use of small molecules, latency reversal agents (LRAs), to induce viral transcription (shock), followed by the clearance of the reactivated cells (kill) (Ait-Ammar et al., 2020; Walker-Sperling et al., 2016). Reactivating HIV-1 latent cells exposes them to being attacked by the host immune system, but native responses may need to be enhanced to achieve full eradication of the reservoir and viral escape mutants in long-term infected individuals may render immune clearance ineffective. Many LRAs act by directly or indirectly activating transcription factors that bind to the HIV LTR and activate transcription, such as NF-kB, NFAT, cIAP1 (cellular inhibitor of apoptosis protein-1), and Sp1.

Several classes of LRAs can be distinguished, such as compounds activating transcription factors: protein kinase C (PKC) agonists, mitogen-activated protein kinase (MAPK) and AKT activators, and second mitochondria derived activator of caspases (SMAC) mimetics. Another major class of LRAs are epigenetic modifiers, which lead to the decondensation of chromatin, allowing transcription factors to bind to the HIV LTR and activate transcription. A third class of LRAs are immune modulators (i.e. CCR5 antagonist), which act on cell surface receptors or pathogen recognition receptors (PRRs) to induce an activated state in the targeted cells. Finally, TAT mimics and enhancers initiate or enhance the effect of the viral transactivator TAT on HIV RNA transcription initiation. (Tioka et al., 2025). Tat is an essential regulatory protein of HIV-1 that is expressed once the virus integrates into the host cell genome and stimulates elongation by the host RNA Pol II (Karn, 2011).

Unfortunately, many compounds able to exert a latency reversal activity in vitro are mitogenic compounds (e.g. 28 phytohaemagglutinin and phorbol myristate acetate, PMA) and cannot be used in vivo (Spina et al., 2013). Alternative approaches for reservoir reactivation include targeting innate immune recognition pathways. Indeed, TLR-7 activation by the agonist GS-9620 has been demonstrated to promote profound reactivation of CD4 T cells via increased levels of type II IFN (Tsai et al., 2017). Undoubtedly, a major challenge in this approach is the ability to achieve broad and efficient latency reversal without eliciting toxic side effects.

Perhaps, combinations of LRAs could have improved effects by acting on different mechanisms; indeed, synergy between multiple combinations of LRAs has so far been identified in vitro (Thomas et al., 2020).

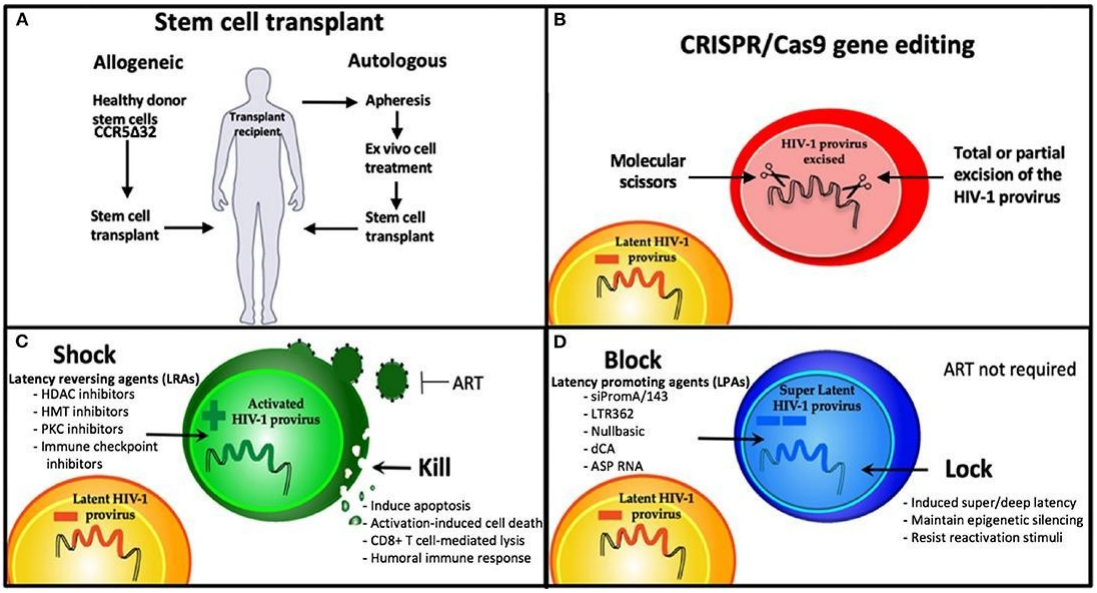


Figure 7. Overview of some strategies for HIV-1 eradication and long-term remission (Ahlenstiel et al., 2020).

Another cure strategy called “block and lock” has been proposed. Rather than using latency reversal, this strategy is aimed to reinforce latency to prevent viral rebound following ART interruption, using small interfering RNAs (siRNAs) to induce transcriptional gene silencing (Mousseau et al., 2015). Tat is a potential target for this type of intervention, since it is required for viral transcription at the onset of replication, thus its silencing may significantly reduce the reactivation capacity of the virus (Chvatal- Medina et al., 2023). Even if the “block and lock” approach may offer an alternative cure mechanism to “shock and kill”, its development is still preliminary and is yet to be tested in human trials.

Another curative strategy is represented by promising gene editing tools, such as CRISPR-Cas9 and zinc finger nucleases. In the context of HIV-1 infection, CRISPR-Cas9 can target different sites, such as the LTR regions, to inhibit viral replication. Gene editing approaches have the advantage of highly specific gene targeting and can produce the desired outcome, mostly without global physiological impact. Nevertheless, off-target effects have been observed in several studies and may affect the safety of these methods (Xiao et al., 2019). This is one of

the reasons why, until now, the use of CRISPR-Cas9 therapy to treat HIV infection remains to be fully addressed. Other obstacles that limit the feasibility of this approach are the genetic diversity of HIV, which may impact on the efficiency of the endonuclease system, and potential off-target activity, which may compromise the function of essential tumor suppressor genes, affect chromosome translocation, or cause other aberrations (Chvatal-Medina et al., 2023).

Finally, therapeutic vaccines and immunization strategies aim to eliminate or significantly diminish viremia rebound by administering the vaccine regimen during sustained ART in patients with suppressed viremia, followed by a period of ART interruption. The idea relies on enhancing HIV-1 specific T cell or antibody response to reduce viral rebound after ART interruption and control the latent reservoir. However, Davenport et al. suggest that, even with highly efficacious vaccines, rebound viremia would likely emerge within 5 weeks following ART interruption (Davenport et al., 2019).

Besides vaccines, also the use of anti-HIV-1 broadly neutralizing antibodies (bNAbs) is being investigated. bNAbs recognize conserved regions of viral gp120 and gp41, neutralizing circulating viruses and engaging the immune system to clear infected cells. bNAbs are being studied for different clinical applications in the context of HIV-1 infection, other than passive immunization. One of their possible additional applications is the use of bNAbs as therapeutic agents in PLWH, for example in the “shock and kill” strategy to clear infected cells after latency reversal (Mahomed et al., 2025; Thavarajah et al., 2024).

However, to achieve clinically significant effects, all these mechanisms must affect most of the latent reservoirs, which is a major challenge considering the variety of anatomical compartments hosting a significant proportion of latently infected cells (Thomas et al., 2020).

Methods to Assess the HIV-1 Latent Reservoir

Measuring the efficacy of HIV-1 interventions requires highly sensitive and accurate assays, but there is currently no consensus on the most appropriate method to use, and several technical challenges limit the ability to accurately measure the size of the latent reservoir. Existing methods to assess it can be divided in two different categories: cell culture-based assays and PCR-based assays (Fig. 8).

Among cell culture-based assays, the viral outgrowth assay (VOA) is considered the gold

standard for measuring replication competent proviruses. In this assay, CD4 T cells are stimulated to reverse latency and drive HIV-1 expression from integrated provirus (Han et al., 2004; Laird et al., 2013).

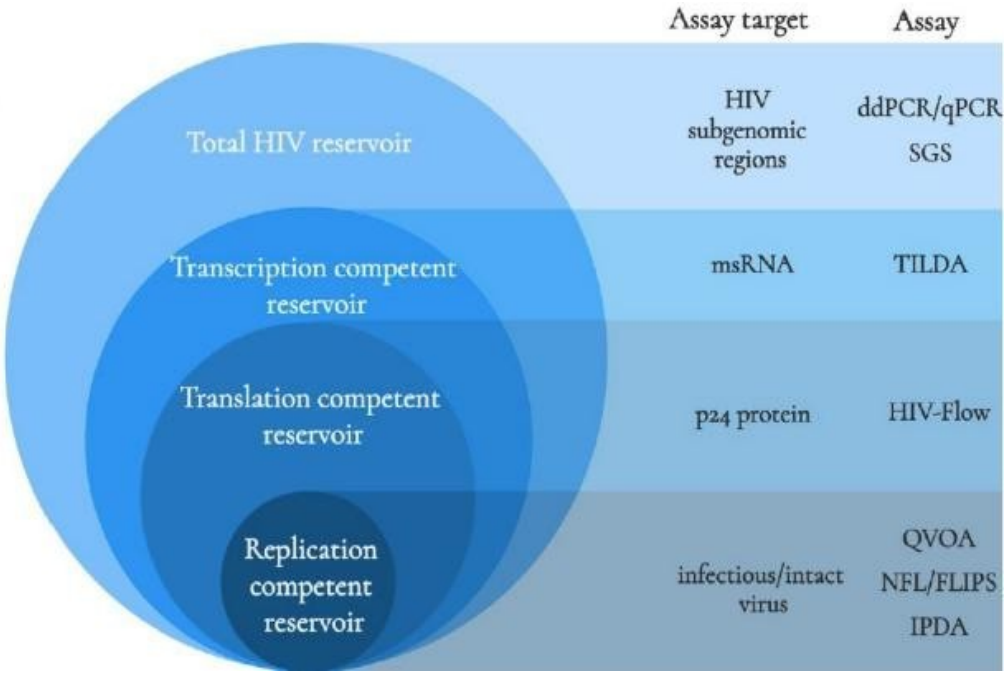


Figure 8. Currently available laboratory methods to investigate the different compartments of the HIV-1 latent reservoir. Adapted from (Gantner et al., 2020).

Following reactivation, viral outgrowth is supported by incubation with CD4 T cells from HIV-1 negative donors for 2–3 weeks and measured via the detection of p24 capsid antigen. Positive cells are quantified and the frequency of latently infected cells with an intact provirus is determined based on Poisson distribution and expressed as infectious units per million cells. The major drawback of VOA is the underestimation of the size of the intact latent reservoirs by 25 to 60-fold due to the presence of non- inducible replication competent viral proviruses (Bruner et al., 2016; Ho et al., 2013). Furthermore, it is expensive, labor and resources intensive and requires a biosafety level 3 facility. Also, extensive analysis of VOA performance using the same samples across different labs has indicated significant variability of results (Rosenbloom et al., 2019). Several improvements of the VOA have attempted to overcome these limitations, including the use of continuous cell lines to improve reproducibility (Badia et al., 2018; Buzon et al., 2014; Davenport et al., 2019; Massanella et al., 2018), the use of qPCR to detect HIV-1 RNA (Laird et al., 2013) or the use of improved p24 ELISA to increase sensitivity (Fun et al., 2017).

Like VOA, another recently developed culture-based assay is the TZM-bl cell-based assay (TZA). This assay uses the TZM-bl cell line, which stably expresses CD4, CCR5, and CXCR4, and carries an integrated copy of the β-galactosidase and luciferase genes under the control of the HIV-1 LTR promoter, allowing the detection of inducible replication-competent HIV-1. This assay is regarded as more sensitive, cost- efficient, and faster than the VOA (Gupta et al., 2017). However, this method lacks validation and inter- laboratory reproducibility data.

Quantification of cell associated HIV-1 RNA (CAR) following CD4 T cell activation may also be used as a surrogate for measuring the size of the inducible latent reservoir (Cillo et al., 2014; Pasternak et al., 2013; Shan et al., 2013). Moreover, as well as CAR, by measuring cell-free HIV-1 RNA (CFR) from culture supernatant, indicative of virus release from cells, may more closely predict replication competence (Cillo et al., 2014; Massanella et al., 2018). However, these methods are prone to false positive results, since cells harboring defective provirus are still able to produce HIV-1 mRNA following T cell activation, despite being unable to generate infectious virions.

The tat/rev induced limiting dilution assay (TILDA), was later developed to enhance accuracy and to reduce limitations of CAR and CFR quantification. TILDA relies on the measurement of tat/rev transcripts from cells plated in limiting dilution, following activation with strong inductors; results obtained from TILDA quantification correlate well with HIV-1 DNA quantification, but not with those obtained from VOA, hence still susceptible to overestimating the size of the latent reservoirs due to the possibility that these transcripts arise from cells with defective HIV-1 genomes (Procopio et al., 2015).

Conversely, PCR-based assays offer a more practical, fast and relatively inexpensive approach to measure the size of the viral reservoir by the quantification of the total amount of HIV-1 DNA (Ho et al., 2013; Thomas et al., 2020). Total HIV-1 DNA quantification has been shown to predict viral rebound (Williams et al., 2014) and differential methods have been improved, offering the potential to identify different DNA forms, such as integrated HIV-1 DNA and non-integrated HIV-1 DNA (2-LTR and 1-LTR circular forms, respectively) (Avettand-Fènoël et al., 2016; Krings et al., 2025). Anyway, several factors affect the accuracy of HIV-1 DNA assays and the reproducibility among different laboratories must be investigated (Vicenti 2022). Another key determinant of the accuracy of HIV-1 DNA quantification assays is the genomic location at which the primers and probes anneal; indeed, primer mismatches in target regions may result in false negatives or target underestimation. Moreover, amplification efficiency may be affected by the DNA input load and by the presence of inhibitory contaminants (Rutsaert et al.,

2018). Digital PCR (dPCR) platforms partially overcome these issues, since they provide absolute quantification of samples independently from an external calibration curve and are less affected by PCR inhibition. For these reasons dPCR is becoming increasingly popular in HIV-1 research and clinical trials (Delporte et al., 2023; Rutsaert et al., 2018). In addition, PCR based assays relying on amplification of short genomic regions to quantify the amount of HIV-1 DNA cannot distinguish between intact and defective provirus and therefore typically overestimate the size of the replication competent latent reservoir (Bruner et al., 2016; Eriksson et al., 2013; Ho et al., 2013). To overcome this limitation, the intact proviral DNA assay (IPDA) has been developed. IPDA consists of a multiplexed dPCR to measure the size of the intact latent reservoir of HIV-1, based on targeting regions frequently affected by deletions or hypermutation in defective genomes (Bruner et al., 2019). Although imperfect, this assay provides a reasonably correct estimation of the amount of replication competent proviruses without the need of T cell stimulation and is thus gaining popularity.

- **Intact Proviral DNA Assay (IPDA)**

More in detail, the IPDA uses a multiplexed dPCR approach targeting two regions of the HIV genome, typically the packaging signal (ψ) at the 5' end and a portion of *env* at the 3' end, along with an additional unlabeled probe to exclude APOBEC3C-hypermutated sequences in the *env* region. If both target regions are present and *env* is not hypermutated, the provirus is more likely to be intact (Fig. 9).

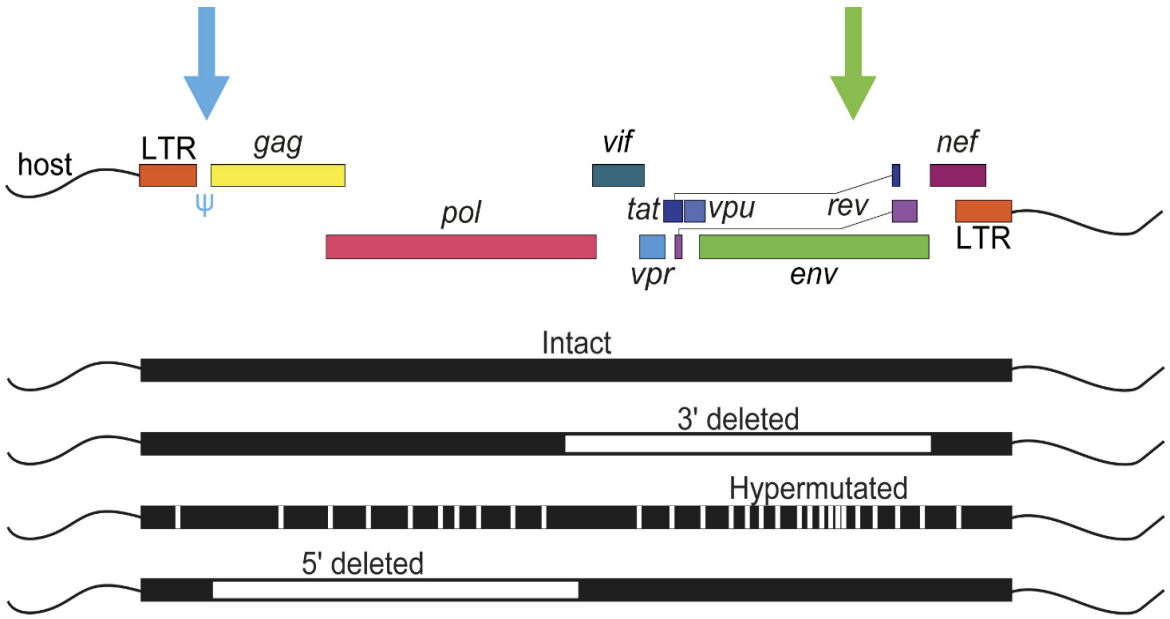


Figure 9. IPDA schematic representation: multiplex dPCRs amplify Ψ and env regions. Adapted from Bruner et al., 2019.

IPDA does not require the induction of latent viruses from infected CD4+ T cells, avoiding one of the principal limitations of the VOA, since many proviruses are non-inducible under standard stimulation protocols, resulting in underestimation of the replication-competent reservoir. Moreover, IPDA is high-throughput, less laborious, and requires fewer cells than the VOA. However, the IPDA also has caveats and limitations, which are critical when designing specific studies and when interpreting results. One of the main limitations arises from the variability of the HIV-1 genome, since mutations in the primer and probe binding sites may result in failure to detect one or both target regions (Fig. 10).

This can lead to underestimation of intact proviruses or complete amplification failure. In addition, also within the same individual, there may be a variety of viral variants, among which some can be detected by IPDA while others cannot, revealing only a subset of intact proviruses (Kinloch et al., 2021).

The original IPDA developed by Bruner and colleagues was developed based on subtype B viruses, which are the majority in high-resource countries, but other regions of the world have predominance of non-B subtypes (e.g. C, A, D, CRFs). Because of the original design, nucleotide variations in primer and probe binding regions are more frequent among non-B subtypes, leading to much higher rates of detection failure or misquantification.

A strategy to overcome this limitation is the sequencing of the probe regions to identify mismatches, designing custom primers and probes. Simonetti and colleagues showed that in cases of mismatches, custom oligos may be designed to detect those variants (Simonetti et al., 2020). Anyhow, it has to be taken into account that, even if both target regions are present and no hypermutation is detected in the env probe region, IPDA does not prove that the provirus labelled as intact is replication competent, since there may be other defects outside the surveyed regions that prevent replication (Gaebler et al., 2019).

Actually, the possibility that a provirus classified as intact by IPDA harbors genome defects in other regions than the amplified ψ and env regions, may lead to an overestimation of intact proviruses. Near full-length (NFL) sequencing or Q4PCR (quadruplex-qPCR + sequencing) studies have indeed shown that only 50- 70% of proviruses classified as intact by IPDA are confirmed as intact (Cicilionytė et al., 2021).

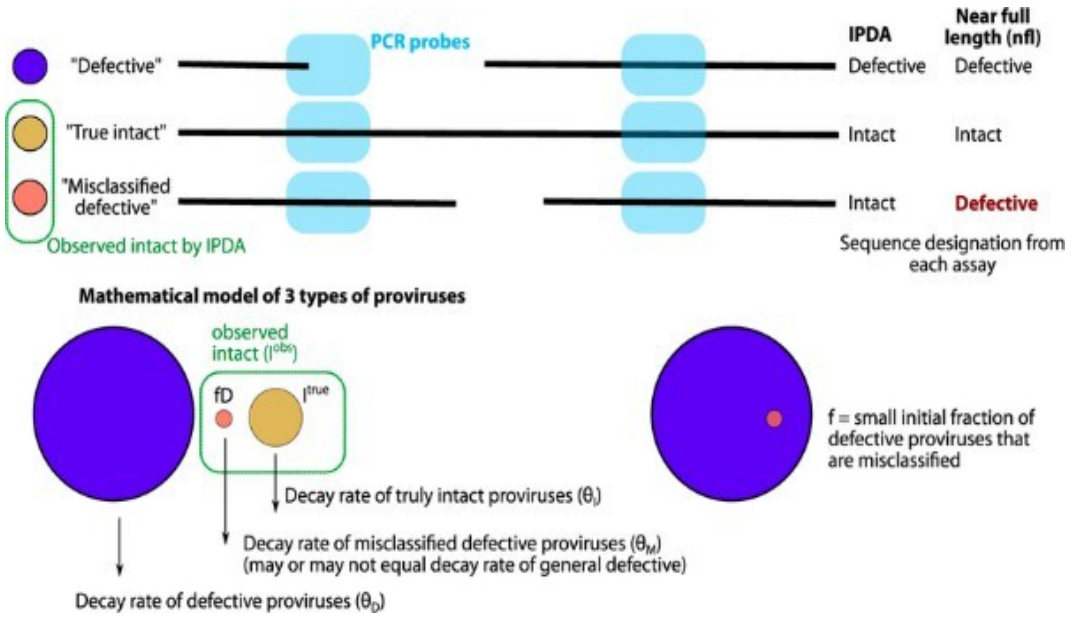


Figure 10. Schematic illustration of misclassified intact proviruses (Reeves et al., 2023).

Another technical limitation is physical fragmentation (shearing) of extracted genomic DNA between the ψ and env target regions, which can cause the miss out of an amplicon even if the provirus is intact, giving a false negative result and underestimating the number of intact proviruses. IPDA protocols limit this misclassification by normalizing the results to a human housekeeping gene amplified with primers that are located at a similar distance on the human genome to that of the ψ and *env* regions on the HIV-1 genome (Bruner et al., 2019). Protocols have been accordingly refined to better control for DNA quality, fragmentation, and shearing between the two targets. Using cellular reference genes with amplicons separated by distances similar to the ψ-*env* distance helps to correct for losses due to shearing. Also, extraction protocols and DNA handling can be optimized to reduce fragmentation (Cicilionytė et al., 2021).

Overall, the IPDA represents a major advance over earlier assays for measuring HIV-1 reservoir, especially considering its throughput, feasibility outside a BSL3 containment facility, cell number requirements, and its ability to detect intact proviruses without induction. However, its reliance on detection of only two regions of the viral genome, and sensitivity to sequence mismatches, still advise to interpret results with caution and further progress to improved IPDA protocols.

AIM OF THE THESIS

The aim of this project was to investigate the impact of treatment simplification from three-drug (3DR) to two-drug (2DR) antiretroviral therapy regimens on the persistence and dynamics of the HIV-1 latent reservoir in virologically suppressed people living with HIV (PLWH).

This work has been conducted as a multicentric collaborative study involving several academic institutions (University of Siena, University of Roma Tor Vergata, University of Modena and Reggio Emilia, University of Milano and University of Torino), enabling a comprehensive analysis, that aims at integrating virological, immunological, and pharmacological data from both peripheral blood and gut-associated lymphoid tissue (GALT).

Within this collaborative study, the specific focus of my doctoral research, carried out at the University of Siena, has been the quantitative and qualitative characterization of the viral reservoir in peripheral blood of 68 enrolled PLWH. This was done by measuring the total and intact cell-associated HIV-DNA and the cell-associated HIV-RNA in CD4+ T cells by digital PCR, investigating its changes after 18 months from treatment simplification to 2DR vs. continuing standard 3DR.

By longitudinally assessing these virological markers over an 18-month follow-up period, the objective was to investigate whether reduced pharmacological pressure influences the size or the dynamic of the HIV latent reservoir in peripheral blood and to provide insights that may support evidence-based decisions on ART simplification, contributing to selecting ideal candidates for this intervention and to personalize antiretroviral treatments.

MATERIALS and METHODS

Samples collection and storage

PBMCs were isolated from whole blood using Leucosep™ tubes (Greiner, #227288) preloaded with Lymphoprep™.

Before sample loading, Leucosep™ tubes were centrifuged for 1 minute at 200 x g to ensure the positioning of the Lymphoprep™ layer below the resin barrier. Whole blood was diluted 1:2 with phosphate-buffered saline (PBS) and transferred into the Leucosep™ tube, without disturbing the resin barrier. Samples were then centrifuged at 800 x g for 30 minutes at room temperature (RT) with no brake.

After centrifugation, plasma was removed and the PBMC layer was carefully transferred into a new 50- mL conical tube, washed with PBS and centrifuged at 400 x g for 10 minutes. Following supernatant removal, a second wash was performed with a centrifugation at 150 x g for 10 minutes. After discarding the supernatant again, the cell pellet was resuspended in complete medium.

Cell number and viability were determined using Trypan Blue (1:5 dilution; 160 µL Trypan Blue and 40 µL cell suspension). Based on cell yield, PBMCs were aliquoted into cryovials to be shipped to the collaborating centers, ensuring to have at least three vials per individual. To allow long term cryopreservation and viability of the PBMCs, an equal volume of serum containing 20% DMSO was added to each cell vial.

$CD4^+$ isolation from peripheral blood mononuclear cells

$CD4^+$ T cells enrichment was performed using the AutoMACS® Neo Separator (Miltenyi Biotec; Fig. 11) in combination with the CD4 MicroBeads, human lyophilized kit (Miltenyi Biotec, # 130-097-048).

After thawing, PBMCs were washed twice with PBS and counted using a hemocytometer. Briefly, PBMCs were resuspended in MACS buffer (PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA) at a maximum concentration of 10^7 cells per 80 µL buffer.

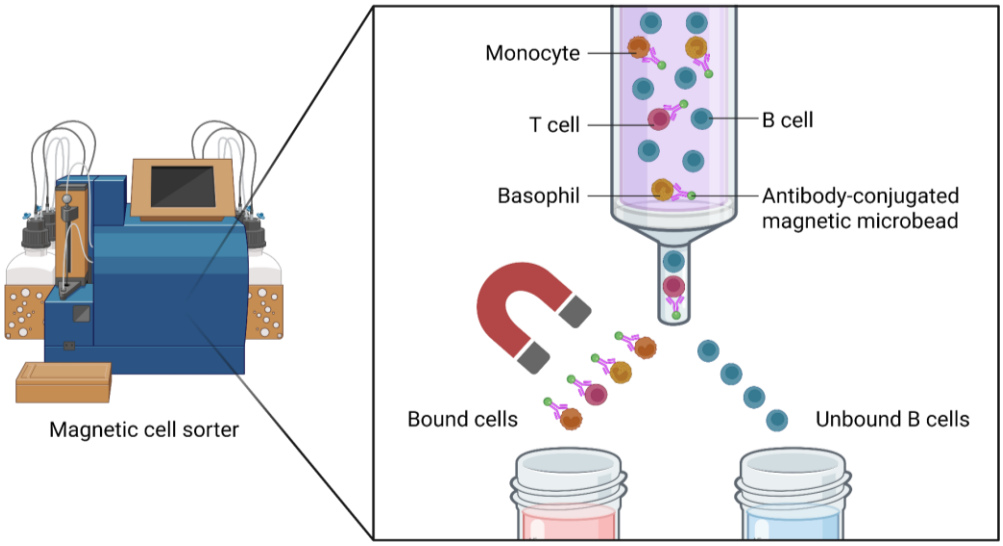


Figure 11. Magnetic sorting of CD4+ T cells with the AutoMACS® Neo Separator (Miltenyi Biotec).

Cells were then incubated with 20 µL of CD4 MicroBeads for 15 minutes at 4 °C with gentle mixing. After incubation, cells were washed with 1 mL of MACS buffer, centrifuged at 300 × g for 10 minutes, resuspended in 500 µL of the same buffer and loaded on the AutoMACS® Neo Separator for positive magnetic selection of CD4+ T cells. The CD4+ positive fraction was collected, centrifuged at 330 x g for 5 minutes, and the resulting cell pellets were stored at -80 °C for subsequent extraction of nucleic acids.

Nucleic acids extraction

To avoid excessive DNA fragmentation (DNA Shearing Index >0.5), nucleic acids were isolated from CD4+ T cells using a manual Cell-Associated RNA and DNA extraction assay. All procedures involving HIV- infected cells were performed under BSL-2 conditions, using appropriate personal protective equipment.

- **Reagents**

The following reagents were used: Calcium Chloride ($CaCl_2$; 1 M; Sigma, Cat. #21115), DNase I Amplification Grade (Merck, Cat. #AMPD1-1KT), EDTA (0.5 M, pH 8.0; Ambion, Cat. #AM9260G), ethanol (100%; Sigma), glycogen type II from oyster (Merck, Cat. #G8751-5G), guanidine hydrochloride (GuHCl; Molecular Biology Grade; Merck, Cat. #G3272-25G),

guanidine thiocyanate solution (GuSCN; BioUltra; Merck, Cat. #50983-50ML), isopropanol (100%; Sigma), Proteinase K (20 mg/mL; Applied Biosystems, Cat. #AM2548), nuclease-free molecular grade water (H_2O; Gibco, Cat. #10977), Tris-HCl (1 M, pH 8.0).

- **Reagents Preparation**

A GuHCl stock solution was prepared with 3 M guanidine hydrochloride, 50 mM Tris-HCl (pH 8.0; adjusted to final pH 7.6), and 1 mM $CaCl_2$, and stored at room temperature (RT). Immediately before use, a GuHCl/Proteinase K working solution was prepared by adding 50 µL Proteinase K to 950 µL GuHCl+. A GuSCN stock solution (~5.7 M GuSCN, 50 mM Tris-HCl, pH 7.6, and 1 mM EDTA) was prepared by adding 2.65 mL of 1 M Tris-HCl (pH 8.0) and 106 µL of 0.5 M EDTA to 50 mL of 6 M GuSCN, and stored at RT. Immediately before use, the GuSCN/Glycogen solution was prepared by adding 30 µL of glycogen to 970 µL GuSCN.

- **Extraction Procedure**

Cell Lysis and Protein Digestion: cell pellets were thawed, immediately lysed by the addition of 100 µL GuHCl+/ProK solution, vortexed and incubated at 42 °C for 1 h.

RNA and DNA Precipitation: after incubation, 400 µL GuSCN/Glycogen solution was added, and samples were vortexed and incubated at 42 °C for 10 minutes. Then, 500 µL of 100% isopropanol (RT) was added, vortexed vigorously for 10 seconds, centrifuged at 21,000 × g for 10 minutes at RT and the supernatant was carefully removed.

Pellet Washing: pellets were washed with 750 µL of 70% ethanol (RT) and vortexed to ensure complete resuspension. After centrifugation at 21,000 × g for 10 minutes (RT), the supernatant was discarded and pellets were air-dried for 5–10 minutes until they became translucent, avoiding over drying.

Resuspension and DNA Processing: dried pellets were resuspended in 70 µL of 5 mM Tris-HCl and incubated at 42 °C for 20 minutes. After incubation, the suspension was divided into two tubes: one for the RNA extraction and one for the DNA fraction. The DNA was incubated ay 42 °C for 2 hours and then stored at 4 °C. Concentrations of the extracted DNA samples were determined using a Qubit fluorometer and recorded in ng/µL.

RNA Processing: the RNA fraction was treated with DNase I at 37 °C for 15 minutes, to degrade DNA and further purified by addition of 200 µL GuSCN, followed by vortexing.

Subsequently, 250 µL of 100% isopropanol (RT) was added, vortexed for 10 seconds, and centrifuged at 21,000 × g for 10 minutes at RT. Pellets were washed with 1 mL of 70% ethanol (RT), centrifuged at 21,000 × g for 5 minutes at RT, and
residual ethanol was removed by aspiration. Pellets were air-dried for 2–5 minutes until they became translucent. RNA was resuspended in 30 µL of 5 mM Tris-HCl and immediately processed for cDNA synthesis.

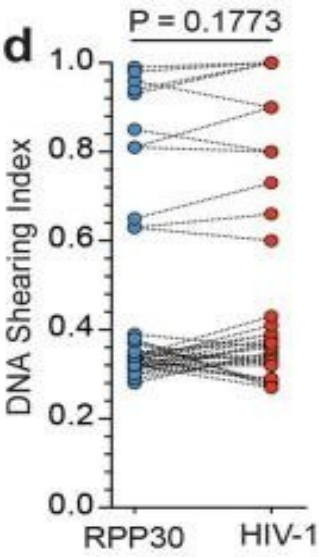
cDNA synthesis

Total extracted RNA (30 µL) from each sample was divided into two aliquots and simultaneously retrotranscribed, as the reverse transcription enzyme that was used supports a maximum input volume of 15 µL of RNA extract per reaction. Fifteen microliters of extracted RNA were added to a mixture of 5 µL of LunaScript RT SuperMix (New England Biolabs, #E3010) and 5 µL of dH_2O in a final volume of 25 µL. Reverse transcription reactions were run in a Veriti™ Thermal Cycler (ThermoFisher Scientific) with an initial step at 25°C for 2 minutes, followed by a reverse transcription step at 55°C for 10 minutes and a final step at 95°C for 1 minute. The two cDNA aliquotes obtained from RNA were then combined to have a final volume of 50 µL and stored at 4 °C for subsequent amplification through dPCR reaction.

Digital PCR

- **DNA Shearing Index**

To measure DNA fragmentation during nucleic acid isolation, which may lead to underestimation of intact HIV-1 proviral genomes, we calculated a DNA shearing index (DSI) using a dPCR reference assay (Bruner et al., 2019) targeting two regions of the human RPP30 gene (RPP30_1 and RPP30_2) located approximately 11 Kb apart, comparable with the length of an intact HIV-1 proviral genome. After data acquisition and analysis performed as described in the dedicated paragraph, DSI was calculated as 1 - (Double Positive Partitions / Average of All Positive Partitions). The shearing fraction was assumed to reflect the degree of shearing affecting the ~10 Kb HIV-1 genome (Fig. 12).



The calculated DSI for each sample was then used to correct the number of HIV-1 double-positive droplets from the intact proviral assay of the same DNA sample as follows: Corrected Double Positive (IP) = Observed Double Positives (IP) / (1 – DSI).

Figure 12. The figure represents the DNA shearing index measured for RPP30 and HIV-1 on JLat DNA samples subjected to different levels of shearing (Bruner et al., 2019).

- **Quantification of Cell-Associated and Intact Proviral HIV-1 DNA**

Quantification of Cell-Associated HIV-1 DNA (CAD) and Intact Proviral HIV-1 DNA (IP) was performed using a multiplex dPCR assay adapted from Delporte et al., 2023 and from Bruner et al., 2019. The 5'-LTR region of the HIV-1 genome was targeted for CAD quantification, while the Ψ and env regions were used for IPDA (Intact Proviral DNA Assay). We optimized the original sets of primers and probes (Tab. 2) described by Bruner to increase coverage across a broader panel of HIV-1 subtypes. In particular, we introduced degenerate bases to improve recognition of additional non-B subtypes.

- **Quantification of Cell-Associated HIV-1 RNA**

To quantify cell-associated HIV-1 RNA (CAR), we employed a dPCR assay targeting the terminal region of the 5'-LTR, upstream of the D1 splice donor site. By positioning primers and probes in this conserved LTR region, the assay likely captures all HIV-1 transcripts, including the unspliced, partially spliced, and completely spliced (Fig. 13) (Pasternak et al., 2013). Indeed, all HIV-1 transcripts share the LTR sequence at their 5' ends, so that this LTR-targeting dPCR assay effectively measures total CAR, providing a global estimate of viral transcriptional activity rather than a subset of transcripts. Consistent with this, several studies support the use of LTR-based assays to capture total HIV-1 RNA in PBMCs including those on virologically suppressed ART-treated individuals (Petrara et al., 2024).

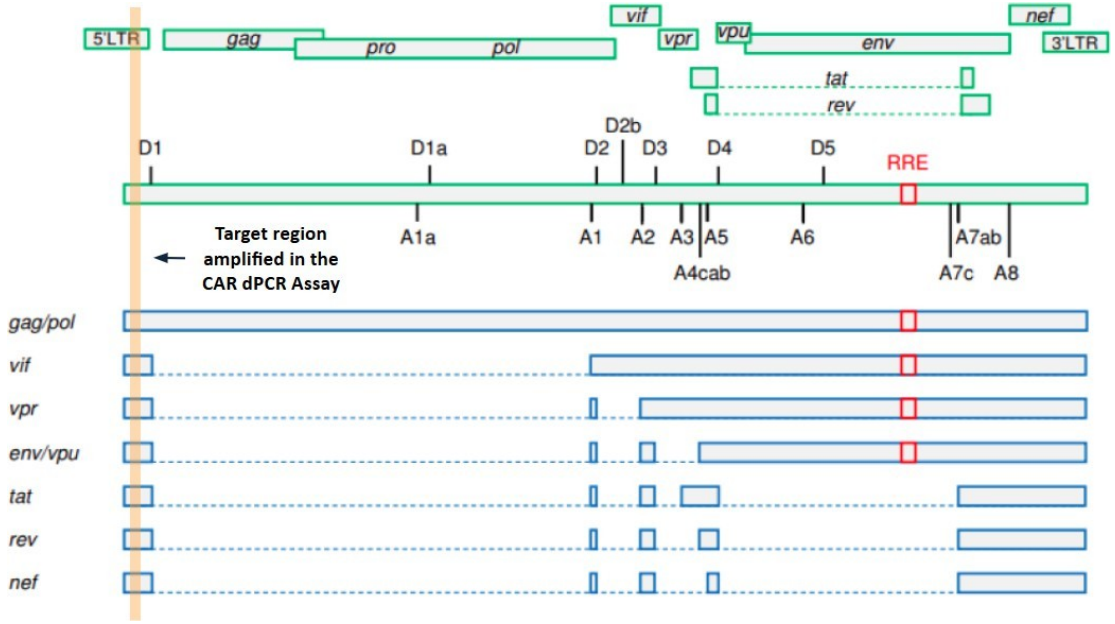


Figure 13. Representation of the target region amplified in the CAR dPCR assay and of HIV-1 splicing sites (Pasternak & Berkhout, 2021).

- **General dPCR workflow**

Digital PCR is a molecular technique that allows the absolute quantification of nucleic acids by partitioning a sample into thousands of individual reactions. Each partition undergoes PCR amplification, and the presence or absence of the target sequence is determined in each partition by the emission or lack of fluorescence, respectively (Fig. 14). The target amount is then estimated by Poisson analysis of the proportion of positive and negative partitions at any given sample input. This approach allows for precise quantification of target molecules without the need of standard curves, making dPCR particularly useful for detecting rare variants and measuring low-abundance targets in complex samples (Coccaro et al., 2020). DNA and cDNA concentrations were quantified using the Qubit dsDNA HS Assay (Thermo Fisher Scientific). A fraction of DNA was diluted to a final concentration of 0.5 ng/µL to be used in the DSI assay.

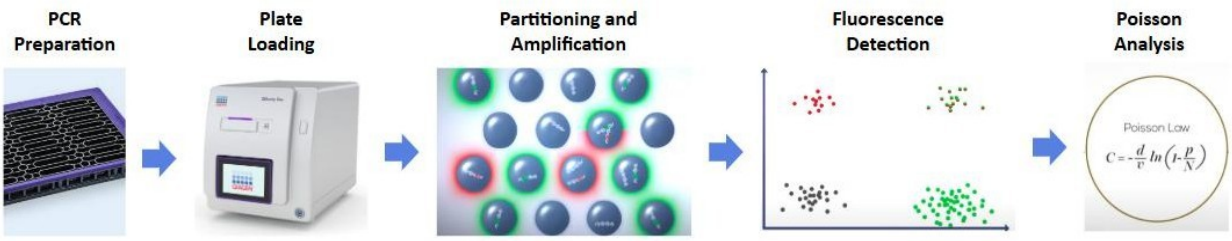


Figure 14. Schematic representation of the dPCR workflow.

dPCR reaction mixes were prepared using the QIAcuity Probe PCR Kit (Qiagen, #250103), according to the manufacturer's instructions and dispensed into a 96-well standard PCR plate. Appropriate volumes of DNA or cDNA were then added to each reaction. For the DSI assay, a maximum of 2 ng per replicate was loaded (2 replicates were performed for each sample), whereas for the CAD+IPDA assay and for the CAR assay, up to 600 ng of DNA or cDNA per replicate were used (a maximum of 10 and 5 replicates were performed for CAD/IPDA and CAR assays, respectively). Each reaction mix with a final volume of 12 µL was subsequently transferred into a QIAcuity Nanoplate 26k 24-well (Qiagen, #250001). Plates were sealed and placed into the QIAcuity reader (Qiagen) for amplification, following the cycling and acquisition conditions detailed in Tables 3 and 4.

Assay	Target	Primer	Sequence	Labelling
DSI	RPP30_1	Fwd	AGCCATCATACCTAGCCTAATTTG	
		Rev	CACCAATCATTCTCCTTCCTTCC	
		Probe	ACATCTCAGACACAA	5'-FAM 3'-MGBNFQ
	RPP30_2	Fwd	TCAGCATGGCGGTGTTTGCA	
		Rev	GCTGTCTCCACAAGTCCG	
		Probe	TTCTGACCTGAAGGCTCTGCGC	5'-HEX 3'-BHQ1
IPDA+CAD	Ψ	Fwd	CAGGACTCGGCTTGCTGARG	
		Rev	GCACCCATCTCTCTCCTTCTAGC	
		Probe	WTTGGCGTACTCACCAG	5'-FAM 3'-MGBNFQ
	ENV	Fwd	AGTGGTGSARAGAGAAAAAAGAGC	
		Rev	GTCTGGCCTGTACCGTCAGC	
		Probe	CCTTGGGTTCTTGGGA	5'-HEX 3'-MGBEQ

		Probe	CCTTAGGTTCTTAGGAGC	Unlabelled
	5’-LTR	Fwd	GCCTCAATAAAGCTTGCCTTGA	
		Rev	GGCGCCACTGCTAGAGATTTT	
		Probe	AAGTAGTGTGTGCCCGTCTG	5’-TEXAS RED 3’-MGBNFQ
CAR	5’-LTR	Fwd	GCCTCAATAAAGCTTGCCTTGA	
		Rev	GGCGCCACTGCTAGAGATTTT	
		Probe	AAGTAGTGTGTGCCCGTCTG	5’-FAM 3’-MGBNFQ

Table 2. Sequences of primers and probes used in the dPCR assays.

Imaging setting	
500 ms	green
500 ms	yellow
200 ms	red
400 ms	crimson

Table 3. Imaging acquisition parameters.

Thermocycler program	
95 °C 2 min	
95 °C 15 sec	40 cycles
56 °C 30 sec	

Table 4. Thermocycler program used for the dPCR assays.

- **Data Analysis**

Data acquisition and analysis were performed using the QIAcuity Software Suite (Qiagen). For assay validation, a minimum of 18,000 events per well was required. In each well, the presence

of both positive and negative events is essential, even at low frequency. For this reason, no more than 600 ng of DNA were loaded per well in the IPDA assay, to avoid saturation of all partitions, which would prevent the application of Poisson statistical analyses.

Preliminary inspection of droplet distribution was carried out in the 1D visualization mode, with the negative control used as a reference for setting the threshold between positive and negative droplets. The results were then analyzed in the 2D visualization mode (Fig. 15), where the threshold set in 1D was automatically transferred and, if necessary, manually adjusted. In this representation, droplet populations were classified as follows:

- **Q1** = Single-positive for Target 1 (yellow)
- **Q2** = Double-positive (blue)
- **Q3** = Double-negative (grey)
- **Q4** = Single-positive or Target 2 (light blue)

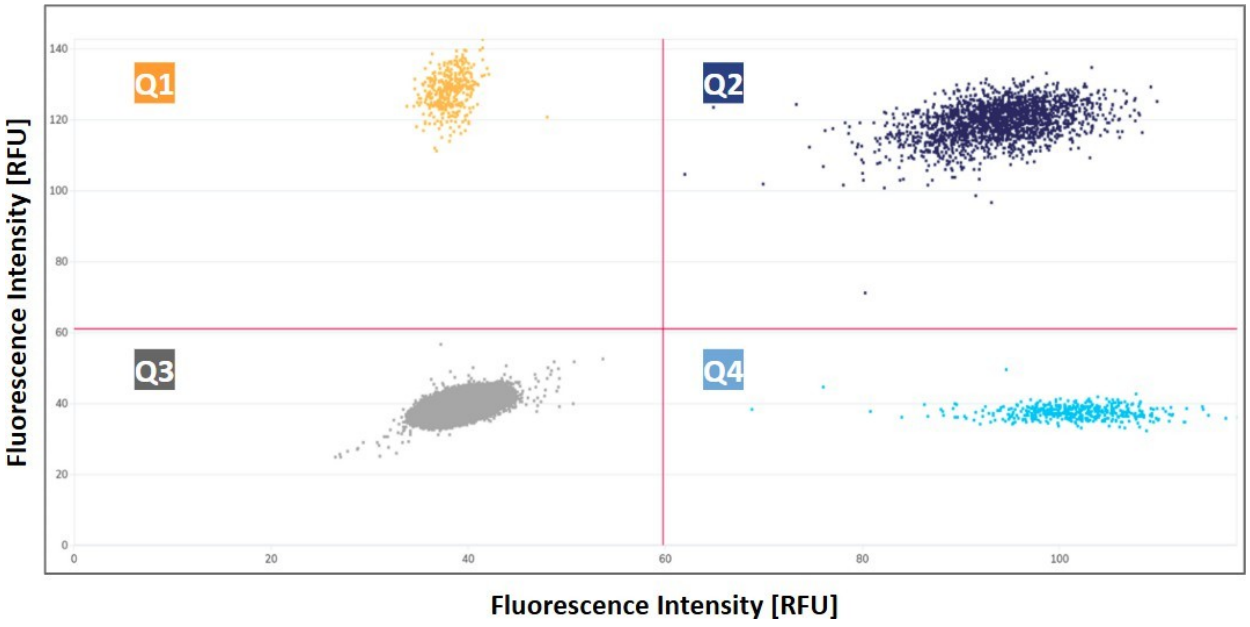


Figure 15. Example of a 2D plot obtained from a multiplex dPCR.

Data interpretation required well separated populations forming distinct clusters. In cases where only a single positive droplet (either single- or double-positive) was detected, the sample was not considered positive. Indeed, under such conditions, the assay was repeated with an increased number of replicates to obtain more robust results.

Once thresholds had been defined and all samples verified, the data were exported under the Multiple Occupancy File format and quantitative analyses were performed using values

expressed in copies/µL for each target. Final IPDA results were reported as the percentage of intact HIV-1 DNA relative to the total HIV-1 DNA, as determined from the Excel worksheet. Final CAD and CAR values were expressed as copies of HIV-1 per 10^6 CD4+ T cells.

Statistical Analysis

Statistical analyses investigated the impact of clinical variables on virological outcomes over time, among the two treatment groups (3-DR and 2-DR). CAD, CAR and IP data were generated at baseline (T0) and after 18 months (T1) and their changes over time were reported as T1-T0 values (ΔCAD, ΔCAR, ΔIP). All statistical analyses were performed using non-parametric methods, as most datasets showed non-normal distributions (Kolmogorov-Smirnov test).

- **Baseline Descriptive Statistics**

Descriptive analyses were performed both on the overall study population and for the two treatment groups, to assess baseline comparability for age, sex, Nadir CD4+ count, CD4+ count, Zenith VL, time under ART.

- **Comparative Analysis Intra- and Inter-Groups**

Group comparison was carried out using the Mann–Whitney U test to evaluate differences between 2-DR and 3-DR groups for each clinical and virological variable. CAD, CAR, and IP were compared to the overall population considering their Δ values, and between the two groups both at T0 and T1. In addition, Wilcoxon signed-rank test was performed to assess whether changes over time in the virological parameters were statistically significant within each treatment group.

- **Spearman's Rank Correlation Analyses**

To explore potential associations between clinical and virological parameters, correlation analyses were performed using Spearman's rank correlation test. Specifically, we investigated the relationships between Zenith VL, Nadir CD4+, T0 CD4+ count, and time under ART with CAD, CAR, and IP at T0.

- **Regression Models for Predictors of Virological Changes**

Linear regression analyses were conducted to identify predictors of changes in the three virological parameters over time. For each delta value (ΔCAD, ΔCAR, ΔIP), an initial univariate linear regression model including all baseline clinical parameters was performed. Variables showing a p-value < 0.1 in the univariate model were subsequently included in multivariate regression analyses. All statistical analyses were performed using SPSS version 30.0 (IBM Corp., Armonk, NY, USA). A two-tailed p-value < 0.05 was considered statistically significant.

RESULTS and DISCUSSION

Study Population

The study population included 67 PLWH on continuous suppressive 3-DR consisting of either 2 NRTIs + 1 INSTI (*n* = 50), 2 NRTIs + 1 NNRTI (*n* = 13), or 2 NRTIs + 1 PI (*n* = 4). All enrolled participants had maintained plasma HIV-1 RNA levels below 50 copies/mL for at least 12 months without any viral blip in the 6 months before enrollment.

All subjects tested negative for HBsAg and HCV, had $CD4^+$ counts greater than 200 cells/mm^3, no current or recent clinical AIDS-defining conditions, and no evidence of drug resistance according to pre-treatment resistance testing.

The majority of participants harbored HIV-1 subtype B (n = 42, 63%), while other subtypes detected included subtype A (n = 2), A1 (n = 1), C (n = 2), F1 (n = 2), G (n = 2) and various CRFs (n = 15). HIV-1 subtype was not available for 1 subject.

One group continued 3-DR (n = 32), the other group (n = 35) switched to a 2-DR (DTG+3TC, n = 19; DTG+RPV, n = 15; CAB+RPV, n = 1). The treatment regimens of the PLWH remaining on 3-DR included 2 NRTIs + 1 INSTI (*n* = 24), 2 NRTIs + 1 NNRTI (*n* = 6), or 2 NRTIs + 1 PI (*n* = 2).

The enrolled population reflects the composition of PLWH in Europe, with a predominance of subtype B (Abecasis et al., 2013) but also a relevant proportion of mixed non-B subtypes (37%), which underscores the importance of continuous monitoring of HIV diversity as mostly derived from migration patterns and globalization (Bbosa et al., 2019).

The high proportion of male participants (94%) also mirrors the demographic composition typical of most European HIV cohorts, largely made of men who have sex with men (Krings et al., 2025). The study population also represents the best-case scenario for ART simplification strategies, in light of prolonged virological suppression, stable immunological profile and lack of drug resistance. All these features indeed contribute to minimizing the risk of virological failure. The distribution of baseline 3-DRs, with predominance of INSTIs based regimens (75%), also aligns with current international guidelines that recommend INSTIs as anchor drugs thanks to their high genetic barrier and tolerability profile (https://clinicalinfo.hiv.gov/en/guidelines, last reviewed September 2024; EACS Guidelines version 12.1, November 2024). Moreover, the transition to DTG-based 2-DRs (97% of the 2-DR group) is in line with the current clinical practice in treatment simplification.

Baseline Demographic and Clinical Characteristics

At baseline, participants in the 2-DR and 3-DR groups were comparable in most demographic and clinical parameters (Tab. 3). No significant differences were observed between groups in terms of sex, $CD4^+$ counts, zenith VL ($p = 0.210$), or time under ART ($p = 0.703$).

PARAMETERS Median (IQR)	**Overall Population**	**3-DR Group**	**2-DR Group**	***p*-value**
Age years	45 (12)	48 (14)	43 (11)	**0.008**
Male %	94	97	91	0.351
Nadir $CD4^+$ cells/µL	368 (280)	285 (369)	445 (275)	**0.007**
T0 $CD4^+$ cells/µL	861 (399)	866 (421)	856 (344)	0.851
Zenith VL log cp/mL	5 (1)	5 (1)	5 (1)	0.210
Timer under ART months	75 (62)	82 (44)	66 (75)	0.703

Table 3 Baseline demographic and clinical characteristics of the study population.

Conversely, the median age was significantly lower in the 2-DR group compared with the 3-DR group, and the nadir $CD4^+$ count was significantly higher in the 2-DR group compared with the 3-DR group, possibly reflecting a longer history of HIV infection and the associated exposure to less effective treatments.

The lower median age in the 2-DR group may also reflect a trend where younger patients are more frequently considered for novel therapeutic strategies, as they show better adherence and greater treatment satisfaction (Lombardi et al., 2020). Lower nadir $CD4^+$ counts may have advised against switching to 2-DR (Dueñas-Gutiérrez et al., 2023), as nadir $CD4^+$ count has an impact on long-term immunological outcomes, reservoir size, and risk of AIDS-related and non-AIDS-related morbidity, even in patients with sustained virological suppression (Martínez-Sanz et al., 2023).

By contrast, patients with higher nadir $CD4^+$ counts typically represent cases with earlier diagnosis and treatment initiation which may be associated with better immune function and lower viral reservoir (Barré-Sinoussi, 1988). Nevertheless, similar $CD4^+$ cell counts and time

under ART at T0 indicate that both groups achieved sustained immune reconstitution and virological control before enrollment. This, together with comparable zenith VL and duration of ART, makes the two groups suitable for the analysis of the role of the surrogate markers of the virus reservoir in the context of treatment simplification.

Virological Parameters at Baseline

At baseline, there were no statistically significant differences in the surrogate markers of HIV-1 reservoir between treatment groups (Tab. 4). However, CAD and CAR showed borderline trends toward higher values in the 3-DR group, again possibly reflecting longer duration of infection and hence delayed start of ART.

	Baseline (T0)				**18 months follow-up (T1)**		
PARAMETERS Median (IQR)	**Overall**	**3-DR Group**	**2-DR Group**	***p*-value**	**3-DR Group**	**2-DR Group**	***p*-value**
CAD Log cp/10^6 $CD4^+$	2.7 (1.2)	3.0 (0.9)	2.6 (1.1)	0.069	3.0 (1.0)	2.9 (1.1)	0.875
CAR Log cp/10^6 $CD4^+$	3.5 (1.3)	3.9 (1.3)	3.7 (1.0)	0.056	3.7 (0.9)	3.7 (1.9)	0.980
IP %	4.0 (14.0)	3.5 (11.0)	8.1 (19.5)	0.193	5.0 (8.5)	0.0 (12.0)	0.539

Table 4. Virological parameters at baseline (T0) and after 18 months (T1). Note: 11 samples (2 and 9 samples in the 3-DR and 2-DR group, respectively) failed to be amplified in IPDA for one (env n = 7; ψ n = 1) or for both (n = 3) targets at T0 or T1.

The median CAD levels observed in the overall population are consistent with previously reported values in virologically suppressed patients on long-term ART, typically ranging from 2.0 to 3.5 log copies/10^6 $CD4^+$ cells (Lombardi et al., 2020). Similarly, the CAR values (3.5 log copies/10^6 $CD4^+$ overall) align with expected levels of residual transcriptional activity in patients with sustained viral suppression, reflecting low-level ongoing viral transcription despite effective suppression of viral replication. Percentage IP values showed considerable inter-patient variability in both groups, as shown by the wide interquartile ranges, however median values were within the expected range for PLWH on long-term suppressive ART, where intact proviruses typically represent only a small fraction (2-10%) of total proviral DNA

(Nühn et al., 2025). The lack of significant difference in IP between groups at baseline suggests comparable reservoir composition despite the differences in CAD. This may support a differential role for IP as compared with CAD, which is relatively easier to assess but represents an ensemble of different HIV-1 DNA forms.

Correlation analysis among the three surrogate markers of the HIV reservoir in the whole study population detected a strong positive correlation between CAR and CAD at T0 ($\rho = 0.653$, $p < 0.001$; Fig. 17A). This is not surprising since larger reservoirs induce higher residual transcription, as already been observed across multiple cohorts (Gärtner et al., 2024).

On the other hand, IP was not correlated to either CAD or CAR, again in line with a possible differential meaning of this subset of HIV-1 DNA (Fig. 17B and 17C). This independence may reflect the complex interplay between proviral integration sites, clonal expansion of cells harboring intact versus defective proviruses, and stochastic factors affecting reservoir composition.

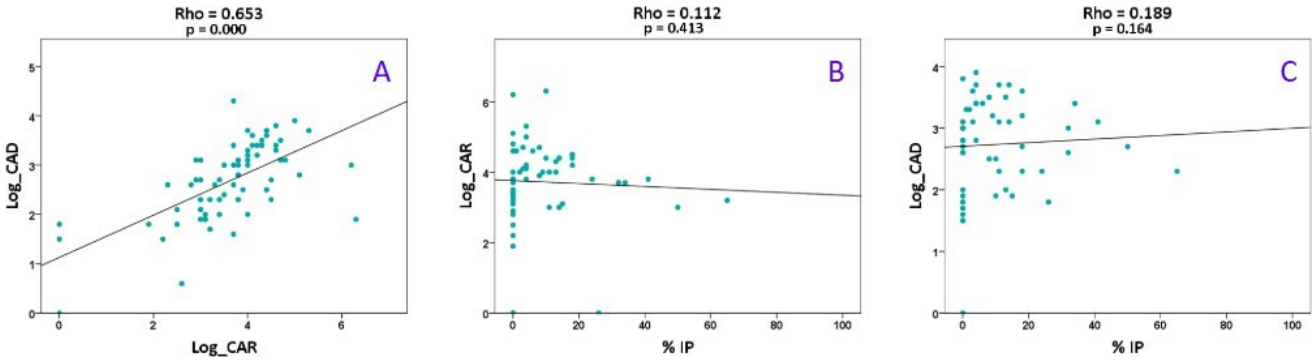


Figure 17. Scatter plots showing the monotonic association between the three virological parameters CAD, CAR and IP. Each plot reports its Spearman's ρ and *p*-values.

Correlation between Virological and Clinical Parameters at Baseline

Analysis of virological and clinical data in the whole study population at baseline detected a positive association between CAR and age (Fig. 18A) and a negative association between nadir $CD4^+$ cell counts and both CAR and CAD (Fig. 18B and 19B, respectively) emphasizing the long-term impact of a history of immunological decline on the HIV-1 reservoir even with durable suppressive ART (Bachmann et al., 2019).

While our study population was middle-aged adults, older age may play a role in favoring residual HIV-1 transcription due to progressive immune senescence, chronic inflammation, and altered T cell homeostasis (Pathai et al., 2014). No significant correlations were observed between IP and any of the clinical variables examined (Fig. 20). This is in line with IP behaving independently of the other virological and clinical markers.

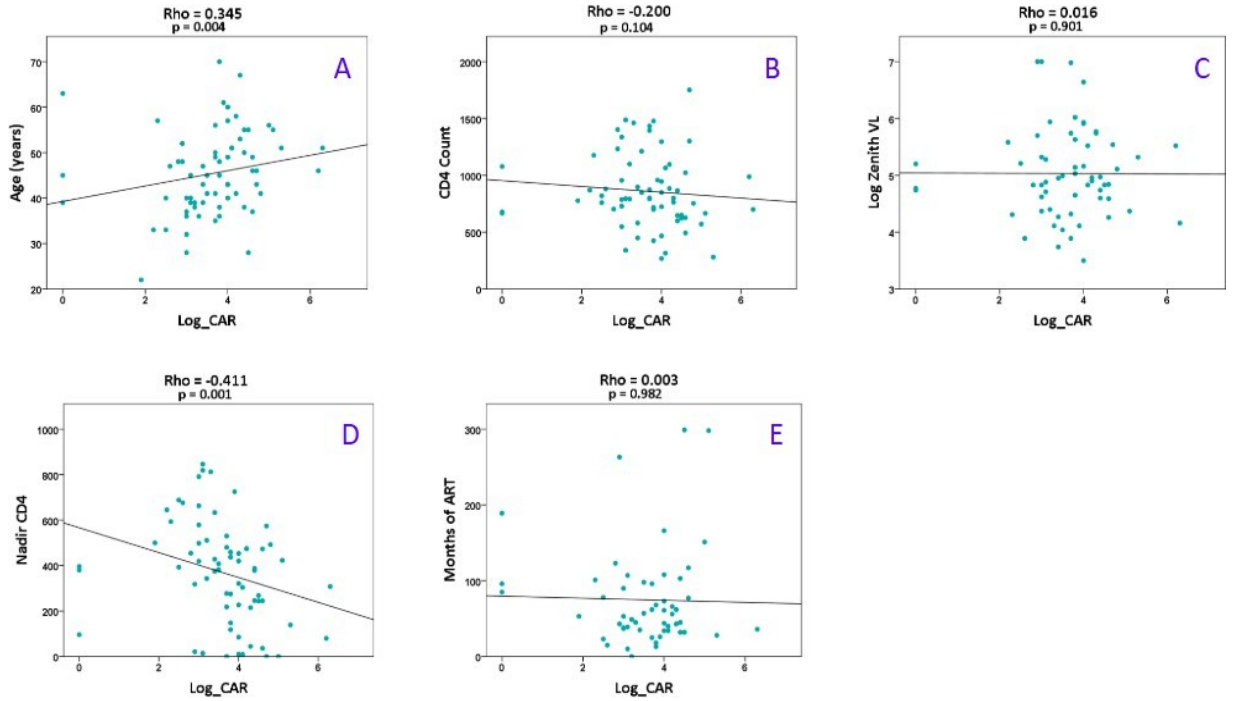


Figure 18. Scatter plots showing the monotonic association between CAR at T0 (X axis) and the various clinical parameters (Y axis). Each plot reports its Spearman's ρ and *p*-values.

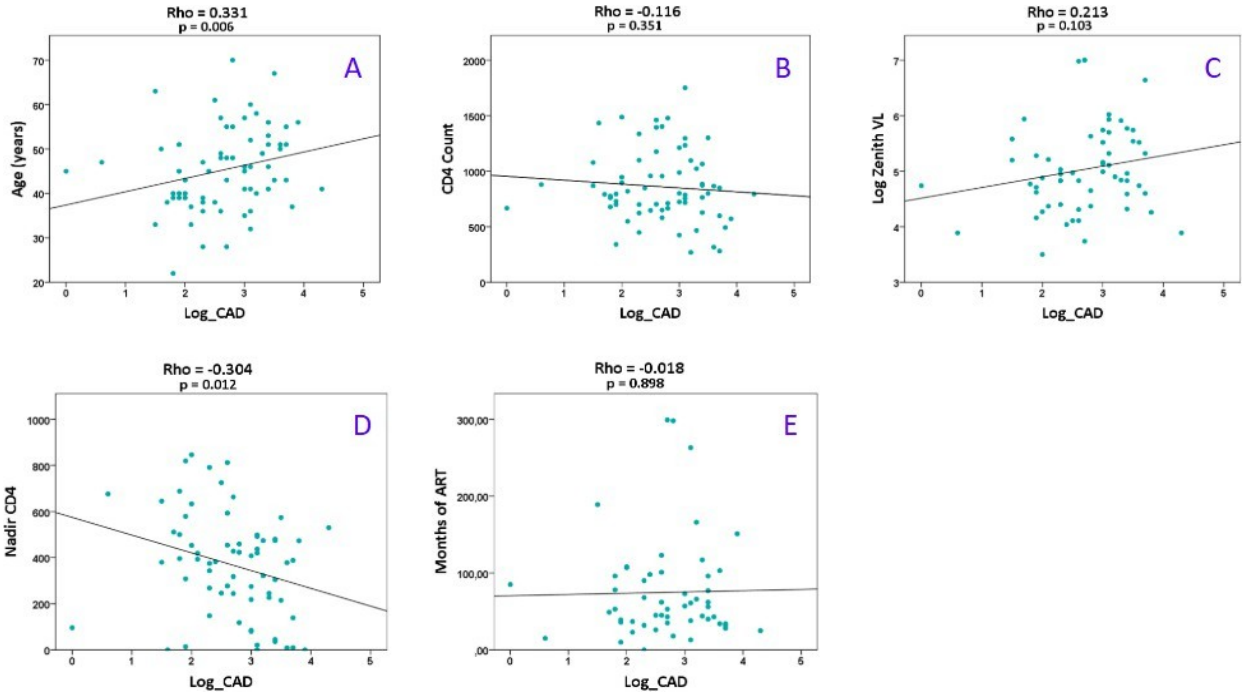


Figure 19. Scatter plots showing the monotonic association between CAD at T0 (X axis) and the various clinical parameters (Y axis). Each plot reports its Spearman's ρ and p-values.

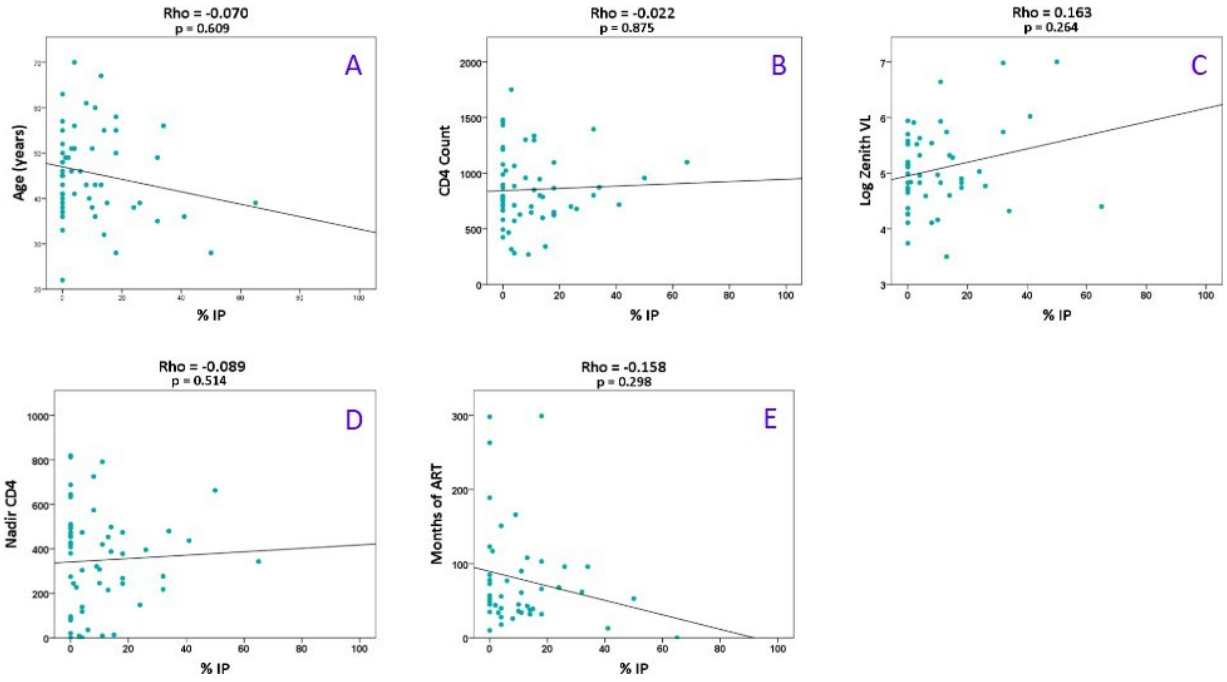


Figure 20. Scatter plots showing the monotonic association between IP at T0 (X axis) and the various clinical parameters (Y axis). Each plot reports its Spearman's ρ and *p*-values.

Changes of virological parameters over time

We next analyzed changes over time in CAD, CAR, and IP within each treatment group (Fig. 16). The overall analysis did not show major changes in the surrogate markers over the 18-month follow-up. However, some changes reached statistical significance, including increased CAR in the 3-DR group and increased CAD associated with decreased IP in the 2-DR group.

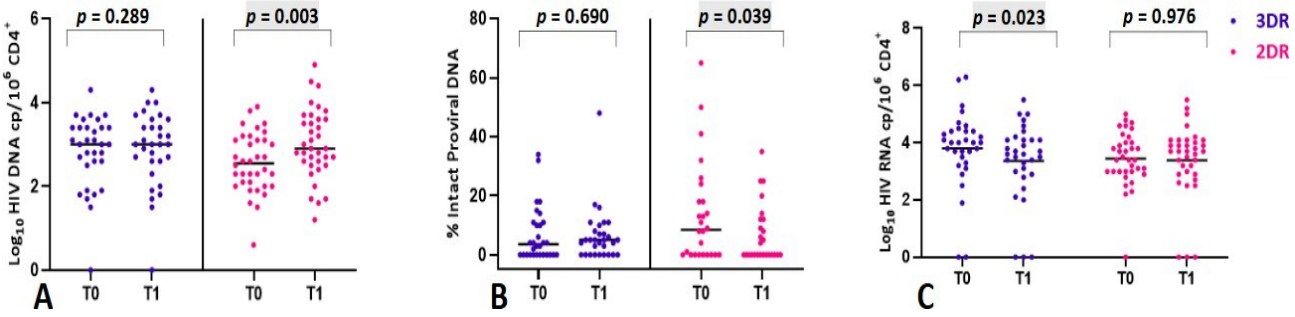


Figure 16. CAD (A), IP (B) and CAR (C) values at T0 and T1 in the 3-DR and 2-DR groups.

When comparing the extent of variation of the three virological markers (ΔCAD, ΔCAR, ΔIP) between the two groups, IP was confirmed to change more in the 2-DR with respect to the 3-DR group ($p = 0.035$) while ΔCAD and ΔCAR were comparable in the two groups.

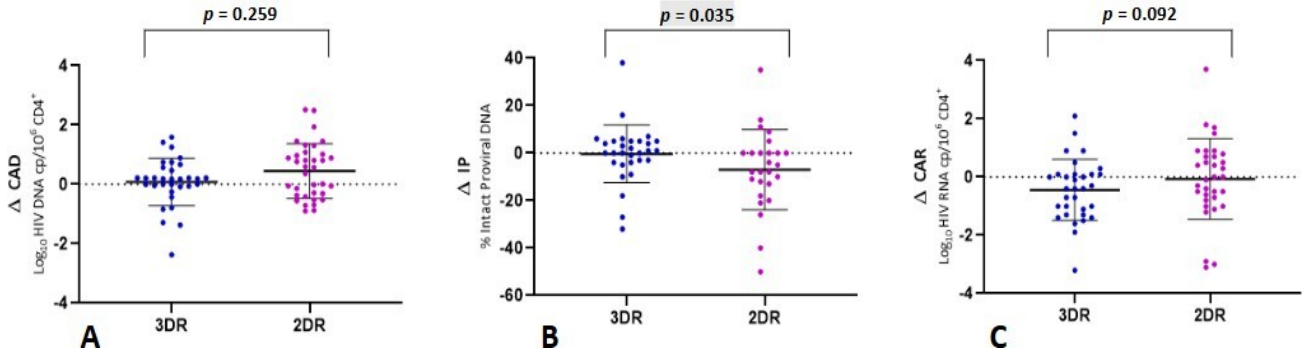


Figure 17. Comparison of 2DR and 3DR for ΔCAD (A), ΔIP (B) and ΔCAR (C), defined as T1 - T0 values.

The decrease in the proportion of IP together with an increase in CAD in the context of continuous suppression of viral replication, as observed in the 2-DR group, points to some mechanism favoring maintenance or expansion of nonfunctional HIV-1 genomes most likely through homeostatic proliferation and/or selective loss of IP. This phenomenon has been documented with a biphasic pattern whereby IP declines for the first decade and then tends to stabilize (Nühn et al., 2025). It may be possible that the longer duration of treatment in our 3-DR group was closer to the end of the first phase IP decay which continued to occur instead in the 2-DR group during the study follow-up. Lower decline in IP has been also associated with lower pre-ART $CD4^+$ cell counts (Nühn et al., 2025), indeed a condition characterizing our 3-DR group.

Predictors of surrogate markers changes of the HIV reservoir over time

Linear regression analyses were conducted to identify predictors of changes in virological parameters over time.

- **Baseline factors associated with ΔCAD**

At univariable analyses, the only factors with a *p* value below the threshold for multivariable testing were the treatment group and the baseline CAD level. In the multivariable model, only CAD level remained a significant positive predictor of ΔCAD (Tab. 5). It is interesting to note that the direction of the effect of baseline CAD changed from negative to positive when entering the variables into the multivariable model, implying a correction effect by treatment

group. The final association between higher CAD at T0 and higher ΔCAD may suggest persistence over time, consistent with a stabilization (plateau) effect. This appears to be in contrast with the long-term dynamics of CAD under prolonged successful treatment, as usually reported as a slow continuous decay down to plateau levels (Bachmann et al., 2019). However, the reversal of the direction of the baseline CAD effect in multivariable analysis when adjusting for treatment groups suggests complex interactions between baseline reservoir size and treatment that need further investigation with larger sample sizes. It must also be considered that the effect of baseline CAD, in either direction, can be partially explained with the statistical phenomenon of "regression to the mean" where individuals with extreme baseline values tend to show more evident changes toward the population average over time (Cummings, 2009).

	UNIVARIABLE			**MULTIVARIABLE**		
FACTOR (for ΔCAD)	**B Coefficient**	**95 % CI**	***p-value***	**B Coefficient**	**95 % CI**	***p-value***
Age per years	-0.010	-0.033; 0.013	0.401			
Zenith VL log copies/mL	-0.150	-0.453; -0.152	0.324			
Treatment Group 3-DR vs. 2-DR	0.364	-0.060; 0.787	**0.091**	0.218	-0.170; 0.606	0.265
Time under ART per month	0.001	-0.003; -0.005	0.514			
Nadir CD4$^+$ increase	0.001	0.000; 0.001	0.260			
T0 CD4$^+$ increase	0.000	0.000; 0.001	0.145			
T0 CAD log copies/10^6 CD4$^+$	-0.524	-0.768; -0.281	**<0.001**	0.499	-0.746; -0.251	**<0.001**
T0 CAR log copies/10^6 CD4$^+$	-0.123	-0.308; 0.063	0.191			
T0 IP %	0.002	-0.012; 0.017	0.767			

Table 5. Univariable and multivariable linear regression of clinical and virological factors associated with ΔCAD. B coefficients, 95% confidence intervals (CI), and *p*-values are shown.

- **Baseline factors associated with ΔCAR**

Similar to ΔCAD, the only variable showing a significant association with ΔCAR at univariable analysis was baseline CAR (Tab. 6). The negative B coefficient indicates that higher CAR levels are more prone to decrease over time (Tab. 6) and may also be explained as a regression to the mean effect. Since no other factor did reach the threshold for multivariable testing, specific effects of treatment were not detected, differently from ΔCAD.

	UNIVARIABLE			MULTIVARIABLE		
FACTOR (for ΔCAR)	**B Coefficient**	**95 % CI**	***p-value***	**B Coefficient**	**95 % CI**	***p-value***
Age per years	-0.019	-0.051; 0.013	0.244			
Zenith VL log copies/mL	0.000	0.000; 0.000	0.130			
Treatment Group 3-DR vs. 2-DR	0.386	-0.205; 0.978	0.197			
Time under ART per month	0.000	-0.005; 0.006	0.926			
Nadir $CD4^+$ increase	0.000	-0.002; 0.001	0.758			
T0 $CD4^+$ increase	0.000	-0.001; 0.001	0.484			
T0 CAD log cp/10^6 $CD4^+$	-0.172	-0.552; 0.207	0.368			
T0 CAR log cp/10^6 $CD4^+$	-0.418	-0.657; -0.179	**0.001**			
T0 IP %	0.010	-0.011; 0.032	0.331			

Table 6. Univariable and multivariable linear regression of clinical and virological factors associated with ΔCAR. B coefficients, 95% confidence intervals (CI), and *p*-values are shown.

- **Baseline factors associated with ΔIP**

In the univariable model, zenith VL, 3-DR vs. 2-DR and baseline IP were negative predictors of ΔIP while baseline CAR was a positive predictor of ΔIP (Tab. 7). At multivariable analysis,

baseline IP and CAR remained significant and maintained the direction of the effect seen at univariable screening. Although a regression to the mean effect may again explain the association between baseline IP and ΔIP, a higher propensity for higher baseline values to decrease over time is in agreement with the expected kinetics of IP, where decreased levels of intact genomes appears to be favored over decrease of the large majority of non-functional genomes (Peluso et al., 2020). Also, the association between higher baseline CAR levels and increasing IP over time points to a role of residual transcription, rather than total and mostly silent HIV genomes, on refueling of the replication competent reservoir. The association between CAR and IP may then be further favored by selective proliferation of transcriptionally active cells harboring IP through homeostatic expansion or antigen- driven responses. Indeed, recent evidence showed that transcriptionally active, clonally expanded cells can harbor replication-competent proviruses, supporting this hypothesis (Imamichi et al., 2025).

	UNIVARIABLE			**MULTIVARIABLE**		
FACTOR (for ΔIP)	**B Coefficient**	**95 % CI**	***p-value***	**B Coefficient**	**95 % CI**	***p-value***
Age per years	0.169	-0.240; 0.578	0.411			
Zenith VL log copies/mL	-6.516	-12.353; -0.679	**0.029**	-2.816	-6.523; 0.892	0.133
Treatment Group 3-DR vs. 2-DR	-6.656	-14.465; 1.152	**0.093**	-1.293	-6.868; 4.282	0.643
Time under ART per month	0.008	-0.056; 0.072	0.797			
Nadir CD4⁺ increase	-0.003	-0.210; 0.015	0.746			
T0 CD4⁺ increase	-0.003	-0.016; 0.009	0.607			
T0 CAD log cp/10^6 $CD4^+$	-0.609	-5.932; 4.714	0.819			
T0 CAR Log cp/10^6 $CD4^+$	2.916	-0.293; 6.124	**0.074**	2.821	0.691; 4.951	**0.011**
T0 IP %	-0.844	-1.024; -0.663	**<0.001**	-0.792	-0.989; -0.595	**<0.001**

Table 7. Univariable and multivariable linear regression of clinical and virological factors associated with ΔIP. B coefficients, 95% confidence intervals (CI), and *p*-values are shown.

CONCLUSIONS

The study presented in this thesis provides reassuring evidence that ART simplification from 3-DRs to 2- DRs in PLWH with sustained virological suppression does not have an impact on the viral reservoir, as measured by the three key surrogate markers including CAD, CAR and IP. Indeed, treatment group did not independently predict any of the virological delta values in multivariable models.

Regression analysis indicated that baseline reservoir characteristics, particularly the initial size and transcriptional activity, are the primary determinants of longitudinal reservoir dynamics during continued suppressive ART and independently from the number of drugs in the successful regimen. The lack of associations with immunological parameters or treatment regimen suggests that once sustained suppression is achieved, reservoir trajectories become relatively independent of these factors, at least over the 18-month observation period studied.

On the other hand, correlation analyses at baseline revealed that immunological history, particularly nadir $CD4^+$ count, significantly influences the size of the reservoir, as measured by CAD levels. As older participants tended to exhibit higher CAR levels, age-related immune senescence and/or chronic inflammation may combine with virus induced immunological impairment to increase the HIV-1 reservoir. This in turn underscores the importance of early ART initiation and prevention of severe immunological impairment to reduce the size of the reservoir.

Since it is based on a relatively low number of subjects, this study must be interpreted as a preliminary assessment of the fate of the viral reservoir following switching from 3-DR to 2-DR. Also, some of the initial treatment regimens included drugs which are rarely included in modern regimens. Nevertheless, the contextual measurement of three key surrogate markers is valuable as the very few studies reported in a similar setting also have limitations (De Scheerder et al., 2025; Dragoni et al., 2022).

Notably, additional data are awaited from the same subjects in the framework of a multidisciplinary study, including markers of the HIV-1 reservoir as measured in gut biopsies and immunoactivation markers measured in peripheral blood. This data will complement the results presented here and help better define the changes, if any, resulting from switching to 2-DR. Providing reassurance on 2-DR strategies, not only in terms of virological suppression as

defined by undetectable plasma HIV-1 RNA but also in terms of lack of detrimental effects on the viral reservoir, is important to consolidate the concept that canonical 3-DR may be not necessary any more in a relevant proportion of PLWH under sustained virological suppression. 2-DR can indeed convey several advantages, including increased tolerability, better convenience (e.g. long-acting 2-DR), reduced toxicity and cost, and lower risk of drug-drug interactions in subjects with comorbidities.

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ABBREVIATIONS

HML	HIV Monitoring Laboratory
HIV	Human Immunodeficiency Virus
DENV	Dengue Virus
WNV	West Nile Virus
ZIKV	Zika Virus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
PROTAC	Proteolysis Targeting Chimera
ART	Antiretroviral therapy
MARISA	Multidisciplinary Assessment of the blood and gut-associated HIV Reservoir and Immunity following Switch from 3-drug to 2-drug Antiretroviral regimens in virologically suppressed patients
AIDS	Acquired Immunodeficiency Syndrome
PLWH	People living with HIV
CRFs	Circulating recombinant forms
LTR	Long terminal repeats
PR	Protease
IN	Integrase
ORFs	Open reading frames
CCR5	Chemokine receptor type 5
CXCR4	C-X-C motif chemokine receptor type 4
NRTIs	Nucleoside/nucleotide RT inhibitors
NNRTIs	Non-nucleoside RT inhibitor
PIs	Protease inhibitor
INSTIs	Integrase strand transfer inhibitors

2-DRs	Two-drug regimens
3-DRs	Three-drug regimens
PrEP	Pre-exposure prophylaxis
LRAs	Latency reversal agents
MAPK	Mitogen-activated protein kinase
PKC	Protein kinase C
SMAC	Second mitochondria derived activator of caspases
bNAbs	Broadly neutralizing antibodies
VOA	Viral outgrowth assay
TZA	TZM-bl cell-based assay
CAR	Cell associated HIV-1 RNA
TILDA	tat/rev induced limiting dilution assay
IPDA	Intact Proviral DNA Assay
GALT	Gut-associated lymphoid tissue
PBMCs	Peripheral Blood Mononuclear Cells
DSI	DNA Shearing Index
CAD	Cell-Associated HIV-1 DNA
CAR	Cell-Associated HIV-1 RNA
IP	Intact Provirus
dPCR	digital PCR
IQR	Interquartile Range
VL	Viral Load
CI	Confidence Interval

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