UNIVERSITA' VITA-SALUTE SAN RAFFAELE

**CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE
IN MEDICINA MOLECOLARE**

Curriculum in Experimental and Clinical Medicine

Evaluation of HIV reservoir in specific populations: people with a multi-drug resistant virus or a viral coinfection

DoS: Professor Antonella Castagna

Second Supervisor: Professor Jintanat Ananworanich

Tesi di DOTTORATO di RICERCA di Camilla Muccini

matr. 015513

Ciclo di dottorato XXXV°

SSD MED/17

Anno Accademico 2021/2022

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

La sottoscritta/*I* Camilla Muccini

Matricola / *registration number* 015513

nata a/ *born at* Cattolica (RN)

il/*on* 14/06/1990

autore della tesi di Dottorato di ricerca dal titolo / *author of the PhD Thesis titled*

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ACKNOWLEDGEMENTS

The development of this study have been possible thanks to the expertise of Dr. Daniela Cesana and the team of the safety of gene therapy and insertional mutagenesis Unit (San Raffaele Telethon Institute for Gene Therapy), in particular Pierangela Gallina and Laura Rudilosso.

I would also thank Dr Laura Passerini (Mechanisms of peripheral tolerance Unit, San Raffaele Telethon Institute for Gene Therapy) for her help in setting up the cellular assay.

«μὴ μὰν ἀσπουδί γε καὶ ἀκλειῶς ἀπολοίμην,

ἀλλὰ μέγα ῥέξας τι καὶ ἐσσομένοισι πυθέσθαι.»

«Ma non fia per questo

che da codardo io cada: periremo,

ma gloriosi, e alle future genti

qualche bel fatto porterà il mio nome.»

(Omero, Iliade)

ABSTRACT

Aim of the study was to characterize intact and defective proviruses as a measure to quantify HIV reservoir in specific populations on virological suppression under ART: people with a multi-drug resistance (MDR) virus enrolled in the PRESTIGIO registry, who hardly achieve and maintain viremia below detection limit, and people with viral coinfections (HCV, EBV and HPV) with or without virus-driven malignancies. In people with coinfections, we also wanted to identify antigen-specific CD4 T cells containing replication-competent proviruses.

Firstly, we evaluated 35 people living with HIV (PLWH): 14 (13 M; 1 F) with a MDR virus and 21 (19 M; 2 F) without any drug resistance mutations. Median age was 57 (54-62) and 48 (40-52) years, respectively (p=0.008). No differences emerged in the quantification of both HIV reservoir and defective proviruses: intact HIV DNA was 11.9 (0-46.5) and 16.7 (0-127.0) copies/10^6 CD4 T cells among people with and without MDR infection, while 5' deleted proviruses were 167.6 (63.2-796.6) and 202.9 (86.9-410.7) copies/10^6 CD4 T cells and 3' deleted proviruses 278.1 (67.0-615.1) and 419.5 (27.4-674.4) copies/10^6 CD4 T cells, respectively.

We also enrolled 17 PLWH with coinfections (8 with HCV, 4 with EBV-driven lymphoma and 5 with HPV-driven cancers), and 11 PLWH without any coinfections and virus-driven malignancy. Median age was 48.8 (40.7-52.0) in PLWH with coinfections and 43.3 (40.6-50.4) in PLWH without coinfections (p=0.288), 93% were male. Median value of intact HIV DNA was 62.1 (14.5-138.3) and 43.7 (7.5-134.8) copies/10^6 CD4 T cells among people with and without a viral coinfections/virus-driven malignancy, while 5' deleted proviruses were 243.9 (86.9-652.0) and 257.9 (116.3-401.2) copies/10^6 CD4 T cells and 3' deleted proviruses 299.4 (95.5-535.9) and 521.4 (20.6-677.8) copies/10^6 CD4 T cells, respectively.

To assess the presence of HIV quasi-species from HCV-, EBV-, HPV- specific CD4 T cells, we tested different culture conditions to evaluate T cells response in vitro in PLWH with a coinfection and then for the detection and expansion of viral-specific CD4 T cells. Even though analyses are still ongoing, we can observe that there is no difference in the quantification of intact proviruses between study populations and control groups. Further steps will estimate the frequency of antigen-specific T cell clones harboring a replication-competent virus and will detect HIV-specific clones in tumor tissue.

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ACRONYMS AND ABBREVIATIONS

ABC	Abacavir
AHI	Acute HIV Infection
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
BIC	Bictegravir
cART	Combined Antiretroviral Therapy
CMV	Cytomegalovirus
CNS	Central Nervous System
DAA	Direct-Acting Antiviral Agents
DNA	Deoxyribonucleic Acid
DRV/c	Darunavir/cobicistat
DTG	Dolutegravir
EBV	Epstein Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
EVASION	Evaluation of the VirAl reServoir in people living with HIV-1 Infection and virus-driven malignancies Or viral coiNfections
EVG/c	Elvitegravir/cobicistat
FDC	Follicular Dendritic Cells
FTC	Emtricitabine
FFPE	Frozen and Formalin-Fixed Paraffin-Embedded
GALT	Gut-Associated Lymphoid Tissue
GI	Gastrointestinal
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
IN	Integrase
ID	Patient Identifier
IFNg	Interferon Gamma
InSTI	Integrase Strand Transfer Inhibitor
IPDA	Intact Proviral DNA Assay
IS	Integration Site
ISH	In Situ Hybridization
LTR	Long Terminal Repeat
MDR	Multi-Drug Resistant
MSD	Major Splice Donor
nFGS	Near Full Genome Sequencing
PHA	Phytohaemagglutinin
PLWH	People Living With HIV
PR	Protease
qPCR	Quantitative Polymerase Chain Reaction
QVOA	Quantitative Viral Outgrowth Assay
RAL	Raltegravir

RM	Rhesus Macaque
RNA	Ribonucleic Acid
RPV	Rilpivirine
RT	Reverse Transcriptase
SIV	Simian Immunodeficiency Virus
TAF	Tenofovir alafenamide
Tcm	Central Memory T Cell
TCR	T Cell Receptor
TDF	Tenofovir Disproxil Fumarate
Teff	Effector T Cell
Tem	Effector Memory T Cell
Tfh	Follicular T Helper Cell
Treg	Regulatory T Cell
Tscm	Stem Central Memory T Cell
3TC	Lamivudine

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1. INTRODUCTION

1.1. HIV INFECTION

The acquired immunodeficiency syndrome (AIDS) is a sever condition secondary to Human Immunodeficiency Virus (HIV) infection. The epidemic beginning dates back to 1981, when the first case of AIDS was described in a homosexual man based in Los Angeles. The virus responsible for this illness was isolated two years later (1,2).

1.2. HIV EPIDEMIOLOGY

Overall, 84 million people have received a diagnosis of HIV infection and about 40 million people since 1981. Globally, people living with HIV (PLWH) were 38.4 million at the end of 2021 (3). Even though prevalence and incidence are significantly different depending on countries, adults living with HIV represent 0.7% of global population (3).

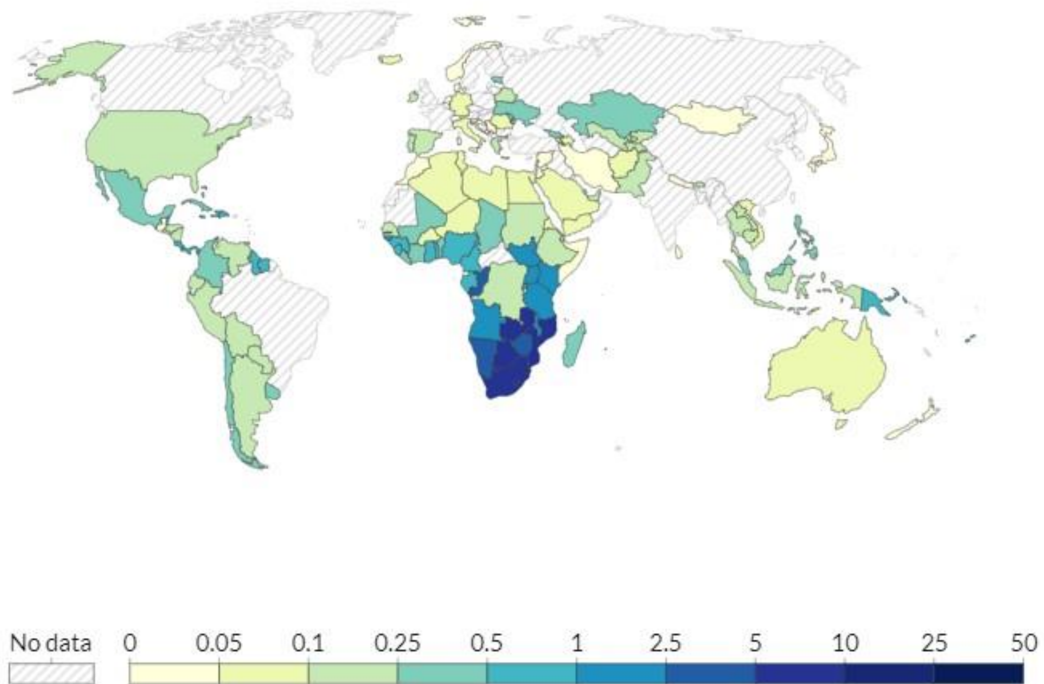


Figure 1. Incidence of HIV per 1.000 uninfected adults, 2020 (UNAIDS) (4)

In 2021, PLWH in Italy were about 140.000; during this year, newly infected people with HIV were 1.200 (90%). Furthermore, deaths both in adults and children due to AIDS were less than 500 (5).

Overtime, the incidence of HIV has declined, mostly by promoting treatment coverage and HIV testing: in fact, it has been described a two-fold global reduction in HIV incidence in the last decade (2000-2018). However, ending the HIV/AIDS epidemic by 2030 is currently a target too hard to achieve. Intervention strategies should be more extensively promoted among key populations to limit HIV transmission in the hard-to-reach settings (6).

Overall, of all PLWH, 85% received HIV diagnosis, 75% were taking antiretroviral drugs and 68% had undetectable viremia in 2021. These data highlight that the obstacles in HIV worldwide eradication are the poor accessibility to HIV testing services and ART coverage. Under this perspective, WHO launched its "treat all" consolidated guidelines in July 2016, which abolished all limitations on eligibility for therapy among PLWH, such as pregnant women, co-infected patients and children (7).

Worldwide, there are two types of HIV, which are distinguished according to the structure of the genome and phylogenetic characteristics into HIV-1 and HIV-2. The most diffused is the HIV-1, while HIV-2 infection accounts for only 5% concentrating mostly in West Africa. Interestingly, HIV-2 infection usually have a more indolent disease progression than HIV-1, but it is intrinsically resistant to certain antiretroviral agents (8). Moreover, 3 distinct virus groups belong to HIV-1 based on env gene sequences: M, that is the predominant group and includes 10 subtypes, N, O and P. (9).

At present, HIV cannot be cured. In fact, antiretroviral drugs can only limit viral replication, suppressing the number of circulating copies of the virus and preventing its transmission. With the current strategies, people with HIV can have healthy and long lives. Starting from 1997, combinations of at least three antiretroviral contributed to decrease HIV RNA levels below the limit of detection (10) and reduced morbidity. The introduction of a combined antiretroviral therapy (cART) transformed a fatal illness into a chronic disease, reducing mortality and extending life expectancy by preventing AIDS and non-AIDS related illnesses (11).

1.3. Course of HIV disease

A severe immunodeficiency caused mainly by the loss of T helper cells in a setting of chronic immune activation is the defining feature of HIV illness. The expression of CD4, the HIV-binding receptor, characterizes T helper cells phenotypically.

Following HIV transmission, specific types of cells, including macrophages and resident CD4 T cells, are the putative cells the virus infects to cross the epithelium of mucosal surfaces (12). Then, the migration to the draining lymph nodes is very rapid, constituting an obstacle to the effectiveness of post-exposure vaccine (13). In the end, CD4 T lymphocytes are infected and spread the infection via bloodstream to other lymphoid tissues.

The period characterized by high levels of HIV RNA and a transient drop of CD4 cells is known as early HIV infection or as primary HIV infection, that can be asymptomatic or clinically similar to a mononucleosis syndrome in the first weeks post-infection.

Humoral and cellular responses begin approximately 3 to 6 weeks after infection, causing the decline of plasma viremia and plasma antigenemia, the stabilization of CD4 cells and the resolution of the clinical presentation. In six months, plasma viremia reaches the viral set point, being together with CD4 T cell nadir predictors of HIV progression (14). In the chronic phase of HIV disease, virus continuously replicates in people not receiving antiretroviral treatment, destroying an average of 10 billion CD4 cells daily (15).

1.4. HIV molecular biology

HIV belongs to the genus Lentivirus, like the simian immunodeficiency virus (SIV).

HIV is a spherical RNA virus with a maximum diameter of 120 nm. The innermost layer of the cell is the genome, followed by the middle nucleocapsid and the outside glycoprotein membrane, which is encased in a lipoprotein envelope.

The envelope is formed of lipoproteins, which the virus codes for the glycoproteins while the lipid component comes from the host cell membrane and is obtained via budding from the plasma membrane. Each viral particle membrane contains glycoprotein heterodimer complexes comprised of bound trimers of the anchoring transmembrane pedicles (gp41) and the projecting spikes on the surface (gp120). Specifically, gp120 interacts with cell surface receptors on receptive host cells to determine the virus's tissue tropism; gp41

forms the lollipop stick, which promotes cell fusion. The envelope surrounds a capsid made of a capsid, matrix and nucleocapsid protein. Protease (PR), reverse transcriptase (RT) and integrase (IN) are 3 fundamental enzymes contained by the capsid. *Nef*, *Vif*, and *Vpr* are additional proteins carried by virus particles.

1.4.1. HIV GENOME

HIV genome is 7 to 11 kb long and consists of 3 principle genes (gag, pol and env), shared among all the retroviruses:

- Matrix protein and core structural proteins are encoded by gag;
- Envelope glycoproteins (gp41 and gp120) are encoded by env;
- RT, PR and IR are encoded by pol.

It has a 5'-cap and a polyadenylated 3'-end at its extremities; in these spots, there are the Long Terminal Repeats, containing both the enhancer region and the viral promoter. Since the Long Terminal Repeat can be bound by cellular transcription factor, this relationship is crucial for the modulation of the transcription (16).

1.5. THE CHALLENGE OF THE HIV-1 LATENT RESERVOIR

ART has significantly decreased mortality in PLWH (17); however, ART alone cannot eradicate HIV infection due to the persistence of a latent viral reservoir, defined as "a cell type or anatomical site in which a replication-competent form of virus accumulates and persists" (18), in spite of long periods of ART-suppressed viraemia (19,20). In fact, viral rebound occurs within weeks in most PLWH who interrupted ART, even though they had maintained undetectable viral load for years (21). Thus, the need for ART is lifelong. A cure for HIV is fundamental to eliminate the pill burden, to avoid toxicity ART-related and to decrease the ART cost that may be hard to sustain, especially in low-income countries. (22,23).

1.5.1. THE SEEDING OF HIV-1 LATENT RESERVOIR

HIV reservoir is established in the first phases of infection. Data from rhesus macaque (RM) SIV model suggest that HIV reservoir is seeded days following mucosal SIV infection, prior to systemic viremia. Initiation of therapy on day 3 post SIV infection arrest the emergence of both viral RNA and proviral DNA in the peripheral blood and also decreased proviral DNA in gastrointestinal mucosa and lymph nodes. Viral rebound, though delayed, nonetheless occurred in all animals following the ART interruption despite 24 weeks of suppressive ART (24).

The formation of the reservoir has been described as a very early event also in human studies: starting ART during acute HIV infection (AHI) did not block the establishment of the reservoir (25). The importance of the reservoir was highlighted by studies of treatment interruption, being clear that even long-term ART was not sufficient to eradicate the virus (26). In particular, even in PLWH under ART who have been suppressed for at least 7 years, the decay of HIV reservoir is slow; in fact, it is reported a half-life of 44 months and a complete eradication would take at least 60 years with cART only, according to computational analyses, suggesting the urgent need for alternative therapeutic strategies (27).

Initiating ART during early HIV infection is associated with multiple beneficial effects including a smaller latent reservoir (28), a reduced viral diversity (29,30) and the preservation of innate as well as T and B cell immune responses (28,31,32). Preserving immune responses and limiting the expansion of HIV reservoir may increase the possibility of virologic control on ART discontinuation.

For instance, the Mississippi child initiated treatment by 2 days of life for 18 months and then was capable to control viremia for 27 months without receiving any antiretroviral drug (33); moreover,14 PLWH from the VISCONTI cohort who started ART within the first 2 months of HIV infection maintained undetectable viral load after treatment discontinuation (34),

However, studies investigating ART interruption have been mostly conducted on individuals with AHI, although chronically infected individuals represent the majority of PLWH.

As mentioned before, antiretrovirals are not a cure, as residual viremia is maintained and viral rebound occurs after treatment suspension.

Three main approaches should be pursued for a definitive cure for HIV, defined as viral replication control without taking ART: an eradication strategy which involves the elimination of all viral reservoirs; a functional cure that aims at controlling replication without eradicating reservoir, or a hybrid cure that consists in the reduction of the reservoir by the boosting of immune responses (35). At present, we have only 5 anecdotical examples of a successful eradication cure, namely the Berlin patient (36), who underwent hematopoietic stem cells transplantation back in 2009 from a donor who was homozygous for CCR5 delta32. The ‘London patient’, the ‘Düsseldorf Patient’, a woman in New York city and the 'City of Hope patient' were treated with similar approaches (37–39).

1.5.2. MECHANISMS OF PERSISTENCE

Different mechanisms allowing the HIV persistence encompass ongoing cycles of viral replication in specific anatomical sites with infection of new host cells, latency and proliferation of cell clones.

1.5.2.1. RESIDUAL REPLICATION

Viral replication occurs especially in sites with a high concentration of cells permissive to infection, a suboptimal drug penetration, an altered tissue architecture and a pro-inflammatory environment, such as primary and secondary lymphoid organs, the central nervous system (CNS), the gastrointestinal (GI) tract and the genital tract.

Another possible mechanism of HIV replication involves immune hyper activation, a generalized state of immune activation induced by microbial translocation, co-infections and inflammation.

However, intensification studies characterized by the addition of raltegravir to the standard 3-drugs therapy showed no impact on residual viremia, suggesting that ongoing replication is not probably the only player sustaining HIV persistence (40). Moreover, the absence of viral phylogenic evolution, the lack of emergence of drug resistance mutations and the detection of identical sequences after long-term ART argue against the role of viral production in HIV persistence (41).

1.5.2.2. *LATENCY*

In the context of HIV infection, latency is characterized by the reversible transcriptional silencing of the provirus into the host cell to escape from the recognition of immune cells and allowing for viral persistence (20).

Latency is a rare and very early event; in PLWH on long-term cART, the frequency of latently infected cells is extremely low, typically around 0.1-10 infectious units per million resting CD4 T cells (42). Of note, only a small fraction carries an integrated provirus which is competent to replication (43). Indeed, resting memory CD4 T cells are recognized as the main cellular reservoir, since they have a long half-life and are less permissive to replication: once infected, the majority dies, but a small proportion survives and reverts back to resting state, persisting as memory cells (44).

The duration of this latent state is limited only by the half-life of the infected cells and of their progeny, in which the virus can persist as mere genetic information. However, latently infected cells can be activated upon antigen or homeostatic stimulation, leading to the reactivation of the viral transcription and production of new infectious viral particles.

1.5.2.3. *PROLIFERATION OF INFECTED CELLS*

The clonal proliferation of HIV-1- infected cells could explain the persistence of low-level viremia, defined as viremia <200 copies/ml), and the onset of viral blips in absence of drug resistance mutations. These clinical scenarios do not improve by intensifying antiretroviral treatment and therefore do not need any therapeutic, showing to be sustained by predominant plasma clones, as emerged by phylogenetic analyses performed in PLWH with low-level viremia (41,45,46).

The major forces driving clonal expansion of HIV-1-infected cells include homeostatic proliferation, integration site-driven proliferation and antigen-driven proliferation.

1.5.2.3.1. *HOMEOSTATIC PROLIFERATION*

Homeostatic proliferation driven by cytokines maintains the repertoire of memory CD4 T cells, such as T central memory (Tcm) and T transitional (Ttm) cells, without inducing virus gene expression and evading immune clearance. Interestingly, it has been found that IL-7 concentrations are inversely associated with the reduction of the reservoir size, thus

suggesting that IL-7 promotes the persistence of HIV-infected CD4 T cells, leading to a qualitative and quantitative stability of the HIV reservoir (47,48).

1.5.2.3.2. INTEGRATION SITE-DRIVEN PROLIFERATION

A considerable proportion of HIV-infected cells in people taking ART have expanded into clones, which were found because their provirus integration sites are identical (45). HIV-1 clones can be defined and followed over time by integration site analysis in that a large fraction of infected CD4 T cells retrieved from PLWH on successful ART shows proviral integration into precisely the same position in the human genome.

Beside from immune-mediated mechanisms, HIV-1 integration may promote aberrant cellular proliferation. Indeed, the recovery of identical integration sites revealed by long-term ART confirmed the existence of cells that are able to proliferate and expand in presence of an integrated virus (46,49). Moreover, HIV integrations were detected in genes linked to with cell proliferation and/or survival, suggesting that cellular proliferation can occur as a consequence of the host gene expression (45). Cesana et al. previously demonstrated that FOXP3-expressing regulatory Tregs have a role in favoring viral persistence in PLWH, taking advantage of insertional mutagenesis mechanisms. (49).

Differently to the immune-mediated mechanisms of expansion, the HIV-1 integration site-driven clonal expansion dynamic should be characterized by a slow but steady increase of HIV-1-infected cells.

1.5.2.3.3. ANTIGEN-DRIVEN PROLIFERATION

Recent works have demonstrated that different antigens can also influence the reservoir proliferation. Memory CD4 T cell proliferation can be driven by antigenic stimulation and by the cross-reactive recognition of other self or foreign peptides presented with MHC class II (50). It is likely that antigen stimulation may activate HIV-1-infected, antigen- specific CD4 T cells and leading to HIV-1 expression and clonal expansion. Therefore, the HIV-infected clones wax as antigen stimulation peaks and wane as the antigen-specific response subsides over time (51), eventually leading to viral blips associated with the stable or intermittent production of viral particles (52). Antigen-driven

proliferation may be explained by the exposure of viral or tumor antigens, as described in more detail later in the Introduction.

1.6. HIV RESERVOIR IN SPECIFIC POPULATIONS

1.6.1. PLWH WITH A MULTI-DRUG RESISTANT VIRUS

Drug resistance is one of the main challenge in the HIV management. (53).
Multi-drug resistant (MDR) HIV is defined as a virus with a diminished susceptibility to multiple drugs belonging to different antiretroviral classes (54) and it has become one of the most complex issues to face in HIV management.
However, the prevalence of people living with a MDR virus has decreased over the last decades due to the availability of an ART that efficiently suppresses viremia and the introduction of new drugs with a higher genetic barrier (55).

For this reason, due to the prolonged history of ART exposure and the broad resistance profile that is often observable, virologic failure on second-line and salvage regimens utilized in ART-experienced PLWH with limited treatment alternatives represents a problem (56).

Although current ART regimens are highly effective, a small number of people harbours a virus resistant to multiclass drugs that lead to a difficult control of HIV replication. Identifying the greatest number of resistance mutations in people with limited treatment options is fundamental to defining a successful personalized regimen.
In order to promote a concerted multinational effort to monitor and prevent the onset of HIV resistance, WHO has recently created a five-year global action plan; strategic objectives are to facilitate the research agenda related to the application and the quality assurance of new tools for HIV-1 drug resistance testing (57).

People with a MDR infection, especially if HIV strain is resistant to all the four principle antiretroviral classes, have a greater incidence of cancers and mortality in comparison with people harboring a wild-type virus (58). Due to the elevated burden of disease in PLWH with a MDR virus, strategies aimed at limiting causes of morbidity are strictly

recommended, mostly in PLWH with a low CD4/CD8 ratio and a history of clinical events.

Even though remarkable progress has been pursued for the approval of new antiretroviral drugs into clinical practice, efforts to guarantee access to drugs with innovative mechanisms of action are necessary to arrest disease progression, reach virological suppression and also improve overall survival and quality of life, especially in PLWH with a virus resistant to the main four antiretroviral classes.

It is not clear if enrichment of HIV reservoir can occur in this subset of PLWH and/or has an impact on disease progression, considering that people with a MDR virus have a longer time of exposure to HIV compared with people with a wild-type strain who rapidly achieved virological suppression. Up to date, few data on describing the dynamics of HIV reservoir in people with MDR infection exists: in fact, once reached viremia below the detection limit, it is not known how rapid is the decay of HIV reservoir in these patients.

1.6.2. PLWH WITH VIRAL COINFECTIONS

HIV shares the same route of infection (i.e. unprotected sexual intercourse and use of intravenous drugs) of many other viruses, including HBV and HCV; for this reason, viral coinfections are really frequent among PLWH.

Viruses like Epstein Barr virus (EBV) are usually established in most PLWH prior to the HIV infection. Although EBV generally leads to a silent infection, EBV can be a cancer promoter of certain types of non-Hodgkin lymphoma, such as diffuse large B-cell and Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma, in particular in PLWH with a low CD4 cells count.

Other oncoviruses are HPV, responsible for cervical, anal, oral cancer and oropharyngeal cancer, and HCV, mainly correlated to the pathogenesis of hepatocarcinoma (59).

Although many coinfections do not have any effect on HIV disease and can be asymptomatic, others can cause different clinical scenarios and lead to viral blips or a sustained increase of viremia, even in PLWH on virological suppression for years (60).

Many researchers have investigated the reasons leading to viral blips during viral coinfections. Although viral blips are only transient spikes in HIV RNA, their occurrence could be explained by mechanisms of HIV persistence, such as the antigen-driven proliferation.

In fact, despite longitudinal analysis on PLWH under ART showed that the size of the reservoir is maintained stable over time using different PCR assays, fluctuating dynamics of infected cells occur, mimicking the typical expansion-contraction phases of adaptive immune responses after antigen stimulation. The chronic and intermittent exposure to viral antigens may account for antigen-driven clonal expansion in vivo, leading to the reported waxing and waning of individual clones of infected CD4 T cells and consequently to the increase and persistence of reservoir (61). In this regard recent works have provided clear evidences that antigens can shape the fate of infected cells contributing to the proliferation of the HIV-1 reservoir. Proviral DNA has been detected in CD4 T cells specific for herpesviruses (62), Candida albicans (63), influenza (64), tetanus (65), and HIV-1 itself (66,67).

Simonetti et al. isolated CD4 T that responded to CMV and Gag from 10 subjects. The authors observed that the proviral populations in CMV-responding cells were mostly large clones, including some that harbor replication-competent proviruses (68). Importantly, TCRb repertoires demonstrated elevated levels of clonality due to an evolving adaptive response. Although proviruses were present in genes related to HIV persistence such as STAT5B, MKL1 and BACH2, these proviruses did not cause infected cells to multiply when stimulated with antigenic stimuli. Paired TCRb and integrated site analysis revealed that HIV-1 infection could occur at either the beginning or end of clonal responses to antigen. This study showed that these cells were infected at rates too high to be explained solely by homeostatic growth alone; in fact, these results highlight that HIV persistence may be also caused by antigen-driven clonal selection, that necessitates further efforts to be eliminated in the pathway to an HIV cure (68).

Similarly, Mendoza et al. isolated CD4 T cells containing clones of replication-competent HIV-1 viruses responsive to antigenic stimulation with peptides derived from EBV: both defective and intact proviral analysis and phylogenetic tree of env sequence were

performed starting from antigen-responding HIV-1 clones; in this work, CD4 T cells containing clones of replication-competent HIV-1 viruses responsive to EBV had proved to be able to produce different viral particles (69).

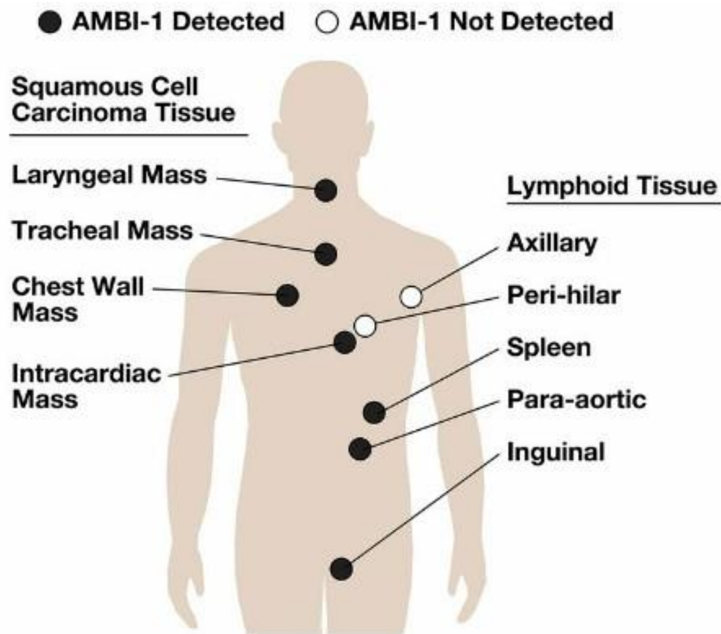
1.6.3. PLWH WITH CANCER

PLWH have a greater risk of developing malignancies compared to general population, considering both AIDS- (such as non-Hodgkin lymphoma, Kaposi sarcoma and cervical cancer) and non-AIDS-related cancers (70).

Chronic inflammation, cytokine dysregulation, persistent antigen stimulation and a higher incidence of coinfections mediated by oncoviruses explain why cancers are more frequent among PLWH (71,72). Moreover, survival appears to be reduced in people with AIDS-defining conditions (73), revealing that immunodepression has a significant effect on the prognosis of the disease. Compared with the general population, PLWH are currently about 500 and 12 times more likely to be diagnosed with Kaposi sarcoma and non-Hodgkin lymphoma, respectively. Moreover, the onset of tumors, including hepatocarcinoma, lung cancers or Hodgkin lymphoma (also known as "non–AIDS-defining cancers" are more frequent in PLWH and one of the main reported cause of mortality in these patients.

Diagnosing and treating cancer early is critical to the impact on prognosis and overall survival, particularly in PLWH who have a higher cancer-related mortality rate than general population (41)

Dynamics of HIV reservoir in people with cancer should be investigated from the diagnosis to the end of treatment in order to establish possible long-term effect of chronic stimulation induced by tumor antigen on viral reservoir. Although it was initially supposed that exclusively cells harboring defective copies could expand clonally (74), Simonetti et al. in 2016 identified for the first time an expanded HIV-infected CD4 T cells clone carrying an infectious provirus (named AMBI-1 and estimated to comprise ca. 1×10^7 cells) able to maintain persistent plasma viremia despite ART at various time points in a subject with squamous cell cancer (41).



Number of HIV DNA Sequences in Different Tissues

	Lymphoid Tissues	Metastatic Lesions	Fold Enrichment	
AMBI-1	6	21	4.4	*p=0.001*
Other Clonal HIV Populations	90	65	0.9	*p=0.69*
Non-Clonal HIV Populations	57	36	0.8	*p=0.40*
Total Sequences	153	122		

Figure 2. HIV-1 sequences detected from tumor and lymphoid tissues in patient with a metastatic squamous cell carcinoma.

The size of AMBI-1, the largest known clone with an intact provirus, changed both with the progression and therapy of tumor in the donor. Then, the cells that carried the AMBI-1 provirus were substantially enriched in the cancer-infiltrating T cells, indicating that the cells in this clone may have been responsive to a cancer-specific antigen or some other cancer-specific factor.

As reported in Figure, the AMBI-1 provirus and the integration site were found in DNA from both lymphoid and malignancy tissues and were present as a larger fraction of all of the HIV DNA sequences in the metastases than in lymphoid tissue. Plasma levels of virus from the clone were constant for months before receiving specific treatment, and did not vary after switching ART . Thus, the stimulus that caused the cells in the AMBI-1 clone

to divide was sufficient to drive expansion to about 9 million cells. Interestingly, it has been demonstrated that the HIV-clones exist since the pre-ART phase (75) and have the potential to replicate under ART leading to the resurgence of the viraemia in a few weeks upon ART discontinuation (45,76).

Considering that the most of studies on HIV reservoir have investigated participants without comorbidities and coinfections, especially people who initiated ART during the first phases of infection, analyzing different HIV populations likely those exposed to a relevant but intermitting antigen stimulation may be a great opportunity to evaluate the dynamics of HIV persistence. Assessing distinct timepoints during the course of viral coinfection or virus-driven malignancy would give us the chance to describe changes in clonal expansion over time, as illustrated in Figure.

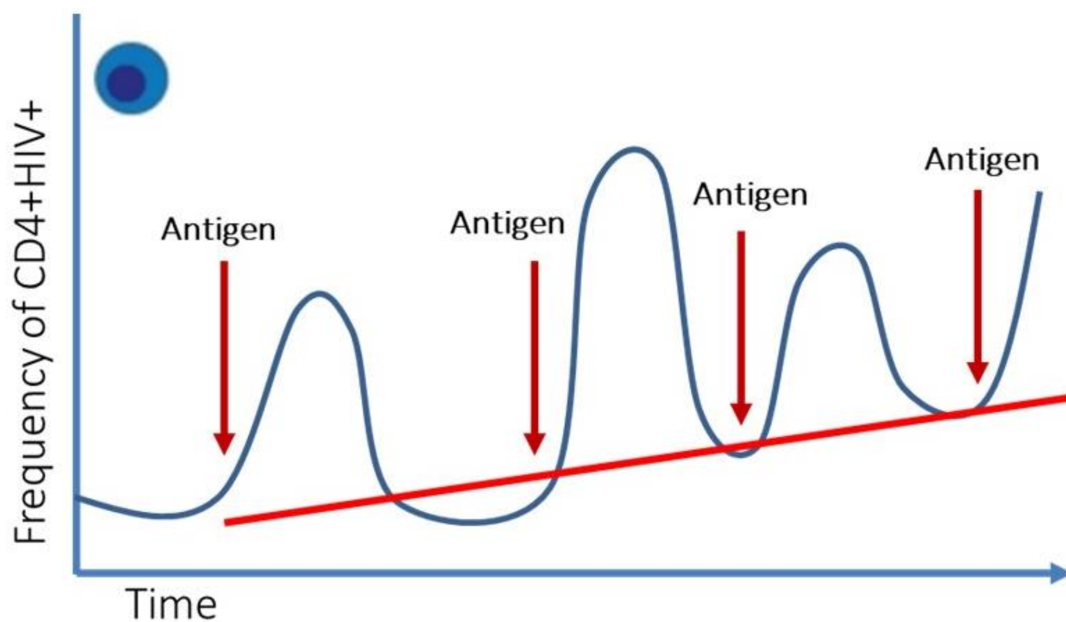


Figure 3. Waxing and waning of clones of infected CD4 T cells due to the exposure to antigens (antigen-driven proliferation).

2. AIM OF THE WORK

Nowadays, there are no data on HIV reservoir in person with multi-resistant HIV infection. Considering that they represent a very fragile population, where virological suppression is really hard to achieve and maintain, is mandatory to understand the role of viral reservoir in disease progression and its dynamics over the course of infection.

In this study, we aimed to quantify HIV reservoir in people with a MDR virus in comparison with people with a wild-type virus. An increased size in HIV reservoir in these patients would contribute to underline the need to find new effective drugs capable of rapidly reducing viremia in people who have currently limited treatment options.

Previous studies have demonstrated that the antigen-driven clonal selection is one of the major factor of HIV-1 persistence.

Here, we wanted to quantify and characterize the viral reservoir size in adult PLWH with active coinfections (HCV, EBV or HPV) +/- virus-driven malignancy (HCV-driven hepatocarcinoma, EBV-driven lymphoma, HPV-driven cancers) exposed to a chronic viral stimulation. We also evaluated whether the presence of coinfections may promote HIV reactivation and thus expansion and persistence of HIV reservoir. This study may reveal the importance of effectively treating both viral coinfections and virus-driven malignancies as soon as possible in order to limit the clonal expansion of HIV infected cells. We believe that it is crucial for HIV specialists to know if delaying diagnosis or treatment of these clinical conditions may represent an additional obstacle to the HIV cure. Furthermore, this study justify further efforts in developing prevention strategies to early detect viral coinfections and cancers in PLWH to not disadvantage these patients in the path towards the HIV eradication.

3. Material and Methods

3.1. PRESTIGIO registry

PRESTIGIO is a multi-center Italian registry (www.registroprestigio.com) that includes forty Infectious Disease Departments and collect clinical and laboratory data of people with a HIV virus harboring a documented resistance to the 4 principal antiretroviral classes. (77)
The aim is to record information about survival, burden of disease and salvage regimens, in terms of efficacy and safety (78).

The Ethical Local Committee approved the PRESTIGIO Registry, that represents the coordinating center, in December 2017 (protocol number 41/int/December 2017) ; all participants provided written informed consent before the enrolment (58).

3.2. EVASION study

EVASION (Evaluation of the VirAl reServoir in people living with HIV-1 Infection and virus-driven malignancies Or viral coiNfections) is a prospective, observational, proof-of-concept study that aims to assess viral reservoir dynamics in adult PLWH coinfected with malignancy-promoting viruses. PLWH under ART for at least 2 years and on virological suppression were enrolled in one of the 3 groups:
1) PLWH with an active HCV infection +/- a HCV virus-driven malignancy;
2) PLWH with an active EBV infection + a EBV virus-driven malignancy;
3) PLWH with an active HPV infection + a HPV virus-driven malignancy.
In all the study participants the coinfection had to be in a replicative phase at time of enrolment.
Participants who have started chemotherapy, radiotherapy or immunotherapy before the enrollment were excluded.
As control group, we included PLWH without any active coinfection, followed at the San Raffaele Hospital in Milan, Italy.
In the context of the project proposed for the Bando della Ricerca Finalizzata, we will design a longitudinal prospective study where blood samples will be collected at week 0, month 6, month 12, month 18 and an additional timepoint if tumor relapse occurs.

The Ethical Local Committee of the San Raffaele Hospital approved the study protocol (on 09/06/2021; approval reference number: EVASION) and all the enrolled patients provided written informed consent.

3.3. LABORATORY PROCEDURES

3.3.1. CELL PURIFICATION, PURITY AND CYTOFLUORIMETRIC ANALYSIS

Naïve CD4 T cells were purified from 70 mL of EDTA peripheral blood of the enrolled subjects from 50 mL of buffy coat of healthy donor by Ficoll-Hypaque gradient. T cells were purified from the PBMCs by magnetic cell purification using the CD4 Isolation Kit (Kit Miltenyi).

Total lymphocytic cellular subpopulations were isolated by FACS sorting procedure starting from CD4 T cells purified using lineage-specific kit (Miltenyi Biotech). PBMC were stained with fluorochrome conjugated antibodies against the following cell surface markers: CD4, CD8, CD14, CD25, CD45RA, CD62L and CD127.

The different T cell subsets were identified by the following markers: T naïve, $CD4^+/CD8^+CD25^-CD45RA^+CD62L^+CD127^+$; T stem central memory, $CD4^+/CD8^+$ $CD25^+CD45RA^+CD62L^+CD127^+$; T central memory, $CD4^+/CD8^+$ $CD25^+CD45RA^-CD62L^+CD127^+$; T effector, $CD4^+/CD8^+CD25^-CD45RA^-CD62L^-$ $CD127^-$; T effector memory, $CD4^+/CD8^+CD25^-CD45RA^-CD62L^-CD127^+$; T transitional memory $CD4^+/CD8^+CD25^+CD45RA^-CD62L^-CD127^+$; and T regulatory cells $CD4^+CD25^+$ $CD45RA^-CD62L^-CD127^-$.

We used flow cytometric analysis to evaluate the purity of each fraction.

In this study, cytofluorimetric analysis was used to assess the relative composition of the specific lymphocytic cellular subpopulations in our groups and compared to healthy donors.

3.3.2. INTACT PROVIRAL DNA ASSAY

IPDA encompasses the use of ddPCR method that distinguishes intact from defective proviruses using two amplicons probes from genomic DNA.

Protocol used for IPDA assay was previously described in detail by Bruner et al. (79).

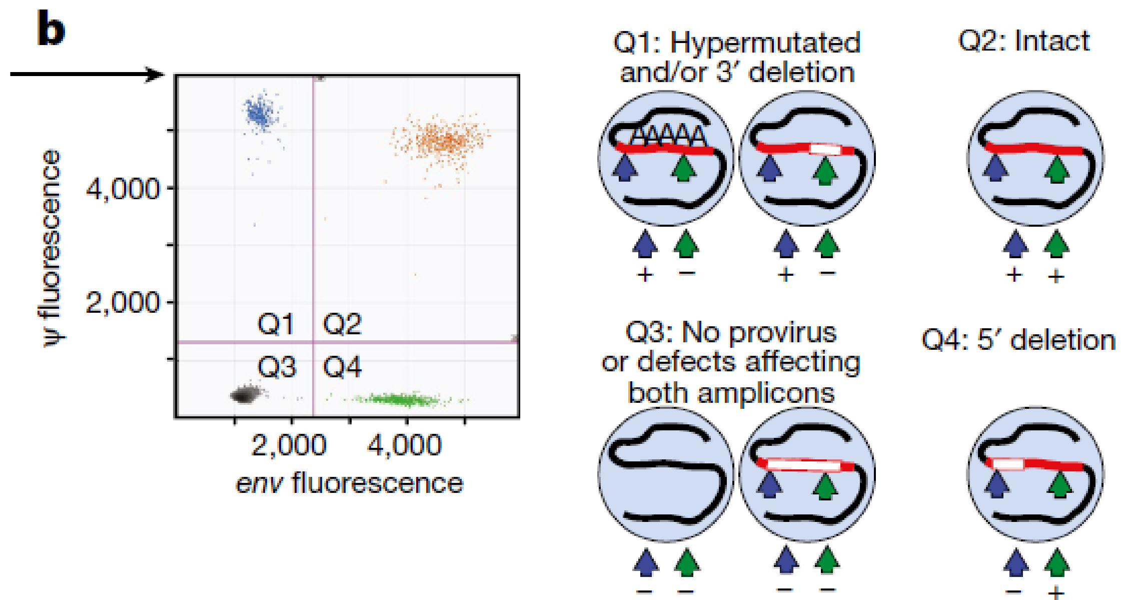


Figure 4. Representative plots of intact and defective proviruses (hypermutated and/or 3'deletion and 5' deletion) measured by IPDA.

3.3.3. *ANTIGEN-SPECIFIC ASSAY*

To identify antigen-responding CD4 T cells, we used cryopreserved PBMCs viably thawed and magnetically selected for CD8-depleted CD4 T cells, that were cultured with auto-APCs together with viral peptides (for HCV/EBV/HPV). After 14 days, a second-round of stimulation was performed and 10 days later CD4 T cells unstimulated were subsequently cultured with auto-APC (negative control), auto-APC + viral peptide and auto-APC + phytohaemagglutinin (PHA, positive control) to evaluate both the proliferation activity (%Ki67+ cells) and the production of type II interferon after stimulation. Supernatants positive for p24 by ELISA were collected.

3.4. STATISTICAL ANALYSIS

The study is defined as exploratory, "proof-of-concept", and the number of patients to enroll was defined on the basis of feasibility criteria. Based on the last 4-year experience of the Infectious Diseases of IRCCS Unit of San Raffaele Scientific Institute, 20 cases/year of HCV coinfection in a replication phase, including 6 cases/year of HCV-

related hepatocarcinoma, 12 cases/years of EBV-related lymphoma and 11 cases/year of HPV-related cancers are followed by our Centre.

Descriptive analyses will be performed according to the study arm and the endpoints will be summarized using medians with interquartile ranges or ranges or 95% confidence intervals (CI) for quantitative variables, and proportions with their 95% CI for qualitative or categorical variables; CIs will be calculated using the exact method. Due to the small sample size, analyses will be mainly descriptive as there will be not statistical power for a formal test of differences between the study groups. The analyses will check for clinically-relevant differences in the main baseline patients' characteristics among study groups; the presence of relevant imbalances in these characteristics among groups will be eventually tested by: chi-square test or the Fisher's exact test (categorical variables) or by the non-parametric Wilcoxon rank-sum test or Kruskal-Wallis test (continuous variables).

Spearman's rank correlation coefficients will be used among different outputs.

Most of the analyses will be performed using GraphPad Prism and R software.

Furthermore, for any other issue that may rise in the course of the experiments we will rely on the support of a statistician working at the Infectious Diseases Unit of IRCCS San Raffaele Scientific Institute

4. RESULTS

4.1. PEOPLE LIVING WITH A MULTI DRUG RESISTANT VIRUS

4.1.1. CHARACTERISTICS OF STUDY POPULATION

In this study, all the PLWH analyzed had undetectable viral load, defined as HIV RNA <50 copies/mL, since at least 6 months.

We evaluated 35 PLWH: 14 [13 males (92.3%); 1 female (7.7%)] people with a MDR virus from the PRESTIGIO registry and 21 [19 males (90.5%); 2 females (9.5%)] people without a MDR virus as control group; median age was 57 (54-62) and 48 (40-52) years, respectively (p=0.008). Years of HIV-1 infection were 31.0 (29.3-32.2) in people with a MDR virus and 13.0 (8.3-22.6) and in those without (p<0.001), while exposure to ART was 27.4 (25.5-29.3) and 8.5 (7.0-16.6) years, respectively (p<0.001). Area under curve of viremia was 12218 (8535-22318) and 5591 (611-12834) copy/years among people with and without MDR infection, while median CD4 count was 629 (287-720) and 768 (414-1026) cell/mcL, respectively. A statistical difference was seen in time spent on undetectable viremia since last positive viremia: 3.5 (1.9-5.5) vs. 6.9 (4.5-7.5) years (p=0.023).

Overall, 14/14 (100%) people with a MDR virus and 16/21 (76.2%) people without a MDR virus were infected with HIV subtype B; HIV subtype data was not available for 5 participants.

Participants' characteristics are reported in Table 1.

Variable		Overall (n=35)	People with a MDR virus (n=14)	People without a MDR virus (n=21)	P-value
Age		50.8 (41.5-57.6)	56.9 (54.0-61.5)	47.5 (39.6-51.5)	0.008
Gender					1.000
	F	3 (8.6%)	1 (7.1%)	2 (9.5%)	
	M	32 (91.4%)	13 (92.9%)	19 (90.5%)	
Years of HIV		23.3 (12.0-30.4)	30.5 (29.3-32.2)	13 (8.3-22.6)	<0.0001
Years of ART		24.2 (7.6-26.7)	27.4 (25.5-29.3)	8.5 (7.0- 16.6)	<0.0001
Nadir CD4+ (cells/mcL)		163 (61-320)	66 (36-147)	260 (132-428)	0.002
Zenith HIV-RNA (copies/mL)		126119 (25541-295000)	230000 (100000-466100)	124500 (15465-227780)	0.219
AIDS diagnosis					0.199
	No	22 (62.9%)	7 (50.0%)	15 (71.4%)	
	Yes	13 (37.1%)	7 (50.0%)	6 (28.6%)	
Years with HIV-RNA<50 copies/mL		5.5 (2.7-7.4)	3.5 (1.9-5.5)	6.9 (4.5-7.5)	0.023
CD4+ (cells/mcL)		664 (404-885)	629 (287-720)	768 (414-1026)	0.081
CD4+%		29.8 (23.3-34.9)	24.9 (23.3-28.8)	33.7 (25.2-37.0)	0.012
CD8+ (cells/mcL)		885 (746 -1116)	918 (878-1116)	836 (655-1030)	0.189
CD8+%		37.2 (33.2-43.9)	42 (35.1-46.8)	36.6 (31.6-41.8)	0.298
CD4+/CD8+ ratio		0.8 (0.6-1.1)	0.7 (0.6-0.8)	0.9 (0.6-1.1)	0.05
Last VL>50 before period with VL<50		141 (74-1502)	106 (57-2646)	166 (90-1372)	0.263
AUC HIV-RNA (copy/years)		9,242 (889-20017)	12.218 (8535-22318)	5591 (611-12834)	0.117
AUC CD4+ (cells/years)		507 (401-723)	462 (351-510)	609 (420- 902)	0.016

Table 1. Characteristics of people with and without a multi-drug resistant virus.

4.1.2. Characteristics of lymphocytic subpopulations

Here, we examined whether the relative composition of lymphocyte cell populations differed between the two groups of PLWH (MDR and non-MDR). As depicted in Figure 5., no statistical differences emerged between people with a MDR virus and those without a MDR virus in the percentage of CD4, CD8 and CD14: CD4% was 21.2 (15.0-26.6) in the first group and 27.8 (17.3 - 36.6) in the second one, CD8% was 26.2 (20.9-35.1) and 25.4 (19.1-31.5), while CD14% was 11.2 (8.6-13.8) and 14.0 (10.5-19.3), respectively.

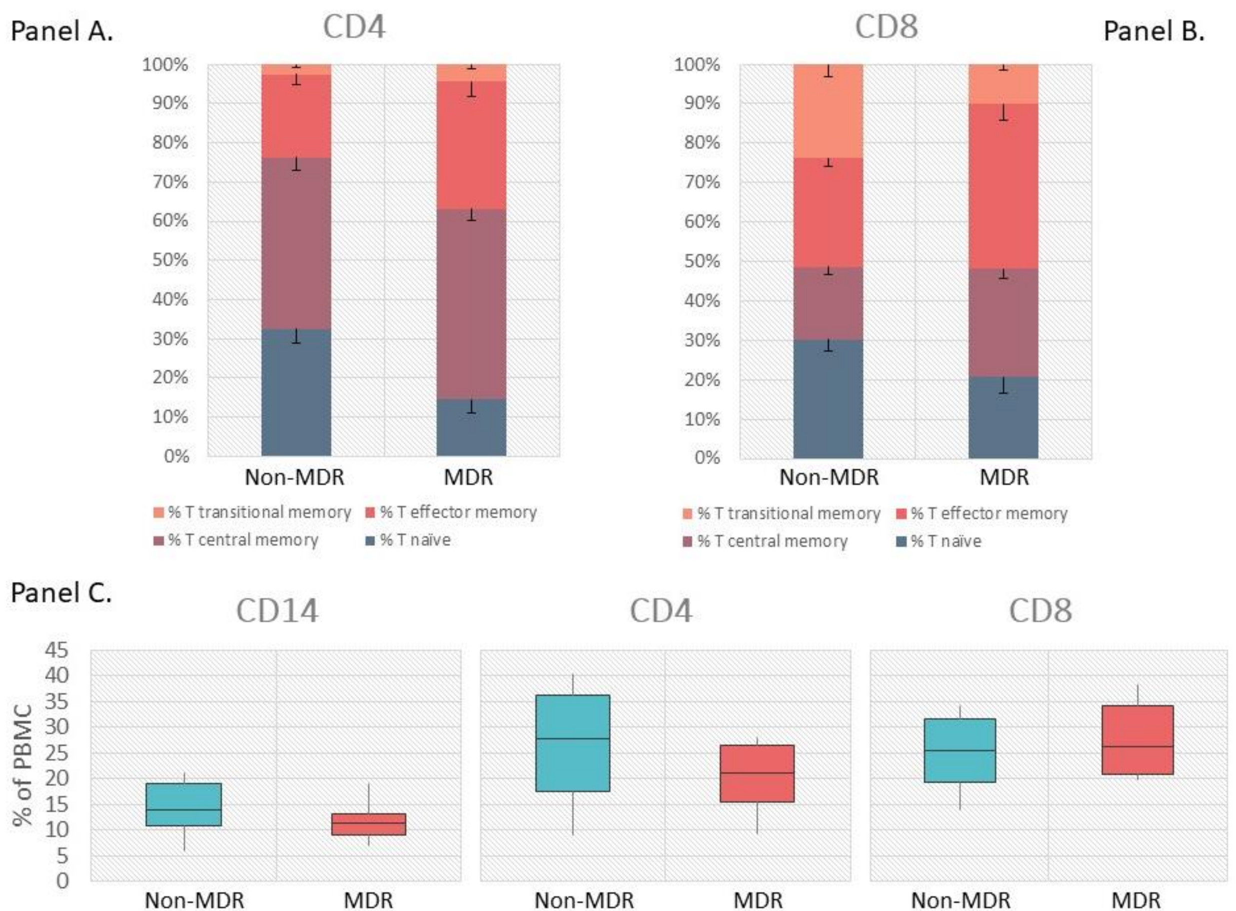


Figure 5. Distribution of lymphocytic subpopulation. Panel A. CD4 T cells subpopulations. Panel B. CD8 T cells subpopulations. Panel C. CD14, CD4 and CD8 T cells percentage of PBMC.

Moreover, percentage of naïve CD4 T cells was significantly greater in the non-MDR group [27.1 (18.9-48.5) vs. 8.8 (6.1-15.3), p=0.002], while percentages of CD4. Tem and Ttm were higher in the MDR group [32.1 (24.8-39.8) vs. 18.2 (13.0-26.2), p=0.017 and 3.8 (2.1-5.3) vs. 1.1 (0.6-1.9), p=0.004, respectively]. Statistical differences were also seen in the composition of CD8 populations: percentages of CD8 Tcm and Tem were greater in the MDR group [26.7 (21.8-33.9) vs. 17.6 (11.9-21.7), p=0.001 and 43.8 (30.3-55.2) vs. 26.0 (22.8- 32.0), p=0.005], while percentage of CD8 Ttm is smaller in this group [8.6 (6.7-11.3) vs. 20.2 (12.4- 29.2), p=0.001].

Percentage of naïve CD8 T cells was only marginally higher in people without a MDR virus compared with people with a MDR virus [29.7 (21.3- 37.2) vs. 17.0 (9.0-23.6), p=0.052)].

Other percentages are reported in Table 2.

Variable	Overall (n=35)	People with a MDR virus (n=14)	People without a MDR virus (n=21)	P-value
%CD4+ T naive	19.9 (9.2-36.3)	8.8 (6.1-15.3)	27.0 (18.9- 48.5)	0.002
%CD4+ Tcm	43.3 (36.0-52.8)	48.5 (36.8-55.2)	41.9 (34.1-47.9)	0.111
%CD4+ Tem	23.5 (13.5-36.9)	32.1 (24.8-39.8)	18.2 (13.0-26.2)	0.017
%CD4+ Ttm	1.9 (0.9-4.4)	3.8 (2.1-5.3)	1.1 (0.6-1.9)	0.004
%CD8+ T naive	23.7 (13.1-36.9)	17.0 (9.0-23.6)	29.7 (21.3- 37.2)	0.052
%CD8+ Tcm	20.4 (15.8-26.7)	26.7 (21.8-33.9)	17.6 (11.9-21.7)	0.001
%CD8+ Tem	30.4 (23.6-41.5)	43.8 (30.3- 55.2)	26.0 (22.8- 32.0)	0.005
%CD8+ Ttm	12.6 (8.7-22.4)	8.6 (6.7-11.3)	20.2 (12.4- 29.2)	0.001

Table 2. Composition of CD4 and CD8 T cells.

4.1.3. Evaluation of HIV reservoir by IPDA

To evaluate the amount of intact replication-competent provirus copies in each patient, IPDA method was performed on MDR and non-MDR group. No differences emerged in the quantification of both HIV reservoir and defective proviruses: median intact HIV DNA was 11.9 (0-46.5) and 16.7 (0-127.0) copies/10^6 CD4 T cells among people with and without MDR infection, while 5' deleted proviruses were 167.6 (63.2-796.6) and 202.9 (86.9-410.7) copies/10^6 CD4 T cells and 3' deleted proviruses 278.1 (67.0-615.1) and 419.5 (27.4-674.4) copies/10^6 CD4 T cells, respectively. These results are consistent with IPDA values reported in other cohorts of HIV patients (80,81)

Means are also reported in Figure 6; among people with a MDR virus, mean intact HIV DNA was 75.8 copies/10^6 CD4 T cells (standard deviation, SD ±142.1), while 5' and 3' deleted proviruses were 407.8 (SD ±465.5) and 547.3 (SD ±682.5) copies/10^6 CD4 T cells; mean values in the control group were 100.0 (SD ±146.1), 489.6 (SD ±640.6) and 563.0 (SD ±672.3) copies/10^6 CD4 T cells, respectively, as illustrated in Figure 6.

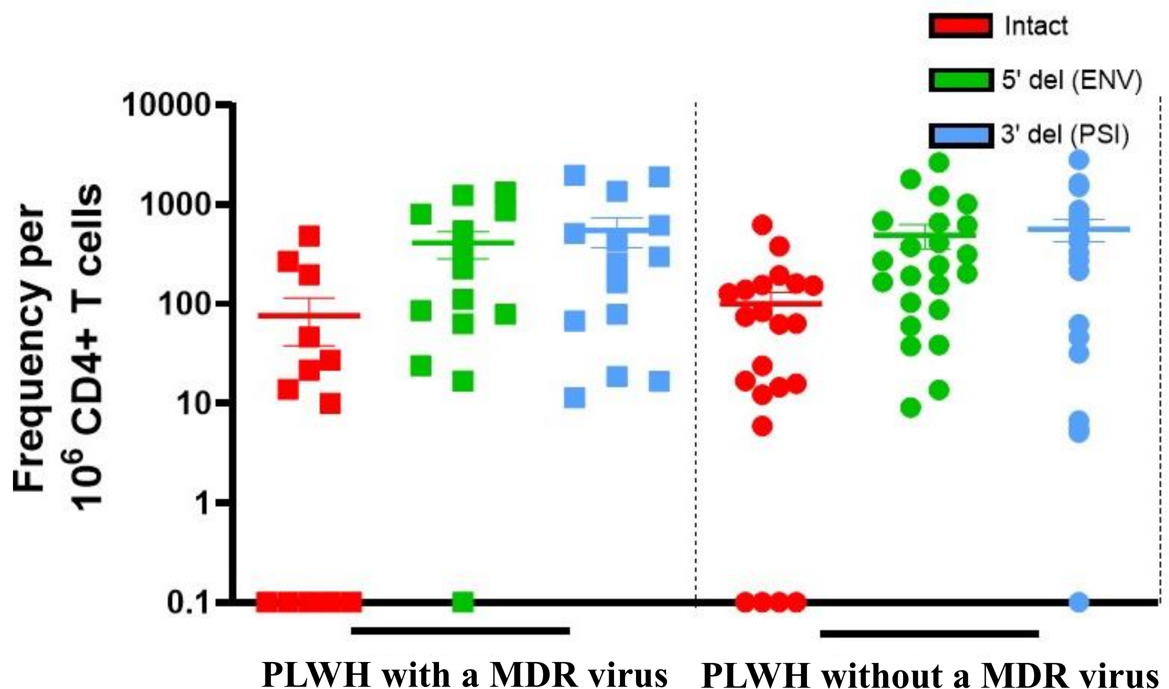


Figure 6. HIV DNA copies/10^6 CD4 T cells as intact and non-replication competent with deletion at 5' (ENV) or at 3' (PSI). Square and circle represent subjects enrolled (N=35), subdivided for the 2 groups (square for PLWH with a MDR virus and circle for those without a MDR virus). Horizontal black line indicate mean values, with standard deviation.

Overall, 6/14 (42.9%) among people with a MDR virus and 5/21 (23.8%) among people with a non-MDR virus had zero copies/10^6 CD4 T cells of intact provirus (Figure 6).
We checked whether the presence of specific mutations in the envelop (env) genomic region of the genome could have influenced the results of the IPDA assay. Data obtained from genotypic resistance testing (Sanger sequencing) showed that for 5 patients mutations present in the env region might have led to underestimate the amount of intact

copies quantified by IPDA. Median values of intact HIV DNA were similar between people with and without mutations in the env region: 13.8 and 10 copies/10^6 CD4 T cells, respectively.

Figure 7. Mutations in the pol region captured by Snapgene, based on historical genotypic resistance tests.

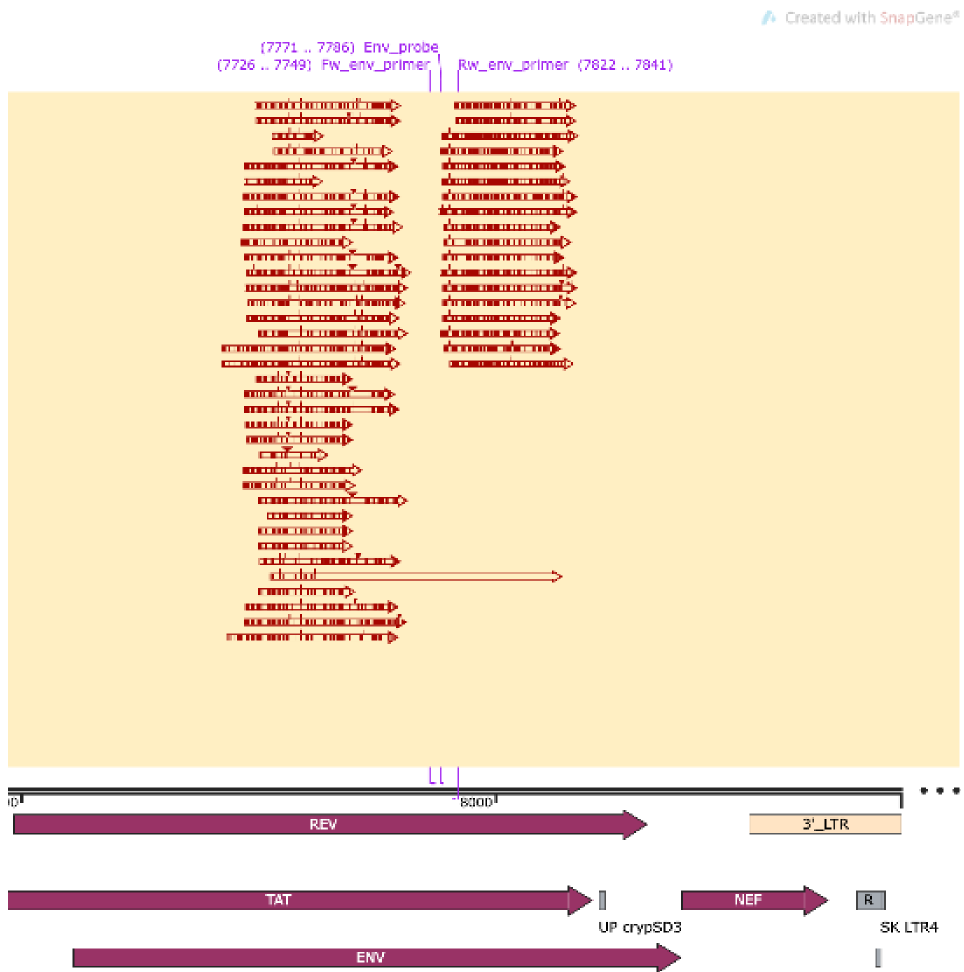


Figure 8. Mutations in the env region captured by Snapgene, based on historical genotypic resistance tests.

4.2. PLWH WITH VIRAL COINFECTIONS AND VIRUS-DRIVEN MALIGNANCY

The chronic and intermittent exposure to viral antigens may account for antigen-driven clonal expansion in vivo, leading to the reported waxing and waning of individual clones of infected CD4 T cells and consequently to the increase and maintenance of viral reservoir (61). For this reason, in our work participants will be enrolled to evaluate HIV reservoir dynamics in adult PLWH with active coinfections +/- malignancy-promoting viruses.

4.2.1. CHARACTERISTICS OF STUDY POPULATION

In the EVASION study, PLWH with viral coinfections (HCV, EBV or HPV) in replication phase +/- a virus-driven malignancy (HCV-driven hepatocarcinoma, EBV-driven lymphomas or HPV-driven cancers) were enrolled. All the PLWH included in the analysis had undetectable viral load, defined as HIV RNA <50 copies/mL, since at least 6 months.
Up to date, 17 PLWH with coinfections (8 with HCV, 4 with EBV-driven lymphoma and 5 with HPV-driven cancers, including anal intraepithelial neoplasia), and 11 PLWH without any coinfections and virus-driven malignancy were enrolled.

Overall, median age was 48.8 (40.7-52.0) in PLWH with coinfections and 43.3 (40.6-50.4 in PLWH without coinfections (p=0.288), 93% were male [15/17 (88%) and 11/11 (100%), respectively]. Median years of HIV infection and of ART were significantly different between the two groups [15.5 (12.0-23.3) vs. 8.3 (7.2-13.8), p=0.01 and 12.5 (9.1-22.9) vs. 7.4 (6.0-7.8), p=0.02 respectively]. No difference was seen between people with and without viral coinfections in the median time of HIV RNA <50 copies/mL [8.7 (5.9-12.4) vs. 6.9 (5.7-7.5) years, p=0.658], median CD4 nadir [229 (143-329) vs. 260 (125-412) cells/mcL] and median CD4 at enrolment [599 (446-828) vs. 961 (632-1471) cells/mcL] (Table 3 and 4).

Other clinical characteristics are reported in Table 5 and 6.

	ID	Age	Gender	CD4+ nadir (cells/mcL)	Years of HIV infection	Years of ART	Years of HIV-RNA <50 copies/mL	HIV subtype	ART at enrollment
PLWH with an active HCV infection +/- a HCV virus-driven malignancy	MCHIV 001	52.7	M	37	22.6	16.6	5.4	B	TAF+FTC+ DRV/c+RPV
	MCHIV 002	69.1	M	132	30.2	24.5	14.0	-	DTG+3TC
	MCHIV 003	41.9	M	177	13.0	9.4	9.0	B	TAF+FTC+RPV
	MCHIV 004	57.6	F	/	33.7	25.7	4.3	B	TAF+FTC+DTG
	MCHIV 005	41.8	M	301	11.5	11.2	7.0	-	TAF+FTC+RPV
	MCHIV 006	33.8	M	338	12.0	6.3	5.9	B	DTG+3TC
	MCHIV 007	49.4	M	490	15.5	9.1	8.8	B	TAF+FTC+BIC
	MCHIV 008	40.7	M	/	21.4	20.4	2.1	-	TAF+FTC+DRV/c
Median (IQR)	-	45.7 (41.5-53.9)	-	239 (143-329)	15.5 (12.5-26.4)	11.2 (9.3-20.6)	7.0 (5.7-8.9)	-	-
PLWH with an active EBV infection + a EBV virus-driven malignancy	MCHIV 009	50.1	M	179	18.5	18.4	13.1	-	TAF+FTC+RPV
	MCHIV 010	52.0	M	13	23.3	23.1	19.8	B	ABC+3TC+DTG
	MCHIV 011	39.5	M	185	1.9	1.8	1.7	B	TAF+FTC+BIC
	MCHIV 012	56.4	M	486	27.4	22.9	14.2	-	TAF+FTC+BIC
Median (IQR)	-	51.1 (47.5-53.1)	-	182 (138-260)	20.9 (14.4-24.3)	20.7 (14.3-23.0)	13.7 (10.3-15.6)	-	-
PLWH with an active HPV infection + a HPV virus-driven malignancy	MCHIV 013	32.7	M	/	10.8	7.1	6.8	B	DTG+RPV
	MCHIV 014	49.3	F	113	27.9	24.9	8.7	-	TAF+FTC+DTG
	MCHIV 015	43.3	M	291	12.3	9.6	9.3	-	TAF+FTC+RPV
	MCHIV 016	34.7	M	439	9.0	8.0	7.6	-	DTG+3TC
	MCHIV 017	48.8	M	273	12.6	12.5	12.4	B	DTG+3TC
Median (IQR)	-	43.3 (34.7-48.8)	-	282 (233-328)	12.3 (10.8-12.6)	9.6 (8.0-12.5)	8.7 (7.6-9.3)	-	-
Overall median (IQR)	-	48.8 (40.7-52.0)	-	229 (143-329)	15.5 (12.0-23.3)	12.5 (9.1-22.9)	8.7 (5.9-12.4)	-	-

Table 3. Participants' characteristics stratified for each group (EVASION study).

ID	Age	Gender	CD4+ nadir (cells/mcL)	Years of HIV infection	Years of ART	Years of HIV-RNA <50 copies/mL	HIV subtype	ART at enrollment
L003	43.0	M	123	2.2	2.1	1.9	-	TDF+FTC+EVG/c
L004	50.8	M	278	16.6	13.8	13.7	-	TAF+FTC+RPV
L005	51.5	M	184	13.0	8.5	8.2	B	TDF+FTC+RPV
L006	50.0	M	58	20.8	5.7	5.5	B	TDF+FTC+RPV
L007	43.3	M	73	7.6	7.6	7.3	-	TAF+FTC+EVG/c
L008	38.6	M	428	7.9	7.4	6.9	B	TAF+FTC+RPV
L009	39.6	M	260	8.3	8.0	7.5	B	TAF+FTC+RPV
L010	47.5	M	396	14.5	7.6	7.4	-	TDF+FTC+RPV
L011	53.4	M	127	11.2	6.7	6.1	B	TAF+FTC+RAL
L012	41.5	M	545	4.2	4.2	3.9	B	ABC+3TC+DTG
L013	38.0	M	530	6.7	6.2	5.9	B	TDF+FTC+RPV
Median (IQR)	43.3 (40.6-50.4)	-	260 (125-412)	8.3 (7.2-13.8)	7.4 (6.0-7.8)	6.9 (5.7-7.5)	-	-

Table 4. Control group's characteristics (EVASION study).

	ID	Active coinfection	Virus-driven malignancy
		HCV RNA (copies/mL)	
PLWH with an active HCV infection +/- a HCV virus-driven malignancy	MCHIV 001	10300	-
	MCHIV 002	3780000	-
	MCHIV 003	2920000	-
	MCHIV 004	2220000	-
	MCHIV 005	700000	-
	MCHIV 006	557000	-
	MCHIV 007	127000	-
	MCHIV 008	446300	-
		EBV DNA (copies/mL)	
PLWH with an active EBV infection + a EBV virus-driven malignancy	MCHIV 009	521000	Non-Hodgkin lymphoma
	MCHIV 010	25000000	Non-Hodgkin lymphoma
	MCHIV 011	494	Hodgkin lymphoma
	MCHIV 012	567	Non-Hodgkin lymphoma
		HPV subtypes	
PLWH with an active HPV infection + a HPV virus-driven malignancy	MCHIV 013	16, 18, 39, 45, 51, 58 (high risk); 6, 44, 53 (low risk)	Anal intraepithelial neoplasia (AIN) grade 2
	MCHIV 014	16	Oral cavity cancer
	MCHIV 015	33, 58, 68 (high risk); 42, 53, 70 (low risk)	AIN 2
	MCHIV 016	18, 31, 35, 53, 68 (high risk)	AIN 1
	MCHIV 017	16, 31, 53, (high risk)	AIN 2

Table 5. Active coinfections and virus-driven malignancies stratified for each group (EVASION study).

ID	HCV antibody	EBV serology	HPV subtypes
L003	Negative	-	-
L004	Negative	-	18 (high risk), 44 (low risk)
L005	Negative	-	-
L006	Negative	IgG positive, IgM negative	Negative
L007	Negative	IgG positive, IgM negative	-
L008	Negative	-	Negative
L009	Negative	IgG positive, IgM negative	Negative
L010	Negative	IgG positive, IgM negative	16, 45 (high risk)
L011	Negative	-	31, 35, 52, 58, 68 (high risk), 44, 53 (low risk)
L012	Negative	-	16, 35, 45, 51, 58, 73 (high risk), 42, 44, 53 (low risk)
L013	Negative	-	33, 35, 39, 52, 59, 68 (high risk), 6, 40, 54, 61, 70 (low risk)

Table 6. HCV antibody, EBV serology and HPV subtype characterization in the control group (EVASION study).

4.2.2. *CHARACTERISTICS OF LYMPHOCYTIC SUBPOPULATIONS*

We characterized lymphocytic subpopulations in PLWH with viral coinfections +/- virus-driven malignancies. Data from this group were compared with PLWH without any coinfection, 2 patients with HCV coinfection and 24 healthy donors (HD).

A significant difference in the average percentage of CD4 T cells in EVASION participants with HCV (17.0% vs. 34.6%, p<0.01) and HPV coinfections (5.4% vs. 34.6%, p<0.5) and also in people with HCV infection (13.0% vs. 34.6%, p<0.0001) in comparison with PLWH without any coinfection; moreover, a smaller average of CD4/CD8 ratio was seen in PLWH with HCV (0.9 vs. 1.4, p<0.01) and EBV coinfections (0.9 vs. 1.4, p<0.05) vs PLWH included in the control group, as shown in Figure 7.

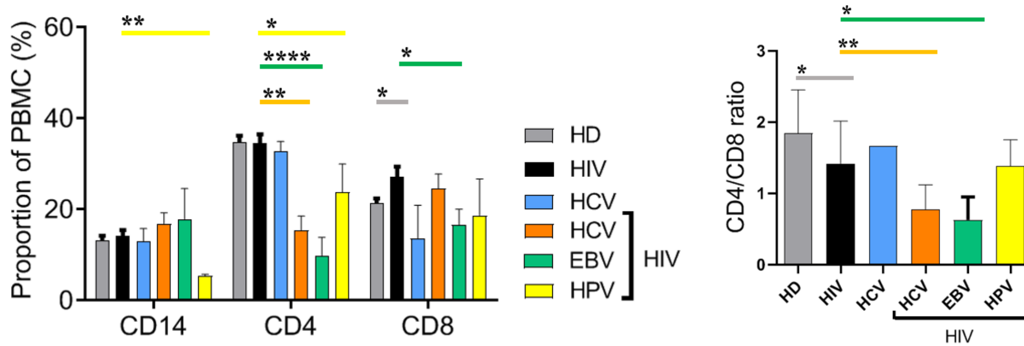


Figure 9. Percentage of CD14, CD4 and CD8 in PLWH with viral coinfections (HCV, EBV or HPV), PLWH without any coinfection, people with HCV infection and healthy donors. Legend: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

No statistical difference emerged in the average of percentage of T regulatory cells among different groups [PLWH with viral coinfections (HCV: 7.1%, EBV:10.0% or HPV: 5.9%), PLWH without any coinfection: 8.6%, people with HCV infection: 6.9% and healthy donors: 9.7%], as reported in Figure 8. As depicted below, a reduction on the average of naïve T cells was observed in PLWH with coinfections, especially in those with HCV coinfection compared with PLWH without infections (21.1% vs. 39.3% p=0.03).

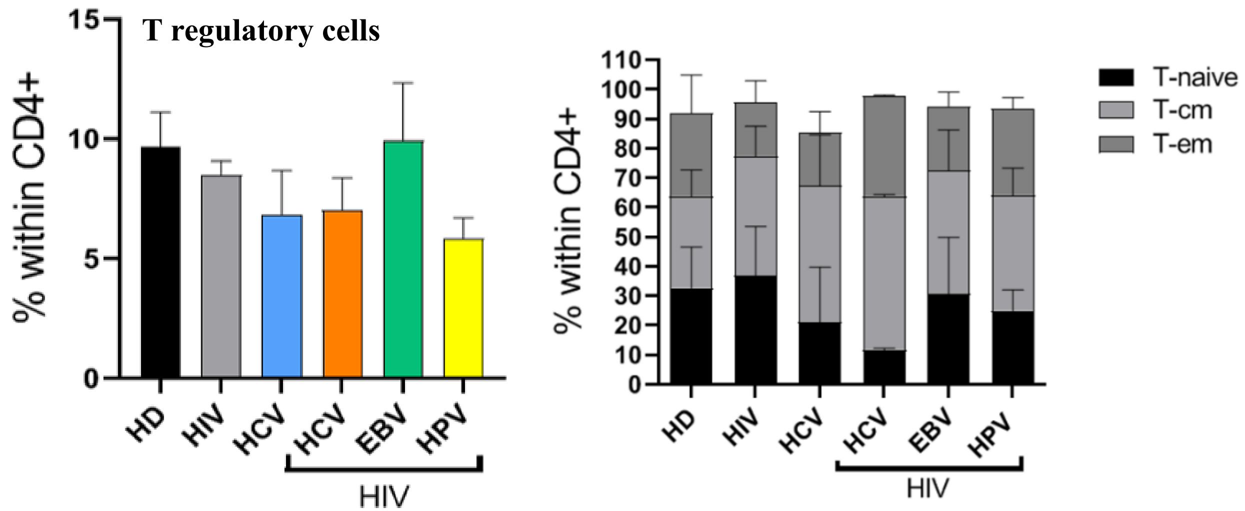


Figure 10. Percentage of T reg, T naive, Tcm and Tem within CD4 cells.

4.2.3. EVALUATION OF HIV RESERVOIR BY IPDA

To quantify intact and defective HIV proviral copies in CD4 T cells, peripheral CD4 T cells were purified to determine the intact proviruses frequency over time using two different approaches based on ddPCR (IPDA) and quantitative PCR assays (quadruplex PCR, q4PCR). Both assays can distinguish and quantify defective and intact copies of the HIV provirus. These assays will be performed at the time of enrolment and every six months to evaluate variation in the size of the reservoir and in the relative contribution of intact and defective copies.

Up to date IPDA assay has been performed at the enrolment time point. No significant difference in the size of the intact and defective proviruses was observed between two groups: median values of intact HIV DNA were 62.1 (14.5-138.3) and 43.7 (7.5-134.8) copies/10^6 CD4 T cells among people with and without a viral coinfections/virus-driven malignancy, while 5' deleted proviruses were 243.9 (86.9-652.0) and 257.9 (116.3-401.2) copies/10^6 CD4 T cells and 3' deleted proviruses 299.4 (95.5-535.9) and 521.4 (20.6-677.8) copies/10^6 CD4 T cells, respectively.

As there was no difference between groups, means are reported in Figure 9; mean intact HIV DNA was 90.9 (SD ±109.2) copies/10^6 CD4 T cells, while 3' and 5' deleted proviruses were 489.3 (SD ±541.8) and 461.6 (SD ±530.92) copies/10^6 CD4 T cells among PLWH with viral coinfections; mean values in the control group were 111.9 (SD ±465.5), 526.0 (SD ±790.4.) and 602.7 (SD ±830.5) copies/10^6 CD4 T cells, respectively, as illustrated in Figure 9. as depicted in Figure 9. Moreover, 2 participants with HCV coinfection, 1 with EBV-driven lymphoma and 2 without any coinfection had zero copies/10^6 CD4 T cells; no mutations in env and pol regions emerged from genotype resistance testing available (Sanger sequencing) performed before starting ART.

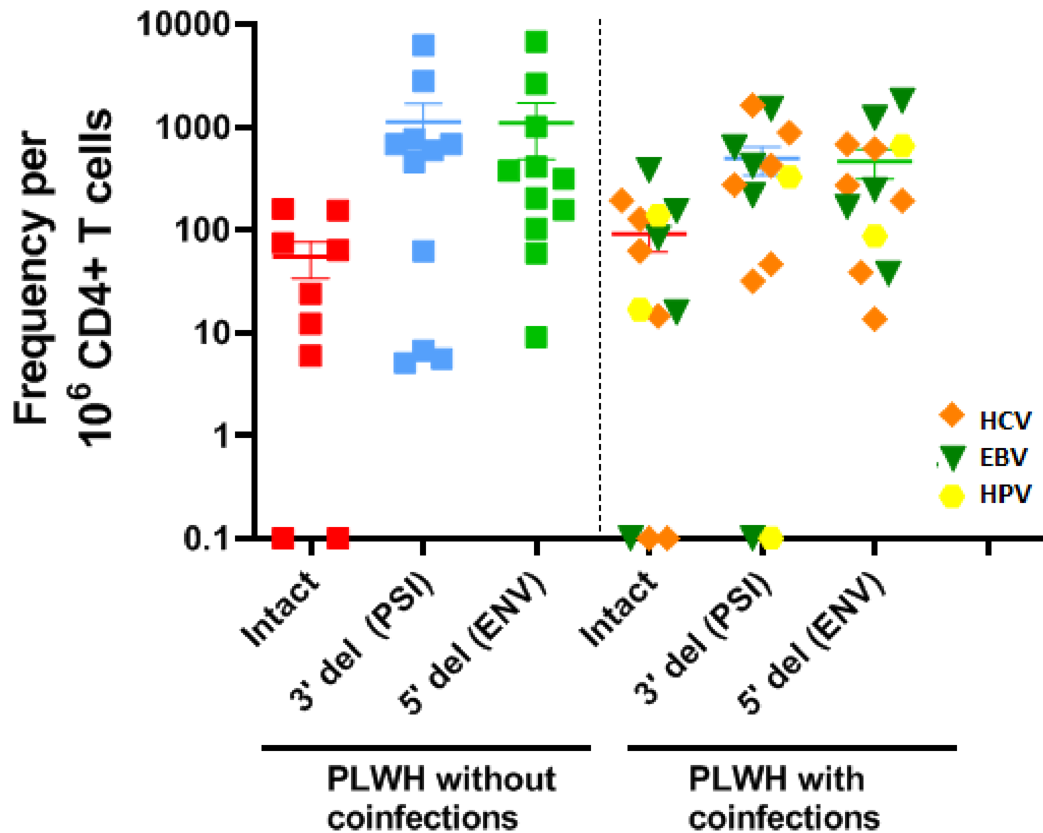


Figure 11. HIV DNA copies/10^6 CD4 T cells as intact and defective proviruses with deletion at 5' (ENV) or at 3' (PSI) among PLWH with (rhombus, triangle and circle) and without viral coinfections (square). Horizontal black line indicate mean values, with standard deviation.

4.2.4. *Identification of HCV-, EBV and HPV- specific CD4 T cells containing replication competent proviruses*

Viral outgrowth assay will be performed on purified CD4 T cells collected from PBMCs depleted from CD8. CD4 T cells will be cultured in presence of patient-specific antigen presenting cells (APCs) loaded with specific viral antigen (HCV, EBV and HPV). In each culture, the presence of replication competent virus will be evaluated by aseessing p24-production in the supernatant by ELISA assay. Only from cultures that resulted positive for p24 expression, we will:

1. quantify the complexity of the viral populations by performing and sequencing HIV-1 env specific libraries. Indeed, env has the most sequence diversity in HIV genome, resulting highly genetically diverse even within a single host. Thus by performing the complexity of the viral variant populations.
2. quantify the frequency and specificity of antigen-responding clones by performing T-cell receptor (TCR) sequencing. T cells express unique antigen receptors produced by random assembly of TCR variable, diversity and joining gene segment, thus T cells with identical TCRs are produced by clonal expansion/proliferation of the same T cell clone. Hence, by performing TCRß sequencing we will use the VDJ rearrangements as T cell barcodes to identify how many different T cell clones will be activated in culture following the viral antigen stimulation.
3. characterize the overall number of HIV infected cells by retrieving viral integration sites (IS) and their relative level of abundance. HIV viral integration sites can be used as distinctive label of each infected cell clone and its progeny, so that different infected cells will harbor HIV viral integration in different position of the genome. Thus, by using specific and sonication-based PCR protocols, we will be able to recover viral integration sites in p24-positive wells. This approach will allow us to identify and quantify how many different T cell clones harboring an integrated copy of HIV are present and eventually expanded in culture.

These combined molecular approaches will allow the detection and eventually estimate the frequency of antigen-specific T cell clones that harbor a replication-competent virus.

To identify and quantify HIV quasi-species from viral (HCV-, EBV-, HPV-) specific CD4 T cells containing replication-competent proviruses, we performed pilot in vitro experiments to design a cellular assay for the detection and expansion of HCV-specific CD4 T cells from peripheral blood samples of PLWH.

Since virus-specific T cells may acquire an exhausted phenotype and lack effector functions during chronic viral infections (82,83), we first tested whether viral antigen-specific T cell proliferation could be detected upon in vitro stimulation of PBMCs from a HIV-negative patient with HCV infection and a healthy donor. To this aim, CD8-depleted PBMCs (responder cells) were cultured with autologous APCs ± HCV peptide pool for 6 days. Polyclonal stimulation (autologous APC + PHA) was used as positive control for T cell proliferation and vitality. We compared results with those obtained upon culture in the presence of anti-IL10R/TGFβ mAbs, to counter-act the inhibitory effect of IL10 and TGFβ, molecules known to contribute to the inhibition of T cell responses during chronic infections (82,83). After 6 days of stimulation, we did not observe any HCV-specific response in the HCV+/HIV- patient, while polyclonal stimulation determined proliferation of memory CD4 T cells and secretion of IFN-γ in the cell culture supernatants both in the HCV+/HIV- patient and healthy donor (Figure 10). Blockade of IL10 and TGFβ signaling did not restore detectable HCV-specific response, suggesting that lack of response to viral antigens was not dependent on inhibitory molecules, but likely on the low frequency of viral antigen-specific CD4 T cells in the starting population.

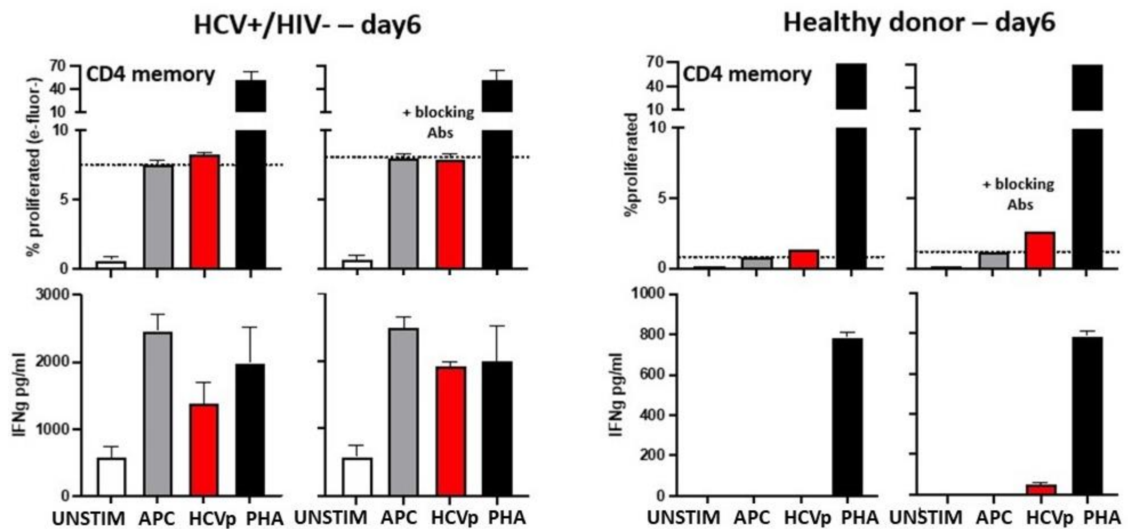


Figure 12. T cell proliferation (upper panels) and IFN-γ production (lower panels) after 6 days of in vitro culture in the presence of the indicated stimuli in a HCV+/HIV-patient and a healthy donor, with or without anti-IL10R/TGFβ mAbs.

Therefore, we prolonged T cell culture up to 14 days and performed a second round of stimulation, in the absence of blocking agents (Figure 11).

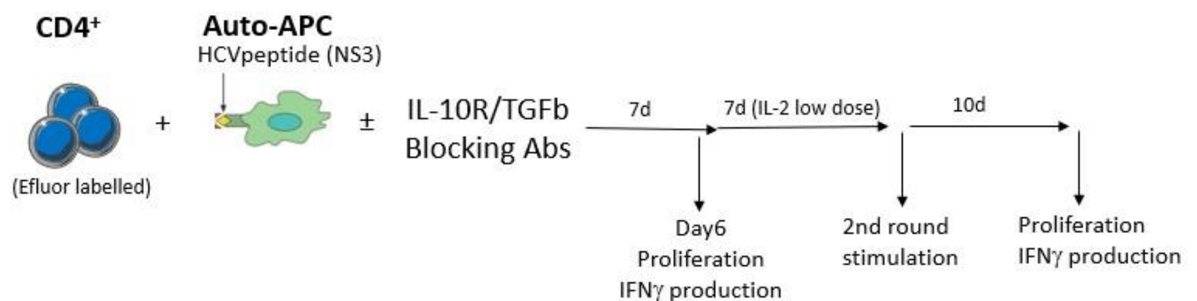


Figure 13. Experimental scheme for the evaluation of viral Ag-specific T cell response in HCV+/HIV- patients vs. healthy donors.

In vitro rechallenge of the resulting HIV-/HCV+ patient-derived T cell line with autologous APCs pulsed with HCV-peptides induced IFNγ-production and T cell proliferation (measured as frequency of cells expressing the ki67 proliferation marker), while HCV-specific T cell response was undetectable in healthy donor-derived primary T cell line (Figure 12).

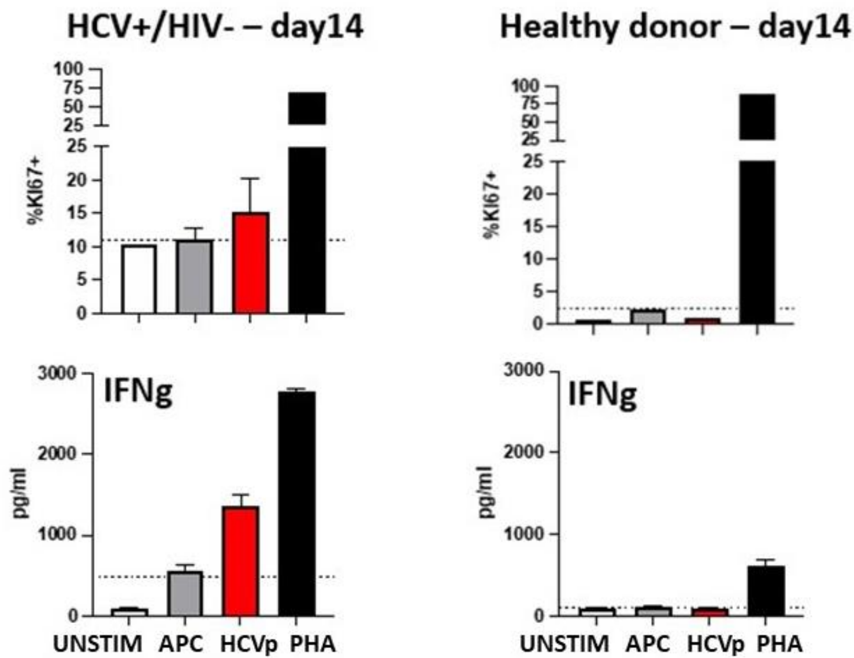


Figure 14. HCV-specific response after two rounds of stimulation with HCV-derived peptide pulsed APCs. T cells from a HCV+/HIV-patient and a healthy donor were expanded in vitro in the presence of autologous APC pulsed with HCV-derived peptides. After 14 days T cells were re-challenged in vitro for 3 days with the indicated stimuli: proliferation (upper panels) was evaluated by flow cytometry based on ki-67 expression; IFN-γ production (lower panels) was measured by ELISA.

We applied the described protocol of viral antigen-specific T cell in vitro expansion (Figure 10) to CD8-depleted PBMCs of 6 study participants (1 with HCV mono-infection and 5 with HCV/HIV coinfection). Upon in vitro re-challenge of T cell lines, HCV-specific T cell proliferation was observed in 3 out of 5 study participants (HCV+/HIV+, MCHIV 03-04-010) and confirmed in the HCV+/HIV- control (Figure 13 and 14). In most of the responders, increased proliferation in response to HCV peptides was accompanied by increased release of IFN-γ in cell culture supernatants (Figure 13). Polyclonal stimulation (PHA) served as positive control for T cell response: lack of proliferation by MCHIV03-04 T cell lines upon PHA stimulation was likely due to premature activation-induced cell death at re-challenge, as suggested by consistent IFN-γ release in cell culture supernatants.

Overall, these findings show that HCV-specific T cells are present in the peripheral blood of HCV+ HIV-infected patients and that these cells can be expanded in vitro upon stimulation with HCV-derived peptides. On the other hand, after in vitro expansion we found low frequency of HCV-specific T cells in the resulting T cell lines (<20%), with high variability among different patients and lack of antigen-specific T cell enrichment in 2 out of 6 T cell lines.

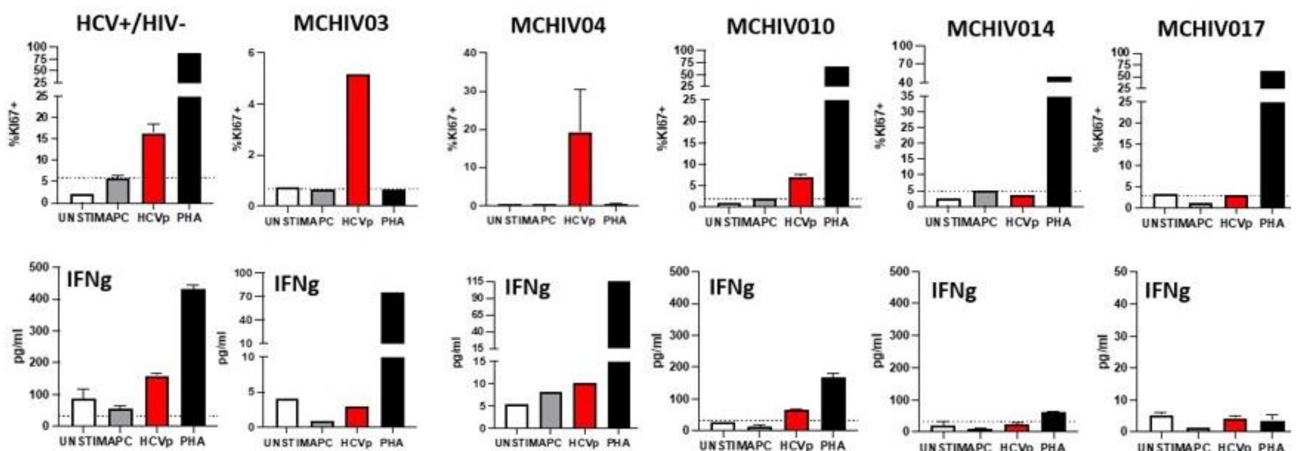


Figure 15. A) Experimental scheme for the evaluation of viral Ag-specific T cell response in study participants. B) T cells from 5 HIV patients with HCV co-infections and 1 control HCV+/HIV-patient a were expanded in vitro in the presence of autologous APC pulsed with HCV-derived peptides. After 10 days T cells were re-challenged in vitro for 3 days with the indicated stimuli: proliferation (upper panels) was evaluated by flow cytometry based on ki-67 expression; IFN-γ production (lower panels) was measured by ELISA.

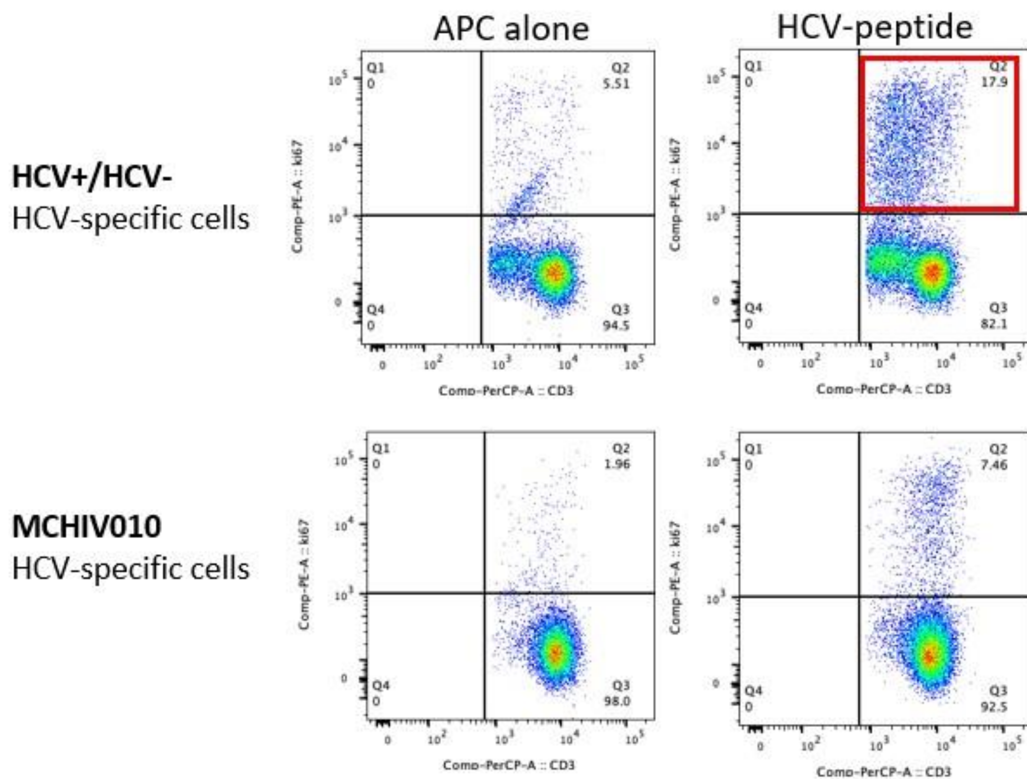


Figure 16. T cell proliferation upon in vitro re-challenge with autologous APCs/HCV-derived peptides. CD4 T cells were expanded in the presence of autologous APC pulsed with HCV-derived peptides. T cell proliferation was measured by flow cytometry based on expression of the proliferation marker ki-67 after 3 days of in vitro re-challenge with autologous APCs alone (left panels) or pulsed with HCV-derived peptides (right panels). Representative plots of HCV-specific cell proliferation in n=1 patient with HCV mono-infection and n=1 patient with HCV/HIV coinfection (MCHIV010) are shown.

Cellular death observed in MCHIV03 and MCHIV04 might have been secondary to the prolonged time of cell culture, the reactivation/new production of HIV virions or cytotoxic effects of HIV proteins. To prevent new infection events, we will further include in culture enfuvirtide, an antiretroviral drug that inhibit the final stage of HIV fusion with the target cell.

To evaluate whether expansion of HCV-specific T cells correlated with reactivation/new production of HIV virions, the presence of replication-competent virus was evaluated by measuring p24-production by ELISA in the cell culture supernatants of primary (day 7, 10 and 14) and secondary stimulation (day 7 and 10) (Figure 10): none of the participants'

samples showed detectable production of p24. Given the low sensitivity of p24 ELISA assay, it cannot be excluded that replication-competent HIV is present in few of the expanded T cells clones. We therefore planned quantification of HIV RNA in these cell culture supernatants.

To address the high variability of findings and the overall low frequency of antigen-specific T cells obtained at the end of our T cell cultures, we tested alternative in vitro assays for the detection of antigen-specific T cell responses. For direct ex vivo characterization of human antigen-specific CD4 T cell response we applied the previously described antigen-specific T cell enrichment (ARTE) assay. This assay allows enrichment of antigen-specific CD4 T cells prior to in vitro expansion, based on cell sorting of CD154 (CD40L) expressing T cells after short-term in vitro exposure to the cognate antigen (84). The verify whether this assay could be efficiently applied to cryopreserved material previously collected from study participants, we performed a pilot experiment using CD8-depleted cryopreserved PBMC from a patient with HIV infection (L012). Briefly, cells were stimulated with a pool of MHC class II-restricted peptides from Cytomegalovirus, Epstein-Barr virus, Influenza virus, and Tetanus toxin (CEFT pool) for 6 hours after overnight resting and, as alternative protocol, overnight after 6 hours resting. As control, cells were left unstimulated or polyclonally stimulated with anti-CD23/28 beads. While polyclonal stimulation induced strong upregulation of CD154, no significant difference in CD154 expression was detected after exposure of T cells to the selected antigens, likely due to high basal expression of CD154 after T cell thawing. Therefore, we did not proceed to analysis and enrichment of cryopreserved samples from our study cohort, but rather opted to apply this technique to fresh ex vivo samples from future patients. As a consequence, this part of the study remains preliminary and will be concluded in the upcoming months.

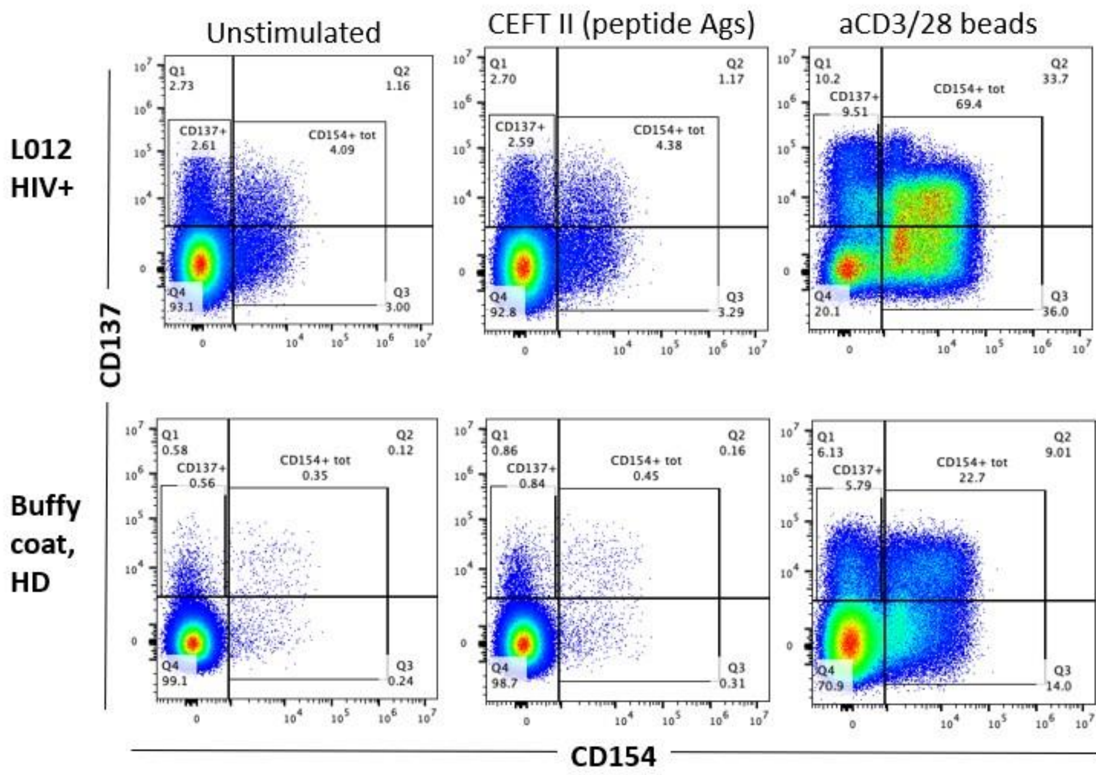


Figure 17. Antigen-specific response stimulating for 6 hours after overnight resting

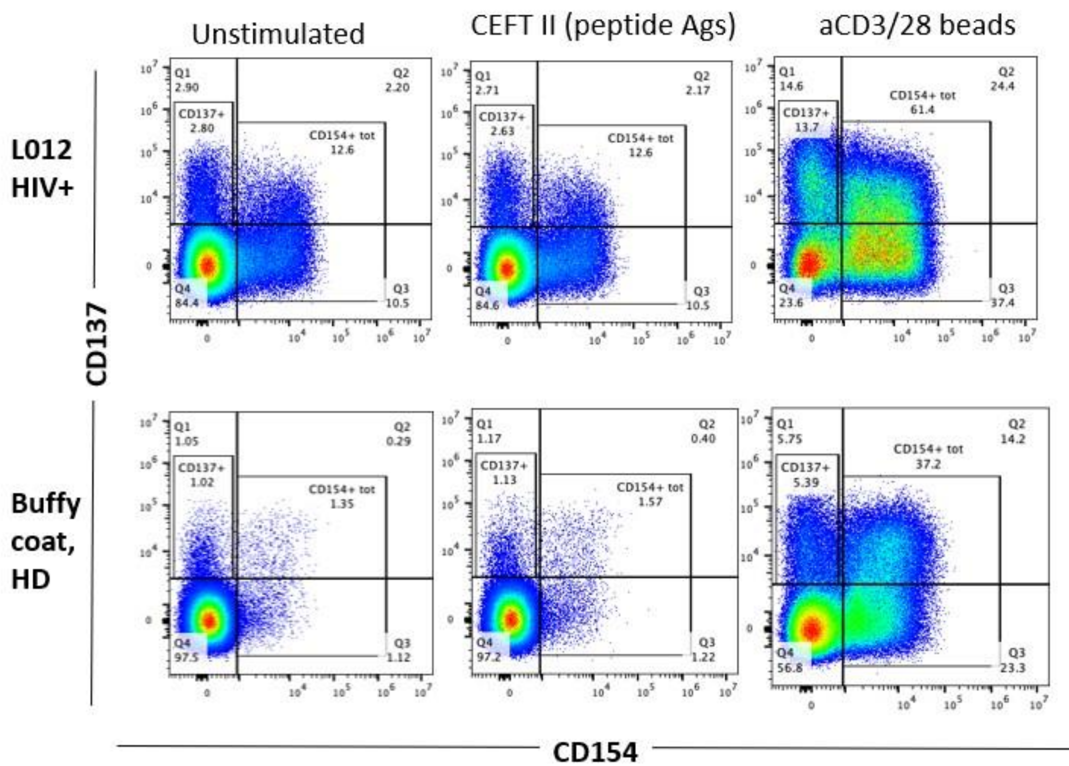


Figure 18. Antigen-specific response stimulating overnight after 6 hours resting.

FURTHER ANALYSIS: TO DETECT HIV IN TUMOUR TISSUE

Given the increased understanding of the important role that tumoral tissues (liver for HCV-driven hepatocarcinoma, lymph nodes for EBV-driven lymphoma, tissues from anal, genital or oropharyngeal tract for HPV-driven cancers) play in HIV persistence, we need more detailed analyses of HIV reservoirs in anatomical "sanctuary sites". Here we want to evaluate if an enrichment of HIV RNA and HIV DNA is occurring in CD4 T cell infiltrating the tumoral tissues induced by the presence a specific co-infective agents.

For this reason, HIV RNA and HIV DNA will be evaluated by a highly specific in situ hybridization (ISH) assay with single-copy sensitivity (DNA and RNA scope) on frozen and formalin-fixed paraffin-embedded-(FFPE) tumoral tissue (HCV-driven hepatocarcinoma, EBV-driven lymphoma, HPV-driven cancers).

Laser capture microdissection (LCM) will be applied to isolate tissue sections resulted positive at RNA/DNA scope assay. From such tissue samples single genome sequencing (env region) will be performed to investigate how many HIV viral variants are present and express at the tumoral site (41). HIV DNA and HIV RNA scope assays will be performed on malignant-derived tissue biopsies (i.e. EBV-driven lymphoma and hepatocellular carcinoma) to detect HIV presence and expression in T cell clones at the reactivation site of the viral coinfection.

5. DISCUSSION

Data discussed in the results section have been obtained over a 3-year PhD program. Due to the COVID-19 pandemic, I had to work full-time in the Infectious Diseases Unit of IRCSS San Raffaele Hospital as a Infectious Diseases specialist. For this reason, both the enrolment of participants and the analyses have been delayed and some experiments are still ongoing.

HIV reservoir is recognized as the major barrier for achieving viral eradication.
Therefore, research has currently focused its attention on exploring, identifying and better describing the mechanisms at the basis of HIV persistence.
Considering that most of the clinical studies aimed at characterizing HIV reservoir are conducted on people with AHI and without comorbidities, our study has included specific populations that usually are not involved in these investigations, such as people with an MDR virus or viral coinfections/virus-driven malignancies.

5.1. HIV RESERVOIR IN PEOPLE WITH A MULTI-DRUG RESISTANT VIRUS

The first part of my PhD thesis aimed to characterize HIV reservoir in people with a virus resistant to the four main antiretroviral classes, previously enrolled in the PRESTIGIO registry.

In this group of patients, a lower percentage of naïve CD4 T cells was observed when compared to PLWH without MDR virus. Loss of naïve CD4 T cells is linked to disease progression (131), and indeed within the group of people with a MDR virus there is a higher proportion of individuals with a history of AIDS and a lower CD4 nadir.

Since recent works have proved that drug-resistant viruses are capable of ex vivo outgrowth just like wild-type viruses and being responsible of viral rebound in vivo at treatment interruption (121), we wanted to quantify replication-competent proviruses in participants enrolled in the PRESTIGIO registry.
Our analyses revealed that the overall levels of intact proviral HIV DNA were similar in PLWH and were not affected by the presence of MDR virus. No differences in median

values of defective proviruses were observed between people with and without MDR virus. Although it has been demonstrated that a smaller size of HIV reservoir is associated with an early treatment initiation in the first phases of infection (122,123), participants from the PRESTIGIO registry had a longer exposure to HIV and ART and fewer years with HIV RNA <50 copies/mL compared with the control group.
Consistently with previous studies (124,125), levels of intact HIV DNA among people with a MDR virus who initiated ART during HIV chronic infection were similar to those described in individuals with AHI (126); nonetheless, we did not evaluate the presence of replication-competent proviruses in tissues and therefore we cannot assume a low viral reservoir in other anatomical compartments.

Moreover, we reported zero copies/10^6 CD4 T cells of intact provirus in 6/14 PLWH with a MDR strain and in 5/21 in PLWH included in the control group. A study from a large cohort including 400 PLWH treated with ART showed that in 6.3% of participants evaluated a failure in the amplicon signal was reported, leading to undetectable IPDA results and demonstrating that this issue was secondary to polymorphisms that were stable over time in people under ART (81).
Although the majority of the participants enrolled had a documented subtype B infection and IPDA has been only validated for this subtype, we could not exclude that these findings were secondary to viral polymorphisms, that may cause the failure of this ddPCR-based assay (127).
Indeed, some mutations in the env probe regions used for IPDA were observed in 5 PRESTIGIO participants. Even if the presence of such polymorphism did not cause any significant difference in the quantification of intact proviruses levels between participants with and without mutations in the env probes region, it is reasonable that they may cause an underestimation of the level of intact HIV DNA copies in subjects harboring a virus with these mutations.
Up to now, Sanger sequencing approach on RNA samples were adopted for identifying HIV drug resistances; however, this approach does not allow the detection of HIV variants presence with a frequency <20% (128). In contrast, next-generation sequencing (NGS) protocols identify HIV genomic variants with higher sensitivity, thus improving the detection of drug resistance mutations present under the 20% threshold (129). Among

20 subjects enrolled in the PRESTIGIO registry (not included in our analysis), 255 resistant variants were detected using NGS, of which 182 were already present in historical genotypic resistance testing. Therefore, 73 (28.3%) additional variants were found by NGS (130).

To overcome the impact of polymorphisms on IPDA results, patient-specific primers and probes for the assay can be designed and set up an assay that includes different HIV regions. During my abroad experience in the course of the PhD program in the laboratory of Professor Linos Vandekerckhove (HIV Cure Research Center, University of Ghent), I helped to validate a 5-plex IPDA combining env, pol, gag, psi and ru5 (data not yet published) that could be helpful in this setting of patients, allowing to select at least 2 regions free of these polymorphisms to estimate the dimension of HIV reservoir in these patients.

Up to now, we are the first team studying HIV reservoir in people with a MDR virus. Despite the small sample size and the need to set up methodologies addressed to participants with HIV strains harboring mutations, these data represent an initial effort to characterize the mechanisms underlying HIV persistence in a perspective of a HIV cure, also in people with limited antiretroviral treatment options available.
Further analyses are needed to better understand the reservoir dynamics in this population, even in different anatomical sanctuary sites.

5.2. HIV RESERVOIR IN PEOPLE WITH VIRAL COINFECTIONS AND VIRUS-DRIVEN MALIGNANCIES

The hypothesis of our study, named EVASION, is that chronic and intermittent exposure to viral antigens may account for antigen-driven clonal expansion in vivo leading to the reported waxing and waning of individual clones of latently infected CD4 T cells, thus favoring the increase and persistence of viral reservoir over time. In the EVASION study, participants with active coinfections and malignancy-promoting viruses have been enrolled to better understand and characterize viral reservoir dynamics. We expect that

PLWH with viral coinfections will have higher levels of intact and defective proviruses, and higher frequency of clonal expanded infected cells when compared to PLWH without any viral coinfections. This study may provide insights into some previously unclear clinical scenarios, such as the onset of viral blips and predominant plasma clones despite good adherence to ART, considering that viral load can be sporadically detected in absence of drug resistance mutations.

Lower CD4 T cells and CD4/CD8 ratio were reported in PLWH with HCV coinfection, as previously described both in long-term non-progressors and in people under ART (85,86) mainly during the first years of coinfection. However, a reduced proportion of naïve T cells was observed in people with HCV mono-infection (85): larger studies are needed to evaluate the impact of coinfections on immune exhaustion in PLWH.

We firstly quantified intact proviruses by IPDA in people enrolled in the EVASION study and in the control group without viral coinfections and malignancies.
No significant differences were seen in the replication-competent reservoir size between PLWH with and without active viral coinfections, even when compare different study groups (people with HCV, EBV and HPV coinfections) separately.
All the participants analyzed were under ART since a median time of 15 years (study group) and 7 years (control group) and on virological suppression for at least 6 months.
Data on the amount of replication-competent proviruses described in our study are consistent with findings previously reported in people with undetectable viremia. In line with recent works that quantified HIV reservoir using IPDA in individuals with HIV chronic infection, we described a greater amount of defective forms in comparison to intact proviruses (126). On the other hand, people treated during AHI have a greater proportion of intact HIV DNA than those who start antiretroviral treatment in the course of chronic infection (133,134).

Nonetheless, our data are in contrast with findings from Lopez-Huertas et al. showing that proviral HIV DNA was significantly increased in people with HIV/HCV coinfection compared to PLWH (87). However, the assay used to quantify the viral reservoir was

different (Alu-LTR PCR) and did not distinguish between defective and intact proviral copies, overestimating the size of HIV reservoir.
Moreover, as mentioned before, IPDA may have some limitations.
Since no major differences were observed among people with and without viral coinfections, we may test the quantitative viral outgrowth assay (qVOA), that can functionally estimate the frequency of CD4 T cells infected with a replication-competent virus (23).
In the EVASION study, although no variation in the HIV reservoir has been detected yet, we will consider performing IPDA in participants with viral coinfection or virus-driven malignancy after receiving the specific treatment, due to the dynamic nature of the latent reservoir.

Furthermore, specific assay may reveal and quantify the presence of the viral reservoir in antigen specific cells; for this reason, we set up in vitro an antigen-specific cell assay to promote the proliferation/activation of antigen-specific T cells and assess the production of HIV particles. Although we are still developing the antigen-specific cell assay, findings from recent works investigating viral antigen-driven proliferation encouraged us to study in detail mechanisms underlying HIV persistence.

Mendoza et al. showed that CD4 T cells that contain clones of replication-competent viruses respond to antigenic stimulation with viral peptides acausing recurrent or chronic infections (101). Similarly, Simonetti et al by combining provirus, integration site, and TCRβ analyses identified many persistent CMV-specific clones carrying replication-competent proviruses, highlighting antigen-driven proliferation as major force in favoring the expansion and persistence of HIV reservoir (93,100). In both cases, authors described a great variability across patients enrolled in the distribution and amount of antigen-specific clones that carry HIV genomes. Moreover, a significant heterogeneity was also described in the recovery of replication-competent virus from antigen-specific cells (100). Finally, it has been shown that high-level of EBV DNA and CMV in peripheral blood cells are associated with a greater of HIV DNA diversity and an increase in HIV proviral integration (114,135). These findings suggest that targeting the replication of EBV and CMV could be an approach to decrease the size of viral reservoir in people receiving ART (135).

EVASION study may be a great opportunity to analyze people with viral coinfections and virus-driven malignancies likely exposed to relevant antigen stimulation, by both characterizing and quantifying viral reservoir by molecular, cellular and HIV integration studies, as demonstrated by the fact that this project has been awarded by "Bando della Ricerca Finalizzata - Giovani Ricercatori (GR-2021-12375370)",

Several questions still remain unsolved and will be the object of future studies. Antigen-driven proliferation can be responsible for a clonal expansion that persists for long time and cause virological rebound, making the pathway towards HIV eradication more challenging.

In conclusion, studying the mechanisms leading to HIV persistence is crucial for planning and developing innovative and effective HIV cure strategies. Findings from this study may highlight the importance of a prompt management of co-infections and virus-driven malignancies to control persistent HIV-1 replication induced by viral and tumor antigen stimulation.

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