

UNIVERSITÉ CATHOLIQUE DE LOUVAIN

SECTEUR DES SCIENCES DE LA SANTÉ

Faculté de pharmacie et des sciences biomédicales

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**USEFULNESS OF PROVIRAL DNA OF HUMAN
IMMUNODEFICIENCY VIRUS TYPE 1 IN
AIDS TREATMENT DECISIONS**

KABAMBA MUKADI Benoît

Promoteur: Pr. Patrick GOUBAU

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LIST OF ABBREVIATIONS

- Ab	antibodies
- Ag	antigen
- AIDS	acquired immunodeficiency syndrome
- Alu	most abundant mobile elements in the human genome.
- APOBEC3G	apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
- ART	antiretroviral therapy
- ARV	antiretroviral
- ATV	atazanavir, Reyataz®
- AUC	area under the curve
- AZT or ZDV	zidovudine, Retrovir®
- Asp-COO-	aspartic acid terminus
- CCR5	CC-chemokine receptor 5
- CD4	cluster of differentiation 4
- CDC	Centers for Disease Control and Prevention
- CNS	central nervous system
- CO-NH	peptide bond or amide bond
- CRF	circulating recombinant forms
- CSF	cerebrospinal fluid
- CXCR4	CXC-chemokine receptor 4
- CYP	Cytochrome P
- D1 to D4	4 immunoglobulin-like domains of CD4
- DHHS	US Department of Health and Human services
- DLV	delavurdine, Rescriptor®
- DNA	deoxyribonucleic acid
- DRV	darunavir, Prezista®
- dsDNA	double-stranded DNA
- EACS	The European AIDS Clinical Society
- eCDC	European Centre of Disease Prevention and Control
- ELISA	Enzyme linked immunosorbent assay
- <i>env</i>	envelope gene
- Env	envelope protein
- ETV	etravirine, Intelence®
- <i>gag</i>	group antigen gene
- FRC	reticular cell

- gp120	surface glycoprotein, 120kD
- gp41	surface glycoprotein, 41kD
- H ₂ O	molecule of water composed of Hydrogen and Oxygen
- HAART	highly active antiretroviral treatment
- HAV	hepatitis A
- HBV	hepatitis B
- HCV	hepatitis C
- HR	heptad repeat region of gp41
- HIV	human immunodeficiency virus
- HLA	human leukocyte antigen
- HPCs	hematopoietic progenitor cells
- EFV	efavirenz, Stocrin®
- EIA	immunoassays
- IC ₅₀	50% inhibitory concentration
- IN	integrase
- INI	integrase inhibitors
- IVDU	intravenous drug users
- kD or kDa	kiloDalton
- LAV	Lymphadenopathy associated virus
- LTR	long terminal repeat
- MA	matrix protein
- MAC	Mycobacterium avium complex
- MCT	mother-to-child
- MEIA	microparticle enzyme immunoassay
- mRNA	messenger RNA
- MHC	major histocompatibility complex
- N36	amino-terminal 36-amino acid fragment derived from gp41 HR1
- <i>Nef</i>	negative regulatory factor gene
- Nef	negative regulatory factor
- NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
- NNRTI	non-nucleoside reverse transcriptase inhibitor
- NRTI	nucleoside reverse transcriptase inhibitor
- NVP	Nevirapine, Viramune®
- 3'-OH	3'-hydroxyl terminus of DNA
- PBMC	peripheral blood mononuclear cell

- PBS	primer binding site
- PCP	<i>Pneumocystis pneumonia</i>
- PCR	polymerase chain reaction
- PEP	Post-exposure prophylaxis
- PHI	primary HIV-1 infection
- PI	protease inhibitor
- PIC	pre-integration complex
- PML	progressive multifocal leukoencephalopathy
- PNV	predictive negative value
- <i>Pol</i>	polymerase gene
- PPV	predictive positive value
- PR	protease
- <i>Rev</i>	regulator of virion expression gene
- Rev	regulator of virion expression
- RNA	ribonucleic acid
- RNase H	ribonuclease H
- RRE	rev response element
- RT	reverse transcriptase
- RTI	reverse transcriptase inhibitors
- RTV	ritonavir, Norvir®
- ssDNA	single-stranded DNA
- SU	subunit
- <i>Tat</i>	trans-activator of transcription gene
- Tat	trans-activator of transcription
- TAR	transactivation response element
- TB	tuberculosis
- TPV	tipranavir, Aptivus®
- tRNA	transfer RNA
- UNAIDS	United Nations Programme on HIV/AIDS
- V1-V5	variable regions 1 to 5
- V3 loop	third variable region of HIV Env
- <i>Vif</i>	virion infectivity factor gene
- Vif	virion infectivity factor
- VL	viral load
- <i>Vpr</i>	viral protein R gene

- Vpr viral protein R
- *Vpu* viral protein U gene
- Vpu viral protein U
- WB Western blot
- WHO World Health Organization
- WT wildtype virus
- YMDD tyrosine, methionine and 2 aspartic acids form the RT catalytic site
- ZDV or AZT zidovudine

SUMMARY

1. INTRODUCTION:

The project aims to establish the value of the quantification and characterization of the HIV-1 provirus. We choose to study the evolution of the provirus in the context of disease and treatment follow-up, including its load and its nucleotide sequence in order to draw a possible prognostic value on long-term therapeutic efficacy. The assumption was that treatment reduces the proviral load and that the emergence of resistance has an impact on the reservoirs and thus on long-term effectiveness.

2. DEVELOPMENT OF THE HIV-1 PROVIRUS DNA QUANTIFICATION

The first objective of the project was to develop a real-time PCR assay to quantify the HIV-1 provirus present in subpopulations of circulating leukocytes, the CD4⁺ cells in comparison to a reference gene, in this case the beta-globin gene. The detection of the amplified DNA was performed with SYBR-Green I. Several primers were synthesized in the highly conserved regions of the viral genome among different subtypes of HIV-1 in order to amplify all strains with equal efficiency. The limit of quantification was 5 copies of provirus by PCR reaction, 50 copies per sample and ranged from 50 to 10⁶ copies. The intra- and inter-assay coefficient of variation calculated on 10 runs ranged from 3.1% to 37.1% for high and low proviral loads, respectively. Quantification of the gene for β -globin was done using the commercial kit "LightCycler-Control Kit DNA" with a limit of quantification of 1.5 pg / μ l (approximately 5 cells) with the intra- and interassay coefficient of variation based on 10 sets of tests ranging from 1.8% to 21%.

The second step consisted in validating this method on some reference strains and clinical samples. Four reference strains of HIV-1 and HIV-2 were obtained from the National Institute for Biological Standards and Control (NIBSC, UK) : a primary isolate of HIV-1 subtype A , the subtype B 8E5 strain, the HIV-1 SE12808 subtype C strain, the HIV-1-ELI subtype D strain, the HIV-2 ROD subtype A strain and the HIV-2 EHO subtype B strain. . The different subtypes of HIV-1 were amplified with the same PCR efficiency while the HIV-2 reference strains were not amplified.

We also tested this pair of primers on 30 samples received for determination of plasma viral load in 10 treatment naïve patients and in 20 individuals who had received antiretroviral therapy. Two samples from patients infected with HIV-2 were included in the study. No statistically significant correlation was found between the DNA proviral load and the plasma viral load or the CD4 + cell count. In the study group, the DNA proviral load was highly

variable among infected patients, regardless of the clinical stage of the disease. Several subtypes of HIV-1 (A, B, C, D, F, ...) from these clinical samples were amplified successfully without recognizing HIV-2.

The assay was also applied to a group of 15 patients undergoing a structured treatment interruption (STI) of at least 2 years. On interruption all patients were on combination therapy of at least 3 anti-HIV drugs including 1 or 2 protease inhibitors. The treatment history showed a mean treatment duration of 5 years and mono- or dual therapy prior to the highly active antiretroviral treatment (HAART). Two years after interruption of treatment, 7 of 15 patients had a plasma viral load <3.3 log copies / ml and 3 had an undetectable viral load. All these 7 patients had a proviral load <2.5 log copies/ 10^6 CD4+ cells, including 4 with undetectable proviral load (<5 DNA copies / PCR). The 8 other patients had a viral load ranging from 3.9 to 4.7 log copies / ml. Three of them had a proviral load <2.5 log copies DNA / 10^6 CD4 + cells while five had a proviral load > 2.5 DNA copies / 10^6 CD4 + cells. We observed a statistically significant correlation (Pearson coeff of 0.578 and an associated p -value of 0.012, Spearman coefficient of 0.545 and an associated p -value of 0.018) between the proviral load and the plasma viral load at steady state. The proviral load and the rebound of plasma viral load were higher in patients who took longer to reach an undetectable viral load after the initiation of the treatment.

3. SEQUENCING OF THE HIV-1 PROVIRAL DNA

We developed a population sequencing assay of the reverse transcriptase and protease genes coding for enzymes targeted by antiretroviral drugs. The nucleotide sequence allows to deduce mutations at the amino acid level known to confer a decrease in viral susceptibility to specific drugs or to a group of drugs.

The error rate of reverse transcriptase (1 mutation per 10,000 bases) due to the absence of the exonuclease 3'5' activity (proof reading) combined with the very high rate of viral replication (10^9 to 10^{10} virions synthesized per day and 10^9 CD4+ T cells regenerated per day) lead to a coexistence of a multitude of variants in a given infected patient, called "quasi-species". An effective anti-HIV-1 therapy is evidenced by an undetectable plasma viral load, but the virus is not eradicated as the provirus remains present in certain "protected areas".

As part of this project, the technique is used to compare amino acid sequences of protease and reverse transcriptase in circulating virions and in provirus. Because of this existence of a "quasi-species", one might see major differences between the sequences of free plasma virus and cell associated provirus. It was also interesting to study the kinetics of emergence of wild sensitive strains in patients with virologic failure. We believe that this aspect of the

characterization of the provirus may have predictive value on the effectiveness of the long-term treatment in patients with undetectable plasma viral load.

The HIV-1 proviral nucleotide sequences from patients enrolled in the third phase of the project, both at the naive stage and after initiation of treatment were prospectively analyzed. The mutations present in the provirus were compared with those of free plasma virus.

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4. FOLLOW UP OF TREATED PATIENTS

The last part of the study consisted in comparing the impact of the regimen with or without protease inhibitors on the provirus. It is known that reverse transcriptase inhibitors act at the first phase of the intracellular viral life cycle by blocking the synthesis of viral DNA, whereas protease inhibitors will act at the level of the infected cell by preventing the maturation process of new virions.

We developed a study protocol in collaboration with the treating clinicians, in order to study the evolution of the proviral load and of the resistance mutations in three groups of patients, the first one receiving combination therapy including a protease inhibitor (PI) associated to nucleoside reverse transcriptase inhibitors (NRTIs), the second group was receiving only reverse transcriptase inhibitors (nucleoside and non-nucleoside reverse transcriptase inhibitors= NNRTI) and the last group comprised untreated patients.

Between May 2002 and July 2007, 69 patients who had not yet received antiretroviral treatment were included in the study. The study was approved by the local ethics committee and informed consent was obtained from each patient. The HIV-1 positive status was confirmed by accepted methods.

The treatment history of all patients was verified. Verification of treatment history can be difficult but might be of importance when studying the resistance mutations in so called naïve patients.

4.1 EVOLUTION OF THE HIV-1 PROVIRAL LOAD

Assuming that residual amounts of provirus represent a part of the viral reservoir, the quantification of the HIV-1 proviral load might permit a better assessment of the long term therapeutic efficacy. It is because of these viral reservoirs that viral production resumes at the end of treatment. Does the quantification of proviral load provide complementary information to that of the plasma viral load in the clinical management of the disease?

Three parameters were monitored before and after initiation of treatment: the proviral load in CD4 + cell, the plasma viral load and the number of CD4 + lymphocytes.

4.1.1 PATIENTS

Of the 69 enrolled patients, 32 patients received a combination of 2 NRTIs + 1 PI, and HIV-1 proviral DNA was successfully amplified for 28 of them. The proviral load was also quantified for 12 patients receiving 2 NRTIs + 1 NNRTI and for 14 of 25 patients who remained naïve.

4.1.2 STATISTICAL ANALYSIS

The impact of treatment on the provirus was assessed by analyzing the mean differences between 3 patient groups before and after treatment. Plasma viral load decreases while the CD4⁺ cell count increases when an effective treatment that controls the viral replication was given.

The Shapiro test was applied to verify normality of distribution. In case of normal distribution, analysis of variance (ANOVA) was performed to compare mean differences between the 3 groups of patients (naïve, NNRTI and PI).

No difference in mean changes in proviral load between the 3 groups of patients was observed. The analysis of the CD4⁺ cell count showed that the mean difference in the number of CD4⁺ cells was statistically different between the NNRTI and PI groups on the one hand and the group of naïve patients on the other hand.

4.2 ANALYSIS OF PROVIRAL DNA SEQUENCES COMPARED TO THE VIRAL RNA

The virus was successfully sequenced for 63 of the 69 persons initially selected, in both plasma and cells. Fifty-eight percent of patients were Europeans and 42% from outside Europe, mainly from Central Africa. Thirty-nine percent of HIV-1 sequences were subtype B. Thirty-two patients received the combination of 2 NRTIs + 1 PI (mostly lopinavir / ritonavir = LPV / r), 12 received 2 NRTIs + 1 NNRTI (mainly efavirenz = EFV) and 25 remained naïve.

The results showed that before treatment, 90 and 66% of detected mutations were respectively present in CD4 cells and plasma. We detected seven key mutations, 4 of them (M184M / V, M184M / I K103K / N M46M / I) were only found in cells. In 40 followed patients, the mutations detected at the naïve stage remained present for at least 1 year after initiation of treatment. New key mutations emerged in the CD4 (M184I, M184M / I and Y188Y / H) during treatment.

This study showed that the proportion of mutations detected in proviral DNA were significantly higher than in plasma. These mutations persisted for at least 1 year, regardless of the type of combination therapy. The pre-existence of resistance mutations did not jeopardize

the effectiveness of treatment when the concerned drug was not included in the scheme. The DNA analysis of HIV-1 might be useful in long-term untreated patients or when viraemia is undetectable

CONCLUSION:

Although the present study has certain limitations, obviously the small number of patients and the heterogeneity of the study population, which need to be taken into account when considering generalization of the results, some interesting findings can be seen at least as directions for further research.

In terms of proviral load, this work did not show a higher impact of certain regimens associating RTIs with or without PIs on the HIV-1 proviral load. The variations were not statistically different between the patient groups, treated or untreated, while the effect of treatment on plasma viral load and CD4+ cell count was obvious. However, the use of the HIV-1 proviral DNA load as a reflection of reservoirs is gaining a new interest in the assessment of new treatment strategies to eradicate HIV infection, which was not subject of this project.

In terms of proviral nucleotide sequencing, analysing the provirus provides information complementary to that of the plasma virus. A significantly higher number of mutations was indeed detected in proviral DNA than in plasma RNA, and these mutations persisted for at least 1 year, regardless of the type of combination therapy. The pre-existence of resistance mutations did not compromise the effectiveness of treatment when the drug in question was not included in the combined therapy. The DNA analysis of HIV-1 might be useful in chronic infections in which wild type virus might overgrow any resistant HIV-1 variant or during simplification of treatment in patients with undetectable viraemia, when treatment needs to be modified because of toxicity or intolerance.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Typical diseases associated with the acquired immunodeficiency syndrome (AIDS) consisting of *Pneumocystis pneumonia* (PCP) and Kaposi sarcoma, unusual at that time, were first described early in 1981 in homosexual men of Los Angeles and New York. This new clinical entity described by Gottlieb [1] comprised extensive mucosal candidiasis and a severe reduction in cellular immunity selectively involving the CD4⁺ T cell and macrophages. The disease was later reported worldwide. Cases started to be seen in heterosexuals, drug addicts, and people who received blood transfusions, proving the syndrome knew no boundaries. In 1983, Françoise Barré-Sinoussi from Luc Montagnier's group at the Pasteur Institute in France isolated a retrovirus (Lymphadenopathy associated virus or LAV) that they believed was related to the immunodeficiency epidemic [2]. Controversy came when Dr. Robert Gallo too claimed that he isolated a retrovirus called HTLV-III, responsible for immunodeficiency outbreaks [3]. Both viruses proved to be identical and an international committee of scientists renamed the virus "Human Immunodeficiency Virus" (HIV) in 1984. The initial description of HIV-2 was reported by Clavel in 1986 [4]. This was the beginning of what has become the biggest health care concern in modern history, with HIV-1 being the major cause of AIDS. Thirty years later the disease still plagues society.

1.2 THE HIV EPIDEMIC

According to the report from the Joint United Nations Programme on HIV/AIDS (UNAIDS) [5], an estimated 33,3 million people were living with HIV in 2009 (Table 1). UNAIDS reported 2.6 million new HIV infections and 1,8 million AIDS-related deaths in that year. The number of new infections has fallen by 19% as compared to 1999 considered as the peak of the epidemic. After decades of increasing mortality, the annual number of AIDS deaths globally has declined in part as a result of the greater access to treatment. Nearly 5,2 million people were receiving antiretroviral treatment in low- and middle-income countries at the end of 2009. The number of people receiving therapy worldwide at the end of 2009 represented 13-fold improvement over 2004. Globally, women account for half of all HIV infections for the past several years. Children younger than 15 years living with HIV were about 2.5 million, with 370,000 new HIV infections in 2009 in this category. It is estimated that more than 90% of children living with HIV acquired the virus during pregnancy, birth or breastfeeding, HIV transmissions that can easily be prevented.

The epidemiology unit of the Belgian Scientific Institute of Public Health, which groups data from the AIDS Reference Laboratories and Centres reported that the diagnosis of HIV infection was made in a total of 24,646 people between the beginning of the epidemic and the end of 2010 [6] and the highest incidence occurred between 2008 and 2010 with 1,196 new diagnosed cases in 2010 (Figure 1). The proportion of people of Belgian nationality among newly diagnosed cases with known nationality increased between 2004 and 2010, from 33.1 to 43.3%. The majority of patients reported heterosexual contact with a decreasing proportion from 67.1% to 49.5% between 2002 and 2010. At the same time, the proportion of homo and bisexual contacts increased from 23.6% to 45.6%. Other routes of transmission, although still existing, declined.

In terms of evolution of the incidence of AIDS cases, a plateau was observed from 1991 to 1995 with an average of 256 cases diagnosed per year. The incidence has sharply declined from 1996 through the introduction of protease inhibitors allowing the highly active antiretroviral treatment. The adjusted incidence (for reporting delays) for the 5 past years fluctuates between 88 and 129 new AIDS cases per year. Only 16 deaths from the disease were reported in 2010 with a total of 1,942 deaths reported between 1983 and 2010 in Belgium [6].

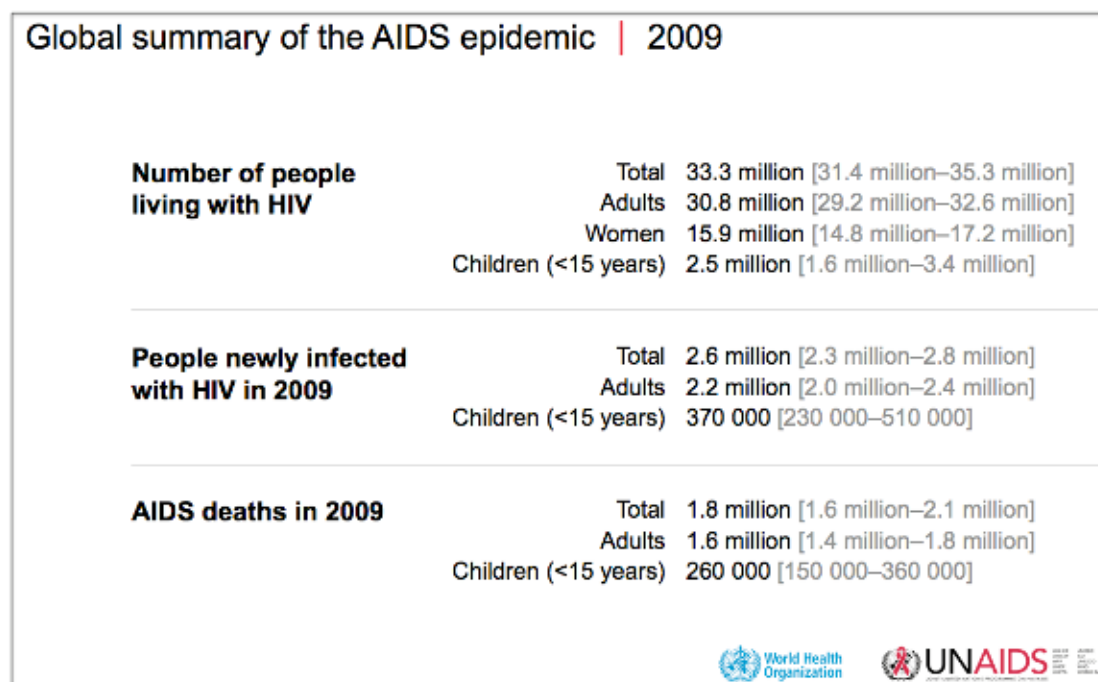


Table 1: Estimation of Adults and children living with HIV in 2009 as reported by the Joint United Nations Programme on HIV/AIDS (UNAIDS) [5]

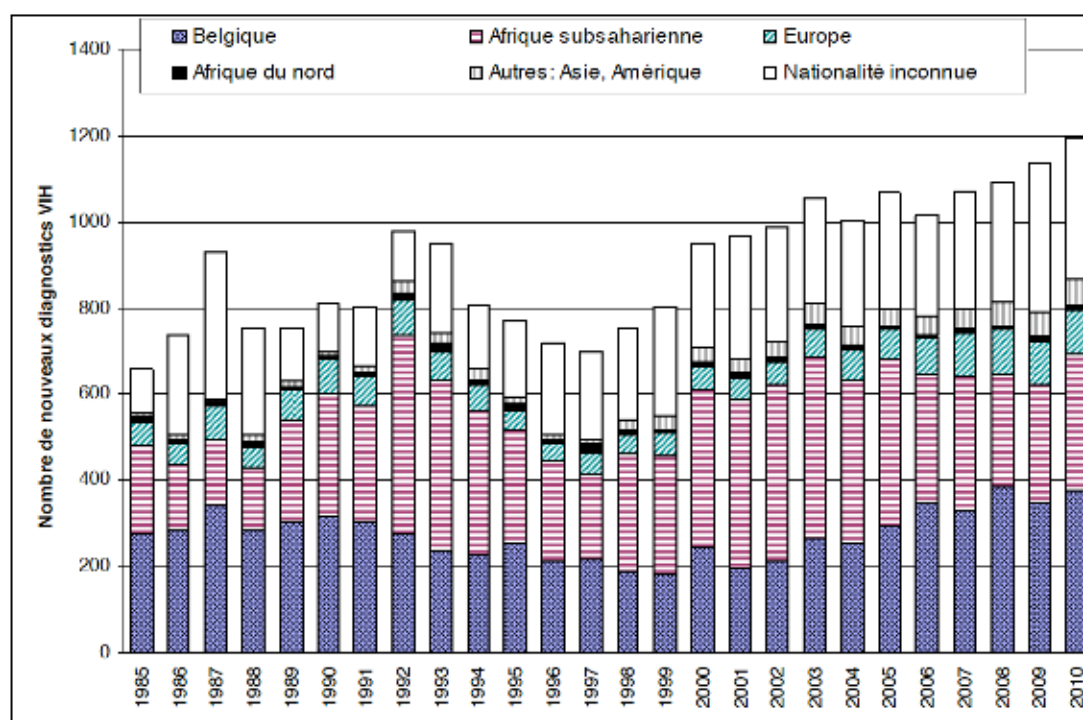


Figure 1: Number of newly diagnosed HIV infections by nationality and year of diagnosis in Belgium as reported by the epidemiology unit of the Belgian Scientific Institute of Public Health [6].

1.3 HIV-1 VIROLOGY

1.3.1 STRUCTURE OF THE VIRION

The electron micrograph (Figure 2) of the budding of HIV-1 particles shows spherical mature virions of approximately 100 nm in diameter characterized by the presence of a conical core. The scheme of the HIV-1 structure shown in the figure 3 represents the icosahedral sphere surrounded by the outer lipid bilayer membrane that contains 72 viral glycoprotein complexes involved in the cell host recognition and the entry process. These glycoprotein complexes contain the extracellular unit gp120 interacting with the cell receptor and co-receptor and the transmembrane unit gp41 that intervenes in the fusion process. The viral envelope, which derives from the host cell membrane also contains host proteins. The internal viral capsid comprises structural proteins such as the major core protein p24, the matrix protein p17, which is tightly bound to the nucleoproteins p7 and p6 that stabilise 2 RNA(+) viral genomes encapsidated in each virion. As others members of the *Retroviridae* family, the HIV-1 genome (Figure 4) contains 3 major genes, the *env* coding for the envelope glycoproteins, the *gag* coding for internal structural proteins and the *pol* gene coding for the viral enzymes, the protease, the reverse transcriptase and the integrase. The HIV- 1 genome comprises a number of other genes, *tat* and *rev* genes are translated early into regulatory proteins: *Tat* activates the transcription process. The accessory genes such as *nef*, *vif*, *vpu* and *vpr* modulate the viral cycle. The viral genome carries at each end long terminal repeat sequences (LTRs), which participate in the insertion of the viral DNA into the host genome and the 5'LTR acts as an RNA pol II promoter.

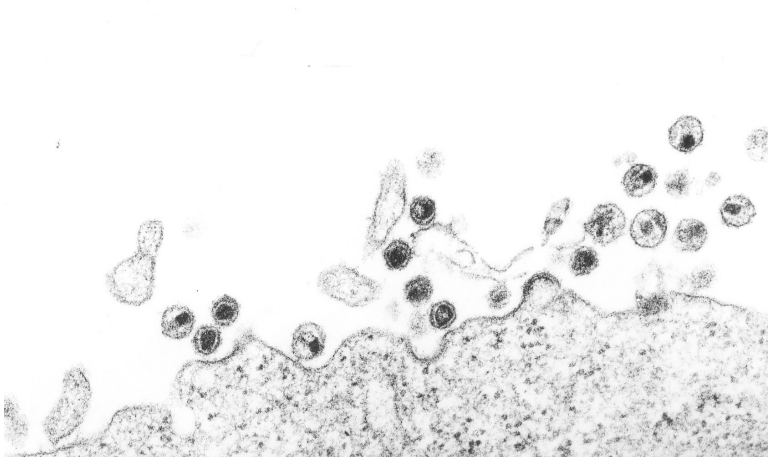


Figure 2: Electron micrograph showing a single HIV-1 particle in the process of budding from infected cultured human PBMC, and several mature virions containing the characteristic conical/bullet-shaped nucleoid [7].

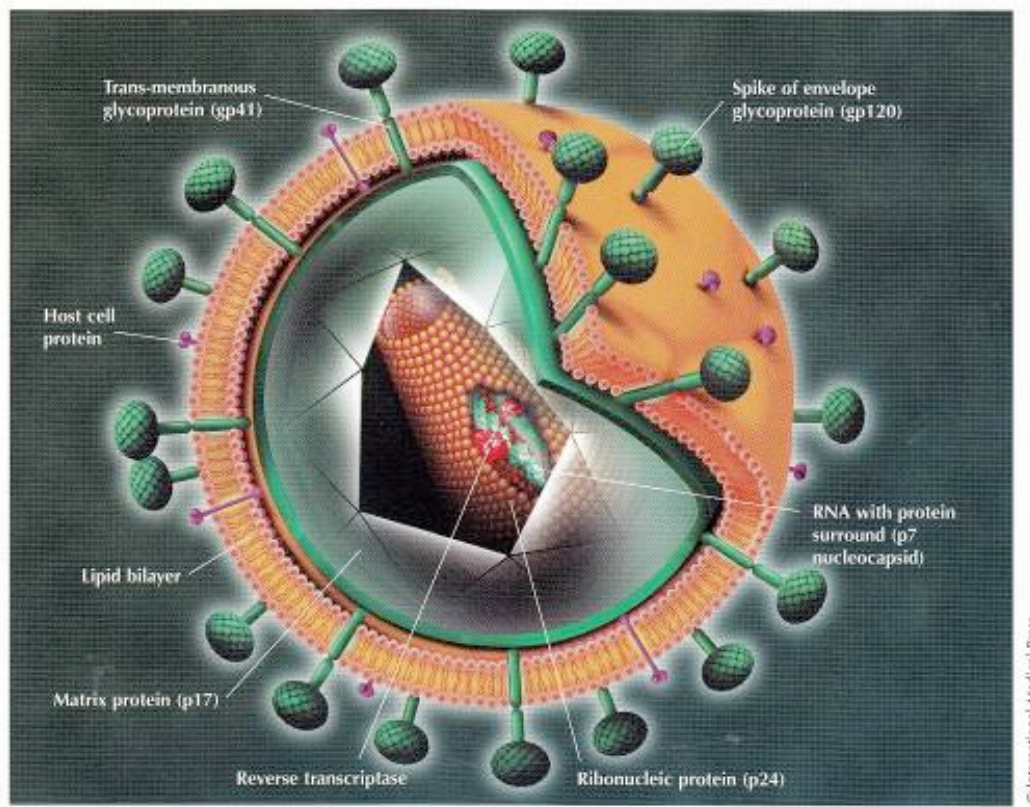


Figure 3: a scheme of the HIV-1 structure. (A) HIV particle showing the bilayer external envelop carrying viral glycoproteins gp120 and gp41 and host cell protein. Internal structural viral proteins are shown, the matrix protein p17 and the core protein p24. Inside the core, the genomic RNA is represented in combination with the nucleoprotein p7 [8].

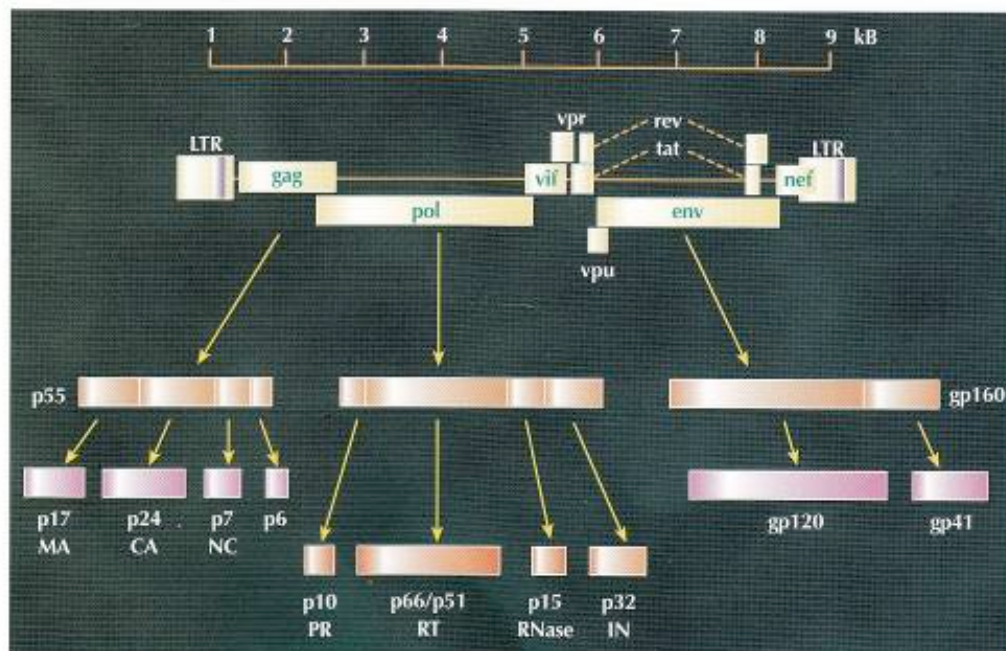


Figure 4: the genome organisation of HIV-1 shows the 3 major genes, *gag*, *pol* and *env* translated into polyproteins, which undergo cleavage by the protease. The *gag-pol* polyproteins are processed into their active enzymes and mature structural proteins by the viral protease while the *env* product is processed by the cell protease [8].

1.3.2 VIRAL REPLICATION CYCLE

1.3.2.1 ENTRY PROCESS

The entry of the HIV into the target cell requires sequential interactions involving viral glycoproteins gp120 and gp41 and cell proteins, mainly the CD4 glycoprotein and the chemokine receptors. The viral glycoprotein consists of 2 units, the homotrimeric gp120 glycoprotein not covalently anchored to the transmembrane trimeric complex gp41 inserted in the viral envelope [9-11]. The gp120 subunit (SU) sequence (550 amino acids) contains five variable regions (V1–V5) [12]. The conserved gp120 regions are involved in the interaction with the gp41 and the target cell. The V1-V4 are the exposed variable regions of the gp120 SU, the hypervariable V3 loop being the major determinant of cell tropism and the coreceptor usage for the entry process. The glycosylation of the gp120 regions and their variability probably modulate its immunogenicity. The gp41 (320 amino acids) contains 3 regions, the C-terminal transmembrane region, the heptad repeat (HR) region comprising 2 domains (HR1 and HR2) that interact with each other forming a hairpin like structure which allows the approximation of the viral and the cellular membranes and the N-terminal region called fusion peptide that is inserted in the target cell membrane [13-16].

The viral replication cycle begins by the recognition of the target cell through the interaction between the HIV envelope gp120 and its primary receptor, the CD4 protein expressed at the cell membrane, leading to the gp120 conformational change [8-10]. The shift in the conformation of gp120 allows its binding to the chemokine receptor, mainly CCR5 or CXCR4 called viral co-receptor which is mostly present at the surface of macrophages or T-helper cells, respectively. The double interaction between the viral gp120 and the CD4 receptor and CCR5 or CXCR4 co-receptor unmasks the viral transmembrane gp41, which inserts its N-terminal fusion peptide into the membrane of the host. The gp41 HR domains are leucine like zippers that form a coiled-coil bundle [15,16,17] resulting in apposition of the viral and target cell membranes that allows the fusion process.

1.3.2.2 HIV LIFE CYCLE

Following the entry process, the HIV core particle is released into the cell cytoplasm where it uncoats and releases the 2 viral genomic RNAs (Figure 5). The transcription of the viral RNA to the double stranded viral DNA (dsDNA) is mediated by the viral reverse transcriptase (RT) in multiple steps. The RT first synthesises a single-stranded DNA (ssDNA) leading to an intermediate hetero-duplex RNA-DNA. The ribonuclease H activity (RNase H) of RT removes the genomic RNA that served as the template and then copies the complementary

strand to produce a dsDNA. The replication process gives rise to a pre-integration complex (PIC) in the cell cytoplasm. HIV-1 RT is a heterodimer protein, consisting of subunits of 66kD (p66, 560 amino acids) and 51kD (p51, 440 amino acids). The p66 contains the polymerase active site and the RNase H activity. The 2 polymerase domains have similar aminoacid sequences but different three-dimensional structures.

The enzyme has a high error rate because of the lack of proofreading ability. The presence of 2 genome copies is thought to favour the occurrence of recombinants.

The PIC is actively imported into the nucleus and the viral integrase (IN) enzyme incorporates the produced viral dsDNA into the chromosome of the infected cell [18,19]. The PIC comprises viral components such as HIV dsDNA, viral integrase (IN), viral matrix (MA), Vpr protein, viral RT, as well as host proteins [20-24]. The HIV viral IN allows the overall integration process by 2 major catalytic activities: an endonucleolytic cleavage of the viral genomic DNA at each 3'-OH extremities called “maturation” and a transfer of the strand allowing the insertion of the processed viral DNA into the cell DNA by a trans-esterification mechanism (Figure 6). The viral integrated DNA called “provirus” persists in the infected cells and serves as template that is translated into messenger RNA and new genomic RNA allowing the production of new virions. The HIV DNA may remain silent (latent) or be actively transcribed depending on the chromosomal loci, the metabolic state of the host cell and its activation state, at least for the CD4 T cells [25-27]. The linear DNA molecule that enters the nucleus is either being integrated as provirus or non-integrated in the genome of target cells. There are different non-integrated DNA species representing the majority of HIV DNA [20,28-32]. The non-integrated forms comprise the 2-LTR circle formed by joining the ends of the linear DNA, the single LTR resulting from homologous recombination between the two LTRs of the 2-LTR circle and the rearranged circular products as a result of autointegration of the viral DNA ends into a pre-existing HIV DNA circle [20,30,33] (Figure 7). The integration or non-integration phenomenon seems to depend more on cellular factors than on the viral integrase.

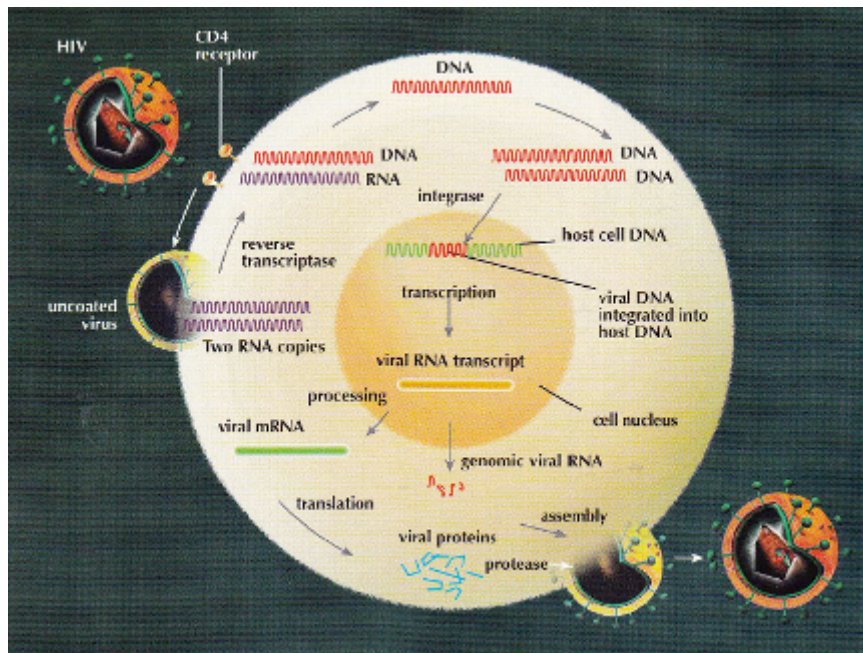


Figure 5: Replication cycle of HIV. Following the release of viral genomic RNAs into the cytoplasm, the viral RT catalyzes the synthesis of a double stranded DNA copy, the provirus which is subsequently integrated into the host cell genome by the viral integrase. The proviral DNA serves as a template for transcription and production of new virions [8].

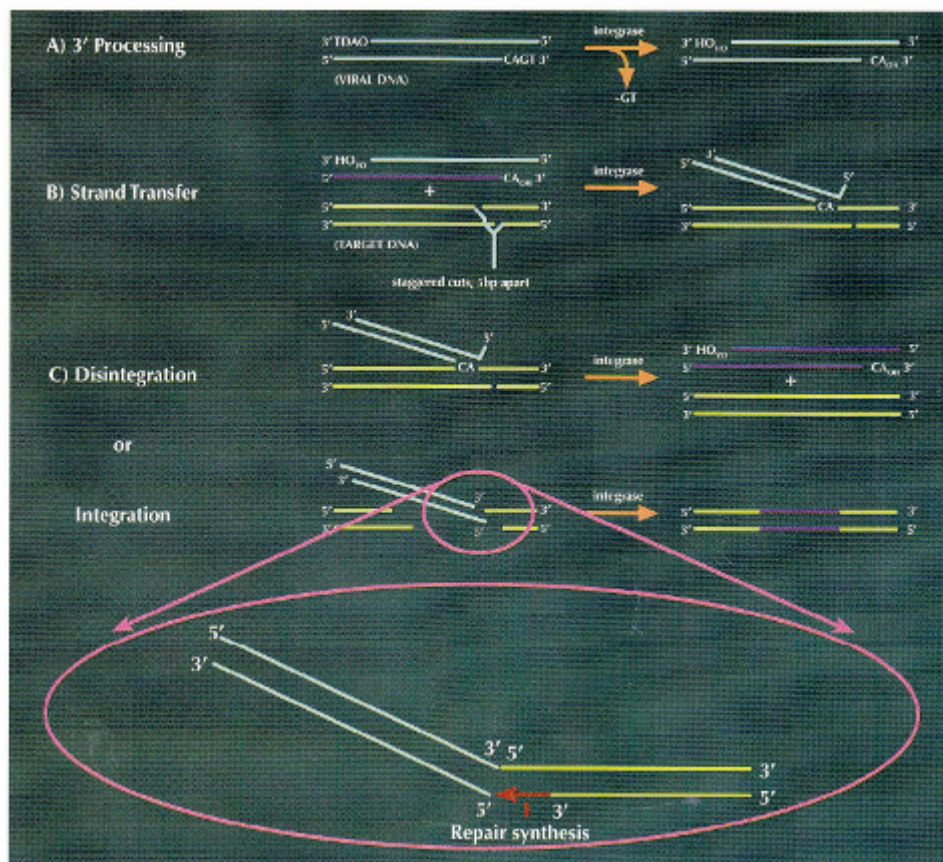


Figure 6: HIV DNA integration process (A) The first step consists of 3'processing of the viral dsDNA by the viral integrase, which removes two nucleotides from each 3'end of the viral DNA. (B) In the strand transfer step, the integrase joins the viral DNA 3'ends to the 5'ends of cell DNA at the site of integration. (C) Integrase may also catalyze the excision of viral DNA [8].

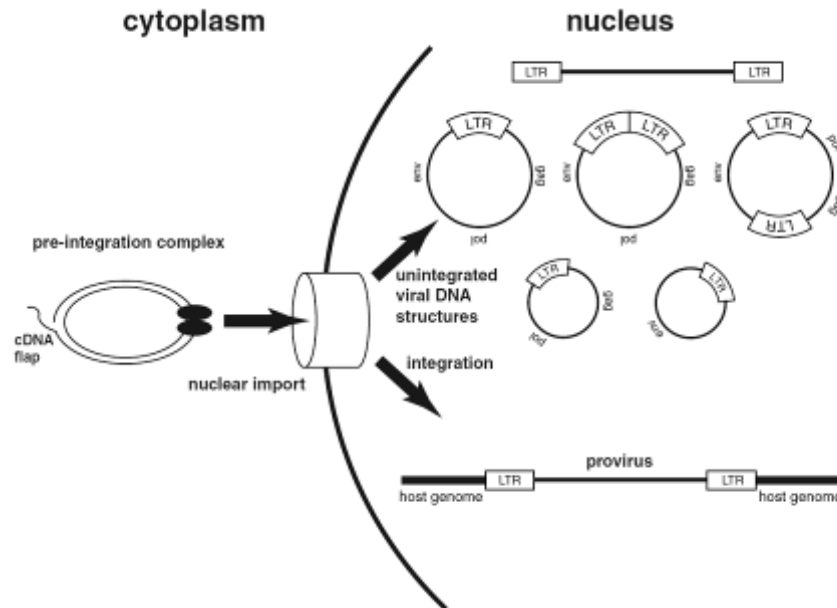


Figure 7: Schematic description of the generation of the multiple HIV DNA forms. After the passage of the pre-integration complex (PIC) through the nuclear pore, the linear HIV DNA is either integrated into the host chromosome, then called provirus, or non-integrated linear or various circular DNA forms. The non-integrated DNA represents the major fraction, particularly the linear form [20].

It has also been demonstrated that in infected patients, the amount of non-integrated HIV DNA was about 100 fold higher than integrated DNA, all DNA forms being present in blood and in tissue [34,35]. Chun *et al* [35] estimated by using PCR that around 10^9 CD4 T cells are infected, 1.2×10^7 of them containing latent provirus and 1.4×10^6 producing new virions after appropriate T cell stimulation. To distinguish between episomal and integrated HIV-1 DNA, usually Alu-HIV PCR assays are performed, where a pair of primers anneal to the LTR HIV-1 sequence and the highly repeated chromosomal Alu elements. PCR targeting the 2-LTR is also performed to analyse the episomal circle HIV-1 DNA. These assays allow kinetic studies of the integrated and non-integrated HIV-1 DNA and assessments of the effectiveness of integrase inhibitors. Furthermore, it may be of interest in long term HAART efficacy [36-38].

Several factors mediate the activation of viral transcription, cellular factors such as NF- κ B as well as early produced viral proteins such as the transcription activating factor (Tat) and the Rev. The Tat promotes the transcription elongation over 1000 times [39-43], while the regulatory protein Rev is involved in the migration and the stabilization of messenger RNA to the cytoplasm [41]. The viral proteins are synthesized in the cytoplasm as polyproteins from different splicing forms of mRNA. The HIV-1 *pol* and *gag* genes are translated into a *gag-pol* polyprotein (p160), which undergoes cleavages into *gag* and *pol* precursor proteins. The HIV

protease processes the 55-kd *gag* polypeptide into 4 smaller functional structural proteins, the major core protein (p24), the matrix protein (p17) and the nucleoproteins (p7, p6) while the *pol* precursor protein is cleaved into viral enzymes, the protease (p10), the reverse transcriptase (p66-p51) and the integrase (p15). The *Env* gene is translated from several partially spliced mRNAs without *gag-pol* coding genes into a large glycoprotein precursor (gp160) that undergoes a cleavage by a cellular protease to produce the 2 major functional viral glycoproteins, gp41 (350 amino acids) and gp120 (550 amino acids), which bind specifically to CD4 protein on target cells. Host cell proteins with certain phospholipids and cholesterol may be incorporated in the viral envelope during the budding process, leading to the release of virions [7,8,44,45]. The HIV aspartic protease (PR) is a homodimer protein consisting of 2 identical peptides of 99 amino acids having an N-terminal proline and C-terminal phenylalanine. The active site harbouring 2 aspartic acids involved in the hydrolysis of the peptide bonds [46-49]. The CO-NH bond of peptide undergoes a nucleophilic attack of 2 H₂O molecules activated by Asp-COO- terminus of the PR active site. The reaction leads to an unstable zwitterionic tetrahedral intermediate, which breaks down into amine and carboxylate acid compounds [50,51]. Thus, the protease intervenes in the cleavage process of precursor viral molecules allowing the generation of infectious viral particles.

1.3.3 CELLULAR RESERVOIRS OF HIV-1 AND THEIR ROLE IN VIRAL PERSISTENCE

To achieve HIV eradication, replication of virus in the whole body has to be inhibited by the treatment. HIV reservoirs are thought to be a mix of latently infected cells and biological compartments that allow persistence of the virus. The central nervous system (CNS), in which the monocyte-derived microglial cells are predominantly infected and the lymphoid organs with infected CD4 T cells are thought to be the most important sanctuary sites, which can harbour viral variants that are genetically distinct from those present in plasma [52-54]. The genitourinary tracts as well has shown distinct viral variants [55]. Other cells also contribute to HIV-1 persistence, such as hematopoietic progenitor cells (HPCs), in which proviral DNA can be found in a subpopulation of HIV-1-infected patients [56]. The HPCs can proliferate and possibly generate clonally infected cells. Thus viral reservoirs are numerous and the cellular and molecular mechanisms of HIV persistence in these different sanctuaries appear varied. Latent HIV reservoirs seem to be established early during primary infection and constitute a barrier to eradication [41,56,200]

The reservoirs influence the evolution of the viral concentration in the body. Potent antiretroviral drugs produce a dramatic drop in plasma viral load (VL) in two weeks, as a result of eliminating free virus and virus from activated cells that have a rapid turnover. In the second phase, the VL declines slowly, as the turnover of long-term reservoirs of chronically or latently infected cells is slower [57,58]. In addition, the plasma VL measure does not measure the virus in the major reservoir and replication sites, such as lymphoid tissue, where the VL levels is more than 1000-fold greater than in the plasma. Testing lymphoid tissue in infected humans is very difficult. For that reason an alternative indirect approach is to test CD4 cells in blood, knowing that 2% of these cells from lymphoid tissue recirculate daily in the blood stream as calculated in rat experiments [59,60].

The ability of the HIV to infect cells latently is a main obstacle to eradication of the infection. Memory infected CD4⁺ T cells have a life span of several years, allowing the virus to persist despite an immune response and a successful highly active antiretroviral therapy (HAART). The memory CD4⁺ cells are considered to be an important part of the reservoir of the latent HIV-1 proviral DNA. They can reactivate and produce infectious virions in the presence of specific antigen or other stimulation. Based on the measurements of the VL, the virus half-life was determined, assuming that the anti-VIH treatment stopped completely the viral replication. Using mathematic modelling [61-64], the virus half-life was calculated in two studies. In the first one, Ho and *al* estimated it to be 2.1 days [61]. Wei and *al* proposed 2 days [62], demonstrating the continuous intensive viral production. Ho and *al* evaluated the minimum rate of HIV-1 production to be 0.68×10^9 virions per day while Wei and *al* calculated this estimation around 1.1×10^8 virions per day. Other parameters were also calculated as the virus clearance rate, the cellular dynamics estimated by the overall steady-state CD4 cell turnover rate (Figure 8). Perelson and colleagues conducted an other study in 1996 [63] with mathematical modelling and nonlinear regression calculating the clearance of plasma virions with a mean half-life of approximately six hours, the mean total HIV- 1 production being 10^{10} virions per day, 15 times greater than previous estimations. Productively infected cells were estimated to have a half-life of approximately 1.6 days.

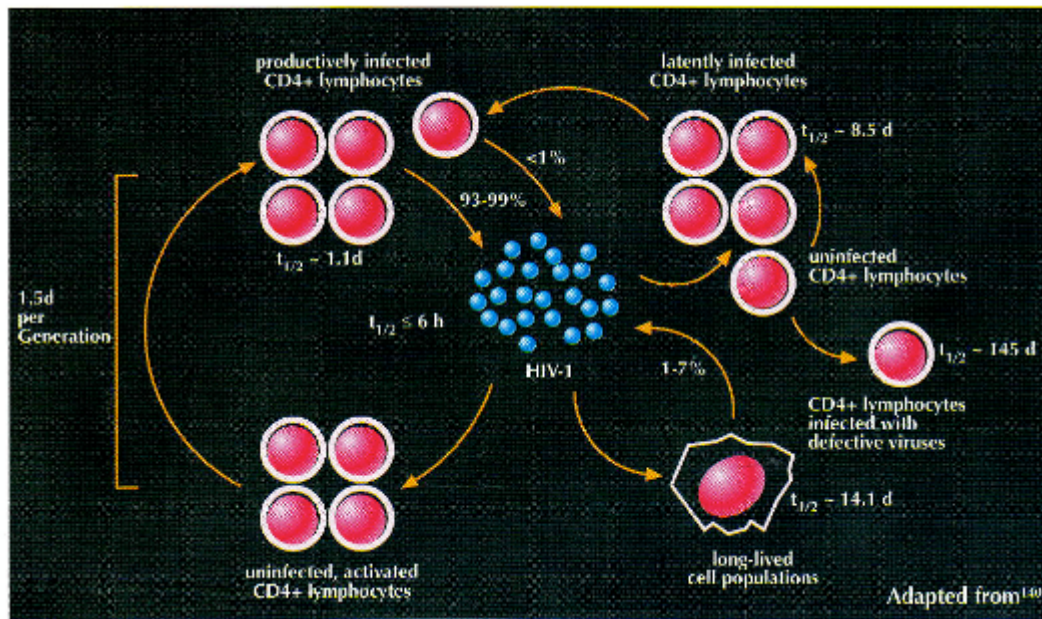


Figure 8: Dynamics in virus production: replication of HIV-1 shown on the left-hand-side accounts for up to 99% of the plasma viremia produced by activated CD4 lymphocytes as well as their destruction. On the right-hand-side, long-life cells (monocytes-macrophages, memory T cells) account for less than 1% of the plasma viral load but are thought to re-ignite high levels of viral replication once therapy is stopped [8].

1.4 THE NATURAL COURSE OF HIV INFECTION

1.4.1 TRANSMISSION ROUTES

The major routes of transmission of HIV comprise any type of sexual contacts, parenteral exposure to blood and products contaminated by blood and mother-to-child (MCT) transmission during pregnancy or by breast-feeding. Factors favouring sexual transmission include the viral load in genital secretions, sexually transmitted diseases, type of sexual relationship, multiple partners, first-stage and late-stage infections in the source patient. Co-transmission of other viruses, particularly in intravenous drug users (IVDU), as hepatitis B (HBV) and hepatitis C (HCV) are frequent, with prevalences of 6-14% and 25-33%, respectively [65-67]. The risk of infection of an HIV-negative person exposed to blood or genital fluids of an HIV-positive individual ranges between 1% and 0,1%, being 10 times and 100 times lower than the transmission rate of HCV and HBV respectively [68]. It is thought that the transmission of HIV through occupational exposure in healthcare personnel is about 0.3% for percutaneous exposures and 0.09% for damaged skin or mucosal membranes [69]. The residual risk in transfusion is estimated to be less than 1/1000.000, as it is controlled by routine screening tests in industrialised countries [70-72], but is up to 5% of HIV transmission in sub-Saharan Africa according to the World Health Organization (WHO) [73]. A third of

MCT occur *in utero*, but the most frequent transmission is at delivery. The transmission rate in untreated, non-breastfeeding populations is about 15 to 20%, with an additional 10% of infections by breastfeeding. When preventive treatment is initiated, the MCT falls to 5-8% (<2% in Europe) [74,75]. Other transmission routes of HIV transmission are notably rare and often found in case reports as transmissions due to blood contact of open wounds or mucosa, after a bite or in newborn children via pre-chewed food by their mothers [76]. Large investigations of other transmission routes of HIV clearly show that daily contacts such as the shared use of toilets or drinking from the same glass, contact via saliva, urine, or infectious blood with intact skin did not show a single transmission of HIV [77].

1.4.2 PATHOGENESIS OF HIV

The HIV-1 infection is a chronic disease that progressively leads to a decline in the functionality and the number of CD4 T-cells by promoting cell death, thus leading to the development of immunodeficiency. Even if the mechanisms of CD4 T cell death are not fully understood, they have been attributed to direct and indirect mechanisms: the review article published by Varbanov M *et al.* [78] indicates that the indirect pathway such as increased apoptosis is the major mechanism of the depletion of CD4 T cells that accompany the chronic immune activation associated with HIV-1 infection. The two other mechanisms of programmed cell death are the necrosis-like programmed cell decay for which less is known about CD4T-cell death and the autophagic programmed cell death which is needed to trigger apoptosis in uninfected cells. Several HIV-1 proteins (Env, Tat, Vpr, Nef, Vpu, PR and Vif) directly modulate CD4 T-cell death by different pathways for both infected and uninfected cells. Other studies stress that the fibrosis induced in the lymphoid tissue plays a role in the T cell depletion and impairs immune reconstitution upon HAART. In a published article in 2010, Zeng *et al.* [79] have identified a mechanism by which immune activation-induced fibrosis of lymphoid tissues leads to depletion of naive T cells in HIV-1 infected patients. Based on an animal model, they showed that during SIV infection in rhesus macaques, naive T cells have restricted access to self-Ag/MHC complex signals and to the survival factor IL-7 present on the fibroblastic reticular cell (FRC) network, resulting in apoptosis and depletion of T cells. In turn, this process removed a major source of lymphotoxin- β produced by T cells, a survival factor for FRCs during SIV infection, resulting in a vicious cycle of depletion of T cells and of the FRC network.

The HIV primary infection (Figure 9) is often subclinical and may not be of much consequence. Some individuals develop shortly after infection, within 2-3 weeks, a so-called

acute retroviral syndrome characterized by maculopapular rash, fever and lymphadenopathy. Some show myalgia, pharyngitis, arthralgia, retro orbital headache, malaise, diarrhea and vomiting. The diagnosis of HIV infection relies on laboratory testing as the symptoms are unspecific, variable and do not last longer than 3 or 4 weeks.

The primary HIV infection is followed by an asymptomatic phase of several years with a median duration of 10 years. The immune system compensates the destruction of CD4⁺ T cells, often maintaining its peripheral blood cell count above 500 cells/mm³. Thereafter symptoms or diseases may occur, an early symptomatic HIV disease preceding the AIDS phase (Figure 9).

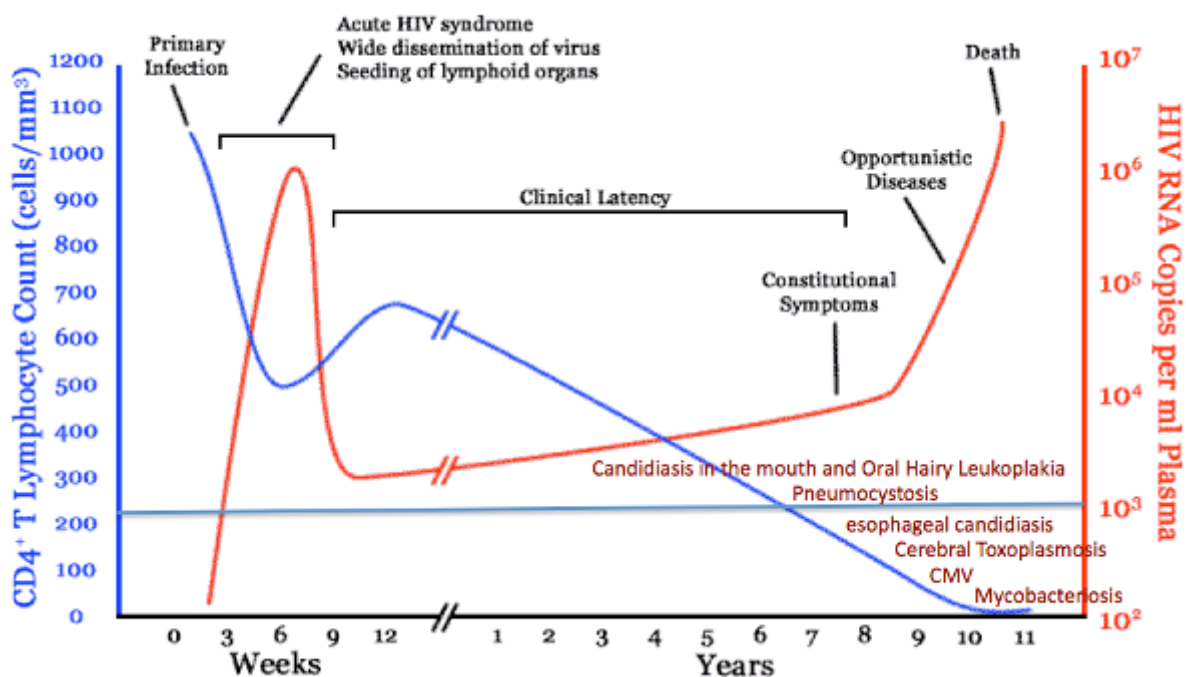


Figure 9: natural history of HIV infection: during primary infection, most individuals are asymptomatic or will develop a flu-like illness, swollen lymph nodes, diarrhea, fever and fatigue. The CD4⁺ cells decline while the viral load increases. The second stage of the disease is asymptomatic, the immune system compensates the CD4⁺ cell losses but the virus remains active. The last stage is symptomatic due to the decline of the immunity (CD4⁺ cells <200/mL), opportunistic diseases occur as thrush, Herpes Zoster and Herpes Simplex primary infections or reactivations, Oral Hairy Leukoplakia, Idiopathic Thrombocytopenic Purpura, Pneumococcal Pneumonia, Pneumocystic Pneumonia, Kaposi's Sarcoma, Tuberculosis, HIV-Related Lymphoma... [80].

In order to classify the disease progression, the 1993 revised CDC classification takes into account the clinical symptoms and the number of CD4 T cell (Table 2). In clinical settings, the disease stage has to be adapted according to the disease progression, as the term of AIDS has not strictly the same meaning. It can be used in the US when the CD4 T cell count is < 200 cells/μl but in Europe it only corresponds to the clinical manifestations.

Symptoms / CD4 T cells	Asymptomatic or acute HIV disease	Symptomatic but not stage A or C	AIDS-defining illness*
> 500/ μ l	A1	B1	C1
200 – 499/ μ l	A2	B2	C2
< 200/ μ l	A3	B3	C3

Table 2: Classification of HIV disease according to the 1993 CDC classification [81]. Details of different categories see table 3.

Diseases of CDC category B are not AIDS-defining (Table 3), however their occurrence is defined as symptomatic stage. Symptoms include fever, unexplained weight loss, recurrent diarrhea, fatigue and headache. Other clinical manifestations like oral hairy leukoplakia, oral thrush, seborrheic dermatitis, folliculitis, recurrent herpes simplex infections, cytomegalovirus and herpes zoster reactivations may occur. Usually, the highly active antiretroviral treatment (HAART) is initiated at this period, as the number of CD4 T cells progressively decreases, as a result of a disturbed cellular immune system and there is an increase in the plasma viral load. AIDS-defining illnesses (Table 3) occur later in the course of HIV infection as the CD4 count falls lower than 200 cells/mm³, eventually lead to death after a variable period of time if HAART is not started. The AIDS related opportunistic infections or malignancy comprise *Pneumocystis jirovecii* pneumonia (PCP), toxoplasma encephalitis, disseminated mycobacterium avium complex (MAC), oesophageal candidiasis, lymphoma and Kaposi sarcoma. Rarely, patients may develop the progressive multifocal leukoencephalopathy (PML), usually fatal viral disease due to JC polyomavirus. In the advanced HIV disease stage, the CD4 cell count is less than 50 cells/mm³ and multiple opportunistic infections and malignancies of CDC category C are present. Survivals among the advanced AIDS patients have dramatically increased with HAART.

Category A

Asymptomatic HIV infection

- Acute, symptomatic (primary) HIV infection
- Persistent generalized lymphadenopathy (LAS)

Category B

Symptoms or signs of diseases that do not fall into Category C but are associated with a disturbed cellular immunity. Among these are:

- Bacillary angiomatosis
- Infections of the pelvis, in particular complications of fallopian tube or ovarian abscesses
- Herpes zoster in the case of more than one dermatome or recurrence in the same dermatome.
- Idiopathic thrombocytopenic purpura
- Constitutional symptoms like fever or diarrhea lasting >1 month
- Listeriosis
- Oral hairy leukoplakia (OHL)
- Oropharyngeal candidiasis (oral thrush)
- Vulvovaginal candidiasis, either chronic (>1 month) or difficult to treat
- Cervical dysplasia or carcinoma in situ
- Peripheral neuropathy

Category C

AIDS defining diseases

- Candidiasis of the bronchia, trachea, or lungs.
- Oesophageal candidiasis
- CMV infections (except liver, spleen and lymph nodes)
- CMV retinitis (with loss of vision)
- Encephalopathy, HIV-related
- Herpes simplex infections: chronic ulcer (>1 month); or bronchitis, pneumonia, oesophagitis
- Histoplasmosis, disseminated or extra-pulmonary
- Isosporiasis, chronic, intestinal, duration >1 month
- Kaposi sarcoma
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic, intestinal, duration >1 month
- Lymphoma, Burkitt
- Lymphoma, immunoblastic
- Lymphoma, primary CNS
- Mycobacterium avium complex or M. kansasii, disseminated or extrapulmonary
- Mycobacterium, other or not identified species
- Pneumocystis pneumonia
- Pneumonia, bacterial, recurrent (>2 within a year)
- Progressive multifocal leukoencephalopathy
- Salmonella Sepsis, recurring
- Tuberculosis
- Toxoplasmosis, cerebral
- Wasting Syndrome
- Cervix carcinoma, invasive

Table 3: Clinical categories of HIV infection according to CDC Classification [82].

1.5 HIV-1 DIAGNOSIS AND FOLLOW-UP

1.5.1 SCREENING TESTS

HIV infection diagnosis is primarily based on indirect tests detecting HIV antibodies (Ab, Fig 10), as a “seronegative” HIV infection is an absolute rarity and irrelevant in practice [83,84]. Screening is not compulsory but should be widely proposed in agreement with the patient and the confidentiality is essential. It is required for all donations (blood and organ tissue) and is broadly available in antenatal clinics. The diagnosis is established by two steps, screening assays that are very sensitive and designed to detect all infected individuals and confirmatory assays that have a high specificity to be performed in case of reactive result of a screening test in order to distinguish individuals who are not infected but who have reactive screening test results from really infected patients. There are numerous commercial systems available for screening, the basic technological principle is the antigen-antibody binding. HIV antibodies are present in a patient’s serum or plasma bind to antigen. A washing process is done to remove the unbound antibodies and an enzyme-labelled second antibody anti-human antibodies called “conjugate” is then added. Finally, a second wash process is performed and a substrate is added. The conjugate allows a change in the substrate’s colour which can be measured photometrically. The prototype assay is the ELISA (enzyme linked immunosorbent assay), performed on a plastic microwell plate. Rapid and modern test systems are highly automated and use microparticles as solid phase coupled with the virus antigens and antibodies, a method referred to as the “microparticle enzyme immunoassay” (MEIA). Any approved screening test detects all known HIV types (HIV-1 and -2), HIV groups and HIV subtypes.

Direct assays include nucleic acid tests (NAT) that look for HIV's genetic material or antigen (Ag) tests that detect viral proteins, essentially the p24 core protein, may also be used for HIV diagnosis. In case of suspected recent HIV infection, the result of viral load measurement can be used for confirmation. Tests that combine the detections of Ab and Ag referred to as “4th generation assays” are intended to cover the “window period” between infection with HIV and the ability to detect antibodies. Screening tests can be carried out using rapid HIV tests: a reactive result must be also confirmed by a confirmatory analysis. Rapid tests are usually based on immunochromatographic methods allowing rapid results within 15 to 30 minutes and the use of whole blood in addition to serum and plasma is suitable to avoid centrifugation step in certain situations. They lack somewhat sensitivity as compared to the usual tests performed in the lab. US and European guidelines recommend confirming all positive HIV

test results, regardless of whether they are from rapid or conventional tests, by a Western blot or Immunoblot confirmatory test [85-87].

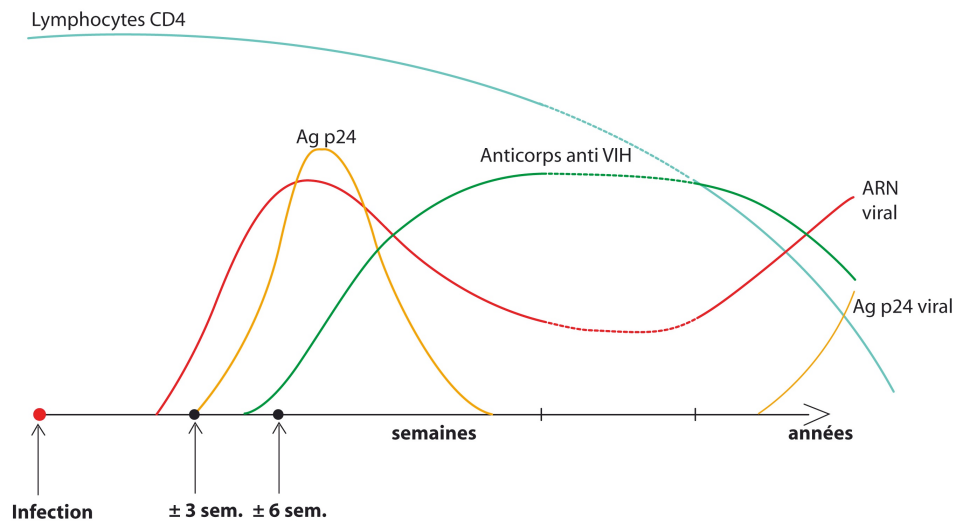


Figure 10: Evolution of HIV parameters that are used for diagnosis. The detection of Ab is the common performed assays but tests based on combine detection of Ab/Ag are becoming the reference [88].

1.5.2 CONFIRMATORY TESTS

(<https://www.wiv-isp.be/epidemie/EPIEN/AIDSEN/ARLEN/index.html>)

All reactive screening test results have to be confirmed by a Western Blot (WB) or an Immunoblot (or Line Blot) analysis. The tests consist of a reaction between viral proteins separately fixed on a test strip, native proteins for WB and synthesized ones for Immunoblot, and anti-HIV antibodies present in patient's sample. The resulting antigen-antibody complex will become visible on the test strip using a conjugate and a corresponding substrate. All HIV proteins are present on a WB strip (one strip for HIV-1 and another for HIV-2) while on the same Immunoblot strip, usually *env* proteins (gp41 and gp120 for HIV1, gp36 and gp105 for HIV-2) and *gag* proteins (p24, p17, p31) are fixed. There is no international consensus for a positive blot result. Generally it is considered positive when at least two or three bands are visible, depending on the commercial test used. WHO criteria demands antibodies against at least 2 *env* proteins while the US FDA recommends p24, p34 and gp41 or gp120/160 reactive bands. Any positive sample should be confirmed on a second serum to avoid misidentification of patient.

1.5.3 SPECIAL SITUATIONS

(<https://www.wiv-isp.be/epidemie/EPIEN/AIDSEN/ARLEN/index.html>)

In some situations, the standard HIV test is not capable of detecting infected individuals.

Primary HIV infection with a “diagnostic window” (the period between transmission and the onset of biological markers of the infection) can lead to a false negative result. During this time, HIV viral load and the likelihood of transmitting the virus are very high. The mean time to detect anti-HIV Ab is 2 to 8 weeks, 97% of persons will develop detectable antibodies in the first 3 months. The 4th generation immunoassays (EIA) combining Ab/Ag can shorten the diagnostic gap earlier than single Ab EIA with an average period of 1 month. In extremely rare cases, it can take up to 6 months to develop antibodies to HIV. We published a case report [89] of failure of one of these fourth generation assays. Surprisingly in this seroconversion case, the Axsym antibody assay was positive 3 days after the first sample, whereas the Vironostika combined assay remained negative. This was due to lack of sensitivity of the antibody detection by this assay, while the antigen detection could have been positive in the earlier stages.

The earliest laboratory infection marker is HIV RNA that is detectable between 10-15 days after infection, approximately 5 to 7 days before the p24 antigen and about 10 to 12 days before seroconversion [90]. However in the context of suspected HIV infection, a negative result doesn't rule out a possible infection. An HIV test should therefore be performed at the earliest 3 weeks after exposure except for legal reasons establishing a status of non-infection before exposure and can not be ruled out until three months after exposure. Early antiretroviral therapy in acute or early HIV Infection is associated with durable virologic suppression but may delay seroconversion much longer [83,91,92].

False positivity due to maternal antibodies transplacentally transmitted to newborns is systematic in the case of HIV-infected mothers, making the detection of antibodies difficult to interpret until the age of 18 to 24 months [93,94]. Diagnosis is carried out by NAT the first HIV PCR should be performed after the first month of life (sensitivity 96%, specificity 99%), then again after the third month because of the nearly 100% sensitivity and specificity. The detection of cell associated Human Immunodeficiency Proviral DNA by PCR is one of the most sensitive non-serologic methods recommended for confirming HIV infection in the neonate. All positive NAT results must however be confirmed by analysing a second sample. However the mother-to-child transmission can be ruled out only if there is no other source of transmission like breastfeeding and the disappearance of maternal antibodies are documented.

Screening tests of infections after an occupational exposure should comprise testing of the source patient for hepatitis B and C and HIV but not delay the potential rapid start of postexposure prophylaxis (PEP). An injury with potentially infectious materials, especially a hollow needle with fresh blood, should lead to an immediate interruption of work and to consult the emergency service. The earlier PEP will be initiated, preferably be started within 2 to 24 hours, the better the chances of success [95,96]. The absence of clinical elements of suspected HIV infection in the index patient and a negative screening test exclude HIV infection with a high predictive negative value (VPN) and PEP should be terminated. An HIV PCR test should be considered only if there is evidence of acute HIV infection of the index patient or of the victim. Currently, a serological follow-up of victims of occupational exposure is recommended, but there is no consensus on duration. Four or 6 months are proposed [95-97] if the index patient has a HIV positive or unknown status. If the exposed patient has been treated, testing at least 6 months or at 12 months after PEP is recommended [98].

1.5.4 FOLLOW-UP

The eradication of HIV is presently not possible in a patient. Infected patients have to deal with a chronic infection and a lifelong treatment and follow-up. To evaluate the patient's virological status and to follow-up treated and untreated patients, the HIV RNA viral load (VL) is a necessary parameter, along with the CD4⁺ cell count, which is a measure of the immunological status. Apart from these, all guidelines on the treatment of HIV infection recommend a number of other tests, including the genotypic resistance testing and in some therapeutic situations, the tropism testing and the drug plasma levels. Resistance testing is discussed in the section 1.6 dedicated to HIV-1 Treatment.

Plasma viral load is the amount of cell-free HIV RNA present in the blood, reflecting the replication of the virus. The CD4 T cell count and the VL have become the most important surrogate markers for the management of HIV infection. HIV RNA load is first of all recommended to evaluate the treatment efficacy, it may be used to diagnose acute HIV-1 infection prior to seroconversion, it predicts disease progression, it correlates directly with the probability of transmission and is a decision factor to start treatment [44]. Several methods have been used for this purpose, presently mostly real-time PCR methods. There is a trend towards increased commercial automated molecular diagnostic systems for detecting and monitoring infections. They allow a higher throughput and standardization of results. It is important to ascertain that assays launched on the market have similar performances in regard

to HIV strain genetic differences, including HIV-1 group M, its subtypes and a number of circulating recombinant forms (CRF), groups O and N. At present, assays dedicated to HIV-2 viral load are homemade owing to the limited market share they represent. Standardized international units/ml either for HIV-1 or for HIV-2 have not been adopted by the clinical practice, but results are reported as copy number or logarithmic copy number/mL of plasma. A VL above 100,000 copies/ml is generally considered to be high for HIV-1 and correlates often with CD4+ cell count drop and subsequent progression to disease. In contrast, the correlation between VL and CD4+ count can vary greatly between individuals, some patients having stable CD4 T cells despite a high VL, others having low CD4+ cell counts although a controlled low VL. Due to method differences and their varying performances to detect subtypes, it is generally not advisable to change from one HIV VL method to another during follow-up of infected individuals. Changes in VL of up to threefold corresponding to a variation of 0.5 Log copy/mL can be considered as irrelevant when performing the same method [44]. VL is also influenced by other factors including vaccinations and intercurrent infections. The studies by David Ho and his group showed the dynamics of HIV infection [61-63], which clearly demonstrate that the VL is a result of dynamic processes of viral production and elimination. The kinetics of VL under ART are biphasic, beginning with a sharp reduction curve representing the clearance of free plasma virus and activated cells that have a rapid turnover, followed by longer phase until the VL drops to a low level. This last phase corresponds to the turnover of less accessible sites or latently infected cells [57]. Plasma VL does not measure directly the amount of virus in the major reservoir and replication sites, such as lymphoid tissue [58].

1.6 HIV-1 TREATMENT

1.6.1 GENERAL REMARKS

Since the introduction of zidovudine (AZT or ZDV) in 1987, there have been dramatic evolutions in the treatment of HIV infected patients. In early years, from 1987 to 1994, monotherapy with AZT and later with other nucleoside reverse transcriptase inhibitors (NRTI). These NRTI, introduced between 1991 and 1994 (zalcitabine or ddC, didanosine or ddI and stavudine or d4T) brought great hope but the Concorde study did not show efficacy in terms of limiting the number of deaths or stopping progression of the disease [99]. Two studies published in 1995-1996 [100,101] showed that combination therapy with two NRTI was more effective than sequential monotherapy. A breakthrough was reached with the

introduction of a completely new class of drugs, protease inhibitors (PI) in 1996 (saquinavir or SQV, zidovudine or ZDV and didanosine or DDV) that allowed new combined therapy, so-called “highly active antiretroviral therapy” (HAART) showing great potential in terms of reducing the frequency of mortality and morbidity [102]. A third antiretroviral (ARV) class was introduced in June 1996, non-nucleoside reverse transcriptase inhibitors (NNRTI) with the licence of nevirapine. Patients went back to live and to work but complaints about increasing side effects such as fat abdomen, buffalo humps, thin legs and faces called “lipodystrophy”. Mitochondrial toxicity was suggested as the cause of lipodystrophy [103]. Other new classes of drugs were launched, making possible new possibilities of combined treatment including entry inhibitors (the anti-gp41 Enfuvirtide or T20, the anti-CCR5 maraviroc or MVC) and integrase inhibitors (raltegravir or RAL). The compliance was improved with new drug formulations combining molecules such as Combivir®, Trizivir®, Kivexa®, Truvada®, Atripla® (Table 4), which aim to reduce the high pill burden and the toxicities. Today HIV infection is considered as a chronic incurable disease, which is controllable with therapy, allowing a normal life expectancy possible. However, there are still some questions about whether antiretroviral therapy (ART) will stand the test of time in terms of resistance to treatment and toxicities such as effects on the heart, kidney, bones and other organs and the impact of aging in HIV population.

1.6.2 ANTIRETROVIRAL DRUGS

There are numerous guidelines Guidelines for the use of antiretroviral agents in HIV-1-infected adults, adolescents and infants and some have been recently updated [96,104-107]. Guidelines provide international standards and they have to be adapted to national antiretroviral therapy programmes according to the local epidemics, the strengths and weaknesses of the health systems, and the availability of financial, human and other essential resources, particularly in low- and middle-income settings.

To date, twenty-three anti-HIV compounds grouped in 5 classes are licensed. AZT molecule (zidovudine, Retrovir®) was the first approved antiretroviral agents on the market in 1987. AZT belongs to the class of nucleoside reverse transcriptase inhibitors that target the HIV enzyme reverse transcriptase. NRTIs are nucleoside analogs missing the hydroxyl group (OH) at 3' position of the deoxyribose molecule. They are called ‘chain terminators’, as they stop DNA synthesis because phosphodiester bridges can no longer be built without the -3'OH. To be active, NRTIs need to be triply phosphorylated intracellularly, as natural nucleosides. The unphosphorylated form is called pro-drug (Figure 11). Overall tolerability of NRTIs is not

bad, even if considerable differences between individual drugs are observed. Short-term complaints consist of fatigue, headache and gastrointestinal problems and the long-term side effects mainly due to mitochondrial toxicity [103] include especially lipoatrophy but also myopathy, myelotoxicity, lactic acidosis, polyneuropathy and pancreatitis.

Trade name	Abbrev.	Drug	Manufacturer
Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs)			
Emtriva®	FTC	Emtricitabine	Gilead
Epivir®	3TC	Lamivudine	GSK/ViiV
Retrovir®	AZT	Zidovudine	GSK/ViiV
Videx®	ddI	Didanosine	BMS
Viread®	TDF	Tenofovir	Gilead
Zerit®	d4T	Stavudine	BMS
Ziagen®	ABC	Abacavir	GSK/ViiV
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
Sustiva® or Stocrin®	EFV	Efavirenz	BMS/MSD
Viramune®	NVP	Nevirapine	Boehringer
Intelence®	ETV	Etravirine	Tibotec
Rescriptor®	DLV	Delavirdine	Pfizer/ViiV
Protease Inhibitors (PIs)			
Aptivus®	TPV	Tipranavir*	Boehringer Ingelheim
Crixivan®	IDV	Indinavir*	MSD
Invirase®	SQV	Saquinavir*	Roche
Kaletra®	LPV	Lopinavir/Ritonavir	Abbott
Norvir®	RTV	Ritonavir	Abbott
Prezista®	DRV	Darunavir*	Tibotec
Reyataz®	ATV	Atazanavir*	BMS
Telzir® or Lexiva®	FPV	Fosamprenavir*	GSK/ViiV
Viracept®	NFV	Nelfinavir*	Roche/Pfizer/ViiV
Entry Inhibitors			
Celsentri® or Selzentry®	MVC	Maraviroc	Pfizer/ViiV
Fuzeon®	T-20	Enfuvirtide	Roche
Integrase Inhibitors			
Isentress®	RAL	Raltegravir	MSD
Combination drugs			
Atripla®	ATP	TDF+FTC+EFV	Gilead+BMS+MSD
Combivir®	CBV	AZT+3TC	GSK/ViiV
Kivexa® or Epzicom®	KVX	3TC+ABC	GSK/ViiV
Trizivir®	TZV	AZT+3TC+ABC	GSK/ViiV
Truvada®	TVD	TDF+FTC	Gilead

Table 4: Overview of antiretroviral drugs licensed up to January 2011 [108].

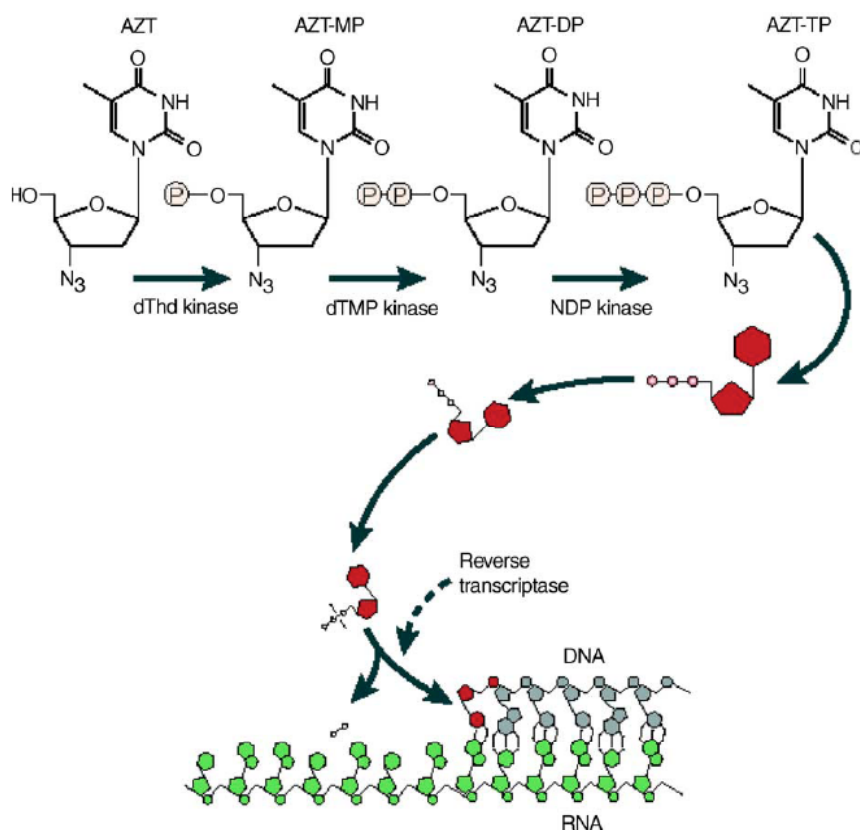


Figure 11: Mechanism of action of azidothymidine (AZT) acting as ‘DNA chain terminator’, after being triple phosphorylated intracellularly [109].

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) constitute the second antiretroviral drug class and the first generation appeared in 1990 with nevirapine (NVP, Viramune®). NNRTIs have overall a similar activity as NRTIs but a different structure. They are powerful non-competitive inhibitors targeting the RT enzyme. They interact with an allosteric site near the active site of the RT leading to conformational changes that inhibit the HIV-1 RT catalytic activity (Figure 12).

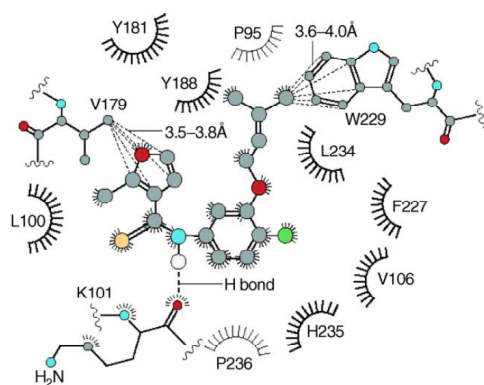


Figure 12: Interaction of a NNRTI (UC781) with an allosteric site of the RT leading to conformational changes that inhibit the HIV-1 RT catalytic activity. The hydrogen bond with K101, and other bonds are shown [109].

No phosphorylation steps are needed for NNRTIs' intracellular activation. They are HIV-1 specific drugs and not effective against HIV-2. They have good diffusion in the central nervous system (CNS), pass the feto-maternal barrier and are concentrated in lymphatic tissue but their genetic drug resistance barrier is low with very high and rapid resistance development against almost the entire class: an example is the single mutation at position 103 (K103N) of the hydrophobic binding site. Despite the problems with resistance, NNRTIs have demonstrated extreme efficacy when combined with NRTIs [110,111]. Thanks to the single dosing and the good overall tolerability, ART regimens containing NNRTIs have become an important choice as first line or simplification therapy. No metabolic disorders or lipodystrophy are associated with NNRTIs but the main observed side effects are rash in the 10-20 days after initiation of therapy containing NVP, delavirdine (DLV, Rescriptor®) or Etravirine (Intelence®) which may lead to discontinuation of treatment because of the risk of Stevens-Johnson or Lyell syndrome, cytolytic hepatitis with NVP or neuropsychological Disorders with Efavirenz (EFV, Stocrin®). NNRTIs are glucuronoconjugated through hepatic metabolism via CYP 450, which can lead to drug interactions with protease inhibitors (PIs) ., EFV inhibits CYP 3A4, 2C9 and 2C19. The new molecule etravirine (ETV, Intelence®) has limited activity against HIV-2. ETV is active against resistant K103N mutants but the efficacy decreases in the presence of multiple NNRTI mutations.

The introduction of HIV protease inhibitors (PIs) in 1996 allowed new strategies for antiretroviral treatment called tri-therapy, associating 2 NRTIs + 1 PI. Protease inhibitors are peptidomimetics that bind competitively to the active site of the viral protease (Figure 13A and B). The HIV PIs prevent the maturation process of viral proteins, which consists of cleavage of polypeptide precursors into functional subunits, the inhibition results in non-infectious viral particles. The structure design of PI molecules is based on the molecular structure of the protease encoded by the virus (Figure 13C). Using computer-aided drug design tools and the crystallographic structure of the protease-IP complex, structure-activity relationship studies allowed development of novel PIs in such a way that they fit exactly into the active site of the HIV protease [112]. PIs are active on infected cells such as activated CD4, on sanctuaries such as lymphatic tissue, cerebrospinal fluid (CSF) and seminal fluid. They are active on HIV-1 and HIV-2 at nanomolar concentrations and have a high genetic resistance barrier against the two virus types but due to their sometimes high pill burden and side effects they are less well tolerated, which leads to difficulties in adherence. PIs are powerful antiretroviral drugs that have revolutionized the treatment of HIV infection [102,113,114]. Even PI monotherapy regimens are being investigated although controversy

about their use as an alternative for treatment simplification remains [115]. PIs are an essential component of HAART with new easy-to-take PIs and boosted combinations with ritonavir (RTV, Norvir®). All PIs are metabolized in the liver by cytochrome P450 (CYP), especially the hepatic isoenzym CYP 3A, but also the intestinal CYP 3A. The CYP P450 has a very high variability between individuals, leading to the need of individual adjustment of the dosage and to a lot of drug interactions which may lead to contraindications. Interactions with CYP inducers such as rifampicin, rifadin, phenytoin and carbamazepine will cause a decrease in plasma concentration of PIs, whereas CYP inhibitors such as ketoconazole and RTV will increase their concentration. RTV has the highest affinity and inhibits the CYP3A, but is also a substrate of other cytochromes such as CYP 2D6, CYP 219, CYP 2C9. Currently, RTV is administered specifically to exploit this property, at low dose of 100mg once or twice a day in combination with other PIs in order to significantly increase or “boost” their maximum concentration (C_{max}), trough levels (C_{trough}) and half-life [116]. This interaction allows treatment simplification by reducing the frequency and number of pills. In some cases a once-daily regimen is possible. Apart from gastrointestinal problems, all PIs used in long-term therapy show side effects, mainly the lipodystrophy associating peripheral lipoatrophy, central hypertrophy and metabolic alterations such as dyslipidemia [117,118]. More or less than 50% of patients have experienced lipodystrophy (33-82%) [119-121]. This is due to an inhibitory effect on the differentiation of adipocytes [118]. NRTIs are responsible for about 14% of lipodystrophy in PI-naïve patients [122], especially d4T molecule, due to mitochondrial toxicity. Atazanavir (ATV, Reyataz®) has a favorable side effect profile by not negatively affecting plasma lipids and has a long half-life, which allows for a once-daily dosing schedule. Studies have shown that switching to a once-per-day ATV containing regimen in patients with virologic suppression who were receiving other PIs has a better maintenance of virologic suppression and an improvement of lipid parameters, [123-126]. Toxicity of PIs also includes other metabolic abnormalities such as lactic acidosis, hyperglycemia in 5-30% of treated patients [127,128] and dyslipidemia. The cohort-TM APROCO study conducted between 1998-1999 reported the presence of an abnormal glucose tolerance in 20-30% of patients treated with IP for 12 or 20 months [128,129]. Dyslipidemia [121,122] is characterized by elevated total cholesterol (10-24% of hypercholesterolemia), elevated LDL cholesterol and elevated triglycerides (43-66% of hypertriglyceridemia) with risks of cardiovascular complications. Other problems encountered with PIs based treatment are dermatological, hematological, hepatic and neurological side effects. Studies conducted before PI approval [130] have revealed that amino acid substitutions in the HIV-1 protease were sufficient to yield cross-resistance to a panel of protease inhibitors.

Darunavir and tipranavir [131,132] are two second-generation PIs on the market which are effective even in the presence of several resistance mutations in treatment-experienced patients as their respective drug-resistance profiles are different with limited cross-resistance to other PIs.

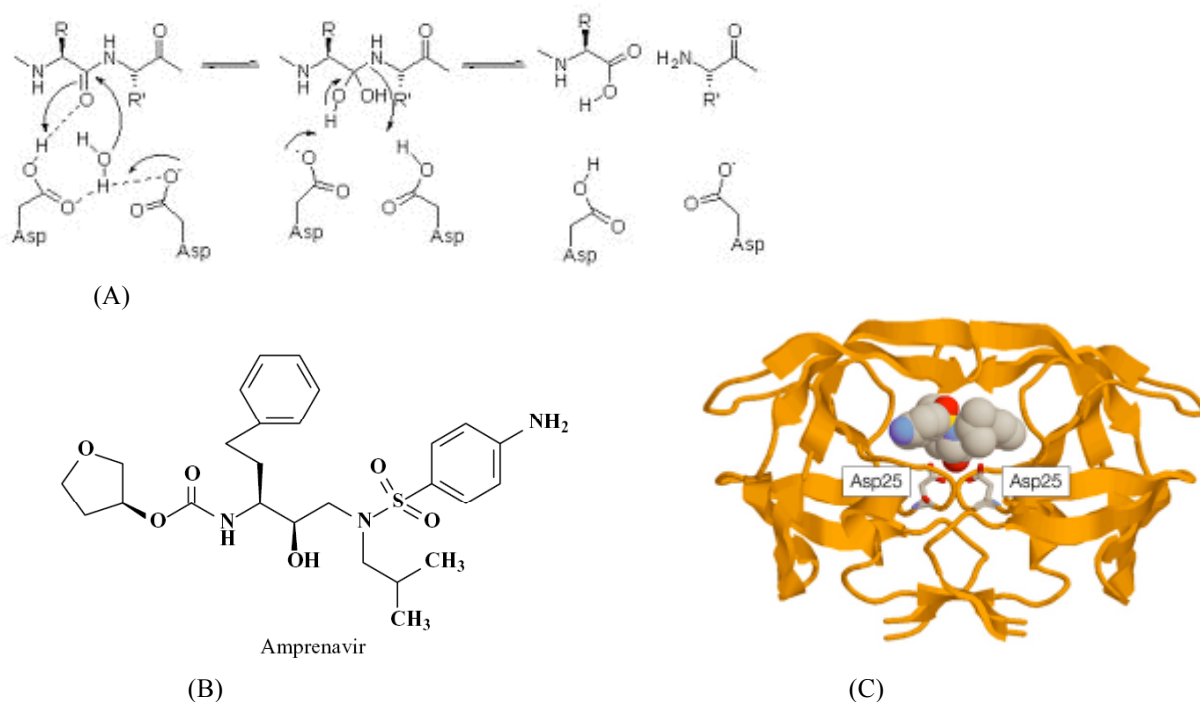


Figure 13: The HIV protease as member of the aspartic protease family has a catalytic site comprising 2 opposite protonated aspartic acids (Asp) in quasi-coplanar conformation, which undergo a nucleophilic attack. The reaction leads to an unstable zwitterionic tetrahedral intermediate, which breaks down into amine and carboxylate acid compounds (A). Here, the peptidomimetic structure of amprenavir that bonds and inhibits the protease (B) and the crystal structure of HIV protease with its inhibitor substrate amprenavir bound (C). The inhibitor is shown as a space-filling model and the protease is represented with ribbons and the two active-site aspartic acids (Adapted from [133-134]).

A new class called “entry inhibitors” have been introduced comprising Enfuvirtide (T20 or Fuzeon® licenced in 2003) a “fusion inhibitor” drug that inhibits the fusion of the viral and cellular membranes by targeting the viral glycoprotein gp41 and maraviroc (MVC or Celsentri® or Selzentry®) a “coreceptor antagonist” which targets the HIV coreceptor CCR5. The third “entry inhibitor” class includes “attachment inhibitors”, which are not yet on the market and are supposed to inhibit the attachment of the virus to the CD4 receptor. However, the development of the CD4 inhibitors requires more potent molecules than existing ones and better pharmacological proprieties [11, 135]. Entry inhibitors should be used in combinaison with other ART drugs and are not approved for use in antiretroviral-naïve persons. Enfuvirtide is a peptide of 36 aminoacids that competes with HR2, thereby inhibiting the

attachment of the latter on HR1 and inhibiting the formation of a hairpin structure that should bring the viral and cell membranes into proximity for fusion

Maraviroc was the first antiretroviral compound that targets a host protein, the chemokine receptor CCR5 and inhibits the binding of the viral gp120 to the CCR5 chemokine. This implies that one must first ensure that the tropism of the virus is R5 before using this antiretroviral [136].

The last ART class comprises Raltegravir (RAL or ISENTRESS®), a viral integrase (INT) inhibitor licenced in 2007. Raltegravir and Elvitegravir inhibit the transport of the PIC from the cytoplasm into the nucleus. [108,137-140].

Raltegravir has shown efficacy in heavily treated patients undergoing treatment failure, but is also now approved (2009) for first line therapy in combination regimens. Maraviroc and Raltegravir came into clinical practice after initiation of this work.

1.6.3 WHEN TO START AND WHAT TO START WITH

As currently it is not yet possible to get rid of HIV infection, the main goal of ART is to control the viral replication resulting in the recovery of the immune system. As a consequence, the maintenance of the best quality of health and life of HIV infected patients is part of the ART objectives. Even if there are many guidelines [104-107, 141] that provide specific recommendations on the initiation and the management of ART, the best time to start remains a subject of controversial debate taking in account the risks of inherent long-term toxicity and of developing drug resistance. First of all it is recommended to assess the readiness of HIV infected patients to start HAART by complete medical history, physical examination, specific and routine laboratory evaluation, vaccination (HAV, HBV, pneumococcal), social and psychological condition. Specific laboratory tests include CD4 T cell count, viral load, genotypic resistance. It is important to focus on the patient's physical and mental well-being for the long-term effective treatment. Three important criteria guide the decision to initiate or to defer treatment, the most important being the clinical assessment and the CD4 T cell count, and to a lesser extent the plasma viral load. The current European guidelines published in October 2011 [141] for the management of naive patients are summarized in table 5 and the drugs to start with in table 6. The EACS guidelines also provide recommendations for specific situations such as starting HAART in tuberculosis (TB)/HIV coinfection, switching strategies for virologically effective treatment in case of

toxicity or for simplification, management of virological failure, treatment of HIV in pregnant women and initiation of post-exposure prophylaxis.

Condition	Current CD4+ lymphocyte count ^(i,ii)	
	350-500	> 500
Asymptomatic HIV infection	C	D
Symptomatic HIV disease (CDC B or C conditions) incl. tuberculosis	R	R
Primary HIV infection	C	C
Pregnancy (before third trimester)	R	R
Conditions (likely or possibly) associated with HIV, other than CDC stage B or C disease:		
HIV-associated kidney disease	R	R
HIV-associated neurocognitive impairment	R	R
Hodgkin's lymphoma	R	R
HPV-associated cancers	R	R
Other non-AIDS-defining cancers requiring chemo- and/or radiotherapy	C	C
Autoimmune disease – otherwise unexplained	C	C
High risk for CVD (> 20 % estimated 10-yr risk) or history of CVD	C	C
Chronic viral hepatitis		
HBV requiring anti-HBV treatment	R	R
HBV not requiring anti-HBV treatment	C/R ^(iv)	D
HCV for which anti-HCV treatment is being considered or given	R ^(vi)	D ^(vi)
HCV for which anti-HCV treatment not feasible	R	C

Table 5: The European AIDS Clinical Society (EACS) guidelines published in 2011 for the clinical management and treatment of HIV-infected adults in Europe without prior ART exposure. Recommendations are graded while taking into account both the degree of progression of HIV disease and the presence of or high risk for developing various types of (co-morbid) conditions [141].

6A

A	B	Remarks
NNRTI	NRTI	
• EFV ⁽ⁱ⁾	ABC/3TC ^(vi) or TDF/FTC	• TDF/FTC co-formulated • ABC/3TC co-formulated • EFV/TDF/FTC co-formulated
• NVP ⁽ⁱⁱ⁾	TDF/FTC	• TDF/FTC co-formulated
Ritonavir-boosted PI		
• ATV/r ⁽ⁱⁱⁱ⁾ • DRV/r ⁽ⁱⁱⁱ⁾ • LPV/r ^(iv)	ABC/3TC ^(vi) or TDF/FTC	• ATV/r: 300/100 mg qd • DRV/r: 800/100 mg qd • LPV/r: 400/100 mg bid or 800/200 mg qd
ITI		
• RAL	TDF/FTC	• RAL: 400 mg bid

6B

Ritonavir-boosted PI	Remarks
• SQV/r	1000/100 mg BID
• FPV/r	700/100 mg bid or 1400/200 mg QD
NRTI	
• TDF-3TC • ZDV/3TC • ddI/3TC or ddI/FTC ^(vii)	ZDV/3TC co-formulated
CCR5 inhibitor	
MVC ^(v)	Only if CCR5 tropic HIV ^(vii)

Table 6: The European AIDS Clinical Society (EACS) guidelines published in 2011: (6A) Initial combination regimen (select drug/s of each column) for antiretroviral-naïve Patients (6B) Alternative regimens [141].

Significant differences can be seen in published guidelines. In CDC A category with CD4 T cell count between 200-350/uL, the American guidelines developed by the US Department of Health and Human services (DHHS) also published in 2011 [104] do recommend ART “but data supporting this are less strong than for patients with <200 CD4 T cells” while the EACS and the World Health Organization (WHO) guidelines simply recommend to treat. WHO guidelines for ART for HIV infection in adults and adolescents are published as a basis for national treatment advisory councils, partners involved in the implementation of HIV care and treatment, and organizations providing technical and financial support to HIV care and treatment programmes focused on settings with limited health systems capacity and resources [106]. However, these guidelines have to be adapted according to the balance between risks and benefits, acceptability and cost. The implementation may not be feasible in some settings.

1.6.4 DRUG INTERACTIONS AND TOXICITY

Drug interactions between anti-HIV molecules, mainly inhibitors and inducers of metabolism via CYP 3A are well known and the general side effects of each drug class have been briefly described under each drug class presentation (see 1.5.4.2 Antiretroviral drugs). Interactions between ARV drugs and other medications are often less well characterized and may be important and severe such as the interactions between RTV or SQV and statins that are lipid lowering agents requiring possible dose adjustment of pravastatin. Simvastatin should be avoided as the area under the curves (AUC) may be elevated by >3000% [142]. Fatal rhabdomyolysis have been reported on co-administration of simvastatin, atorvastatin and PIs such as atazanavir, lopinavir and nelfinavir [108,143]. Many other drugs or unproblematic products may interact with ARV and may be avoided, including certain contraceptives, garlic capsules that may reduce the SQV or DRV plasma concentrations or vitamin C can influence IDV plasma levels [144-146]. Alcohol and drugs such as the methadone, amphetamines, e.g., 3,4 methylene-dioxymethamphetamine (MDMA or ecstasy), the popular narcotic gamma hydroxybutyric acid (GHB) and the cannabis or marijuana with its psychoactive chemical compound, the tetrahydrocannabinol (THC), can interact with ART even resulting in more serious consequences [147-149]. By contrast, RAL seems to not have such interaction effects [150].

Combining ART with other medications that have the same toxicity pattern can lead to additive toxicities, one must be cautious in the choice of therapy. Drug causing myelotoxicity (anaemia, neutropenia) such as AZT, caution is required with other myelotoxic drugs, e.g., valgancyclovir or cotrimoxazole. When treating hepatitis C with interferon and ribavirin,

pharmacokinetic interactions with ARV drugs are described, almost exclusively due to the combination of ribavirin and NRTIs [151]. Nephrotoxic medications should also be avoided in combination with TDF, IDV and possibly also atazanavir [108]. Potential allergy-inducing ARV drugs such as nevirapine, efavirenz and abacavir, but also fosamprenavir and darunavir should be avoided in combination with cotrimoxazole or other sulphonamides used as anti-infectious prophylaxis, in order to be able to clearly identify the causative agent for a drug-induced exanthema. Immediate ART switching due to acute side effects is not required in all cases. Gastrointestinal side effects, some allergic reactions or relatively mild CNS disorders occurring at the start of the ART often improve spontaneously or are symptomatically treated. However, in some cases discontinuation or changing of ART is required (Table 7).

Side effects that almost always require discontinuation/change of ART

- Severe diarrhea, which persists despite loperamide even after several weeks (usually with nelfinavir, lopinavir/r, fosamprenavir/r, saquinavir/r)
- Severe nausea, which persists despite metoclopramide, which requires continuous treatment or leads to significant weight loss (usually AZT, ddI)
- Polyneuropathy (d4T, ddI, possibly also 3TC) often resolves very slowly
- Severe anemia (AZT)
- Severe, progressive muscular weakness (d4T, ddI)
- Pancreatitis (ddI, ddI+TDF, d4T+ddI, in rare cases lopinavir/r)
- Lactic acidosis (most often d4T+ddI, but also all other NRTIs)
- Severe allergies with involvement of mucous membranes, fever (typically abacavir, all NNRTIs, more rarely fosamprenavir or darunavir)
- Renal failure (tenofovir, indinavir), nephrolithiasis (indinavir)
- Hepatotoxicity with transaminases >5 x normal values (nevirapine, tipranavir)
- Jaundice (nevirapine, atazanavir, indinavir, tipranavir)
- Rhabdomyolysis (raltegravir)
- Severe repetitive onychitis (indinavir, possibly also 3TC)
- Psychosis (efavirenz, possibly also AZT)

Table 7: Severe side effects leading to ART switch or discontinuation [108].

In the last few years concerns over long-term toxicities such as lipodystrophy and dyslipidemia have conducted to so called “treatment simplification” mostly consisting in changing treatments including PIs that are virologically successful to PI sparing combinations. The switch strategy is virologically safe in most cases, since the viral load is controlled and there is no emergence of resistance. It is also used in order to improve the adherence with reduced pill burden that allows a simplified dosage.

1.6.5 TREATMENT RESISTANCE

Treatment failure is usually linked to 3 factors: first of all there are patient-related causes, including poor adherence to treatment, which is the primary cause of treatment failure, secondly there are drug-related causes, mainly potency, toxicity and dosage and finally causes

related to the virus, mainly drug resistance. Among virus related causes, the assessment of the CCR5 coreceptor usage is a prerequisite in Maraviroc-associated treatment. Two established assays, the genotypic and the phenotypic resistance tests have been developed to measure sensitivity of the HIV to ARV drugs [152].

Resistance to AZT described by Larder and *al* in 1989 [153-155] was the first data suggesting correlation between the emergence of viral resistance and clinical outcomes. As at that time, no test was available to assess the viral replication under drug treatment, clinical progression was the primary indication of possible drug resistant virus. They demonstrated that a recombinant mutant with mutations introduced by site-directed mutagenesis in the HIV RT maintained enzyme activity but had a reduced ability to recognize inhibitors such as AZT-triphosphate. In another article, they studied the genetic basis of the resistance. They identified 5 amino acid substitutions that could predict resistance to AZT by comparing RT nucleotide sequences of sequential sensitive and resistant isolates obtained at the initiation and during therapy. Cohen and *al* have shown that antiretroviral treatment guided prospectively by phenotypic resistance assay led to the increased use of active antiretroviral agents and was associated with a significantly better virological response [156].

Phenotypic resistance tests directly quantify virus sensitivity to a given drug by measuring the viral replication in cell culture under the selective pressure of increasing ARV drug concentrations compared to viral replication of wildtype (WT) virus. The phenotypic results are expressed as IC₅₀ or IC₉₀ (inhibitory concentration) values, that are the required drug concentrations inhibiting respectively 50% or 90% of viral replication in cell cultures (Figure 14). Another way to report the viral sensitivity is in the form of the fold-change value as a ratio of the IC₅₀ of a strain divided by the IC₅₀ of a WT reference virus. The fold-change value is compared to the cut-off value that is the maximum fold change for a strain classified as sensitive. Approved phenotypic assays use recombinant virus composed of a standard reference virus encompassing patient-derived plasma HIV RNA genes of interest such as PR and RT genes, more recently, integrase and envelope sequences. Commercial phenotypic resistance tests include Antivirogram™ (Tibotec-Virco Group NV, Belgium), PhenoSense™ (Monogram Biosciences, South San Francisco), PhenoTec™ (InPheno, Switzerland), Phenoscript™ (Viralliance, France). European guidelines [157] recommend to consider phenotyping for new drugs, in heavily pretreated patients, and for HIV-2 where genotyping is not easily interpretable and to use a clinical cut-off if available, or otherwise a biological cut-off. It is recommended to take into account the clinical context, the therapy and resistance histories.

The American DHHS guidelines [104] recommend in most situations genotypic testing because of the faster turnaround time, lower cost, and enhanced sensitivity for detecting mixtures of wild-type and resistant virus. However, for patients with a complex treatment history, DHHS guidelines state that results derived from both assays might provide critical and complementary information to guide regimen changes.

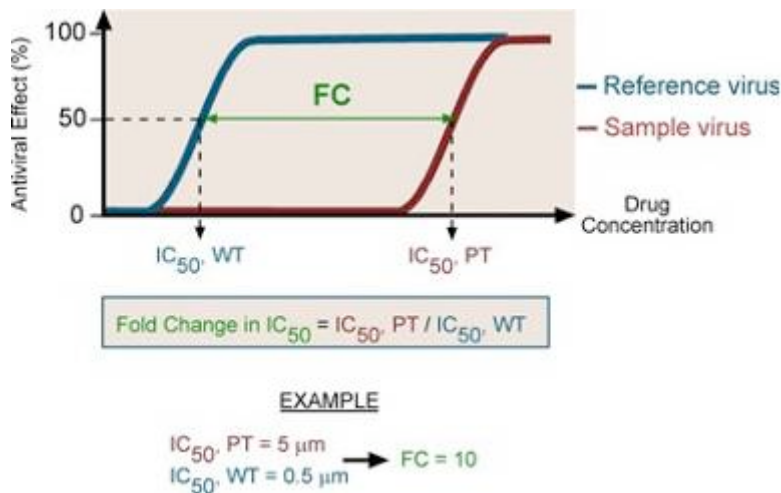


Figure 14: shows the relationship between the antiviral effect (Y-axis) and drug concentration (X-axis) of WT and mutant strains of HIV and increasing drug concentrations. The shift of the WT sigmoidal concentration curve (blue curve) to the right patient's (PT) mutant curve (red curve) illustrates that greater concentrations of drug are needed (IC_{50}) to reach the same level of inhibition [158].

Both phenotypic and genotypic methods require a minimum amount of virus to perform the test, usually more than 1,000 viral copies/ml. They also need clinical interpretation, which may vary depending on the test performed and the algorithm used. Genotypic assays search for known mutations in the viral genome that have shown an impact on viral susceptibility to certain drugs in vitro and selected in vivo in patients failing HIV treatment including these drugs. These mutations are determined by the direct population sequencing of HIV amplicons that detects all possible resistance-associated mutations or by specific hybridization techniques with wild-type or mutant oligonucleotides that test only a limited subset of the positions in which resistance mutations may occur. Interpretation of resistance mutations is performed at amino acid level after translation of the obtained nucleotide sequences. Standard population-based genotypic tests only detect minor viral mutants representing at least 20% of the total virus population depending on the tests used. More sensitive methods, such as oligonucleotide hybridization, and ultrasensitive methods such as allele-specific real-time PCR, single genome sequencing with detection limits of 10% to <0.1–5%, respectively, are available in some laboratories with questions about the clinical relevance of minority variants. There are numerous genotypic assays for predicting resistance or sensitivity of HIV to

specific antiretroviral drugs, including “homebrew” methods, established and commercial ones. The assays accredited by the FDA are HIV-1 TruGene™ (Siemens Healthcare Diagnostics) and the ViroSeq™/ABI Prism® 3100 Genetic Analyzer (Abbott Molecular / Applied Biosystems and Celera). The main limitation of the genotype testing is the clinical interpretation of detected mutation patterns, given the number of existing algorithms, the complexity of the available data and the ongoing acquisition of new data. The report of a genotype assay is a list of the identified resistance mutations, including an interpretation of the degree of resistance to particular drugs. The most popular drug resistance interpretation algorithms include the HIVdb (Stanford University Medical Center, CA, USA), the RetroGram (Virology Networks BV, Utrecht, the Netherlands), the RegaInst (Rega Institute for Medical Research and University Hospitals Leuven, Katholieke Universiteit Leuven, Belgium), the ANRS AC11 (Agence Nationale de Recherches sur le SIDA, France, see table 8), the HIV-GRADe (developed by a German standardisation initiative) and the International AIDS Society–USA (IAS–USA) drug resistance mutations list. These algorithms or lists are updated regularly and most of the non-commercial ones can be used via convenient web services.

ANRS - AC 11 : RESISTANCE GROUP		
GENOTYPE INTERPRETATION: NUCLEOSIDE AND NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS		
	Mutations associated with resistance	Mutations associated with « possible resistance »
ZDV	• T215Y/F • At least 3 mutations among : M41L, D67N, K70R, L210W, T215A/C/D/E/G/H/I/L/N/S/V, K219Q/E [1, 2, 3, 4] • Q151M • Insertion at codon 69	• T215A/C/D/E/G/H/I/L/N/S/V [1, 2, 3, 4]
3TC/FTC	• M184V/I • Insertion at codon 69	• K65R [11, 12, 16] • Q151M
ddl	• At least a score of + 2 among: M41L + T69D + 215Y/F + K219Q/E – K70R – M184 V/I [5, 14, 15, 17, 18] • L74V/I [19] • Q151M • Insertion at codon 69	• K65R [11, 12]
d4T	• V75A/M/S/T • T215Y/F [6] • At least 3 mutations among : M41L, D67N, K70R, L210W, T215A/C/D/E/G/H/I/L/N/S/V, K219Q/E [4, 7, 14, 15] • K65R [30, 31, 32] • Q151M • Insertion at codon 69	• T215A/C/D/E/G/H/I/L/N/S/V [4, 7]
ABC	• At least 4 mutations among : M41L, D67N, M184V/I, L210W, T215Y/F [8, 19, 29] • K65R [9, 11, 12] • L74V/I [24, 25, 26, 27, 28, 29] • Y115F • Q151M • Insertion at codon 69	• 3 mutations among : M41L, D67N, M184V/I, L210W, T215Y/F [8, 19, 29]
TDF	• At least 6 mutations among: M41L, E44D, D67N, T69D/N/S, L74V/I, L210W, T215Y/F [13, 20, 33] • K65R [9, 10, 11, 12] • Insertion at codon 69 • K70E [21, 22, 23]	• 3, 4 or 5 mutations among: M41L, E44D, D67N, T69D/N/S, L74V/I, L210W, T215Y/F [13, 33]

ZDV: zidovudine, 3TC: lamivudine, FTC: emtricitabine, ddl: didanosine, d4T: stavudine, ABC: abacavir, TDF: tenofovir

A

ANRS - AC 11 : RESISTANCE GROUP
GENOTYPE INTERPRETATION: NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

	Mutations associated with resistance	Mutations associated with « possible resistance »
EFV	• L100I • K101E • K103H/N/S/T [1] • V106M [2] • E138K [12, 13] • Y181C/I • Y188C/L • G190A/C/E/Q/S/T/V • P225H • M230L	
NVP	• A98S (for HIV-1 subtype C only) [3] • L100I • K101E • K103H/N/S/T [1] • V106A/M [2] • Y181C/I • Y188C/H/L • G190A/C/E/Q/S/T/V • M230L	• E138K [13]
ETR	• At least 4 among: V90I, A98G, L100I, K101E/H/I/P/R, V106I, V179D/F/I/L/M/T, Y181C/I, G190A/S, M230L [4, 7, 8, 9, 10, 11] • E138K [12, 13] • Y181V [5, 6] • Y181C+H221Y [7]	• 3 mutations among: V90I, A98G, L100I, K101E/H/I/P/R, V106I, V179D/F/I/L/M/T, Y181C/I, G190A/S, M230L [4, 7, 8, 9, 10, 11] • E138A/G/Q/R [5, 6, 7, 8]
RPV	• E138K [12, 13]	• E138G/Q/R [13] • Y181C/I/V [13] • H221Y [13]

EFV: efavirenz, NVP: nevirapine, ETR: etravirine, RPV : rilpivirine

B

ANRS - AC 11 : RESISTANCE GROUP
GENOTYPE INTERPRETATION: PROTEASE INHIBITORS

	Mutations associated with resistance	Mutations associated with « possible resistance »
IDV	• M46I/L • V82A/F/M/S/T [11] • I84A/V [8] • L90M and at least 2 among : K20M/R, L24I, V32I, M36I, I54V/L/M/T, A71V/T, G73S/A, V77I	• L90M
SQV/RTV 1000/100 mg BID	• G48V • At least 4 mutations among: L10F/I/M/R/V, I15A/V, K20I/M/R/T, L24I, I62V, G73S/T, V82A/F/S/T, I84V, L90M [9]	• 3 mutations among: L10F/I/M/R/V, I15A/V, K20I/M/R/T, L24I, I62V, G73S/T, V82A/F/S/T, I84V, L90M [9]
NFV	• D30N • I84A/V [8] • N88S/D • L90M	• V82A/F/S/T and at least 2 among: L10I, M36I, M46I/L, I54V/L/M/T, A71V/T, V77I [1]
FPV/RTV 700/100 mg BID	• I50V • V32I and I47A/V [2, 13, 14] • At least 4 mutations among: L10F/I/V, L33F, M36I, I54A/L/M/S/T/V, I62V, V82A/C/F/G, I84V, L90M [2, 20]	
LPV/r	• At least 6 mutations among : L10F/I/R/V, K20M/R, L24I, L33F, M46I/L, I50V, F53L, I54M/L/T/V, L63P, A71I/L/V/T, V82A/F/S/T, I84V, L90M [3, 4, 5, 21] • I47A [15, 16] • L76V [18, 19]	• 4 or 5 mutations among: L10F/I/R/V, K20M/R, L24I, L33F, M46I/L, I50V, F53L, I54M/L/T/V, L63P, A71I/L/V/T, V82A/F/S/T, I84V, L90M [3, 4, 5, 21]
ATV/RTV 300/100 mg QD	• I50L [6] • At least 3 mutations among: L10F/I/V, G16E, L33F/I/V, M46I/L, D60E, I84V, I85V, L90M [7, 12, 22]	
TPV/RTV 500/200 mg BID	• At least a score of + 3* : 36I/L/V – 53L/W/Y + 58E + 69I/K/N/Q/R/Y + 89I/M/R/T/V [10, 23]	• A score of + 2* : 36I/L/V – 53L/W/Y + 58E + 69I/K/N/Q/R/Y + 89I/M/R/T/V [10, 23]
DRV/RTV 600/100 mg BID	• At least 4 mutations among: V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V, L89V [17, 24, 25, 26]	• 3 mutations among: V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V, L89V [17, 24, 25, 26]

IDV: indinavir, SQV: saquinavir, NFV: nelfinavir, RTV: ritonavir, FPV: fosamprenavir, LPV: lopinavir, ATV: atazanavir, TPV: tipranavir, DRV : darunavir

C

ANRS - AC 11 : RESISTANCE GROUP
GENOTYPE INTERPRETATION: FUSION INHIBITOR

ENF T20	Mutations associated with resistance • G36A/D/E/S/V [1, 2, 3, 4, 5, 6, 7] • V38A/E/K/M • Q40H/K/P/T • N42D/T • N43D/H/K/S • L44M • L45Q/M
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ENF (T20): enfuvirtide

D

GENOTYPE INTERPRETATION: INTEGRASE INHIBITORS

	Mutations associated with resistance	Mutations associated with « possible resistance »
RAL	• T66K [10] • E92Q [1, 2] • F121Y [10] • G140A/S [7] • Y143C/G/H/R/S [1, 3, 4, 5, 9] • Q148E/G/H/R [1, 2] • V151L [10] • N155H/S/T [1, 2, 10] • E157Q [2]	• 3 mutations among : V72I, L74M, T97A, V151I, G163R, S230R [8, 10]
EVG	• T66I/A/K [6] • E92Q [6] • F121Y [10] • E138K • G140C/S • P145S [10] • S147G • Q148H/R/K [6] • V151L [10] • N155H/S/T [6, 10]	

RAL: raltegravir, EVG: elvitegravir

E

Table 8: The French ANRS (National Agency for AIDS Research) AC11 Resistance group provides HIV-1 genotypic drug resistance interpretation's algorithms (update from May 2011- Version n°20) guiding the choice of antiretroviral treatment. The rules are presented as tables listing specific anti-HIV drugs and associated mutations conferring genotypic resistance or possible genotypic resistance. (A) Nucleosidic RTI's table (B) NNRTI's table (C) PI's table (D) Fusion inhibitor's table and (E) INI's table [159].

Guidelines published in 2011 [104,160] for using HIV resistance testing recommend to perform the assay in ART-naïve patients with acute or chronic HIV infection on the earliest sample, in treated patients with virologic failure, in patients with suboptimal suppression of viral load and in HIV-infected pregnant women. The European HIV Drug Resistance Guidelines updated in May 2011 recommend resistance testing for integrase and fusion inhibitors if such drugs were included in the failing regimen and to consider testing for CCR5 tropism at virologic failure or when changing the therapy. In the latter case the test can be performed on DNA in absence of detectable viral load or on the last detectable plasma RNA. European experts also recommend to test the earliest detectable RNA in NNRTI's failing combined therapy, to test a source patient when postexposure prophylaxis is initiated and to

consider resistance testing for HIV-2 after treatment failure. When performing resistance assays is not possible, guidelines recommend storing samples. It is advised that laboratories that perform HIV resistance testing participate to external quality control programs and HIV clinicians and virologists take part in continuous education and discuss problematic clinical cases.

Clinical usefulness of Genotype testing before making changes to therapy has been shown in several prospective, controlled studies. In the VIRADAPT prospective study [161], 108 patients that underwent treatment failure were enrolled. At month 3 and 6, respectively 29% and 32% of patients in the genotypic group had HIV-1 RNA under detection level compared to 14% in the control group at each time. The study was discontinued in the 6th month, due to a difference in benefit between the 2 groups in favour of the Genotype test group. Other studies such as CPCRA 046, Havana or Narval [162-164] confirmed that more significant decreases in viral loads were achieved in patients whose physicians guided the choice of ARV drugs on information about existing mutations than those in whom ART was changed without knowing the mutation resistance pattern. A meta-analysis study [165] of published studies (total of 2258 participants) showed increased efficacy in term of virological outcome when therapy modification was guided by genotype or virtual phenotypic testing even if the conclusion of the article was that the evidence for benefit of antiretroviral resistance assays is sparse and limited to small short-term improvements of virologic response. In addition, the clinical relevance of resistance testing in new drugs such as RAL, the integrase inhibitor or MVC, the CCR5 antagonist has not been proven. Transmission of virus harbouring mutations has been already described in treatment-naïve patients in different regions. A prevalence of 10,4% of primary resistance mutations in 2208 new HIV diagnoses from 19 European countries was observed in the European CATCH study, a substudy of SPREAD (Strategy to Control Spread of HIV Drug Resistance), between 1996 and 2002 [166]. Vercauteren *et al* [167] showed that the transmission of drug resistance (TDR) appeared to stabilize in Europe, with no time trend in the overall TDR or in NRTI resistance, but for PI and NNRTI resistance, they observed a decrease in the transmission that was statistically significant after an initial increase.

1.7 LITERATURE REVIEW OF HIV PROVIRUS

The HIV provirus results from the transcription of the viral RNA to the double stranded viral DNA (dsDNA) mediated by the viral RT and subsequently integrated into the host cell genome by the viral integrase. The provirus serves as a template for production of new virions. The reservoir of the latent HIV-1 proviral DNA is constituted by long living infected cells and importantly restricted to some biological compartments that are not easily accessible to drugs, making it impossible to eradicate the virus. The HIV life cycle and the role of reservoir in viral persistence have been extensively explained under paragraph 1.3.2.2 and in chapter 1.4, respectively. Some characteristics that have already been described are important to be reminded. The proviral DNA is not totally integrated, the amount of non-integrated HIV DNA was found to be about 100 fold more predominant than integrated DNA [34,35]. To distinguish between episomal and integrated HIV-1 DNA, Alu-HIV PCR assays or PCR targeting the 2-LTR are performed allowing kinetic studies of the integrated and non-integrated HIV-1 DNA. These assays are also used to assess the effectiveness of integrase inhibitors. Ninety-five to 99% of infected cells are CD4⁺ T cells [168], and memory infected CD4⁺ T cells have a long life span. Around 10^9 CD4 T cells are infected, 1.2×10^7 of them containing latent provirus and 1.4×10^6 producing new virions after appropriate T cell stimulation [35]. About 2% of lymphocytes in healthy young men [59] circulate in peripheral blood and it has been shown that in a rat there is a continuous circulation of small lymphocytes between the peripheral blood and the lymph nodes with a 3 minutes cycle [60]. At present, the HIV-1 viral load and the drug resistance mutations in infected patients are performed on plasma viral RNA. The HIV-1 proviral DNA could be an alternative matrix for monitoring the long term efficacy of HIV drugs, measuring the impact of the HAART on the reservoirs and detecting archived resistance mutations. A number of articles consider the detection of drug resistance mutations in HIV-1 provirus with conflicting results. Some authors have described the presence of key mutations conferring resistance to reverse transcriptase (RT) inhibitors and major mutations in the protease (PR) region in proviral DNA that were not present in the plasma virus [169-172]. By performing direct sequencing, other authors found that mutations associated with drug resistance in treatment-naïve patients remained compartmentalized in plasma and PBMCs [172] while others observed concordance [172,173] with an early establishment of the viral reservoir during primary HIV-1 infection (PHI) [173] and therefore the persistence of possibly acquired resistance mutations. In contrast some showed that mutations detected in the plasma were not present or were infrequently present in the proviral DNA. Those discrepancies persisted for more than 3 years

[174]. An article published in 2011 [175] compared RT and PR sequences of 134 and 141 viral DNA with 443 and 462 corresponding plasma RNA, respectively. The mean rates of concordance between DNA and RNA genotypes were low (46.7% for NRTIs, 26.3% for NNRTIs and 43.7% for PIs). As it was difficult to amplify the archived mutated DNA, authors concluded that in the context of low or undetectable viraemia, the history of the classical RNA genotyping during previous periods remains the gold standard for choosing future treatments.

Quantification of specific DNA is another measurement of the HIV-1 provirus studied in the literature. As HIV-1 proviral DNA exists in different forms and there is no standardisation in performed assays, articles on the quantification are not always comparable. Some assays are conducted on PBMCs while others use purified CD4⁺ cells. CD4⁺ cells normally represent 30 to 50% of the PBMCs but may be lower in HIV infected individuals. There might be some concern about sensitivity and accuracy of assays quantifying low-copy-number samples which is often the case, ranging from 1 to < 5 log₁₀ HIV-1 DNA copies (median 2–3 log₁₀) in 10⁶ PBMC equivalents [176-186]. Methods quantifying the HIV-1 DNA proviral load by sequence-specific hybridisation probes are more specific but the very high variability of HIV sequences makes the design difficult. A sequence-independent detection with SYBR Green I might be more sensitive but less specific. The PCR format based on SYBR Green I that binds to double-stranded DNA molecules requires an extra analysis of melting temperature curves to ensure the specificity of the signal. The HIV-1 proviral load is reported as a relative quantification against a reference gene that may influence the final result, for example by inhibiting PCR reaction due to high amount of the reference gene. Having identical PCR efficiencies for target and reference genes in sample, standard, and calibrator is one of the experimental prerequisites for PCRs that may have a major impact on accuracy.

Gibellini et al. [185] developed a quantitative detection of HIV-1 proviral DNA in peripheral blood mononuclear cells by SYBR green qPCR technique on LightCycler technology. They evaluated the DNA reservoir that represents one of the major drawbacks to the total eradication of HIV-1. It is also an important diagnostic marker in the diagnosis of HIV-1 infection of newborns of HIV-1 seropositive women. PCR results of this study showed that five copies of the HIV-1 genome were detected while no amplification was obtained on any PBMC blood donors.

Kostrikis and al. [176] reported that the quantitation of HIV-1 DNA forms in peripheral blood cells may predict the progression of the disease. In this study, they quantified the baseline concentration of HIV-1 DNA forms that have undergone the second template switch (STS DNA) and 2-long-terminal-repeat DNA circles in PBMCs samples. In 130 patients included

with haemophilia, the median baseline levels of STS HIV-1 DNA were significantly higher in patients who progressed to AIDS during the 16 years of follow-up (1,017 [235 to 6,059] and 286 [31 to 732] copies per 10^6 PBMC, respectively; $P < 0.0001$).

Sarmati et al. [187] studied the relation between the HIV DNA load in cells and immunological parameters in 163 patients under HAART with undetectable viremia. Patients with low HIV DNA (133 copies/ 10^6 PBMCs) had higher pre-HAART ($P < 0.001$) and current ($P < 0.005$) CD4 counts and a prolonged duration of treatment ($P < 0.001$). At adjusted analysis, prolonged treatment was independently associated with lower ($P < 0.006$) and undetectable ($P < 0.001$) HIV DNA values.

Sabine Yerly et al. and the Swiss HIV Cohort Study [189] assessed the value of HIV proviral DNA to predict the viral rebound and viral setpoint after structured treatment interruptions. They found that in patients under HAART with long-term undetectable viraemia, the pre-HAART plasma viraemia and the baseline proviral DNA level were significantly associated with the viraemia setpoint during scheduled treatment interruptions. They concluded that as pre-HAART viraemia may not be available, proviral DNA measured at the time of scheduled treatment interruption, can help to identify patients likely to reach a low viraemia setpoint after treatment interruption.

Another study conducted by Sarmati et al. [190] evaluated the predictive value of the total HIV-1 DNA quantitation for long-term success of simplified treatment. Among 62 patients with effective HAART containing PI before being enrolled in a prospective non-randomized cohort with simplified PI-sparing regimen, patients with proviral DNA levels below the median value (226 copies/ 10^6 PBMCs) had a significant higher CD4 cell count at nadir ($P = 0.003$) and at enrolment ($P = 0.001$). Eighteen month after treatment simplification, a reduced risk of experiencing virological failure or blip was correlated with HIV-1 DNA levels below 226 copies/ 10^6 PBMCs at baseline (OR 0.002, 95% CI 0.001–0.46, $P = 0.025$). Authors also observed a correlation between sustained undetectable plasma viral load with low proviral DNA load before treatment simplification.

Vitone et al. [188] quantified HIV-1 proviral DNA in 93 treated patients by SYBR green qPCR in PBMCs. The proviral DNA load, ranging from 14 to 1847 copies per 10^6 PBMCs was not correlated with plasma RNA levels or CD4 cell count. In 32 followed patients with persistently undetectable viremia, a decrease of 0.5 log or more in proviral DNA levels was significantly associated with a definite increase in CD4 lymphocyte counts. They concluded that measuring proviral DNA levels could represent an adjunct prognostic marker to plasma HIV-1 RNA load, especially in patients with undetectable HIV-1 RNA levels.

De Rossi et al [191] conducted a study in seven different laboratories aiming to standardize

an HIV-1 proviral DNA quantification method of the most relevant circulating subtypes. PCR designed in the gag gene in combination with the human telomerase reverse transcriptase (hTERT) housekeeping gene showed Inter-laboratory results not statistically different and minor variations in HIV-1 DNA amounts and in different HIV clades, with a good agreement among participating laboratories. Such standardization allows the comparison of proviral DNA studies in order to approach the real meaning and usefulness of this parameter for clinical management of HIV-1 infected individuals.

An article from Re et al. in 2011 [192] studied therapeutic information from the total and 2-LTR proviral HIV-1 DNA load in addition to the input provided by plasma RNA determination in the follow-up of infected individuals. Two sex- and age-matched groups of selected acutely infected subjects were studied from the acute phase on. Nineteen naive individuals not treated during the study showed no significant variation at any time in the total and 2-LTR HIV-1 DNA ranging respectively from 896 ± 731 to 715 ± 673 copies/ 10^5 PBMC and 94 ± 10^5 to 65 ± 44 copies/ 10^6 PBMC. Twenty treated patients demonstrated a significant reduction in both parameters, ranging from 997 ± 676 to 262 ± 174 copies/ 10^5 PBMC for total DNA and 116 ± 55 to 26 ± 35 copies/ 10^5 PBMC for 2-LTR DNA, together with a CD4+ T cell count increase and RNA load decrease.

Another therapeutic parameter is the coreceptor tropism determination in order to use a coreceptor antagonist such as Maraviroc (Celsentri®) in combined therapy. Verhofstede and al. [193] recently described the bulk V3 sequencing based on proviral DNA compared to plasma RNA, followed by geno2pheno interpretation for genotypic coreceptor tropism testing (GTT). Studied groups consisted of 165 patients with a viral load of >500 HIV-1 RNA copies/mL with available plasma RNA and proviral DNA, and 126 patients with low viral load of <500 copies/mL. On the last group, study was conducted on current proviral DNA and on pretreatment plasma RNA. GTT was compared to the phenotypic tropism testing (PTT) results. In the simultaneous plasma RNA- cell DNA comparison, concordance in predicting coreceptor tropism was high, ranging from 95.2% to 96.4% depending on the interpretative cut-off (false positive rate) respectively set at 10 or 5%. In the second group comparison of cell DNA to pretreatment plasma RNA showed a concordance of 88.1% and 90.5% at cut-off of 10 and 5%, respectively. When comparing GTT to PTT, the overall concordance of RNA ranged from 82% to 83% and of DNA was 85% indicating an interesting approach to predict coreceptor tropism in patients with low or suppressed viraemia.

CHAPTER 2

OUTLINE OF THE THESIS

2.1 RESEARCH SETTING

The work presented in this thesis was conducted within the AIDS Reference Laboratory (ARL) of the Université Catholique de Louvain (UCL) located in Brussels, Belgium.

Missions of Belgium AIDS Reference Laboratories are specified in an agreement with the National Institute for Health and Disability Insurance (NIHDI) that has a key role in the social security system in Belgium. The ARL of UCL has set up three programs to meet its obligations: (1) the confirmation or exclusion of the presence of HIV (2) the development, adaptation and application of techniques to monitor and predict the outcome of the AIDS and major associated illnesses, in treated or untreated patients (3) the quality evaluation of selective kits for the diagnosis of acquired immunodeficiency, before or after market implementation. In addition, the ARL has developed research programs, including the study of the effect of treatment on the proviral DNA of HIV-1, the study of the impact of the integration step in the viral cycle of HIV-2, the clinical follow-up of HIV-2 infected patients in term of effectiveness of antiretroviral therapy and genetic changes involved in resistance to treatment and the study of variability of the cytoplasmic tail of the HIV-2 transmembrane glycoprotein.

2.2 AIMS OF THE THESIS

The project aimed to establish the value of the quantification and the characterization of HIV-1 provirus in the management of anti-HIV treatment in clinical settings. It is known that the HIV proviral DNA persists in infected cells even after a prolonged successful therapy. Treated patients may experience treatment failure or viral load blips and antiretroviral treatment simplification or interruption may lead to plasma viral RNA rebound. Assuming that effective anti-HIV-1 treatment has an impact on the reservoirs that leads to the reduction of proviral load and emergence of drug related resistance, differences in long-term effectiveness could be observed with different combined anti-HIV therapies. In order to draw a possible prognostic value of the provirus characterization, we chose to characterize the evolution of the provirus by the type of treatment, including its load and its nucleotide sequence.

The HIV provirus is a result of the transcription of the viral RNA to double stranded viral DNA (dsDNA) mediated by the viral RT and subsequently integrated into the host cell

genome by the viral integrase. The provirus serves as a template for production of new virions. The reservoir of the latent HIV-1 proviral DNA is constituted by long-life infected cells and situated in some biological compartments that are not easily accessible to drugs, making it impossible to eradicate the virus. The majority of infected cells (95-99%) are CD4+ T cells. Memory T cells have a long life span.

2.3 STUDY POPULATION

New HIV-1 infection diagnosis between May 2002 and July 2007 were included at two Belgian sites, the Department of Infectious Diseases of *Cliniques universitaires Saint-Luc* in Brussels and the Department of Infectious Diseases of *Cliniques Saint-Joseph* in Liège. Sixty-nine patients were enrolled in the study after obtaining an informed consent, 32 were later-on treated with PI containing regimen, 12 received NNRTI containing regimen and 25 remained untreated.

2.4 METHODS

The implemented study in collaboration with clinicians was prospective and observational. The first objective of the PhD project was to develop a real time PCR that quantifies the HIV-1 provirus present in subpopulations of circulating CD4 + leukocytes compared to a reference gene, in this case the beta-globin gene. The detection of the amplification was performed with the SYBR-Green I. The published method [196] designed in the highly conserved part of the *gag* gene showed a limit of quantification of 5 copies of provirus by PCR.

In the second part the developed assay was applied on 30 samples received for RNA viral load determinations and on retrospective samples from 15 patients undergoing 2 years of structured treatment interruption (STI).

The third part of the project consisted in following up the proviral load and the resistance mutations in three groups of patients, before and after PI- or NNRTI-based therapy initiation in 69 initially drug-naïve patients. The first group included 32 patients receiving antiretroviral therapy with a PI combined to NRTIs, the second group of 12 individuals received only RTIs (2 NRTI and 1 NNRTI) and a control group comprised 25 untreated patients. The study was approved by the local ethic committee and informed consent was obtained from each patient. The HIV-1 positive status of each patient was confirmed by accepted methods. We published a letter on the diagnostic aspects of the HIV infection [89]. For each selected patient, we checked the historical data on possible treatment

CHAPTER 3

REVIEW OF OUR RESEARCH

1. HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) PROVIRAL DNA LOAD IN PURIFIED CD4+ CELLS BY LIGHTCYCLER® REAL-TIME PCR

Authors:

Benoît Kabamba-Mukadi¹, Philippe Henrivaux², Jean Ruelle¹, Nicole Delferrière¹, Monique Bodéus¹ and Patrick Goubau¹

Authors' affiliation:

¹Laboratoire de référence SIDA, Université Catholique de Louvain, Avenue Hippocrate 54, B1200 Bruxelles, Belgium

²Service de Médecine interne, Clinique Saint Joseph, Liège, Belgium

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Technical advance

Open Access

Human immunodeficiency virus type I (HIV-I) proviral DNA load in purified CD4⁺ cells by LightCycler® Real-time PCR

Benoît Kabamba-Mukadi¹, Philippe Henrivaux², Jean Ruelle¹, Nicole Delferrière¹, Monique Bodéus¹ and Patrick Goubau*¹

Address: ¹Laboratoire de référence SIDA, Université Catholique de Louvain, Avenue Hippocrate 54, B1200 Bruxelles, Belgium and ²Service de Médecine interne, Clinique Saint Joseph, Liège, Belgium

Email: Benoît Kabamba-Mukadi - Benoit.Kabamba@mblg.ud.ac.be; Philippe Henrivaux - philippe.henrivaux@skynet.be; Jean Ruelle - Jean.Ruelle@mblg.ucl.ac.be; Nicole Delferrière - Nicole.Delferriere@mblg.ud.ac.be; Monique Bodéus - bodeus@mblg.ud.ac.be; Patrick Goubau* - goubau@mblg.ucl.ac.be

* Corresponding author

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Abstract

Background: The human immunodeficiency virus type I (HIV-I) proviral DNA persists in infected cells, even after prolonged successful HAART. In the present study, a relative quantification assay of HIV-I proviral DNA by LightCycler® real-time PCR based on SYBR Green I detection was developed in comparison to the number of purified CD4⁺ cells as estimated by the quantification of the β -globin gene.

Methods: The ability of the designed gag primers to quantify HIV-I Group M and the PCR efficiency were assessed on HIV-I reference isolate subtypes A, B, C and D. The 8E5 cell line containing a single defective copy of HIV-I proviral DNA was used as a standard for both the HIV-I target gene and the β -globin reference gene. The assay was applied on thirty consecutive patient samples received for RNA viral load determinations and on retrospective samples from fifteen patients undergoing 2 years of structured treatment interruption (STI).

Results: The lower limit of quantification was 50 HIV-I DNA proviral copies per CD4⁺ cell sample. The dynamic range was from 50 to 10⁶ HIV-I DNA copies per CD4⁺ cell sample with intra- and inter-assay coefficients of variability ranging from 3.1% to 37.1%. The β -globin reference gene was quantified down to a limit of 1.5 pg of DNA/ μ l (approximately 5 cells) with intra- and interassay coefficients of variability ranging from 1.8% to 21%. DNA proviral load varies widely among HIV-I infected patients. Proviral load and plasma viral load rebound were high in STI patients who took longer to achieve an undetectable plasma viral load under therapy. A statistically significant correlation was observed between DNA proviral load and RNA steady state viral load in STI patients (p-value = 0,012).

Conclusion: We have developed a fast, sensitive and specific relative quantification assay of HIV-I proviral DNA in purified CD4⁺ cells. The assay enables the monitoring of HIV-I proviral load, which may be useful to monitor therapy efficacy especially in patients with undetectable plasma RNA viral load, and allows the exploration of viral reservoirs.

Background

At present, HIV-1 infected patients are followed by monitoring plasma HIV-1 RNA viral load, allowing follow up of the immediate effects of treatment, and CD4+ T cell count, allowing initiation or reinitiation of the therapy as immune function decreases [1]. The HIV-1 proviral DNA load could be an alternative viral marker, as it is known that proviral DNA persists in infected cells, even after prolonged successful HAART as evidenced by undetectable plasma RNA viral load. A decline in DNA might indicate a long-term impact of the HAART on the reservoirs and the long-term effectiveness of the treatment [2-11]. But data regarding the decline in DNA are sometimes conflicting. Some authors noted decreased levels after one year of antiretroviral triple combination therapy [12] and others reported stable HIV-1 DNA levels over several years in PI-ART naive patients [13,14]. Several assays for the quantification of HIV type 1 proviral DNA in peripheral blood mononuclear cells (PBMC) have previously been reported, and many of them are based either on the principle of conventional PCR requiring post PCR analysis or on real-time PCR on total PBMC, using specific probe detection [9,10,15-23]. Recently, a multiplex real-time PCR for quantification of HIV-1 DNA and the human albumin gene in CD4+ cells has been published [23] using TaqMan probes and an Epstein-Barr virus (EBV) standard curve. Sequence-specific hybridisation probes provide the most specific real-time analysis of amplified target sequences but the very high variability of HIV sequences within subtypes, even in HIV variants in a given patient, led us to choose a sequence-independent detection with SYBR Green I. One of the experimental prerequisites for relative quantification consists in having identical PCR efficiencies for both target gene and reference gene in sample, standard, and calibrator. PCR efficiency may vary between EBV standard PCR and HIV-1 PCR. We therefore used the 8E5 cell line containing a single defective copy of HIV-1 proviral DNA as a standard for both the HIV-1 target gene and the β -globin reference gene in order to carry out a relative quantification. Another recent publication described a real-time PCR based on LightCycler® technology revealed through SYBR green fluorochrome to quantify the HIV-1 proviral DNA load [24]. The article describes quantification in PBMC of HIV-1 seropositive patients. As 95 to 99% of infected cells are CD4+ cells [25], we developed a relative quantification assay of HIV-1 proviral DNA in purified CD4+ cells on the LightCycler® by real-time PCR compared to cell quantification by β -globin PCR.

Methods

Primer design and selection

Several primers were designed in the *gag-pol* junction region with low variability on the basis of a consensus sequence obtained by aligning complete sequences of

HIV-1 subtypes A to J from the Entrez nucleotides database [26]. The Vector NTI® Suite (InforMax, Bethesda, USA) software was used for the design of primers. Selection of primer sets was first based on a specific signal without primer dimer formation when testing DNA from 8E5 cell dilution series and negative controls. Primer and $MgCl_2$ concentrations were optimised by combining primer concentrations ranging from 0.3 mM to 1.2 mM and $MgCl_2$ concentrations from 2 mM to 5 mM. The selected primers were tested on DNA samples obtained from HIV-1 subtypes A, B, C, and D reference strains and from clinical samples including subtypes B and non-B. We also tested the absence of signal with HIV-2 DNA. The final primer set selection was based on the efficiency of the PCR reaction. Differences in efficiency may have a major impact on the calculation of the initial amount. In theory, the optimum efficiency in PCR is two, meaning that every PCR product is replicated once every cycle. In reality, however, many PCR parameters influence PCR efficiency which is calculated according to the formula $E = 10^{-1/\text{slope}}$. The LightCycler® Software calculates the slope of the standard curve by plotting crossing points against the logarithm of concentration for each standard point of a 10-fold dilution series. A slope value of -3.33 indicates the maximal PCR efficiency.

Reference strains and patients

Four different reference strains of HIV-1 and 2 of HIV-2 from the National Institute for Biological Standards and Control (NIBSC, UK) were used in this project: HIV-1 Primary isolates-Uganda (HIV-1 subtype A, NIBSC repository reference ARP177.5) [27], 8E5/LAV (HIV-1 subtype B, NIBSC repository reference ARP110) [28], HIV-1 SE12808/ SE14784 (Subtype C, NIBSC repository reference ARP197.1, ARP197.2) and HIV1-ELI (HIV-1 subtype D, NIBSC repository reference EVA117) [29], HIV-2 ROD reference strain (subtype A, NIBSC repository reference EVA121.1) [30,31] and HIV-2 EHO reference strain (subtype B, NIBSC repository reference EVA132) [32].

Samples from 15 patients undergoing 2 years (start 2001) of structured treatment interruption (STI) and 30 consecutive clinical samples received for RNA plasma viral load determinations were included in the study. Informed consent following the Helsinki declaration was obtained from each patient. The HIV-1 seropositive status was confirmed according to the accepted methods. HIV-1 RNA plasma viral load was performed using the HIV-1 AmpliCor™ Monitor 1.5 (Roche, Branchburg, NJ, USA). The viral RNA and the proviral DNA sequences were determined by using an in house RT-PCR method applied on reverse transcriptase and protease genes [adapted from [33]]. HIV RNA was extracted from plasma (QIAmp Viral Mini Kit™, QIAGEN, Leiden, The Netherlands) and a direct cycle sequencing was used with BigDye terminator chemistry

on an ABI Prism 310 (Applied Biosystems, Foster City, USA).

CD4⁺ cells were isolated from 10 ml of patient EDTA whole blood samples by an immunomagnetic method using anti-CD4 coated magnetic beads (Dynabeads M450 CD4, Dynal AS, Oslo, Norway) according to the manufacturer's protocol and were stored at -80°C until use. The complete process takes approximately 3 hours and the purity of the CD4⁺ cell preparation was about 99% as estimated by Becton Dickinson FACS Flow Cytometer technology (data not shown). HIV-2 clinical samples were obtained from patients who were diagnosed with HIV-2 infection at our AIDS reference laboratory. The 8E5 cell line, the CD4⁺ blood donor cells infected with HIV-1 reference strains, and the H9 cell line infected with HIV-2 reference strains were cultured in RPMI 1640 medium supplemented with 10% FetalClone 1[®] (HyClone, Logan, Utah, USA), 1% Glutamine 200 mM and 0.1% Gentamycin 50 mg/ml. 8E5 cells were counted by the Coulter automated haematology analyser and diluted to 10⁶ cells per aliquot stored at -80°C.

DNA purification

DNA was extracted from purified patient CD4⁺ cells diluted in 200 µl PBS, using the High Pure[®] PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. To concentrate the HIV-1 target gene in patient samples, DNA was eluted in a volume of 50 µl. DNA from the T-lymphoblastoid 8E5 cells, and CD4⁺ blood donor cells infected with HIV-1 reference strains were eluted in 200 µl. The 8E5 cell DNA was purified from 10⁶ cells. A negative control (template replaced by Nuclease-free water) was included in each PCR run. Particular attention was paid to using DNase and RNase free materials. Depending on the number of samples, the whole DNA purification process required approximately 1 hour.

HIV-1 DNA real time PCR assay

A series of ten-fold dilutions of 8E5 DNA corresponding to 2.5 × 10³ to 2.5 HIV-1 DNA copies per µl (2.5 × 10³ to 2.5 cells/µl) was included in each experiment in order to generate an external standard curve. The PCR mixture (total volume 20 µl) in nuclease free water contained 2 µl of LightCycler[®] FastStart DNA Master SYBR Green 1, a ready-to-use "Hot Start" reaction mix (Roche Diagnostics GmbH, Mannheim, Germany), final concentrations of 4 mM MgCl₂ and 0.5 µM of each primer and 5 µl of purified DNA or negative control. All samples were analysed in duplicate. The amplification protocol for HIV-1 on the LightCycler[®] was as follows: a 10 min denaturation step at 95°C for polymerase activation, a "touch down" PCR step of 10 cycles consisting of 10 seconds (s) at 95°C, 10 s at 65°C, and 30 s at 72°C, followed by 40 cycles consisting

of 10 s at 95°C, 10 s at 55°C, and 30 s at 72°C. The fluorescence was measured at the end of each elongation step. The next step was a slow heating (0.1°C per s) of the amplified product from 65°C to 95°C in order to generate a melting temperature curve. This curve served as a specificity control. The entire cycling process including data analysis took less than one hour and was monitored using the LightCycler[®] software program (version 3.5). Second derivative maximum mode was chosen with baseline adjustment set in the arithmetic mode.

A fragment from the human β-globin gene was amplified in parallel with the HIV-1 *gag* gene to quantify the total number of investigated cellular genomes. The β-globin real-time quantitative PCR was performed using the LightCycler[®] Control Kit DNA (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. The PCR mixture (total volume 20 µl) in nuclease free water contained 2 µl of LightCycler[®] FastStart DNA Master SYBR Green 1 and 2 µl of LightCycler[®] Control Kit DNA primer mix, 2 µl of 10-fold dilution of purified DNA or negative control, and a final concentration of 4 mM MgCl₂. The DNA control from the kit corresponds to 30 ng of human genomic DNA. The same 8E5 DNA standard samples corresponding to 2.5 × 10³ to 2.5 cells/µl were included in each experiment in order to generate an external standard curve as for the HIV-1 experiment. All samples were run in duplicate. A melting temperature step of β-globin amplicons was performed.

To validate the use of the 8E5 cells as standards, we first performed a real-time PCR quantification of the human β-globin gene using the DNA control of the kit to generate a standard curve. The values of the 8E5 cell count as performed by the Coulter automated haematology analyser were compared with the values obtained by extrapolating the cell count from the β-globin real-time PCR quantification. In the second step, 8E5 cells were used as standards and we compared the kit DNA control value obtained from the LightCycler[®] quantification with the theoretical value.

Relative quantification was carried out using the LightCycler[®] Relative quantification Software (version 1.0). The calculation of data is based on the crossing point (Cp) values obtained by the LightCycler[®] Software. Results are calculated as the target/reference ratio of the sample divided by the target/reference ratio of the calibrator. This corrects for sample inhomogeneity and variability of detection. The result of the HIV-1 proviral quantification was expressed as log₁₀ number of DNA copies per 10⁶ CD4⁺ cells.

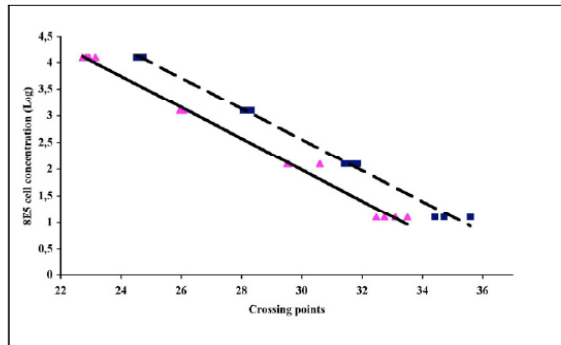


Figure 1
the HIV-1 and the human globin standard curves created by a ten-fold dilution series of 8E5 cell DNA show comparable slopes, indicating equal PCR efficiency. The LightCycler Software calculates the slope of the standard curve by plotting crossing points against the logarithm of concentration for each standard point. Each standard point was run in five replicates. (—) HIV-1 DNA standard curve, (---) Human globin standard curve.

Statistical analysis

Statistical analysis was performed using the parametric Pearson correlation test or the nonparametric Spearman correlation method. The critical p -value required to reject the null hypothesis (there is no proof of significant correlation between the variables) and accept the alternative hypothesis is equal to or less than 0,05.

Results

Primer selection

Out of several primers designed in the HIV-1 *gag-pol* junction region, the sequences of the selected HIV-1 *gag* forward primer BK1F and the reverse primer BK1R were respectively: 5'-GTA ATA CCC ATG TTT TCA GCA TTA TC-3' and 5'-TCT GGC CTG GTG CAA TAG G-3'. These amplify a 181-bp amplicon in the *gag* region of low variability among HIV-1 subtypes.

Standard validation

The use of 8E5 cells as standards was validated. As shown in Figure 1, the HIV-1 and the β -globin curves generated by using 8E5 cells had comparable slopes, indicating equal PCR efficiency. The comparison of the 8E5 cell count as performed by the Coulter automated haematology analyser with the values obtained by real-time PCR quantification of β -globin genes showed similar results, as did the comparison of the DNA control sample value obtained from LightCycler® quantification with the theoretical value (Figure 2).

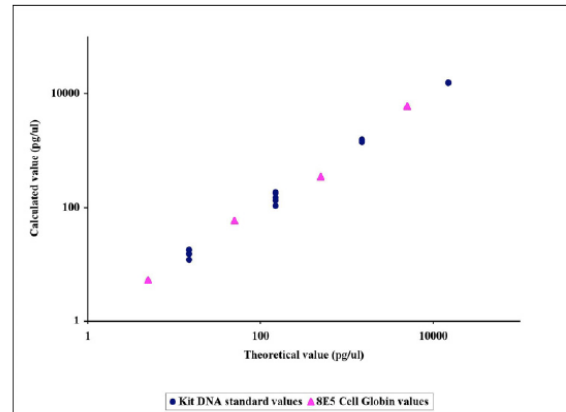


Figure 2
Validation of the use of 8E5 cells as standards for human globin PCR. The figure shows similar results of the 8E5 cell count as performed by the Coulter automated hematology analyzer compared with the values obtained by real-time PCR quantification of the human globin gene. The comparison of the theoretical and calculated values of the DNA control sample from LightCycler Kit shows equal results. Each standard point was run in five replicates.

Specificity of the assay

As described in the methods section, the specificity of the PCR reaction was tested with DNA purified from the 8E5 cell line carrying a single copy of subtype B proviral DNA per cell, with CD4+ blood donor cells infected with HIV-1 subtype A, C, and D reference strains and with H9 cells infected with HIV-2 ROD (subtype A) and EHO (subtype B) reference strains. Clinical samples from HIV-1 or HIV-2 infected patients were included. The subtyping results based on proviral protease sequences showed 65% of subtype B viruses and 35% of non-subtype B viruses, which included subtypes A, C, D, F1 and CRF AE [34].

HIV-1 amplicons produced in the PCR reaction had the same melting temperature of 85.30°C, with a slight variation depending on the HIV-1 subtypes and on the type of initial sample, indicating that the signal was specific. The melting temperature of β -globin amplicons was about 86°C. Furthermore, the specificity of the HIV-1 amplification was confirmed by the presence of the expected band size on the electrophoresis gel (agarose 2%) carried out with the PCR products obtained from standards, reference strains, and patient samples. Direct sequencing of amplified products using the PCR primers showed the expected HIV-1 sequences (data not shown). No amplification was obtained with HIV-2 DNA purified from patient samples and from reference strains belonging to HIV-2 subtypes A

Table 1: PCR efficiencies of HIV-1 group M amplification

Sample concentration:	Subtype A	Subtype B	Subtype C	Subtype D
Log dilution	Mean Cp ^a	Mean Cp	Mean Cp	Mean Cp
-2	26,16	22,90	24,61	26,13
-3	29,50	26,02	28,10	29,27
-4	32,82	29,75	31,79	32,86
-5	36,72	32,98	34,83	36,72
Slope ^b	-3,50	-3,37	-3,43	-3,54
R ^{2c}	0,99	0,99	0,99	0,99

(a) Mean of crossing point values calculated using two replicates of each point in 3 runs.

(b) The LightCycler Software calculates the slope of the curve by plotting crossing points against the logarithm of concentration for each point of a 10-fold dilution series. A slope value of -3.33 indicates the maximal PCR efficiency.

(c) The LightCycler Software also calculates the coefficient of correlation R².

and B. The four HIV-1 reference strains obtained from NIBSC were amplified. The results showed that the assay amplified DNA purified from HIV-1 reference isolate subtypes A, B, C, and D with similar PCR efficiency (Table 1).

Sensitivity and reproducibility of the assay

Ten replicates of 1 copy, 5 copies, and 10 copies of DNA purified from 8E5 cells were tested in the PCR reaction. The detection of one HIV-1 DNA copy per PCR failed in most cases while five and ten copies were always detected. The lower limit of quantification was set at 5 HIV-1 DNA proviral copies per PCR or 50 HIV-1 DNA proviral copies per CD4+ cell sample. A HIV-1 DNA proviral load below 50 copies per CD4+ cell sample was reported as undetectable. The dynamic range was from 50 to 10⁶ HIV-1 DNA proviral copies per CD4 lymphocyte sample. We tested the intra and inter-assay reproducibility of our technique with a series of ten-fold dilutions of 8E5 DNA from 12.5 × 10³ to 12.5 HIV-1 DNA copies per PCR. The intra-assay reproducibility was evaluated using five replicates of each point and the inter-assay reproducibility was calculated on 10 runs. The intra- and inter-assay coefficients of variability of the HIV-1 DNA copy number ranged from 3.1% for high provirus concentrations to 37.1% for low concentrations. The quantification of the human β-globin reference gene by using the "LightCycler® Control Kit DNA" enabled a lower limit of detection of 1.5 pg/μl (approximately 5 cells) with intra- and interassay coefficients of variability ranging from 1.8% for high DNA concentrations to 21% for low DNA concentrations. We observed an inhibition of the human β-globin reference gene amplification at high amounts of the cellular genome. The inhibition was shown by a maximum fluorescence of the amplification curve, which was lower than the maximum fluorescence of the standard points. In our assay, this problem was solved by diluting (10-fold) DNA from patient samples. The influence of the type of sample on

the PCR reaction was tested by diluting the standard 8E5 cells in HIV seronegative plasma from a blood donor before DNA extraction. A slight difference (about 1 Cp) was observed within the range of 12.5 to 12.5 × 10³ HIV-1 DNA copies per PCR between the Cp values obtained for 8E5 cells diluted in PBS buffer as compared to 8E5 cells diluted in the plasma, in both HIV-1 and β-globin assays. We also successfully tested the quantification of both HIV-1 and β-globin genes without the need to include a standard curve in every run by loading an external standard curve generated in a different run.

HIV-1 DNA viral load in clinical specimens

In order to test our quantitative real-time PCR assay on DNA from patient CD4+ cell samples, the assay was first applied on thirty consecutive patient samples received for RNA viral load determinations regardless of whether or not they were receiving antiretroviral therapy. Of the 30 patients, 10 were treatment naive while 20 were antiretroviral-experienced adults (Table 2). If we put all patients (N = 30) together, no significant correlation was found between DNA relative proviral load and either plasma RNA viral load or CD4+ cell count (Figure 3 and Figure 4). The only observed correlative tendency was between DNA relative proviral load and plasma RNA viral load in the naive patients (Figure 5), but this was not statistically significant (p-value >0,05 by Pearson test). DNA proviral load varies widely among HIV-1 infected patients in the same or at different disease stages.

The assay was also applied on samples from fifteen patients undergoing 2 years of STI (Table 2). At the start of the STI, all patients were receiving at least a triple-therapy regimen including two nucleoside reverse transcriptase inhibitors (NRTIs) plus 1 or 2 PIs. All STI patients had received previous monotherapy or dual therapy before HAART with a mean total treatment time of five years. At

Table 2: Patient characteristics

	Naive group	Treated patients	STI patients
Number	10	20	15
Genders			
Males	5	8	8
Females	5	12	6
Transsexuals			1
Mean years old	42	42	43
Geographic origin			
Europeans	5	12	12
Other/Unknown	5	8	3
Mean range follow up			
Years ^a	4 (range 1 – 11) ^a	7.5 (range 1 – 15) ^a	6 (range 2 – 7) ^a
CD4+ count			
cells × 10 ⁶ /l	Median 391 (range 109–805)	Median 338 (range 1 – 790)	Median 717 (range 216 – 1356)
<200 cells × 10 ⁶ /l (Number) ^b	1	6	0
Plasma HIV-RNA			
Log ₁₀ copies/ml	Median 4.8 (range 2.7 – 5.4)	Median 4.4 (range 1.7 – 5.9)	Median 3.2 (range 1.7 – 4.7) ^c
<50 copies/ml (Number) ^b	0	5	3
HIV-DNA			
Log ₁₀ copies/10 ⁶ CD4 cells	Median 3.0 (range 2.6 – 3.7)	Median 3.3 (range 2.2 – 4.6)	Median 2.9 (range 2.1 – 4.0)
<5 copies/PCR (Number) ^b	6	12	5

(a) Number of years between HIV-1 infection diagnosis and DNA proviral load testing.

(b) Number of patients with viral or proviral load under limit of quantification or with CD4+ count <200 cells 10⁶/l.

(c) Steady state viral load after STI.

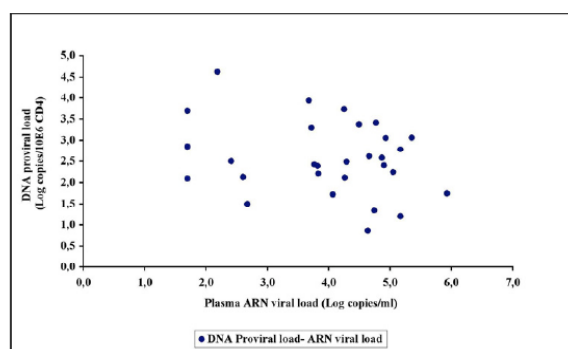


Figure 3

No correlation was found between DNA proviral load and plasma RNA viral load in thirty consecutive unselected patient samples received for RNA viral load determinations.

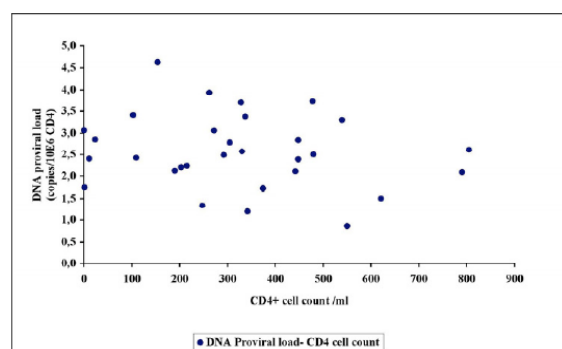


Figure 4

No correlation was found between DNA proviral load and CD4+ cell count in thirty consecutive unselected patient samples received for RNA viral load determinations.

the initiation of STI, 11 patients out of the 15 had an HIV-1 RNA viral load of less than 50 copies/ml; four had a baseline HIV-1 RNA viral load of less than 572 copies/ml (median of 2 log copies/ml) and the mean CD4+ cell count was 748 × 10⁶ cells/l. Out of the 15 STI patients, seven had a steady state viral load less than 3.3 log copies/ml, including three patients with undetectable HIV-1 RNA

viral load (<50 copies/ml), while eight patients had a high HIV-1 RNA viral load ranging from 3.9 to 4.7 log copies/ml. All seven patients with a low steady state plasma viral load showed a DNA proviral load under 2.5 log copies/10⁶ CD4+ cells, including four patients with a proviral load under the limit of quantification (<5 DNA copies/PCR). Of eight patients with a high steady state plasma

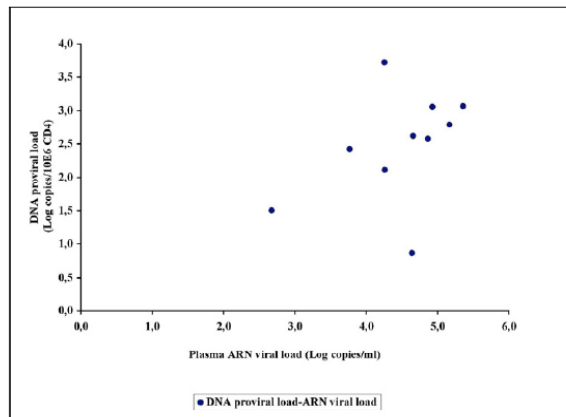


Figure 5
Correlative tendency between DNA relative proviral load and plasma RNA viral load in the naive patients but not statistically significant (p-value >0,05 by Pearson test)

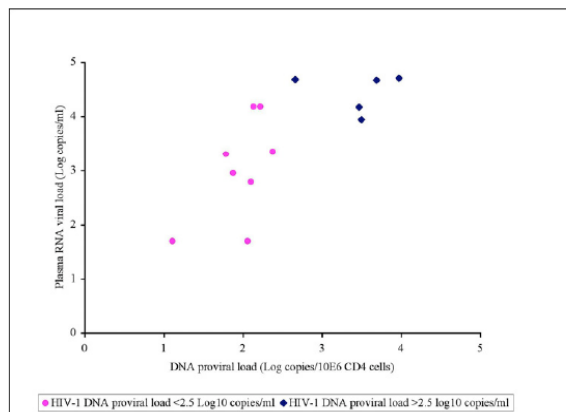


Figure 6
The assay was also applied on samples from 15 patients undergoing 2 years of STI. A statistically significant correlation was observed between DNA proviral load and RNA steady state viral load in STI patients. (p-value = 0,012, Pearson correlation test)

viral load (>3.9 log copies/ml), three showed a DNA proviral load under 2.5 log copies/10⁶ CD4+ cells while five had a DNA proviral load above 2.5 log copies/10⁶ CD4+ cells. A statistically significant correlation was observed between DNA proviral load and RNA steady state viral load in STI patients (Figure 6). The Pearson's coefficient of

correlation was about 0,578 with an associated p-value of 0,012. When applying the nonparametric Spearman correlation method, the associated p-value was about 0,018. Proviral load and plasma viral load rebound were high in STI patients who took longer to achieve an undetectable plasma viral load under therapy.

Discussion

A relative quantification assay of HIV-1 proviral DNA in purified CD4+ cells was developed on the LightCycler® real-time PCR instrument expressed per 10⁶ CD4+ cells as derived from estimated by the quantification of the β -globin gene. In healthy individuals, CD4+ cells represent approximately 30 to 50% of the PBMC and their fraction may be considerably lower and variable over time in HIV-infected patients. Theoretically, to improve the sensitivity and accuracy of an assay for low-copy-number samples, a larger amount of DNA should be added to the PCR reaction mix when purified from PBMCs than directly from CD4+ cells. High amounts of the cellular genome may lead to PCR inhibition. In addition, published data show low proviral loads, ranging from 1 to <5 log₁₀ HIV-1 DNA copies (median 2–3 log₁₀) in 10⁶ PBMC equivalents [14–17,19,20,22–24]. Methods quantifying the HIV-1 DNA proviral load in PBMC may therefore lead to undetectable proviral loads. However, in any case a direct comparison between DNA load in CD4+ cells and PBMCs could be useful as the CD4 isolation adds extra work (i.e. costs), which should be weighed against the clinical benefit. Sequence-specific hybridisation probes provide the most specific real-time analysis of amplified target sequences but the very high variability of HIV sequences within subtypes, even in HIV variants in a given patient led us to choose a sequence-independent detection with SYBR Green I. The ability of SYBR Green I to bind double-stranded DNA molecules with emission of a fluorescent signal allows the PCR reaction to be monitored. As SYBR Green I binds to all double-stranded DNA molecules, a melting temperature curve needs to be constructed to ensure the specificity of the signal. Thus specific primer selection and PCR optimisation is crucial to avoid primer dimer formation. We tested the influence of possible inhibitors in the DNA sample. A slight variation of the melting temperature (about 1°C) and the Cp values (about 1Cp) was observed, depending on the HIV-1 subtypes and on the type of initial sample (EDTA whole blood or culture supernatant). The specificity of the HIV-1 amplification was confirmed by the presence of the expected band size on the electrophoresis gel carried out with the PCR products from standards, reference strains, and patient samples. Direct sequencing of amplified products showed the expected HIV-1 sequences. No amplification was obtained with HIV-2 DNA. The ability of the assay to quantify HIV-1 Group M and the PCR efficiency were assessed on HIV-1 reference isolate subtypes A, B, C,

and D. The results showed similar PCR efficiencies. Particular attention should be given to the quantification of the β -globin gene. We observed an inhibition with a high amount of the reference gene. Diluting (10-fold) DNA from patient samples resolves this problem. The lower limit of quantification is five HIV-1 DNA proviral copies per PCR and 1.5 pg of cellular DNA/ μ l (approximately five cells) for the human β -globin reference gene quantification. The dynamic range of HIV-1 DNA quantification is between 50 and 10^6 proviral copies per CD4 with coefficients of variability of the HIV-1 DNA copy number ranging from 3.1% for high provirus concentrations to 37.1% for low concentrations. Our method shows acceptable technical sensitivity and specificity. Quantification could be performed without the need to include a standard curve in every run by loading an external standard curve generated in a different run. Relative quantification was carried out by using the LightCycler® Relative quantification Software (version 1.0). The result of HIV-1 proviral quantification was expressed as $\log_{10}$ number of DNA copies per 10^6 CD4+ cells. The crossing points that are calculated by the LightCycler® Software are a function of the amplification efficiency. Efficiency differences have a major impact on the accuracy of initial amount calculation. The assay developed met one of the experimental prerequisites for PCRs, HIV-1 target gene and β -globin reference gene, i.e., having identical PCR efficiencies in sample, standard, and calibrator.

The assay was successfully tested on 30 consecutive unselected patient samples. No significant correlation was found between DNA relative proviral load and either plasma RNA viral load or CD4+ cell in this group, overall. Similar observations have been reported in the literature [9,24]. DNA proviral load varies widely among HIV-1 infected patients in the same or at different disease stages.

The assay was also applied on samples from 15 patients undergoing 2 years of SII. In fact, of the 15 SII patients, all seven patients with a low steady state plasma viral load showed a DNA proviral load under 2.5 log copies/ 10^6 CD4+ cells, including four patients with a proviral load under the limit of quantification (<5 DNA copies/PCR). Of the remaining eight patients presenting a high steady state plasma viral load (>3.9 log copies/ml), three showed a DNA proviral load under 2.5 log copies/ 10^6 CD4+ cells while five had a DNA proviral load above 2.5 log copies/ 10^6 CD4+ cells. We observed that in patients who needed a change in antiretroviral treatment to achieve an undetectable plasma viral load or who took longer to achieve an undetectable viral load under treatment, the proviral load was >2.5 log copies/PCR and the plasma viral load rebound was high. However, a large and prospective study is needed to confirm the value of this observation and

particularly to assess the predictive value of proviral load in the setting of STI.

Conclusion

We have developed a fast, sensitive and specific assay, which enables the monitoring of HIV-1 proviral load in CD4+ cells by LightCycler® real-time PCR based on SYBR Green I quantification. This should enable us to evaluate prospectively the proviral load as a prognostic marker in therapy and in the evaluation of treatment interruptions.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

BKM conceived of the study and designed it together with PH and PG. BKM developed the HIV-1 DNA real-time PCR and performed the assay and sequencing reactions. PH assembled the clinical samples. ND did the CD4+ cell preparation and the all the viral culture work. BK and PG drafted the manuscript. JR and MB reviewed the manuscript. All authors contributed to the final version of the manuscript, read and approved it.

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2. HIV-1 PROVIRAL RESISTANCE MUTATIONS: USEFULNESS IN CLINICAL PRACTICE

Authors:

Benoît Kabamba-Mukadi¹, Armelle Duquenne¹, Philippe Henrivaux³, Flora Musuamba², Jean Ruelle¹, Jean-Cyr Yombi³, Monique Bodéus¹, Bernard Vandercam³ and Patrick Goubau¹

Authors' affiliation:

¹AIDS Reference Laboratory, Université Catholique de Louvain, Avenue Hippocrate 54, B1200 Brussels, Belgium

²LBCM Unit, Université Catholique de Louvain, Avenue Hippocrate 54, B1200 Brussels, Belgium

³Department of Infectious Diseases, Université Catholique de Louvain, Avenue Hippocrate 10, B1200 Brussels, Belgium

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ORIGINAL RESEARCH

HIV-1 proviral resistance mutations: usefulness in clinical practice

B Kabamba-Mukadi,¹ A Duquenne,¹ P Henrivaux,³ F Musuamba,² J Ruelle,¹ J-C Yombi,³ M Bodéus,¹ B Vandercam³ and P Goubau¹
¹Université Catholique de Louvain, AIDS Reference Laboratory, Brussels, Belgium, ²Université Catholique de Louvain, LBCM Unit, Brussels, Belgium and ³Université Catholique de Louvain, Department of Infectious Diseases, Brussels, Belgium

Objectives

Transmitted HIV strains may harbour drug resistance mutations. HIV-1 drug resistance mutations are currently detected in plasma viral RNA. HIV-1 proviral DNA could be an alternative marker, as it persists in infected cells.

Methods

This was a prospective study assessing the prevalence and persistence of HIV-1 drug resistance mutations in DNA from CD4 cells before and after protease inhibitor (PI)- or nonnucleoside reverse transcriptase inhibitor (NNRTI)-based therapy initiation in 69 drug-naïve patients.

Results

Before therapy, 90 and 66% of detected mutations were present in CD4 cells and plasma, respectively. We detected seven key mutations, and four of these (M184M/V, M184M/I, K103K/N and M46M/I) were only found in the cells. When treatment was started, 40 patients were followed; the mutations detected at the naïve stage remained present for at least 1 year. Under successful treatment, new key mutations emerged in CD4 cells (M184I, M184M/I and Y188Y/H).

Conclusions

The proportion of mutations detected in the DNA was statistically significantly higher than that detected in standard RNA genotyping, and these mutations persisted for at least 1 year irrespective of therapy. The pre-existence of resistance mutations did not jeopardise treatment outcome when the drug concerned was not included in the regimen. Analysis of HIV-1 DNA could be useful in chronic infections or when switching therapy in patients with undetectable viraemia.

Keywords: CD4 cells, HIV-1, mutation, naïve patients, provirus, resistance

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Introduction

The efficacy of initial therapy for HIV infection may be jeopardized by the presence of drug resistance mutations, which reduce the probability of durable suppression of viral replication. Transmission of resistant HIV-1 strains and reduced drug susceptibility of viruses in untreated patients have been documented [1–4]. Key studies have demonstrated that clinical benefits result from determining viral drug susceptibility before initiating or changing therapy [1,2,5–12]. Based on some publications [13–17]

suggesting that testing for drug resistance is even indicated for newly acquired HIV infection, because the proportion of drug-resistant viruses in new HIV infections is increasing, recent international guidelines recommend resistance testing in cases of primary or recent HIV infection [18]. The panel of experts that prepared these guidelines recommends resistance testing when the prevalence of mutations in naïve patients exceeds 5% to 10% or where there is a strong suspicion of transmission of resistance. In other cases, the guidelines suggest that resistance testing should be carefully considered and, if not performed, they recommend storing the earliest available sample so that testing can be conducted at a later date if necessary. Either way, testing should not delay treatment. Importantly, the level of drug resistance and of acquisition of drug-resistant

Correspondence: Mr Benoît Kabamba-Mukadi, Clinique Universitaire St Luc – UCL, Microbiology – Unit of Virology, Avenue Hippocrate 10, 1200 Brussels, Belgium. Tel: + 3227646726; fax: + 3227646933; e-mail: benoit.kabamba@uclouvain.be

virus may be strongly dependent on the patient's origin, even in a small country such as Belgium, and these factors should be taken into account when considering resistance testing [19–27]. Switching antiretroviral agents for reasons other than virological failure, most frequently to improve convenience or because adverse events require discontinuation, has become standard practice in the management of HIV infection.

At present, HIV-1 drug resistance mutations are detected by analysing plasma viral RNA. However, it is possible that HIV-1 proviral DNA could be used as an alternative marker, as it is known that proviral DNA persists in infected cells, even after prolonged highly active antiretroviral therapy (HAART) that has been demonstrated to be successful on the basis of an undetectable plasma RNA viral load. Data are accumulating regarding the detection of HIV-1 drug resistance mutations in proviral DNA. Some authors have noted the presence of key mutations in proviral DNA which were not present in plasma viral RNA [28–31]. Using direct sequencing, Bona *et al.* [30] assessed the prevalence of mutations associated with drug resistance in cell-free and cell-associated strains in treatment-naïve patients [28–30]. These authors observed that key mutations conferring resistance to reverse transcriptase (RT) inhibitors were found more frequently in proviral DNA than in plasma viral RNA. In addition, major mutations in the protease (PR) region were only found in peripheral blood mononuclear cells (PBMCs). Wang *et al.* [31] showed that drug resistance mutations remained compartmentalized in plasma and PBMCs. In contrast, in therapy-naïve patients, these authors observed a tight concordance between the HIV strains in plasma and PBMCs. Another publication in 2006 [32] described the early establishment of the viral reservoir in patients acquiring resistant strains at primary HIV-1 infection (PHI) and the persistence of resistance-associated mutations with identical profiles in paired HIV RNA and PBMC HIV DNA. Usuku *et al.* [33] followed the changes in drug resistance mutations in a patient receiving HAART. Mutations detected in the plasma were not present or were infrequently present in the proviral DNA. The discrepancy persisted for more than 3 years. It is important to emphasize that the peripheral blood pool of lymphocytes represents about 2% of the total number of lymphocytes in normal young adult men [34]. Schnuda *et al.* [35] showed that the small blood lymphocytes recirculate continuously between the peripheral blood and the lymph nodes in the rat, with each cycle having a duration of less than 3 min.

In this article, we report the results of a prospective study assessing the prevalence and persistence of HIV-1 drug resistance mutations in proviral DNA from purified CD4 cells compared with those in plasma viral RNA before therapy initiation in treatment-naïve patients. We also

evaluated the evolution of HIV-1 drug resistance mutations in proviral DNA before and after therapy initiation, and plasma RNA mutation patterns in patients remaining treatment-naïve. As 95 to 99% of infected cells are CD4 cells [36], and in order to confirm the utility of resistance testing in provirus, we used direct sequencing of HIV-1 proviral DNA in purified CD4 cells to follow the evolution of drug resistance mutations in treated and untreated patients and compared the findings to those obtained from HIV-1 viral RNA using the ABI 310 Prism (Applied Biosystems, Foster City, California). We further chose not to use cloning but direct population sequencing as this is routinely used in clinical settings.

Materials and methods

Patients

Between May 2002 and July 2007, genotypic resistance testing was performed on cell-free and cell-associated virus from 69 patients who were not receiving treatment (Table 1). The study was approved by the local ethics committee and informed consent was obtained from each patient. HIV-1 seropositive status was confirmed according to accepted methods. The therapeutic histories of all patients were checked by asking specific questions when they signed the informed consent form and by consulting their clinical records. When documented histories were absent, we contacted the physicians responsible for the patients' care. This confirmed each patient as HIV drug naïve. Checking the therapeutic histories of all patients can be difficult but is important when studying drug mutations in treatment-naïve patients.

Virus was successfully sequenced for 63 of the 69 selected individuals at baseline, both in plasma and in cells. Fifty-eight per cent of the patients were European and 42% non-European, mostly from central Africa. Thirty-nine per cent of the sequenced HIV-1 viruses were subtype B. Thirty-two of the patients subsequently received combination therapy with two nucleoside reverse transcriptase inhibitors (NRTIs) + lopinavir/ritonavir (LPV/r), 12 received two NRTIs + efavirenz (EFV) and 25 remained therapy-naïve. Patients were followed for at least 1 year after initiation of therapy (Table 1).

Purified CD4 cells and reference strain

CD4 cells were isolated from 10 mL of whole blood [collected from patients in ethylenediaminetetraacetic acid (EDTA)] by an immunomagnetic method using anti-CD4 coated magnetic beads (Dynabeads M450 CD4; Dynal AS, Oslo, Norway) according to the manufacturer's protocol

Table 1 Patient characteristics

	All patients	Naïve group	NNRTI group	PI group
Number of patients				
Total	69	25	12	32
> 12 months of follow-up	40	8	10	22
Gender				
Male (%)	49	56	33	50
Female (%)	48	40	67	47
Unknown (%)	3	4		3
Age (years) [mean (range)]	44 (20–68)	41 (27–55)	44 (20–68)	47 (29–65)
Geographic origin				
European (%)	58	48	58	47
Other/unknown (%)	42	52	42	53
Duration of follow-up (months) [mean (range)]*	28 (11–44)	24 (12–41)	34 (17–44)	25 (11–44)
CD4 count (cells/ μ L)				
Baseline [median (range)] [†]	338 (6–1460)	616 (177–1111)	238 (168–284)	253 (46–1128)
Follow-up [median (range)] [‡]	–	476 (245–826)	396 (194–549)	402 (146–1021)
Mean change (range) [§]	–	– 154 (–533 to 102)	+ 154 (26–365)	+ 209 (–226 to 470)
Wilcoxon test <i>p</i> -value for mean change [¶]	–	0.07	0.002	0.0001
Plasma HIV RNA (log ₁₀ copies/mL)				
Baseline [median (range)] [†]	5.27 (2.6–5.70)	4.00 (1.70–4.82)	4.09 (2.41–4.67)	4.44 (1.70–5.70)
Follow-up [median (range)] [‡]	–	4.27 (3.47–5.23)	1.80 (1.7–2.75)	1.82 (1.70–4.05)
Mean change (range) [§]	–	+ 0.08 (–0.6 to 1.4)	–	–
Wilcoxon test <i>p</i> -value for mean change [¶]	–	0.24	–	–
Patients with viral load < 50 copies/ml (%)	–	–	90	91
HIV-1 subtype (%)				
B	39			
Non-B	61			

*Number of months between the baseline sample and the last follow-up sample sequenced.

[†]Sample tested at baseline (before therapy initiation for treated patients).

[‡]Sample tested after at least 12 months of follow-up.

[§]The changes in CD4 cell count and viral load were calculated per individual in patients with follow-up samples.

[¶]The critical *p*-value required to reject the null hypothesis (no statistically significant difference) and accept the alternative hypothesis is ≤ 0.05 .

NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

and were stored at -80°C until use. The purity of the CD4 cell preparation was approximately 99% as estimated using Becton Dickinson FACScan Flow Cytometer technology (Franklin Lakes, New Jersey, USA) (data not shown). The 8E5 cell line was cultured in RPMI-1640 medium and the cells were counted using a Coulter automated haematology analyser, diluted to 10^6 cells per aliquot and stored at -80°C . The 8E5 cell-free virus present in the culture supernatant was diluted and stored in aliquots of 30 000 HIV-1 RNA copies/mL.

HIV-1 plasma viral load

The HIV-1 RNA plasma viral load was assessed using the Versant HIV-1 RNA 3.0 assay (Siemens Medical Diagnostics Solutions, Tarrytown, NY, USA) according to the manufacturer's instructions. This assay is based on branched DNA (bDNA) technology. It requires 1 mL of sample and has a dynamic range of 50–500 000 copies/mL. Plasma samples and the 8E5 cell culture supernatant were frozen at -80°C until tested for HIV-1 RNA viral load. A viral load result of < 50 copies/mL was regarded as 50 copies/mL when calculating the mean viral load of a given patient group.

Nucleic acid extraction and direct sequencing

DNA was extracted from purified patient CD4 cells diluted in 200 μ L of phosphate-buffered saline (PBS), using the High Pure[®] PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. To concentrate the HIV-1 target gene in patient samples, DNA was eluted in a volume of 50 μ L. DNA from 10^6 T-lymphoblastoid 8E5 cells, used as positive control, was eluted in 200 μ L. A negative control (in which the template was replaced with nuclease-free water) was included in each polymerase chain reaction (PCR) run. Particular attention was paid to using DNase- and RNase-free materials. Depending on the number of samples, the whole DNA purification process required approximately 1 h. HIV RNA was extracted from plasma and from the 8E5 cell culture supernatant using the QIAmp Viral Mini Kit[™] (Qiagen, Leiden, the Netherlands) according to the manufacturer's directions. Viral RNA and proviral DNA genotypic antiretroviral drug resistance mutations were identified using an in-house reverse transcriptase–polymerase chain reaction (RT-PCR) method applied to the RT and PR genes (adapted from Schmit *et al.*

[37]). Direct cycle sequencing with BigDye terminator chemistry was carried out on the ABI Prism 310 sequencer (Applied Biosystems). In cases of PCR failure, samples were analysed using a TRUGENE HIV-1 Genotyping Kit (Siemens Medical Diagnostics Solutions). The Stanford mutation list (last updated 09/01/09), excluding polymorphisms and drug-selected mutations without known significance, was used to identify mutations [38].

Statistical analysis

Statistical analysis was performed using JMP statistical software version 7.0.1 (SAS Institute, Cary, NC). The χ^2 test is a nonparametric statistical test used in this case to determine whether the proportion of mutations detected in DNA differed from that detected in RNA or in follow-up DNA samples. In addition, the kappa statistic was used to estimate the agreement between detection of mutations in DNA and detection of mutations in RNA or follow-up DNA samples. Changes in CD4 cell count and viral load were calculated per individual in patients with follow-up samples taken during the study period. The Shapiro test was performed to evaluate the normality of the viral load and CD4 cell count distributions. If the distribution was normal, a paired Student's *t*-test was used to determine whether the mean difference was statistically different from 0. Otherwise, a Wilcoxon nonparametric test was applied. A logistic regression was performed to assess the relationship between the appearance of new mutations and the time elapsed between sample collections. The critical *P*-value required to reject the null hypothesis (that there is no proof of a significant correlation between the variables) and accept the alternative hypothesis was 0.05.

Results

HIV-1 RNA viral load and CD4 cell count

The characteristics of 69 selected patients at the time of inclusion in the study are presented in Table 1. The 69 treatment-naïve patients had a mean viral load of 5.27 (range 2.6–5.70) $\log_{10}$ copies/mL and a mean CD4 lymphocyte count of 338 cells/ μ L (range 6–1460 cells/ μ L). Twenty-five patients remained drug-naïve and eight of these had follow-up samples taken during the study period. After a mean follow-up time of 24 (range 12 to 41) months, the mean viral load change was 0.08 (range –0.6 to 1.4) $\log_{10}$ copies/mL, which was not statistically different from 0 (Wilcoxon test associated *P* = 0.42). The mean decrease in CD4 cell count of 174 (median –154; range –533 to 102) cells/mL was not statistically significant by the Wilcoxon

test (*P* = 0.07) (Table 1). After EFV-based therapy initiation in the nonnucleoside reverse transcriptase inhibitor (NNRTI) group, 10 patients were followed for at least 12 months and showed a mean increase in CD4 cell count of 173 (median 154; range 26 to 365) cells/mL, which was statistically significant (Wilcoxon test associated *P* = 0.002). Ninety per cent of patients in this group (patient number 16 being the only exception) achieved an undetectable viral load (<50 RNA copies/mL) (Table 1). The protease inhibitor (PI) group comprised 32 individuals, of whom 22 had at least 1 year of follow-up with a mean of 25 months after therapy initiation. The plasma viral load decreased to an undetectable viral load (91% of patients with <50 RNA copies/mL) in 20 of the 22 patients with follow-up. Patients 21 and 37 were exceptions, as viral load was detectable. In this PI group, the mean increase in CD4 cell count was 162 (median 209; range –226 to 470) cells/mL, which was statistically significant (*t*-test associated *P* = 0.0001) (Table 1).

Resistance mutations detectable in plasma and CD4 cells from therapy-naïve patients

Of the resistance mutations detected in the 61 patients with sequenced virus (of the 69 selected patients) at the therapy-naïve stage, 90% were present in CD4 cells and 66% in the plasma. Fifty-five per cent of PR mutations (*n* = 20) and 56% of RT mutations (*n* = 9) were present simultaneously in CD4 cells and plasma. The proportion of mutations detected in the DNA and the proportion detected with standard RNA genotyping were statistically significantly different by the χ^2 test (*P* < 0.0001). We can therefore conclude that the difference in detected mutations is not attributable to chance. The kappa coefficient was 0.71, which means that there was substantial agreement between the two methods in naïve patients [39]. One patient (patient 7) had the M46L PR key mutation in both plasma and cells, while patient 33 had the M46M/I mixed population only in the plasma (3% of 61 patients). The M46I or L mutation confers high resistance to indinavir (IDV). Eight per cent of patients (*n* = 61) had at least one RT mutation in the plasma while 15% had at least one RT resistance mutation in CD4 cells. Seven key mutations were detected in different patients (11.5% of the 61 patients and 10% of all included patients) and four of these (M184M/V, M184M/I, K103K/N and M46M/I) were only found in the cells (data shown for followed patients). For the 40 patients with follow-up samples (see Tables 2 and 4 below), three of the key mutations detected at the naïve stage were present in the RT and PR genes (M46L, M46M/I and K103K/N) of patients 7, 33 and 37. The K103K/N mixed population was not found in the plasma. One treatment-naïve patient (patient 9

Table 2 Evolution of drug resistance mutations in eight of 25 untreated patients detected using a direct sequencing assay of HIV-1 proviral DNA in purified CD4 cells compared with HIV-1 plasma viral RNA

Patient no.	Treatment	Viral load (copies/ml)	CD4 count (cells/ μ l)	Subtype	Follow-up (months)*	Plasma RT mutations	Proviral RT mutations	Plasma PR mutations	Proviral PR mutations
1	Naïve	4106	[†] 180	C	0	Wild type	Wild type	Wild type	Wild type
1	Naïve	< 50	–		39	Wild type	Wild type	Wild type	Wild type
2	Naïve	170 506	[†] 177	?	0	[†] Wild type	Wild type	[†] Wild type	Wild type
2	Naïve	42 534	205		18	Wild type	Wild type	Wild type	Wild type
3	Naïve	59 810	655	B	0	Wild type	[†] Wild type	Wild type	[†] Wild type
3	Naïve	25 023	445		12	Wild type	M184I	Wild type	Wild type
4	Naïve	60 024	[†] 605	A	0	Wild type	Wild type	L10V	L10V, G73G/S
4	Naïve	54 275	245		41	Wild type	Wild type	L10V	L10V, G73G
5	Naïve	11 574	855	G	0	Wild type	Wild type	Wild type	Wild type
5	Naïve	14 656	479		29	Wild type	Wild type	Wild type	Wild type
6	Naïve	2924	468	B	0	Wild type	Wild type	Wild type	Wild type
6	Naïve	65 804	369		27	Wild type	[†] Wild type	Wild type	Wild type
7	Naïve	6151	1111	B	0	Wild type	T215L	M46L	M46L
7	Naïve	1541	578		17	T215L	Not amplified	M46L	M46L
8	Naïve	28 700	768	B	0	Wild type	[†] Wild type	Wild type	[†] Wild type
8	Naïve	22 049	826		14	Wild type	Wild type	Wild type	Wild type

*Elapsed time between two samples, starting from zero for the baseline sample.

[†]Nucleotide sequence obtained using the Bayer Kit or another sample because of sequencing failure.

Mutations in bold: discrepant mutations between plasma and cells.

Mutations in italic: key mutations conferring high resistance.

PR, protease; RT, reverse transcriptase.

in Table 3) had virus with an RT resistance profile (67N, 70R and 219Q) in both CD4 cells and plasma. Global analysis of the resistance revealed identical results in 93% of CD4 cells and plasma.

Twenty-five patients remained therapy naïve, and eight of these untreated patients were followed. The genotyping results for both the RT and the PR resistance mutations in plasma and CD4 cells from these patients are shown in Table 2. One of the eight patients had one revertant RT resistance mutation (T215L in patient 7), while two patients had a PR mutation, including one key mutation (M46L in patient 7). Although one patient (patient 3) showed a new key RT mutation (M184I) after 12 months, which was present only in the cells, follow-up data for resistance mutations in the plasma and CD4 cells demonstrated stable mutation patterns. Patients 3 and 7 showed mutations in the second sample that were not detected in the first sample; this was probably a result of the known low sensitivity of direct sequencing for detecting minor populations.

Resistance mutations detectable in plasma and CD4 cells in the NNRTI group

The genotyping results for RT and PR resistance mutations in plasma and CD4 cells from the NNRTI-treated patients are shown in Table 3. There were 12 NNRTI-treated patients, most of whom received EFV, and follow-up data were available for 10 of them for a mean time of 34 months (range 17–44 months). Four of these 10 patients had at least one secondary RT resistance mutation (patients 9, 14, 16

and 17), while three patients had one PR mutation in both plasma and cells (patients 11, 13 and 18). All detected PR mutations were secondary mutations, which are not directly relevant for drug resistance. Viruses from patients 9 [mutations D67N, K70R and K219Q, conferring resistance to zidovudine (ZDV) and stavudine (d4T)] and 16 (mutation M41L) showed the same NRTI-correlated resistance mutations in plasma samples and in CD4 cells. Patient 9 was successfully treated with a combination of lamivudine (3TC), tenofovir (TDF) and EFV, and had an undetectable plasma viral load for 30 months. Three patients (patients 12, 13, and 15) showed one or two more mutations in the PR gene in CD4 cells than in plasma. However, follow-up analysis of resistance mutations could only be performed in the provirus as the plasma viral load was undetectable in most cases. All mutations detected at the therapy-naïve stage remained present and there were no additional mutations, with the exception of patients 11 and 17, in whom the key RT mutations Y188Y/H and M184M/I were only detected in the CD4 cells after 42 and 17 months of follow-up, respectively. The Y188Y/H mutation confers resistance to all NNRTIs. Patient 16 was treated with 3TC + TDF + EFV and showed a detectable plasma viral load (550 RNA copies/mL) after 36 months of follow-up without additional mutations. Overall, comparison of the amino acid sequences from the CD4 cells obtained at baseline and the plasma at baseline and the CD4 cells obtained during the follow-up showed comparable mutation patterns, particularly in the RT gene, with two new discrepant key mutations.

Table 3 Evolution of drug resistance mutations in 10 of 12 patients receiving nonnucleoside reverse transcriptase inhibitor (NNRTI)-based highly active antiretroviral therapy (HAART) detected using a direct sequencing assay of HIV-1 proviral DNA in purified CD4 cells

Patient no.	Treatment	Viral load (copies/ml)	CD4 count (cells/ μ l)	Subtype	Follow-up (months)*	Plasma RT mutations	Proviral RT mutations	Plasma PR mutations	Proviral PR mutations
9	Naïve	46 617	264 [†]		0	D67N, K70R, K219Q	D67N, K70K/R, K219Q	Wild-type	Wild-type
9	3TC + TDF + EFV	<50	345 [†]		30	–	D67N, K70K/R, K219Q, T69T/S	–	Wild-type
10	Naïve	257 [†]	–	B	0	Wild type	Wild type [†]	Wild type	Wild type [†]
10	3TC + ABC + EFV	<50	415		22	–	Wild type	–	Wild type
11	Naïve	29 932	176 [†]	CRF_01 AE	0	Wild type	Wild type	L10L/I	L10L/I
11	3TC + ABC + EFV	<50	526		42	–	Y188Y/H	–	L10L/I
12	Naïve	4260	270	C		Wild type	Wild type	Wild type	Wild type
12	3TC + ABC + NVP	<50	487 [†]		37	–	Wild type	–	Wild type
13	Naïve	44 990	284	B	0	Wild type	Wild type	L10I	L10L/I
13	3TC + TDF + EFV	<50	363		18	–	Wild type	–	L10L/I
13	ddl + 3TC + EFV	<50	239		44	–	Wild type	–	Wild type
14	Naïve	37 904	–	?	0	E44D	E44D	Wild type	Wild type
14	3TC + ABC + EFV	<50	549		41	–	E44D	–	Wild type
15	Naïve	19 572	279	B	0	Wild type	Wild type [†]	Wild type	Wild type [†]
15	3TC + ABC + EFV	<50	422		34	–	Wild type	–	Wild type
16	Naïve	12 904	–	D	0	M41L	M41L	Wild type	Wild type
16	3TC + TDF + EFV	556	–		36	–	M41L	–	Wild type
17	Naïve	32 941	168 [†]	A	0	T69S	T69S [†]	Wild type	Wild type [†]
17	3TC + TDF + EFV	<50	194		17	–	T69S, M184M/I	–	Wild type
18	Naïve	4051	225 [†]	CRF_02 AG	0	Wild type	Wild type [†]	L10I	L10I [†]
18	3TC + ABC + EFV	<50	389		33	–	Wild type	–	L10I

*Elapsed time between two samples, starting from zero for the baseline sample.

[†]Nucleotide sequence obtained using the Bayer Kit or another sample because of polymerase chain reaction (PCR) or sequencing failure.

–, not tested because of undetectable plasma viral load or lack of sample.

Mutations in bold: discrepant mutations between plasma and cells.

Mutations in italic: key mutations conferring high resistance.

PR, protease; RT, reverse transcriptase; 3TC, lamivudine; ABC, abacavir; ddl, didanosine; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir.

Resistance mutations detectable in plasma and CD4 cells after PI-based therapy

Table 4 shows the genotyping results for the RT and PR resistance mutations in plasma and CD4 cells from patients undergoing PI-based HAART for whom follow-up data were available. Twenty-two of the 32 patients, most of whom received LPV/r-based therapy, were followed for a mean time of 25 months (range 12–44 months). At the therapy-naïve stage, four (18%) of the 22 patients for whom amino acid sequences were obtained had at least one RT resistance mutation, while nine patients had at least one PR mutation. Patient 37 had a key RT mutation, K103K/N, which was present only in the cells and not in the plasma and which confers high drug resistance to all NNRTIs, while three patients had secondary mutations (T69T/S in the plasma for patient 26 and K70K/R in the cells for patients 24 and 32), which are not relevant for drug resistance. In addition to the K103N mutation conferring resistance to NNRTIs, virus from patient 37 also had the V82V/L mixed population, which confers a reduced response to tipranavir (TPV) boosted with ritonavir (r). One patient of 32 (patient 36) receiving an LPV/r-based regimen showed an increase in plasma RNA viral load from <50 to 11 300 copies/mL

after 18 months of follow-up, with an additional mutation. The additional M184I mutation was observed in the plasma RNA but not in the proviral DNA, and confers high-level resistance to 3TC. This patient was treated with d4I, abacavir (ABC) and LPV/r combination therapy for 1 year before being changed to a 3TC + TDF + LPV/r regimen because of poor compliance. Patient 33 had the M46M/I mixed population in the PR gene at the therapy-naïve stage. The plasma viral load was undetectable under HAART in most cases, but follow-up analysis of the proviral resistance mutations showed the presence of mutations detected at the therapy-naïve stage without additional mutations, except in the sequence from patient 36. Overall, comparison of resistance mutation patterns in CD4 cells with plasma RNA data or follow-up data for CD4 cells revealed similar results for the RT and PR genes, with one or two discrepant mutations.

Analysis of DNA resistance evolution and comparison with RNA

The analysis of DNA resistance evolution in all treated patients showed that the proportion of new mutations was 22% ($n = 6$) ($P < 0.0001$ for the difference from 0), and

Table 4 Evolution of drug resistance mutations in 22 of 32 patients receiving protease inhibitor (PI)-based highly active antiretroviral therapy (HAART) determined using a direct sequencing assay of HIV-1 proviral DNA in purified CD4 cells

Patient no.	Treatment	Viral load (copies/ml)	CD4 count (cells/ μ l)	Subtype	Follow-up (months)*	Plasma RT mutations	Proviral RT mutations	Plasma PR mutations	Proviral PR mutations
19	Naïve	260 567	289	?	0	Wild type	Wild type	Wild type	Wild type
19	d4T + ABC + LPV/r	150	259		11	–	Wild type†	–	Wild type†
20	Naïve	> 500 000	131†	CRF_03 AB	0	Wild type	Wild type†	L10I	L10I†
20	3TC + ABC + LPV/r	< 50	460		44	–	Wild type	–	L10I, L10V
21	Naïve	86 406	323	?	0	Wild type†	Wild type†	Wild type†	Wild type†
21	d4T + ABC + RTV + FPV	80	414		15	–	Wild type	–	Wild type
22	Naïve	2403	347†	CRF_02 AG	0	Wild type	Not amplified	Wild type	Not amplified
22	ZDV + 3TC + LPV/r	< 50	603		27	–	Wild type	–	Wild type
23	Naïve	32 279	50	B	0	Wild type	Wild type	Wild type	Wild type
23	ZDV + 3TC + LPV/r	< 50	146		13	–	Wild type	–	Wild type
23	3TC + TDF + EFV	< 50	185		25	–	Wild type	–	Wild type
24	Naïve	14 856	517†	B	0	Wild type	K70K/R	Wild type	Wild type
24	ZDV + 3TC + LPV/r	< 50	291		32	–	Wild type†	–	Q58E†
25	Naïve	35 014	45†	B	0	Wild type	Wild type†	Wild type	Wild type†
25	3TC + TDF + LPV/r	< 50	308		42	–	Wild type	–	Wild type
26	Naïve	166 659	1128	?	0	T69T/S	Wild type	L10L/V	Wild type
26	ZDV + 3TC + LPV/r	< 50	1021		8	–	Wild type	–	Q58E
26	Stops taking treatment	57			32	–	Wild type†	–	Q58E†
27	Naïve	20 517	187	A	0	Wild type	Wild type	Wild type	Wild type
27	ZDV + 3TC + LPV/r	< 50	509		13	–	Wild type†	–	Wild type†
28	Naïve	51 614	284†	B	0	Wild type	Wild type	Wild type	Wild type
28	ZDV + 3TC + LPV/r	< 50	185		19	–	Wild type	–	Q58E
29	Naïve	192 453	10	CRF02	0	Wild type	Wild type	Wild type	Wild type
29	ZDV + 3TC + RTV + FPV	< 50	204		16	–	Wild type	–	Wild type
30	Naïve	154 697	59†	G	0	Wild type	Wild type	Wild type	Wild type
30	3TC + TDF + RTV + APV	< 50	341		26	–	Wild type†	–	Wild type†
31	Naïve	1614	446	F	0	Wild type	Wild type	L10I, L10V	L10I, L10V
31	ZDV + 3TC + LPV/r	< 50	765		18	–	Wild type	–	L10V
32	Naïve	49 231	211	G	0	Wild type	K70K/R	Wild type	Wild type
32	3TC + TDF + RTV + APV	< 50	258		26	–	Wild type	–	Wild type
33	Naïve	457 496	302	CRF01	0	Wild type	Wild type	M46M/I	Wild type
33	ZDV + 3TC + RTV + FPV	< 50			19	–	Wild type	–	Wild type
34	Naïve	> 500 000	4	G	0	Wild type	Wild type	L10I	L10I
34	3TC + ABC + LPV/r	< 50	187†		17	–	Wild type	–	L10I
35	Naïve	215 771	225		0	Wild type	Wild type	Wild type	Wild type
35	3TC + ABC + LPV/r	< 50	501		37	–	Wild type	–	Wild type
36	Naïve	> 500 000	51	CRF_02	0	Wild type	Wild type	L10V	Wild type
36	d4T + ABC + LPV/r	< 50	386		13	–	Wild type	–	L10V
36	3TC + TDF + RTV + APV	11 319	362		18	M184I	Wild type	L10V	L10V
37	Naïve	44 934	272	CRF_06 CPX	0	Wild type	K103K/N	Wild type	V82V/L
37	3TC + d4T + LPV/r	< 50	496		11	–	Wild type	–	Wild type
38	Naïve	5872	200	B	0	Wild type	Wild type	Wild type	Wild type
38	3TC + ZDV + LPV/r	< 50	472		21	–	Wild type	–	Wild type
39	Naïve	56 117	64	B	0	Wild type	Wild type	Wild type	Wild type
39	3TC + TDF + LPV/r	< 50	534		42	–	Wild type	–	Wild type
40	Naïve	1119	334	CRF_02 AG	0	Wild type	Wild type	Wild type	Wild type
40	TDF + FTC + LPV/r	3618	347†		33	Wild type	Wild type	Wild type	Wild type
40	TDF + FTC + LPV/r	< 50	–		35	–	Wild type	–	Wild type

*Elapsed time between two samples, starting from zero for the baseline sample.

†Nucleotide sequence obtained by Bayer Kit or on another sample due to sequencing failure.

–, not tested because of undetectable plasma viral load or lack of sample.

Mutations in bold: discrepant mutations between plasma and cells.

Mutations in italic: Key mutations conferring high resistance.

ABC, abacavir; APV, amprenavir; ddI, didanosine; d4T, stavudine; EFV, efavirenz; FPV, fosamprenavir; FTC, emtricitabine; LPV/r, lopinavir boosted with ritonavir; NVP, nevirapine; PR, protease; RT, reverse transcriptase; RTV, ritonavir; TDF, tenofovir; 3TC, lamivudine; ZDV, zidovudine.

these included three new key mutations. However, the appearance of new mutations was not correlated with the time elapsed between sample collections. A logistic regression was performed and a *P* value of 0.34 [unitary

odds ratio (OR) 1.03; global OR 3.24] was obtained. All the other covariates (patient characteristics and use of antiretroviral therapy) were found not to influence the incidence rate of new mutations. The comparison of pre-HAART RNA

genotyping with post-treatment DNA sequencing gave calculated prevalences of detected mutations of 59 and 78%, respectively. The proportion of detected mutations (19%) in the DNA was significantly higher than in the pre-HAART RNA by the χ^2 test ($P < 0.0001$), with moderately good agreement between the two methods in terms of the number of detected mutations (kappa coefficient 0.56). A kappa coefficient of 0.50 indicated moderately good agreement in terms of predicted drug activity between the pretreatment RNA and pretreatment DNA mutation profiles, and a kappa coefficient of 0.40 indicated only fairly good agreement between the pretreatment RNA and post-treatment DNA mutation profiles, as a result of the accumulation of new mutations.

Discussion

Genotyping for HIV-1 drug resistance mutations is routinely performed on a plasma sample. At present, guidelines do not recommend HIV-1 drug resistance testing on cellular proviral DNA. The proviral compartment archives the various strains, either wild-type or drug-resistant, that arise during infection. The long-term persistence of archived drug-resistant DNA may jeopardize the efficacy of targeted drugs, and represents the 'resistance potential' profile of a patient [40]. This is important when switching antiretroviral agents or initiating treatment in patients without available historical data or conserved samples. Only a small percentage of lymphocytes (about 2% of all lymphocytes in the body) stay in the blood for a short time, before migrating to lymphoid and nonlymphoid organs. Eighty-five percent of re-circulating lymphocyte pool cells entering the lymph system are from the blood while about 15% are from the lymph. These data are mostly derived from animal experiments [34]. They underline the fact that an absence of resistance mutations in blood lymphocytes does not exclude the possibility that resistance is present. There is an increasing body of literature on the possible utility of assessing drug resistance mutations in the provirus. Our data are in accordance with previous observations and indicate the practical feasibility of sequencing the provirus. As reported by Bona *et al.* [30], we also found more drug resistance mutations, particularly key mutations, in the cell proviral DNA than in the plasma. Based on the Stanford mutation list, excluding polymorphisms and drug-selected mutations with no known significance, the proportion of mutations detected in the DNA was significantly higher than the proportion detected using standard RNA genotyping by the χ^2 test. At the therapy-naïve stage, we detected seven key mutations in the RT and PR genes in different patients (10% of all included patients), and four of these (M184M/V, M184M/I, K103K/N and M46M/I) were only found in the cells. Three key

mutations (K103K/N, M46L and M46M/I) were found in different patients, for whom the follow-up was possible (4.3% of 69 patients included in the study). The K103K/N was not found in the plasma. At the time of study inclusion, 8% of patients had at least one RT mutation in the plasma, while 15% had at least one RT resistance mutation in CD4 cells. One therapy-naïve patient had virus with an RT resistance profile (67N, 70R and 219Q) in both CD4 cells and plasma. Before initiating treatment, PR gene sequencing showed that the percentage of patients with viruses carrying at least one PR mutation was 25% for CD4 cells and 23% for plasma. Wang *et al.* [31] and recently Ghosn *et al.* [32] reported a tight concordance of resistance profiles in paired HIV RNA and PBMC HIV DNA. Our own results demonstrate that at baseline only 55% of PR mutations and 56% of RT mutations were simultaneously present in CD4 cells and plasma, with substantial agreement between the two methods as assessed using kappa statistics. In their study, Usuku *et al.* [33] noted the persistence of a discrepancy between plasma and PBMCs for more than 3 years. In this study, the comparison between pretreatment amino acid sequences from CD4 cells and the plasma compartment and the comparison between pretreatment CD4 cell samples and follow-up CD4 cell samples showed a statistically significant proportion of new mutations of 22%, although the appearance of new mutations was not correlated with the time elapsed between tests. One of the 40 patients with follow-up samples had a key RT resistance mutation present in cells but not in plasma. Thirty-eight per cent of patients had RT mutations and 17 of the 40 patients with follow-up (43%) had one or two PR mutations in cells and plasma, but most of these were not relevant for drug resistance. In the NNRTI group, two patients (patients 11 and 17) of 10 who received at least 12 months of EFV-based HAART showed new key mutations (Y188Y/H and M184M/I), while one (patient 36) in the PI group and one naïve patient (patient 3) had a new key RT mutation (M184I). All new key mutations except one (in patient 36) were only present in the CD4 cells. Patient 36, who received d4T, ABC and LPV/r combination therapy for 1 year before changing to a 3TC, TDF and LPV/r regimen, showed a new key mutation (M184I) after 18 months of follow-up in the plasma RNA but not in the proviral DNA. Thus, monitoring of the evolution of drug resistance mutations in treated patients by direct sequencing of HIV-1 proviral DNA in purified CD4 cells revealed new mutations, with moderately good agreement between pre- and post-treatment DNA mutation patterns. In patients who remained treatment-naïve, almost no evolution was observed in mutations detected in plasma RNA or cell DNA. After therapy initiation we noted the persistence of HIV-1 drug resistance mutations in proviral DNA from purified

CD4 cells compared with plasma viral RNA at baseline. In our small cohort, 30 of 32 treated patients showed an undetectable plasma viral load after at least 12 months and up to 44 months of follow-up. Patients with pre-existing resistance mutations had a good response to all types of HAART, but none of them underwent combination therapy with the targeted drug. One interesting question was whether the DNA test might be useful to guide therapy switches in patients with suppressed viral load. This was addressed by comparing the prevalences of detected mutations in pretreatment RNA and post-treatment DNA (59 and 78%, respectively). A statistically significant proportion of mutations (19%) were detected in the DNA compared to the pretreatment RNA. The data demonstrated that sequencing DNA is possible and the recommended RNA sequencing might miss some mutations. In the comparison of pretreatment RNA with post-treatment DNA using kappa statistics, a moderately good agreement was found in terms of mutations detected and only a fairly good agreement in terms of predicting drug activity because of the accumulation of new mutations in the DNA. In patients with detectable viraemia, no new DNA mutations were detected and the viral loads were too low to enable RNA genotyping to be performed (patients 16, 19 and 21 with 556, 150 and 80 copies/mL, respectively). Therefore, we could not conclude that the standard method had underestimated the accumulation of mutations as the test was only possible on cell DNA samples. Transmission of drug-resistant HIV-1 strains and reduced susceptibility of viruses derived from untreated patients have been documented. Although wild-type virus may outgrow any initially resistant virus in patients with chronic asymptomatic HIV infection, some key mutations were consistently detected in this study. More resistance mutations were detected in the provirus in CD4 cells than in the virus in plasma and these mutations persisted for at least 1 year of follow-up with or without therapy, but the overall pattern of resistance was fairly similar in plasma and cells. HIV-1 proviral DNA would in our hands be most useful for making decisions, when changing therapy, on the best alternative treatment for patients with undetectable plasma viral load.

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3. EFFECT OF TREATMENT ON THE HIV-1 PROVIRAL DNA LOAD

Authors:

Benoît Kabamba-Mukadi¹, Armelle Duquenne¹, Philippe Henrivaux³, Flora Musuamba², Jean Ruelle¹, Jean-Cyr Yombi³, Monique Bodéus¹, Bernard Vandercam³ and Patrick Goubau¹

Authors' affiliation:

¹AIDS Reference Laboratory, Université Catholique de Louvain, Avenue Hippocrate 54, B1200 Brussels, Belgium

²LBCM Unit, Université Catholique de Louvain, Avenue Hippocrate 54, B1200 Brussels, Belgium

³Department of Infectious Diseases, Université Catholique de Louvain, Avenue Hippocrate 10, B1200 Brussels, Belgium

Unpublished data – Original research

UNPUBLISHED DATA - ORIGINAL RESEARCH

EFFECT OF TREATMENT ON THE HIV-1 PROVIRAL DNA LOAD

B Kabamba-Mukadi,¹ A Duquenne,¹ P Henrivaux,³ F Musuamba,² J Ruelle,¹ J-C Yombi,³ M Bodéus,¹ B Vandercam³ and P Goubau¹

¹Universite' Catholique de Louvain, AIDS Reference Laboratory, Brussels, Belgium, ²Universite' Catholique de Louvain, LBCM Unit, Brussels, Belgium, ³Universite' Catholique de Louvain, Department of Infectious Diseases, Brussels, Belgium

ABSTRACT**BACKGROUND**

The presence of the HIV reservoirs is the most significant barrier to cure HIV infection. The quantification of the HIV-1 proviral load might permit a better assessment of the reservoirs, thus the long-term therapeutic efficacy. In this study, we evaluated the impact of treatment associations with and without protease inhibitors on proviral load in CD4 + cells.

METHODS

The proviral load in CD4 + cell was monitored before and after initiation of treatment in 69 naïve patients in parallel with recommended parameters for the clinical management of the disease, the plasma viral load and the CD4 + cell count. We analyzed the mean differences of proviral load between 3 patient groups, the first receiving a drug regimen including a non nucleoside reverse transcriptase inhibitor (NNRTI), the second including a protease inhibitor (PI) and the last constituting an untreated control group.

RESULTS

The patients were followed for a mean of 29 months (from 12 to 69 months). No difference in mean changes in proviral load between the 3 groups of patients was observed. The analysis of the CD4+ cell count showed that the mean difference in the number of CD4+ cells was statistically different between the NNRTI and PI groups on the one hand and the group of naïve patients on the other hand. Eighty-three percent and 86% of patients had undetectable plasma viral load in the NNRTI and PI groups, respectively.

CONCLUSION

This work did not show a higher impact of certain regimens targeting viral reverse transcriptase and protease on the HIV-1 proviral load. The variations were not statistically different between the patient groups, treated or untreated, while the effect on plasma viral load and CD4+ cell count was obvious. However, the use of the HIV-1 proviral DNA load as a measure of the reservoirs is gaining a new interest in the assessment of new treatment strategies aiming at eradicating HIV infection or in scheduling treatment interruption, even if the latter is no more recommended..

KEYWORDS: HIV-1, proviral load, DNA, provirus, viral load, CD4 cell count

INTRODUCTION

To achieve HIV eradication, replication of virus in the whole body has to be inhibited by the treatment. It is known that the HIV proviral DNA persists in infected cells even after a prolonged successful therapy. HIV reservoirs are thought to be a mix of latently infected cells and biological compartments called “sanctuaries”. These reservoirs allow persistence of the virus, thus preventing the HIV eradication. The ability of the HIV to infect latent memory T cells that constitute the important part of the HIV reservoir is the most significant barrier to cure HIV infection. HIV replication might resume after antiretroviral therapy (ART) simplification or interruption because of the reservoirs, particularly memory CD4⁺ cells that can reactivate and produce infectious virions in the presence of specific antigen or other stimulation.

Measuring proviral DNA load as a reflection of reservoirs is therefore a parameter extensively studied in the literature to evaluate the impact of anti-HIV therapies [1-9]. HIV-1 proviral DNA exists in different forms and as there is no standardisation of assays detecting different DNA forms, work on the quantification is not always comparable. On one hand, some assays quantify total proviral DNA while others evaluate the integrated HIV DNA, on the other hand some assays are conducted on PBMCs while others use purified CD4⁺ cells. CD4⁺ cells normally represent 30 to 50% of the PBMCs but may be lower in HIV infected individuals. There might be some concern about sensitivity and accuracy of assays quantifying low-copy-number samples which is often the case, ranging from 1 to < 5 log₁₀ HIV-1 DNA copies (median 2–3 log₁₀) in 10E6 PBMC equivalents [1-9]. Methods quantifying the HIV-1 DNA proviral load by sequence-specific hybridisation probes are more specific but the very high variability of HIV

sequences makes the design difficult. A sequence-independent detection with SYBR Green I might be more sensitive but less specific. Having identical PCR efficiencies for target and reference genes in the sample, standard, and calibrator is one of the experimental prerequisites for PCRs that may have a major impact on accuracy. The published real-time PCR (qPCR) assay used in this study complies to PCR prerequisites and showed that five copies of the HIV-1 genome were detected while no amplification was obtained on any PBMC from blood donors [10]. We reported an interesting observation in patients undergoing structured treatment interruption (STI) [10]: proviral load and plasma viral load rebound were high in patients who took longer to achieve an undetectable plasma viral load under therapy. A statistically significant correlation was observed between DNA proviral load and RNA steady state viral load in STI patients (p-value = 0,012). HIV patients stop ART for various reasons, such as to be more time off therapy or to reduce side effects. It must be underlined that so far, large research studies have not shown certain benefits of discontinuing therapy in view of avoiding HIV disease progression, minimize drug resistance or transmission of HIV. STI thus cannot presently be recommended outside controlled clinical trials [11,12].

In the present article, we prospectively studied the value of the quantification of HIV-1 provirus (total integrated and unintegrated DNA) in the management of anti-HIV treatment in clinical settings. Assuming that effective anti-HIV-1 treatment has an impact on the reservoirs leading to the reduction of proviral load and emergence of drug related resistance, differences in long-term effectiveness could be observed with different combined anti-HIV therapies. It is known that reverse transcriptase inhibitors act at the first phase of the intracellular viral life cycle by blocking the

synthesis of viral DNA, while the protease inhibitors prevent the maturation of new virions released by infected cells. In order to draw a possible prognostic value of the HIV-1 proviral DNA quantification, we studied the impact of combined ARV on HIV-1 proviral load before and after the initiation of HAART including a boosted protease inhibitor (PI) or a non nucleoside reverse transcriptase inhibitor (NNRTI) in naïve patients. Data on the detection of drug resistance mutations on proviral DNA for the same groups of patients were published elsewhere [13].

METHODS

We first developed a SYBR-Green I real-time PCR assay quantifying the provirus HIV-1 present in subpopulations of leukocytes circulating CD4 + against β -globin gene as a reference gene [10]. The published method was prospectively applied to naïve patients before and after initiating highly active antiretroviral therapy (HAART) to compare the impact of the antiretroviral (ARV) regimen with or without protease inhibitors on the provirus characteristics. In parallel to the assessment of the evolution of the HIV-1 proviral load in enrolled patients, we also monitored two recommended parameters (plasma viral load and CD4 +cell count) before and after initiation of treatment.

PATIENTS

A prospective observational study protocol was developed in collaboration with clinicians and approved by the local ethics committee. Informed consent was obtained from each patient. Sixty-nine therapy naïve patients were enrolled in the study between May 2002 and July 2007 (Table 1). After treatment initiation, patients were divided in three groups. The first group included 32 patients receiving antiretroviral therapy with a protease

inhibitor combined to nucleoside reverse transcriptase inhibitors. Of these, 28 patients were followed during the study. The second group of 12 followed individuals received only reverse transcriptase inhibitors (2 NRTI and 1 NNRTI). The last group comprised 25 patients remaining untreated as a control group of which 14 patients could be followed. The HIV-1 positive status of each patient was confirmed by accepted methods. The historical data on possible previous treatment were reviewed for all patients. Checking treatment history can be difficult but is important in the context of studies on HIV naïve patients. We published a letter on the diagnostic aspects of the HIV infection [14].

PURIFIED CD4 CELLS AND REFERENCE STRAIN

CD4 cells were isolated from 10 mL of whole blood [collected from patients in ethylenediaminetetraacetic acid (EDTA) by an immunomagnetic method using anti-CD4 coated magnetic beads (Dynabeads M450 CD4; Dynal AS, Oslo, Norway) according to the manufacturer's protocol and were stored at -80°C until use. The purity of the CD4 cell preparation was approximately 99% as estimated using Becton Dickinson FACScan Flow Cytometer technology (Franklin Lakes, New Jersey, USA) (data not shown). The 8E5 cell line used as standard for HIV proviral quantification was cultured in RPMI-1640 medium and the cells were counted using a Coulter automated haematology analyser, diluted to 10E6 cells per aliquot and stored at -80°C. The 8E5 cell-free virus present in the culture supernatant was quantified, diluted and stored in aliquots of 30 000 HIV-1 RNA copies/mL used as local intra-run positive control.

HIV-1 PLASMA VIRAL LOAD

The HIV-1 RNA plasma viral load was assessed using the Versant HIV-1 RNA 3.0 assay (Siemens Medical Diagnostics Solutions, Tarrytown, NY, USA) according to the manufacturer's instructions. This assay is based on branched DNA (bDNA) technology. It requires 1mL of sample and has a dynamic range of 50–500 000 copies/mL. Plasma samples and the 8E5 cell culture supernatant were frozen at -80°C until tested for HIV-1 RNA viral load. A viral load result of <50 copies/mL was regarded as 50 copies/mL when calculating the mean viral load of a given patient group.

STATISTICAL ANALYSIS

The impact of treatment regimen on proviral levels was assessed by analyzing the mean differences between three parameters (plasma RNA load, CD4+ cell count and cell DNA load) before and after treatment. Plasma viral load decreases as the CD4+ cell count increases in effective treatment

that controls viral replication. An assessment of the normality of data is a prerequisite for many statistical tests and was performed by the Shapiro test. In case of normal distribution, analysis of variance (ANOVA) was performed to compare mean differences in the three groups of patients (PI, NNRTI and naive). Changes in CD4 cell count and viral load were calculated per individual in patients with follow-up samples taken during the study period. Statistical analysis was performed using JMP statistical software version 7.0.1 (SAS Institute, Cary, NC).

RESULTS

We published [13] the analysis of the comparison of HIV-1 resistance mutations detected in proviral DNA sequences with plasma viral RNA sequences and pre-treatment sequences. We showed that detecting resistance mutation in HIV-1 DNA could be useful in chronic infections or when switching therapy in patients with undetectable viraemia.

PATIENT CHARACTERISTICS

	All patients	Naive group	NNRTI group	PI group
Number of patients				
Total	69	25	12	32
> 12 months of follow-up	54	14	12	28
Gender				
Males (%)	49	56	33	50
Females (%)	48	40	67	47
Unknown (%)	3	4		3
Age [years] [mean (range)]	44 (range 20-68)	41 (range 27-55)	44 (range 20-68)	47 (range 29-65)
Geographic origin				
Europeans (%)	58	48	58	47
Other/Unknown (%)	42	52	42	53
Duration of follow-up [months] [mean (range)]^a	29 (range 12 - 69)	22 (range 12 - 41)	42 (range 14 - 69)	26 (range 16 - 54)
CD4+ count Age [cells/uL]				
Baseline [median (range)] ^b	338 (range 6-1460)	616 (range 177-1111)	238 (range 168-284)	253 (range 46 - 1128)
Mean change (range) ^c	-	(-)13 (range -1427 - 534)	(+)102 (range -173 - 365)	(+)203 (range -165 - 731)
Plasma HIV RNA [Log copies/mL]				
Baseline [median (range)] ^b	5.27 (range 2.6 - 5.87)	4.00 (range 1.70 - 4,82)	4.09 (range 2.41- 4,67)	4.44 (range 1.70 - 5.70)
Mean change (range) ^c	-	0,06 (range -1,29 - 1.91)	-	-
Patients with VL>50 copies/mL (%)			83	86
HIV-1 Subtype (%)				
B	39			
Non-B	61			

(a) Number of months between the baseline sample and the last follow-up sample sequenced.

(b) Sample tested at baseline (before therapy initiation for treated patients)

(c) The changes in CD4 count and viral load have been calculated per individuals in patients with follow-up samples

Table 1: Patients characteristics

RESULTS OF PROVIRAL LOAD

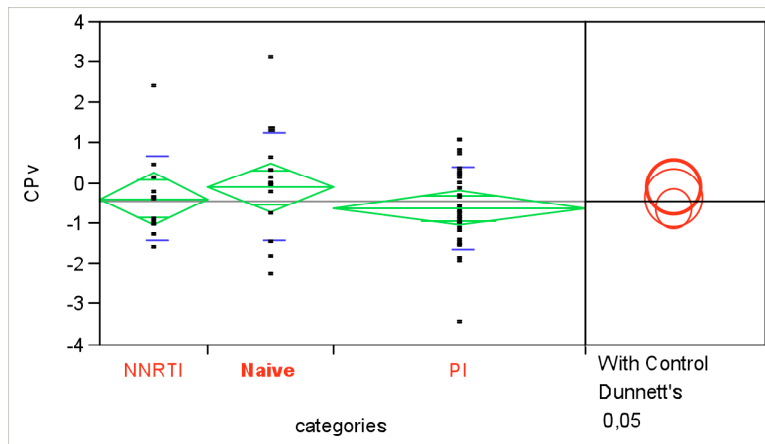


Figure 1: comparison of mean differences of proviral load in the 3 followed groups of patients.

Data of proviral load were normally distributed by Shapiro test. The analysis of variance showed no difference in mean changes of proviral load in the study period between the PI group and the NNRTI one. And also no difference was observed between treated patients and the group of untreated patients (p-value of 0,385) by ANOVA test (Figure 1).

NUMBER OF CD4+ CELL AND PLASMA VIRAL LOAD

CD4+ cell data were normally distributed and the average differences in the number of CD4 were statistically different between treated groups with or without PI on one hand and the untreated patient group on the other hand (p-value of 0,0151) by ANOVA test (Figure 2).

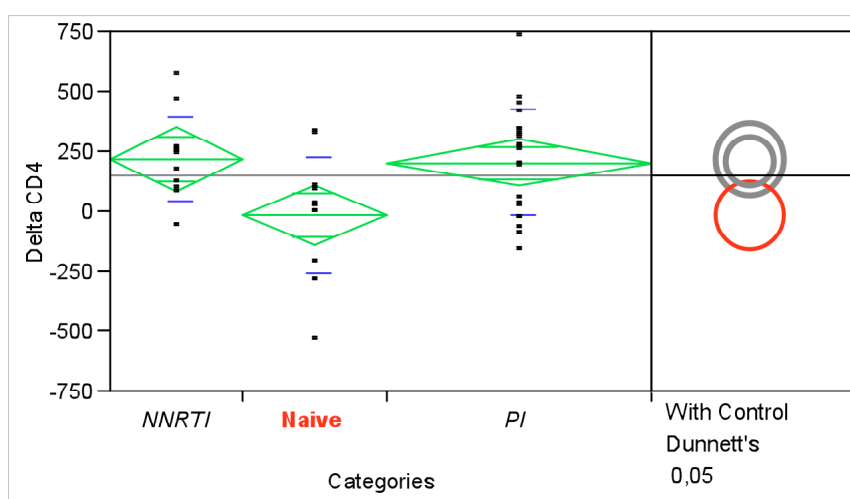


Figure 2: analysis of the variance of the CD4+ cell count in the 3 groups showed statistically differences in mean changes between treated and untreated patients

The HIV-1 RNA viral load (VL) data were normally distributed. The characteristics of 69 selected patients at the time of inclusion in the study are presented in Table 1. The 69 treatment-naïve patients had a mean viral load of 5.27 (range 2.6–5.70) $\log_{10}$ copies/mL. When treatment was initiated, 25 patients remained untreated of whom 14 could be followed. After a mean follow-up time of 24 (range 12 to 41) months, the mean viral load change in untreated individuals was 0.06 (range -1.29 - 1.91) $\log_{10}$ copies/mL, which was not statistically different from 0 (Table 1). In the NNRTI group, 12 patients were followed for at least 12 months and 83% of them achieved an undetectable viral load (<50 RNAcopies/mL) (Table 1). The protease inhibitor (PI) group comprised 32 individuals, of whom 28 had at least 1 year of follow-up, 86% of them having an undetectable viral load (<50 RNAcopies/mL) (Table 1).

DISCUSSION AND CONCLUSION

Based on the assumption that some anti-HIV-1 regimens might affect the HIV proviral DNA representing a part of the viral reservoir, the quantification of the HIV proviral load may allow a better assessment of the long-term therapeutic efficacy and bring additional information to that of the plasma RNA load in monitoring the disease. It is because of this viral reservoir that viral production resumes at the end of treatment. This work, which followed patients for at least 1 year, does not show that HIV-1 proviral load is a better parameter for the assessment of anti-HIV regimens targeting RT and PR on HIV-1 proviral DNA, the variation in proviral load was not statistically different between the three patient groups (naïve, NNRTI or PI). No difference was observed between treated and untreated patients and no trend of

decrease of the proviral DNA load was noticed. On the contrary, the treatment showed as expected an effect on CD4+ cell count that statistically significantly increased in treated patients while the plasma VL was undetectable in about 85 % of them. Even if the quantification of the HIV proviral load did not bring additional information to that of the plasma RNA load in monitoring the disease, we reported on the same group [13] that the sequencing of the provirus provides additional information to that of plasma virus in terms of proportion of drug resistance mutations and detection of new mutations.

How can we explain the stability of the total proviral load over the study period? Is the kinetics of the proviral DNA reduction very slow or should we analyze the really integrated proviral DNA instead of the total DNA?

Data concerning the impact of HAART on the kinetics of proviral DNA load are conflicting. Aleman et al. [15] studied levels of HIV-1 RNA and DNA in 58 patients. They showed that in comparison with HIV-1 RNA, a much slower decline in HIV-1 DNA levels was seen. They observed that the proviral load was stable in 10 untreated patients while a significant decline was noticed in treated patients, markedly more under triple therapy than double therapy. They concluded that studies are needed to interpret changes in HIV-1 DNA load as markers of viral replication during efficient antiretroviral combination therapy. Siliciano et al. [16] have studied how stable the latent reservoir is and whether its persistence reflects replenishment by low-level viremia. They estimated that the reservoir decay was very slow with a half-time of 44 months in treated patients with undetectable viremia for as long as 7 years. They concluded that eradication is unlikely. In our study, the mean of follow-up duration was 29

months (range 12-69), which might be too short to observe a significant decrease in proviral DNA.

This overall rather negative results of our study measuring proviral DNA load as a reflection of reservoirs could receive a renewed interest. New interest in quantifying proviral load has indeed recently emerged in treatment strategies aiming at eradicating all HIV infected cells. Drugs such as histone deacetylase inhibitors, methylation inhibitors, cytokines such as IL-7 or activators of nuclear factor kappa B (NF- κ B) such as prostratin that might revert the latently infected memory T cells to activated T cells are being explored to achieve HIV eradication [17]. In their article dedicated to the review of these new strategies, Lewin and Rouzioux [17] also focused on methods currently used to quantify persistent virus and key issues for successfully moving from laboratory research to clinical trials. They reported that total HIV DNA quantifies integrated and non-integrated DNA as well as latent and defective virus, but there is a strong correlation between total HIV DNA and integrated HIV DNA in patients on HAART. Therefore cell-associated HIV DNA is likely to be a good surrogate marker of the total number of latently infected cells [17,18]. A small study [19] published in 2011 suggested that total HIV DNA may not be an ideal reflect of the reservoir in all patients as three of seven patients examined had an excess of unintegrated DNA. But as assays quantifying separately the proviral DNA forms are less reproducible and sensitive, the quantification of total cell-associated HIV DNA is likely to be the most feasible tool to evaluate the frequency of infected cells in large-scale clinical trials and cohorts.

HIV-1 proviral DNA might be an interesting parameter in patients undergoing structured treatment interruption (STI) in order to identify patients in which plasma viral load might be

controlled after therapy interruption. We reported [10] that proviral load was high in STI patients that experienced a high plasma viral load rebound. In the Swiss HIV Cohort, Sabine Yerly et al. [20] found that in patients under HAART with long-term undetectable viraemia, the pre-HAART plasma viraemia and the baseline proviral DNA level were significantly associated with the viraemia setpoint during scheduled treatment interruptions. They concluded that as pre-HAART viraemia may not be available, proviral DNA measured at the time of scheduled treatment interruption, can help to identify patients likely to reach a low viraemia setpoint after treatment interruption. Another study conducted by Sarmati et al. [21]

evaluated the predictive value of the total HIV-1 DNA quantitation for long-term success of simplified treatment. Among 62 patients with effective HAART containing PI before being enrolled in a prospective non-randomized cohort with simplified PI-sparing regimen, patients with proviral DNA levels below the median value (226 copies/ 10^6 PBMCs) had a significant higher CD4 cell count at nadir ($P=0.003$) and at enrolment ($P=0.001$). Eighteen month after treatment simplification, a reduced risk of experiencing virological failure or blip was correlated with HIV-1 DNA levels below 226 copies/ 10^6 PBMCs at baseline (OR 0.002, 95% CI 0.001–0.46, $P=0.025$). Authors also observed a correlation between sustained undetectable plasma viral load with low proviral DNA load before treatment simplification.

In conclusion in terms of proviral load, this work did not show a higher impact of certain regimens associating RTIs with or without PIs on the HIV-1 proviral load as a quantification of reservoirs. However, the use of the HIV-1 proviral DNA load as a reflection of reservoirs is gaining a new interest

in the assessment of new treatment strategies to eradicate HIV infection or might be useful in scheduling STI even if not recommended, which were not part of this project. It remains an open question whether these perspectives will reactivate interest in this and similar studies. At least it gives our laboratory the possibilities to react swiftly to new demands in this context.

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CHAPTER 4

DISCUSSION AND CONCLUSION

Due to small sample size, one must be cautious before generalizing these findings. Sixty-nine patients from two centres were included in the study but only in 40 patients could the detection of resistance mutations at proviral level be followed. Despite this, detected resistance mutations seemed stable throughout the follow-up with a higher presence at the cellular level. The second limitation was the heterogeneity of the study population due to the use of an observational study protocol: this needs to be taken into account in the evaluation of results. Even so we believe that our findings have some practical consequences and give a hint to further research.

The virological follow-up of HIV-1 infected patients is presently almost exclusively performed on cell-free plasma virus. The HIV-1 cell associated provirus has been extensively studied as an alternative viral marker, as it persists and archives pre-treatment wild-type or drug resistant strains in infected cells, even after prolonged successful HAART. The provirus serves as a template for production of new virions. Therefore it may reignite viral replication when stopping HAART. The circulating HIV-1 T-cell associated provirus may reflect the effects of the HAART on the reservoirs in term of reservoir reduction and archived drug resistance mutations. For these reasons it could be an alternative for monitoring the long term efficacy of HIV drugs and for immediate treatment shift in successfully treated individuals. The conclusions drawn from published data on either the quantification or sequencing aspects of the HIV-1 proviral DNA are conflicting. It is important to remind that data from animal experiments show that only approximately 2% of total lymphocytes circulate in the blood for a short time, before migrating to organs [59,60].

At present, except for the genotypic assays to predict CXCR4 co-receptor usage that can be done on HIV RNA, as well as on HIV DNA [194,195], HIV-1 drug resistance testing and viraemia on cellular proviral DNA are not recommended by guidelines. Concerning the feasibility of detection of HIV-1 drug resistance mutations at proviral level, we observed in our study that the prevalence of mutations was higher in provirus compared to plasma virus [196]. Our published data were based on the Stanford mutation list that excludes polymorphisms and drug-selected mutations with no known significance. These data were in accordance with previous observations demonstrating that key mutations conferring resistance in RT and PI genes were more often present in cell associated HIV DNA than in the plasma free virus [169-172] and indicated the practical feasibility of the assay. Among the 69 patients included in the study at therapy naïve stage, four key mutations (M184M/V, M184M/I, K103K/N and M46M/I) out of the seven detected in the RT and PR genes in different patients (10% of all included patients) were only found in the provirus. At baseline 8% and 23% of the included patients had respectively at least one RT or PR mutation in the plasma, while 15%

and 25% had at least one RT or PR resistance in the provirus, respectively. In contrast to data published by Wang et al. in 2007 [172] and Ghosn et al. in 2006 [173] showing a tight concordance of resistance profiles in paired HIV RNA and PBMC HIV DNA, we observed that 55% of PR mutations and 56% of RT mutations were simultaneously present in CD4 cells and plasma at baseline. Another aspect of resistance testing on provirus is the persistence of detected mutations at baseline and the evolution of mutation patterns during follow-up. Usuku et al. in 2006 [174] also noticed the persistence of discrepant mutations between plasma and PBMCs for more than 3 years. We studied the evolution of the amino acid sequences by comparing the pre-treatment CD4 cell and plasma results to the post-treatment amino acid sequences. We observed a proportion of 22% of new mutations in follow-up CD4 cell samples which was statistically significant, but this was not correlated with the time elapsed between tests.

In the NNRTI group, two patients out of 10 who underwent at least 12 months of EFV-based HAART showed new key mutations (Y188Y/H and M184M/I), while in the PI group and the control group, one patient in each group showed a new key RT mutation (M184I). All new key mutations except one were only present in the provirus, confirming the preceding results. In one patient a new key mutation (M184I) was detected in the plasma but not in the provirus after 18 months of follow-up. He was under d4T, ABC and LPV/r combination therapy for 1 year before switching to 3TC, TDF and LPV/r association. In the control group of patients who remained treatment-naïve, almost no drug resistance pattern evolution was observed in plasma RNA or cell DNA, in contrast to treated patients. In our small cohort with a follow-up time of 12 to 44 months, plasma viral load was undetectable in 30 of 32 treated patients and pre-existing resistance mutations did not jeopardize the response to all types of HAART as none of them comprised the targeted drug.

To address the question of using DNA sequences in patients with undetectable viral load, we compared the prevalences of resistance mutations in pretreatment RNA and post treatment DNA. Fifty-nine percent and 78% of total mutations were respectively present in pretreatment RNA and post treatment DNA, with a statistically significant proportion of mutations (19%) detected in the DNA. This observation showed that sequencing DNA is possible and the recommended RNA sequencing might miss some mutations. In contrast Usuku et al. [174] showed that mutations detected in the plasma were infrequently present in the proviral DNA. Those discrepancies persisted for more than 3 years. Recently, in 2011, Wirden et al. [175] compared RT and PR sequences of 134 and 141 viral DNA with 443 and 462 corresponding plasma RNA, respectively. They observed a low concordance rate between

DNA and RNA genotypes about 46.7% for NRTIs, 26.3% for NNRTIs and 43.7% for PIs. They encountered difficulties to amplify the archived mutated DNA and they concluded that the previous classical RNA genotyping remains the gold standard for choosing future treatments in the context of low or undetectable viraemia. We also observed a imperfect agreement between pretreatment RNA and post-treatment DNA using kappa statistics in terms of predicting drug activity. This is due to the accumulation of new mutations in the provirus DNA. This accumulation could of course not be detected in plasma RNA, because this was too low or undetectable.

Another aspect of the HIV drug resistance that has been extensively documented is the transmission of drug-resistant strains to untreated patients. Although it is admitted that wild-type virus may outgrow any initially resistant strain in chronically infected patients in absence of treatment, we consistently detected some key mutations that were more present in CD4 cell than in the plasma and these mutations persisted for at least 1 year of follow-up with or without therapy. Wang et al. [172] reported that mutations associated with drug resistance in treatment-naïve patients remained compartmentalized in plasma and PBMCs while Ghosn et al. [173] observed an early establishment of the viral reservoir during primary HIV-1 infection (PHI). Therefore there is a possibility of archiving an acquired resistance strain. These authors reported a concordance in detected mutations between plasma RNA and cell DNA compartments.

In clinical practice, we do feel that detecting drug resistance mutations in HIV-1 proviral DNA would be most useful for decisions on the best alternative treatment for patients with undetectable plasma viral load, when changing therapy. In the context of a high risk of transmission of resistance, HIV DNA genotyping could also be an alternative to detect archived resistant strain when the genotyping was not performed at the time of diagnosis and if no plasma was stored.

The other objective of the project was to establish the clinical usefulness of the quantification of HIV-1 provirus in the context of anti-HIV treatment. As the HIV provirus persists in infected cells even after a successful therapy, we hypothesized that effective anti-HIV-1 treatment could have an impact on the DNA reservoirs allowing a better long-term effectiveness. One interesting question was “do different combined anti-HIV therapies with or without PI have a different impact on detectable provirus?”. Viral production resumes from the viral reservoir when the treatment is stopped but at a different pace in different patients. As no standardisation of the tests exists and as provirus is present in integrated and unintegrated forms, comparing results between articles is difficult. Some assays are conducted

on PBMCs while others use purified CD4⁺ cells. In term of analytical performance, there might be important differences in sensitivity and accuracy between assays particularly in case of low-copy numbers. As the HIV-1 proviral load is reported as a relative quantification against a reference gene, the qPCR amplifying the reference gene might impact the final result unless its PCR efficiency is similar to that of the target gene. Kostrikis and al. [176] reported that the quantitation of HIV-1 DNA forms in peripheral blood cells may predict the progression of the disease as he observed that the median baseline levels of HIV-1 DNA were significantly higher in patients who progressed to AIDS during the 16 years of follow-up. Our work does not show that HIV-1 proviral load is a better parameter than plasma viral load for the assessment of the impact of anti-HIV regimens including RTIs with or without PIs on HIV-1 reservoirs [197-198]. The variation in proviral load either expressed as logarithmic copy number per 10⁶ CD4⁺ cells or per mL of whole blood was not statistically different between the three patient groups: naive, NNRTI and PI. Treatment showed an effect on the rise of CD4⁺ cell counts. .

To eradicate HIV infection, replication of the virus in the whole body has to be inhibited by the treatment. The ability of the HIV to infect latent memory T cells is the most significant barrier to cure HIV infection. Measuring proviral DNA load as a reflection of reservoirs is therefore gaining new interest to evaluate the impact of new treatment strategies aiming at eradicating all HIV infected cells. The choice of antiretrovirals or treatment intensification by additional antiretrovirals have failed to demonstrate a significant impact on latent reservoirs. The memory CD4⁺ cells considered to be an important part of the reservoir can reactivate and produce infectious virions in the presence of specific antigen or other stimulation. Drugs that potentially might revert the latency of memory T cells to activated T cells such as histone deacetylase inhibitors, methylation inhibitors, cytokines such as IL-7 or activators of nuclear factor kappa B (NF- κ B) such as prostratin [199,200] are being explored to achieve HIV eradication. In their article of 2011, Lewin SR and Rouzioux C. [199] review the advantages and disadvantages of methods currently being used to quantify persistent virus.

In conclusion, the quantification of global proviral DNA be it in isolated CD4 or in PBMC as a whole doesn't help in patient management or in the choice of treatment targeting viral RT and PR. HIV proviral load may be of interest to evaluate the impact on latent reservoirs of a number of new approaches that aim to eradicate HIV reservoirs. Some of these are entering clinical trials. Sequencing the proviral DNA has however certain practical advantages, allowing to detect archived sequences and on the other hand allowing an assessment of drug sensitivity in patients in whom a switch of medication is necessary for toxicity reasons or other.

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