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Impact of Integrase Polymorphisms and Minor Quasispecies in HIV-1 Infected Individuals Naive or Treated with Strand-transfer Integrase Inhibitors: a Refined Analysis by Cloning and 454-Pyrosequencing Techniques.

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Abstract

Background: Raltegravir (RAL) is a very potent and effective strand-transfer integrase-inhibitor (InSti), recently FDA-approved for use also in first-line highly active antiretroviral therapy (HAART) regimen. Nowadays, by the available literature, the knowledge about the role either of integrase (IN) polymorphisms or HIV-1 minor quasispecies on virologic response and development of resistance to raltegravir is still poor. Therefore, the aim of this study is to explore the presence of InSti resistance mutations in HIV-1 quasispecies present in InSti-naïve patients and to evaluate their impact on *in vitro* phenotypic susceptibility to InSTIs, on replication capacities and on virologic response to raltegravir, by 3 different approaches: Cloning, population sequencing and ultra-deep 454 pyrosequencing (UDPS).

Methods: For the clonal approach, the RT-RNase H-IN region was PCR amplified from plasma viral RNA obtained from 49 HIV-1 subtype B-infected patients (21 drug naïve and 28 failing HAART not containing InSTIs) and recombined with an HXB2-based backbone with RT and IN deleted. Recombinant viruses were tested against raltegravir and elvitegravir and for replication capacity. Three-hundred forty-four recombinant viruses from 49 patients were successfully analyzed both phenotypically and genotypically.

For the population sequencing approach, we analyzed 206 multi-experienced patients that received raltegravir plus optimized background therapy (OBT) from eight clinical centres within Italy and France. HIV-1 RNA and IN genotypes were assessed at baseline and at failure. For 177 patients, viremia values at 24-week were available. The prevalence of baseline integrase mutations was calculated in the overall population, and in the responding and non-responding patients at 24 weeks. For polymorphisms with prevalence >5%, the codon usage of mutated amino acids were also considered. Logistic regression analyses (uni- and multivariate) were performed to investigate if baseline integrase polymorphisms and other variables (such as: baseline HIV-1 RNA, drugs in co-usage and/or subtype) were independent predictors of virologic response.

For the UDPS approach a sub-group of 27 patients treated with raltegravir were genotyped by UDPS at baseline and at failure. IN phenotyping was also performed at baseline and during treatment for failing patients.

In all three approaches, all IN mutations, with particular attention to known InSti resistance associated mutations, have been analyzed. The cut-off limit of reliable detection for UDPS was considered >0.1% (≥50 reads).

Results: Regarding clonal analysis, the majority of clones were not phenotypically resistant to InSTIs: 0/344 clones showed raltegravir resistance, and only 3 (0.87%) showed low-level elvitegravir resistance. No primary resistance mutations for raltegravir and elvitegravir were found as major or minor species. Secondary mutations, such as T97A and G140S, found rarely and only as minority quasispecies, were present in the elvitegravir-resistant clones. A novel mutation, E92G, although rarely found in minority quasispecies, showed elvitegravir resistance.

Regarding the analyses of baseline IN mutations, among the 206 patients genotyped by population sequencing, 186 (90.3%) patients were infected by HIV-1 subtype B versus 20 (9.7%) infected by non-B viruses (4A, 1C, 2D, 5F, 2G, 5CRF_02AG, 1CRF_12BF2). At week 24, 70% of patients achieved virologic response (71.3% [114/160] and 58.8% [10/17] infected by B and non-B viruses, respectively, p=NS). At baseline, all major raltegravir resistance mutations were completely absent, and secondary mutations (L74M, T97A, G140A, V151I, N155S, G163R) were present at very low frequency (≤1%). The presence at baseline of these secondary resistance mutations, as well as all other polymorphisms (with the exception of T125A, specific codon GCA, "see below") did not statistically influence the virologic response among patients starting raltegravir (Fisher test, Benjamini-Hockberg correction). By multivariate logistic regression, the independent predictors of worse virologic response were: baseline viremia (OR=0.42 [CI:0.3-0.7], p=0.0003), AZT or D4T co-usage (OR=0.31 [CI:0.1-0.9], p=0.04) and baseline presence of polymorphism T125A (specific GCA codon, that is consensus sequence for subtypes A, C, D, G and for CRF02_AG) (OR=0.30 [CI: 0.1-0.7], p=0.006). Such prevalence of T125A (specific GCA codon) was higher in patients infected with non-B subtype (13/20 [65%]) vs B subtype (35/186 [19%]) (OR=0.12 [CI:0.05-0.33], P=0.00003), with a greater consistence among failing patients (6/7 [86%] non-B subtype vs 14/46 [30%] B subtype, OR=0.07 [CI:0.01-0.52],p=0.009).

Regarding UDPS analyses, among >200,000 IN sequences analyzed, no minor variants of primary raltegravir mutations with a prevalence of >0.1% were found at baseline. The secondary mutations such as T97A, F121Y and V151I secondary mutations, were rarely found at baseline, in both failing- and success-group of patients, with a frequency ranging from 0.3 to 99% of viral species. Independently of the sequencing method, the presence of secondary-resistant species at baseline was not associated, at failure, with evolution at the same amino acid position or to specific primary raltegravir resistance mutations. Raltegravir phenotypic resistance has never been observed at baseline. At failure, all patients carrying primary mutations N155H, Q148H/R or Y143R, in presence of secondary (L74M, T97A, E92Q, G140S, V151I, E157Q, G163R, S230R) and novel (E92A, T112A) mutations, showed fold changes on susceptibility to RAL >30-100. Interestingly, in 1 patient, we found the combination of two primary mutations at failure, Y143C and N155H, by both population sequencing and UDPS. These mutations appeared at failure for >80% on same haplotypes, and showed a very high phenotypic resistance, particularly for raltegravir (FC raltegravir = 1255.3; FC elvitegravir = 625.3).

Conclusion: By classic and ultra sensitive genotyping (and phenotyping) methods, pre-existing raltegravir resistance is a rare event in InSti-naïve patients, and when present, is confined to a restricted minority of secondary variants only. At baseline, only T125A mutation (specific GCA codon), higher prevalent in non-b subtype viruses, was associated with

poorer virologic response to raltegravir. This finding in non-B subtypes is intriguing and further research is warranted. The clinical implications and relevance of this polymorphism is still to be determined.
Overall, this study suggests that at this point IN genotyping in all patients before raltegravir treatment may not be cost-effective and should not be recommended until evidence of transmitted drug resistance to InSTIs or the clinical relevance of IN minor variants/polymorphisms is determined.

Riassunto

Introduzione: Raltegravir è un potente ed efficace inibitore dell' integrasi (IN) di HIV-1, recentemente approvato dall'FDA anche nei regimi HAART di prima linea. Dagli studi attualmente disponibili in letteratura, il ruolo dei polimorfismi naturali e delle quasi specie minoritarie dell'integrasi sul responso virologico agli InSti e sullo sviluppo di resistenza durante il fallimento è ancora poco chiaro. Pertanto questo lavoro mira a verificare la presenza di mutazioni di resistenza agli InSti nelle quasispecie naturali di HIV-1 in pazienti naive a tali inibitori e a valutare l'impatto sulla suscettibilità fenotipica *in vitro*, sulla capacità replicativa virale e sul responso virologico a raltegravir utilizzando tre diversi approcci: il metodo clonale, il sequenziamento di popolazione e il pirosequenziamento massivo 454 (Ultra-deep 454 Pyrosequencing [UDPS]).

Metodi: Per l'approccio clonale, le sequenze di RT-RNAse H-IN sono state amplificate tramite PCR da campioni di plasma da 49 individui infetti da HIV-1 sottotipo B (21 naive al trattamento e 28 in fallimento a regimi antiretrovirali non includenti gli InSti) e ricombinate con un vettore di espressione contenente lo stipite di HIV-1 HXB2D deleto della regione RT-IN. I virus ricombinanti ottenuti sono stati testati per la suscettibilità a raltegravir ed elvitegravir e per la capacità replicativa. Da 49 pazienti sono stati ottenuti 344 cloni ricombinanti testati genotipicamente e fenotipicamente *in vitro*.

Per l'analisi con il sequenziamento di popolazione, sono stati analizzati 206 pazienti multi-trattati, provenienti da 8 diversi centri clinici italiani e francesi, che iniziavano il trattamento con un regime contenente raltegravir. Il genotipo dell'IN e la viremia sono stati effettuati prima e durate l'inizio della terapia. Alla 24esima settimana di trattamento erano disponibili valori di viremia per 177 pazienti. La prevalenza delle mutazioni è stata calcolata nella popolazione totale, nei pazienti che hanno raggiunto il successo virologico e nei i pazienti che hanno fallito alla 24esima settimana di trattamento. Per i polimorfismi con una prevalenza maggiore del 5% è stato anche considerato l'uso specifico dei codoni codificanti le mutazioni. Per valutare se i polimorfismi ed altre variabili (la viremia, i farmaci co-somministrati e il sottotipo) fossero predittori indipendenti di successo virologico, è stata effettuata un'analisi di regressione logistica (uni e multivariata).

Per l'analisi con l'UDPS un sottogruppo di 27 pazienti trattati con raltegravir è stato genotipizzato al baseline e al fallimento. Inoltre è stato effettuato il test fenotipico delle popolazioni virali al fallimento.

Per tutti e tre gli approcci sono state analizzate tutte le mutazioni dell' integrasi con particore attenzione alle mutazioni di resistanza note. In particolare per l'UDPS, il rilevamento delle mutazioni è stato considerato attendibile osservando una prevalenza >0.1%(>50 varianti).

Risultati: Dall'approcio clonale, la maggior parte dei cloni testati non ha mostrato resistenza fenotipica agli InSti: 0/344 cloni hanno mostrato resistenza per raltegravir e solo 3 cloni (0.87%) hanno mostrato bassi livelli di resistenza per elvitegravir. Non è stata osservata alcuna mutazione di resistenza primaria per raltegravir e/o elvitegravir. Nei cloni resistenti a elvitegravir sono state trovate alcune mutazioni secondarie, come la T97A e la G140S. Inoltre è stata osservata una nuova mutazione, E92G, anch'essa in quasispecie minoritaria, associata a resistenza fenotipica a elvitegravir.

Tra i 206 pazienti analizzati con l'approccio di sequenziamento di popolazione, 186 (90.3%) sono risultati infetti da sottotipo B mentre 20 (9.8%) sono risultati infetti da sottotipi non B (4A, 1C, 2D, 5F, 2G, 5CRF_02AG, 1CRF_12BF2). Alla 24esima settimana di trattamento con raltegravir il 70% dei pazienti ha raggiunto il successo virologico (il 71.3% [114/160] e il 58.8% [10/17] infetti da virus di sottotipo B e non-B rispettivamente, p=NS). Al basale non è stata trovata alcuna mutazione di resistenza primaria mentre le secondarie (L74M, T97A, G140A, V151I, N155S, G163R) hanno mostrato una bassa frequenza (≤1%). La presenza al basale di tali mutazioni secondarie, come di altri polimorfismi (con l'eccezione della T125A, codone GCA, "vedi sotto") non hanno influenzato statisticamente il responso virologico dei pazienti che hanno iniziato raltegravir (Fisher test, correzione di Benjamini-Hockberg). Dall'analisi di regressione logistica multivariata, i predittori indipendenti di negativo responso virologico erano: la viremia al basale (OR=0.42 [CI:0.3-0.7], p=0.0003), la co-somministrazione di AZT or D4T (OR=0.31 [CI:0.1-0.9], p=0.04) e la presenza al basale del polimorfismo T125A (specifico codone GCA, che è sequenza di riferimento per i sottotipi A, C, D, G and for CRF02_AG) (OR=0.30 [CI: 0.1-0.7], p=0.006). La prevalenza di questa mutazione, T125A(specifico codone GCA) risulta più alta nei pazienti infetti da sottotipi non-B (13/20 [65%]) vs B subtype (35/186 [19%]) (OR=0.12 [CI:0.05-0.33], P=0.00003) con una maggiore discrepanza tra i pazienti in fallimento (6/7 [86%] non-B subtype vs 14/46 [30%] B subtype, OR=0.07 [CI:0.01-0.52],p=0.009).

Dall'approccio UDPS, al baseline, tra più di 200000 sequenze dell'IN analizzate, non è stata trovata alcuna variante minoritaria con resistenza primaria a raltegravir con una frequenza > 0.1%. Le mutazioni secondarie T97A, F121Y e V151I sono state trovate raramente e indifferentemente in pazienti in successo e/o in fallimento, con una frequenza compresa tra lo 0.3-99% delle specie virali. Indipendentemente dal metodo di sequenziamento utilizzato, la presenza di varianti resistenti secondarie al basale non correlava al fallimento, né con l'evoluzione alla stessa posizione amminoacidica, né con lo sviluppo di mutazioni primarie. Al basale non è stata osservata resistenza fenotipica a raltegravir, tuttavia al fallimento, i pazienti che hanno sviluppato le mutazioni primarie N155H, Q148H/R o Y143R, associate ad altre mutazioni secondarie (L74M, T97A, E92Q, G140S, V151I, E157Q, G163R, S230R) o non note (E92A, T112A) hanno mostrato un ridotta suscettibilita a raltegravir (Fold change >30-100). Di particolare interesse, in 1 paziente in fallimento, è stata trovata la combinazione delle mutazioni primarie N155H e Y143C, sia utilizzando il sequenziamento di popolazione sia l'UDPS. Queste mutazioni, apparse al fallimento nell'80% degli aplotipi del

paziente, sono associate ad alta resistenza fenotipica particolarmente spiccata per raltegravir (FC raltegravir = 1255.3; FC elvitegravir = 625.3).

Conclusioni: Dai saggi fenotipici e di genotipizzazione classici e ultra sensibili, la resistenza pre-esistente a raltegravir nei pazienti naive agli InSti è un evento raro e quando presente risulta confinato soltanto in quasi specie minoritarie secondarie. Al basale, solo la presenza della mutazione T125A(GCA), più prevalente nei sottotipi non-B, è risultata associata a un inferiore responso virologico a raltegravir. Questa osservazione nei sottotipi non-B è intrigante e necessita di ulteriori investigazioni. L'impatto clinico e la rilevanza di questo polimorfismo devono comunque ancora essere determinati.

In conclusione, questo studio suggerisce che, allo stato attuale, effettuare il genotipo dell'integrasi in tutti i pazienti prima dell'inizio di raltegravir, potrebbe avere un rapporto costo-beneficio spostato verso il costo e non dovrebbe essere raccomandato, almeno fino a quando non si abbiano evidenze di resistenza trasmessa agli InSti o sia chiarita la rilevanza clinica dei polimorfismi e quasispecie minoritarie naturali.

1. Introduction

1.1 HIV

The central dogma of molecular biology states that in biological cells the information flow follows the scheme

(Crick, 1958; Crick, 1970). Replication (DNA to DNA), transcription (DNA to RNA), and translation (RNA to protein) occur in all living cells, while reverse transcription occurs only in cells infected with retroviruses or hepadnaviruses (Hepatitis B virus). Retroviruses carry their genome information in the form of two positive sense (5'→3' direction) RNA copies. The diploid nature of their genome is unique among viruses. Replication can be accomplished only in host cells by converting their RNA to DNA and incorporating the viral genes into the host genome.

Retroviruses were traditionally divided into three subfamilies, based mainly on pathogenicity rather than on genome relationship (oncoviruses which cause neoplastic disorders, spumaviruses which give cytopathic effect in tissue culture but apparently not associated with any known disease, and lentiviruses which induce slowly progressing inflammatory, neurological and immunological diseases). In the last decade, the international committee on the taxonomy of viruses has recognized seven distinct genera in the Retroviridae family (Table 1.1)(Fields, et al., 1996).

Table 1.1. Retroviruses genera

New name	Examples	Morphology
Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus (RSV)	C-type
Betaretrovirus	Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (MPMV) Jaagsiekte sheep retrovirus (JSRV)	B-, D-type
Gammaretrovirus	Murine leukemia viruses (MuLV) Feline leukemia virus (FeLV) Gibbon ape leukemia virus (GaLV) Reticuloendotheliosis virus (RevT)	C-type
Deltaretrovirus	Human T-lymphotropic virus (HTLV)-1, -2 Bovine leukemia virus (BLV) Simian T-lymphotropic virus (STLV)-1, -2, -3	—
Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1	—
Lentivirus	Human immunodeficiency virus type 1 (HIV-1) HIV-2 Simian immunodeficiency virus (SIV) Equine infectious anemia virus (EIAV) Feline immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus	Rod/cone core
Spumavirus	Human foamy virus (HFV)	Immature

The retrovirus family is divided in 7 genera: the Alpharetroviruses, Betaretroviruses, Gammaretroviruses, Deltaretroviruses and Epsilonviruses (all of which used to be classified as one genus, the oncoviruses), the Lentiviruses (which includes HIV) and the Spumaviruses.

The human immunodeficiency virus (HIV), identified in 1983, is a member of the Lentivirus genus which is exogenous, non-oncogenic retrovirus causing persistent infections leading to chronic diseases with long incubation periods (lenti for slow). Like the human T-cell leukemia virus (HTLV) family of primate onco-retroviruses, lentiviruses are complex retroviruses(Cullen, 1991). The significant characteristic of the complex retroviruses is the ability to regulate their own expression via virally encoded protein factors not found in other retroviruses. This property has been proposed to be essential for the long-term association of the complex retroviruses with the host and the generation of chronic active infections. The lentiviral complexity is reflected in their replication cycle, which reveals intricate regulatory pathways, unique mechanisms for viral persistence (Tang, et al., 1999) and the ability to infect non-dividing cells.

1.1.1 Morphology

The HIV virion is a spherical virus particle of about 100 nm in diameter (Fig. 1.1). The viral envelope consists of a lipid bilayer derived from the host cell membrane during release of the newly produced particles from an infected cell. Embedded in the viral envelope are proteins from the host cell as well as viral protein complexes composed of the transmenbrane glycoprotein gp41 (TM) and the surface glycoprotein gp120 (SU). These trimeric TM-SU complexes constitute the characteristics spike of the virion that are involved in cell recognition and entry.

A matrix shell compromising ca. 2000 copies of the matrix p17 (MA) lines the inner surface of the viral membrane. In the center of a mature HIV particle resides the cone-shaped capsid (or cone). The capsid is made of ca. 2000 copies of the viral capsid protein p24 (CA). It encloses two single strands of the HIV RNA genome stabilized as a ribonucleoprotein complex with ca. 2000 copies of the nucleocapsid protein p7 (NC). Additionally, the capsid contains the three virally encoded enzymes, reverse transcriptase, protease, and integrase as well as accessory proteins such as nef, vif, vpr. There are three additional accessory proteins rev, tat, vpu, that are not packaged into the virion. High resolution three-dimensional information is available for all HIV proteins (Frankel, et al., 1998; Turner, et al., 1999)

1.1.2 Genome

The genome of HIV has a length of approximately 9.2 kbp. Like all retroviruses it contains the characteristics:

5’- gag – pol – env - 3’

motif consisting of the three structural genes *gag*, *pol*, and *env* (Fig. 1.2). The *Gag* (group antigen) gene encodes the large precursor polyprotein p55 that is cleaved in four proteins: the matrix p17, the "core" capsid p24, the nucleocapsid p7 and the p6(Freed, 1998). The *pol* (polymerase)gene encodes the synthesis of three viral enzymes: protease p10, reverse transcriptase/ribonuclease H

complex p51 and p66, integrase p32. The *env* (envelope) gene directs the production of an envelope precursor protein gp160, which undergoes cellular proteolytic cleavage into the outer envelope glycoprotein gp120 and the transmembrane glycoprotein gp41.

The RNA genome is flanked by two short redundant (R) sequences at both termini with adjacent unique sequences, U5 and U3, found at the 5' and 3' ends, respectively. In addition, HIV has at least six more genes encoding viral proteins with regulatory functions (*tat* and *rev*) or accessory functions (*nef*, *vif*, *vpr* and *vpu*) (for reviews see(Cullen, 1991; Emerman, et al., 1998; Kleim, et al., 1996; Piguet, et al., 1999; Pollard, et al., 1998; Trono, 1995).

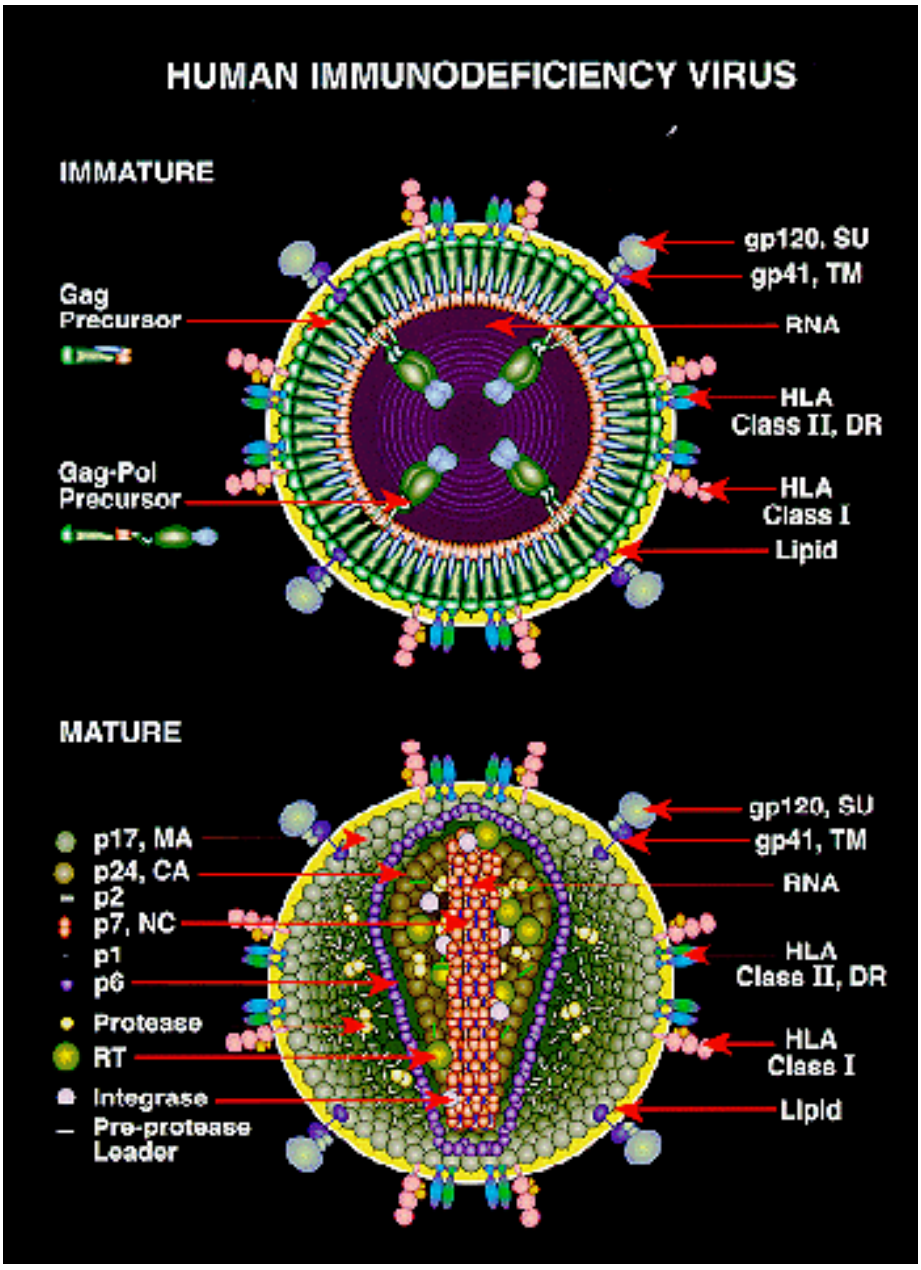


Fig.1.1. The immature and mature forms of HIV-1. Typical lentivirus particles are spherical, about 80-110 nm in diameter, and consist of a lipid bilayer membrane surrounding a conical core. The two identical single-stranded RNA (ssRNA) molecules, of about 9.2kB each, are associated with the nucleocapsid proteins p7gag (NC). They are packed into the core along with virally encoded enzymes: reverse transcriptase (RT), integrase, and protease. P24gag comprises the inner part of the core, the capsid (CA). The p17gag protein constitutes the matrix (MA) which is located between the nucleocapsid and the virion envelope. The viral envelope is produced by the cellular plasma membrane and contains the protruding viral Env glycoproteins: gp120 surface glycoprotein (SU) and gp41 transmembrane protein (TM). (from: http://tolomeo.files.wordpress.com/2008/11/hiv.gif)

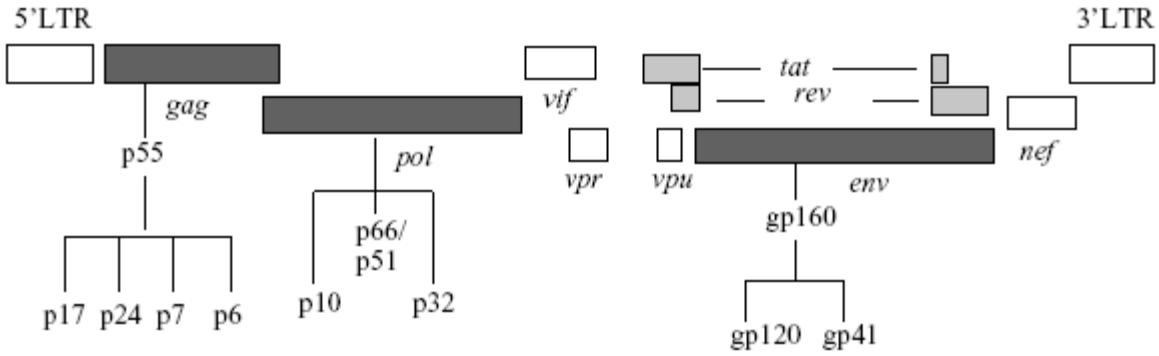


Fig. 1.2. HIV genomic organization. Like all other retroviruses, HIV has three structural genes *gag*, *pol* and *env* (heavily shaded), which are flanked by the long terminal repeats (LTR's). In addition it has six more genes, including two regulatory genes *tat* and *rev* (stippled) and four accessory genes *nef, vif, vpr* and *vpu* (white).

1.1.3 Replication

The HIV replication cycle begins with the recognition of the target cell by the mature virion. The major targets for HIV infection are cells bearing the HLA class II receptor, CD4, on their cell surfaces. These include T-helper lymphocytes and cells of the monocyte/macrophage lineage including microglia cells in the brain. The virus-CD4 binding occurs via specific interactions between the viral outer envelope glycoprotein gp120 and the amino-terminal immunoglobulin like domain of CD4(Dalgleish, et al., 1984; Klatzmann, et al., 1984). These interactions are sufficient for binding but not for infection. Subsequently the virus glycoprotein gp120 interacts with additional cell-surface proteins to promote fusion of the viral and cellular membranes. These coreceptors have recently been identified to be members of the chemokine receptor family and include CXCR4 and CCR5(Alkhatib, et al., 1996; Deng, et al., 1996; Moore, 1997). The initial binding of HIV to the CD4 receptor is mediated by conformational changes in the gp120 subunit, followed by a conformational change in the gp41 subunit, induced by the chemokine receptors, that allows fusion and subsequent entry of HIV. Various strains of HIV differ in their use of chemokines coreceptors. There are strains of HIV known as T-tropic strains, which selectively interact with the CXCR4 chemokine coreceptor of lymphocytes, while M-tropic strains of HIV interact with the CCR5 chemokine coreceptor of macrophages and dual tropic HIV strains that infect both cell types

(Littman, 1998; Moore, 1997). HIV-1 infection of CD4 negative cells, such as neural cells, has also been reported (Clapham et al., 1989; Harouse et al., 1989; Kozlowski et al., 1991; Kunsch et al., 1989) but the mechanisms of HIV entry are still unclear. Membrane fusion is followed by an uncoating event that allows the intracellular reverse transcription. The viral RNA is transcribed in the cytosol into double stranded DNA by the reverse transcriptase (Hansen, et al., 1987; Muesing, et al., 1985)This enzyme have three enzymatic activities: RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and ribonuclease H (RNase H). The reverse transcription process takes place within a large nucleic acid-protein complex known as the preintegration complex (PIC) by the assistance of the accessory protein Vif (Schwedler, et al., 1993)and the nucleocapsid protein NC(Allain, et al., 1994). Once synthesized, the viral DNA is transported to the nucleus of the infected cell as part of the PIC that appears to include tightly condensed viral nucleic acids and the integrase, p17, reverse transcriptase, and Vpr proteins. In contrast to other retroviruses, that require cell division and concomitant breakdown of the nuclear envelope to gain access to the nuclear compartment, the lentiviral PIC is actively imported into the nucleus during the interphase (Bukrinsky, et al., 1992; Lewis, et al., 1994). Nuclear import of the PIC seems to be directed by the accessory protein Vpr (Fouchier, et al., 1997; Heinzinger, et al., 1994), the Gag matrix protein p17 (Bukrinsky, et al., 1993; Schwedler, et al., 1994) and the integrase(Gallay, et al., 1997). Vpr does not contain a conventional nuclear localization signal but appears to function by connecting the PIC to the cellular nuclear import machinery (Fouchier, et al., 1998; Popov, et al., 1998). The ability of lentiviruses such as HIV-1 to utilize active transport mechanisms for translocation of the PIC into the nucleus, allows these viruses to infect non-dividing cells such as differentiated macrophages, quiescent T lymphocytes and possibly neurons. In the nucleus, integrase catalyzes covalent integration of the viral DNA into the host genome, where it resides permanently as a provirus. An important modification as a result of reverse transcription and integration is the duplication of the U5 and U3 sequences in the LTR, such that the provirus now is flanked by tandemly repeated sequences U3-R-U5 with important regulatory functions. The regulation of the HIV transcription

involves a complex interplay between cis-acting DNA and RNA elements present within the chromatin-associated proviral LTRs, cellular transcription factors and the viral regulatory protein Tat (transcriptional transactivator).

The regulation of the HIV transcription involves a complex interplay between cis-acting DNA and RNA elements present within the chromatin-associated proviral LTRs, cellular transcription factors and the viral regulatory protein Tat (transcriptional transactivator). In an arrangement similar to that of several inducible cellular promoters, the HIV-1 promoter, which is located in the U3 region of the 5'LTR, contains a TATA box and binding sites for several cellular DNA-binding transcription factors, such as NF-kB, Sp1 and TBP(Jones, et al., 1994). It is highly inducible and responds to the activation status of the infected cell. NF-kB is the major inducible cellular activator. It is well established that many cells in the lymphoid tissue of infected individuals are latently infected (Pantaleo, et al., 1993), even though the viral replication in the body is always active. In resting T-cells, the activity of the HIV promoter is minimal, leading to viral quiescence in infected primary cells. Therefore, viral activation is associated with cell activation. The transcription of the provirus by the cellular RNA polymerase II results in a primary transcript that may serve three distinct functions: 1) it constitutes genomic RNA that is incorporated into the virion; 2) it serves as template for translation (Gag and Gag-Pol); 3) it functions as the precursor RNA for the production of diverse subgenomic mRNAs (Fig 1.3).

As mentioned before, HIV encodes two essential regulatory proteins Tat and Rev, which increase viral gene expression at the transcriptional and post-transcriptional levels, respectively. HIV mRNA expression is biphasic and can be divided into early (Rev-independent) and late (Rev-dependent) stages (Kim, et al., 1989). First, shortly after the infection of cells, multiply spliced (~ 2kb) RNA species are formed from the primary transcript and three proteins are produced: Tat, Rev and Nef, therefore referred as early gene products(Schwartz, et al., 1990). Tat [for reviews see (Cullen, 1998; Emerman, et al., 1998; Rubartelli, et al., 1998), greatly increases transcription from the HIV promoter, by binding to a cis-acting target sequence, the trans-activator response element (TAR),

which is located at the 5' end of the nascent viral RNA transcript (Berkhout et al., 1989; Dingwall et al., 1989). Tat recruits two cellular factors to this complex: cyclin T and cyclin-dependent protein kinase-9 (Cdk9). Cyclin T is proposed to bind directly Tat and to increase its affinity for the TAR RNA(Wei, et al., 1998). Cdk9 phosphorylates the RNA polymerase II transcription complex and thus stimulates transcriptional elongation (Wei, et al., 1998). Rev (regulator of expression of the virion), which accumulates during the early phase of expression, initiates late gene expression by binding a unique RNA element located in the *env* coding region of HIV-1, the so called Rev-responsive element (RRE). This interaction promotes the stability and transport of unspliced (~ 9 kb) and partially spliced (~ 4 kb) HIV-1 mRNAs out of the nucleus. These mRNAs are responsible for the production of the viral enzymes and structural proteins (Daly, et al., 1989; Felber, et al., 1989; Hadzopoulou-Cladaras, et al., 1989; Hammarskjold, et al., 1989; Malim, et al., 1989). Therefore Gag, Pol, Env, Vif, Vpr, and Vpu proteins are referred to as late HIV-1 proteins.

The Nef (negative factor) protein play various functions. In particular, it enhances viral expression in quiescent cells and mediates lymphocyte chemotaxis and activation at sites of virus replication (Kestler, et al., 1991; Koedel, et al., 1999; Miller, et al., 1994; Swingler, et al., 1999). The Env precursor polyprotein (gp160) is synthesized in the endoplasmatic reticulum (ER) where it is glycosylated and appears to oligomerize to a trimeric structure posttranslationally (Wyatt, et al., 1998; Wyatt, et al., 1998). Thereafter, it is cleaved to produce the non-covalently associated (gp41 TM - gp120 SU)3 trimeric glycoprotein complex, which is transported to the cell membrane for virus assembly. Vpu is thought to enhance this process and inhibit a premature trapping of CD4 to Env in the ER by binding CD4 molecules, which are also synthesized in the ER, and directing them to the ubiquitin-proteasome degradation pathway (Margottin, et al., 1998; Schubert, et al., 1998; Strebel, et al., 1988; Willey, et al., 1992; Willey, et al., 1992). Similarly, the accessory protein Nef facilitates the routing of CD4 from cellsurface and Golgi apparatus to lysosomes, resulting in endosomal degradation and preventing inappropriate interaction with Env (Aiken, et al., 1994). In addition, both Vpu and Nef can down-regulate expression of MHC class I molecules. The

downregulation of CD4 and MHC class I molecules on the surface of infected cells also helps infected cells to evade immune responses of the host, such as killing by cytotoxic T lymphocytes (Collins, et al., 1998; Kerkau, et al., 1997).

During synthesis of the Gag polyprotein by ribosomes, a translational frameshift may occur, resulting in generation of smaller amount of Gag-Pol precursor polyproteins, which associate with the Gag polyprotein at the cellular membrane. The N-terminally myristoylated MA domain of the Gag/GagPol polyproteins directs insertion of the Gag precursors into the cellular membrane and interacts with the cytoplasmic tail of gp41 resulting in the anchoring of Env to the viral particle (Dorfman, et al., 1994). Approximately 1200 to 2000 copies of Gag precursor bud to form an immature particle, which encapsidates two copies of the unspliced viral RNA genome, by the ability of NC to interact with nucleic acids. Vif and Vpu proteins have been reported to play a role in packaging of the nucleoprotein core and in virion release, respectively (Höglund, et al., 1994; Lamb, et al., 1997). Concomitantly or immediately following the external budding, the cleavage of the Gag/Gag-Pol polyproteins by the virally encoded PR produces the structural proteins MA, CA, NC as well as the independent enzymes PR, RT and IN. This final step primes new virus particles for the next round of infection and is termed maturation.

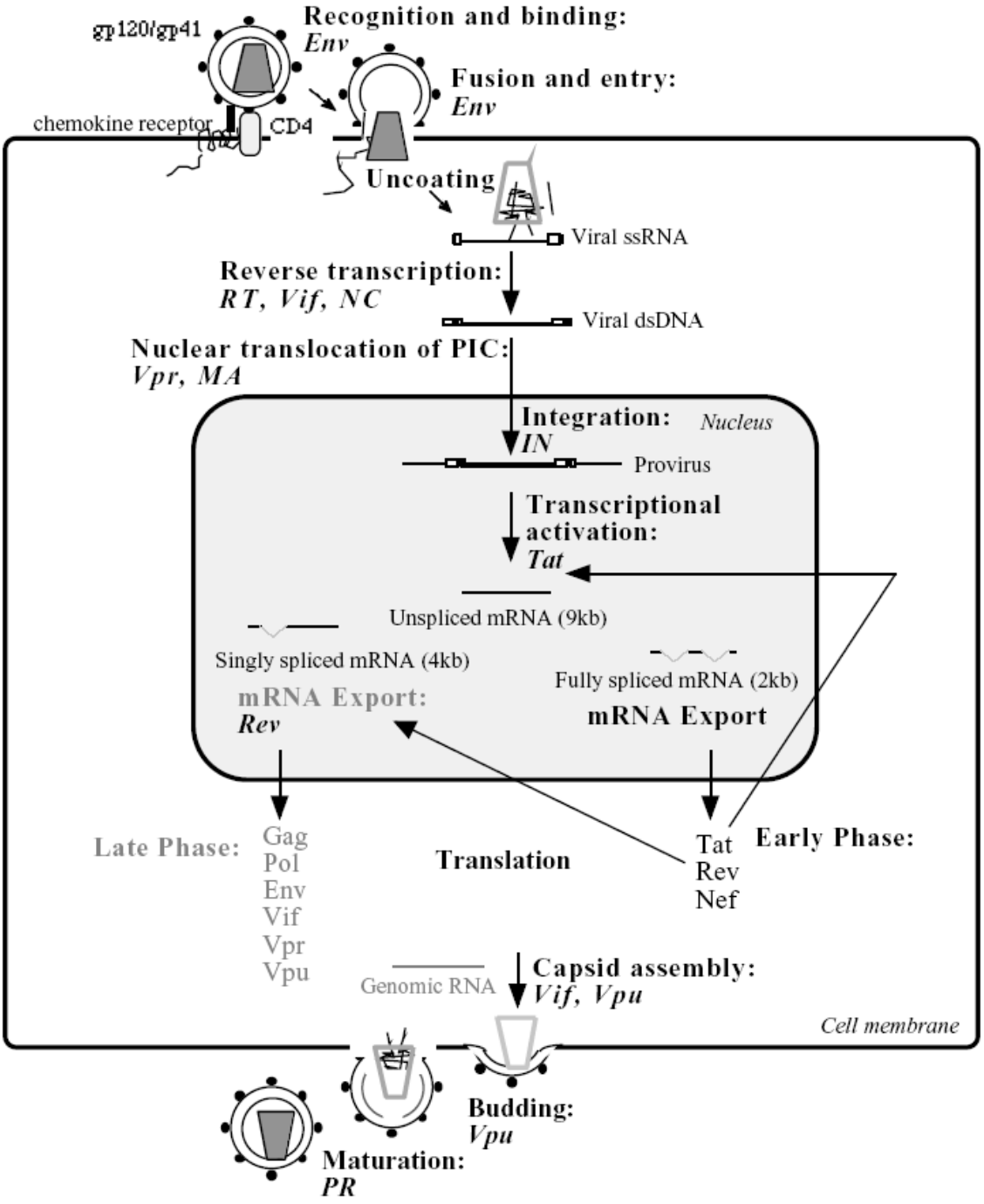


Figure1.3. Replication cycle of HIV-1. Each fundamental step is presented in bold. Names in *italic* refer to viral gene products involved in the specific steps. HIV-1 gene expression is stimulated by HIV-1 Tat and Rev, which act at transcriptional and post-transcriptional levels, respectively, and can be divided into two phases. The early phase is Rev-independent and the later phase is Rev-dependent (text in gray). Rev stabilizes and mediates export of singly spliced and unspliced RNA transcripts out of the nucleus into the cytoplasm.
Modified from Ceccherini-Silberstein, 2001 (http://edoc.ub.Muenchen.de/archive/00000533/01/Ceccherini-Silberstein_Francesca.pdf).

1.1.4 Pathogenesis

AIDS

HIV infection has been associated with the acquired immunodeficiency syndrome (AIDS). A diagnosis of AIDS is made whenever a person is HIV-positive and have:

- CD4+ T cell count below 200 cells/mms
- CD4+ T cells account for fewer than 14% of all lymphocytes
- Diagnosis with one or more of the 25 AIDS defining illness, including various opportunistic infection, brain and nerve disease, certain cancers, and wasting syndrome

Approximately 10% of HIV-infected patients progress to AIDS within the first 2 to 3 years of infection, while for approximately 40% this progression is observed over a period of 10 years. 10% to 17% of HIV-infected patients may be AIDS free, some with no evidences of disease progression. These variations in responses may be due to differences in the degree of stimulation of the immune system by infection with the other pathogens as well as to viral factor, such as deletions in the nef gene or altered cell tropism (Kupfer, et al., 1998).

Course of infection

Schematically, the course of infection can be divided into an acute, an asymptomatic, and symptomatic phase (Fig. 1.4). The acute phase accounts for the first 5-10 weeks of infection and is characterized by high virus production, and activation of lymphocytes in lymphonodes. Up to $5x10^3$ infectious particles per ml of blood plasma may be found in the first days after infection. This viremia is curtailed within a few weeks and level off at the beginning of the asymptomatic phase to the so-called virological set point, that is a predictor of disease progression. During this CD4+ cells numbers decrease at a low steady rate, while virus replication remains constant at a low rate. The duration of the asymptomatic phase may last between 2 and 20 years. The end stage of disease, when the patient develops AIDS, is characterized by CD4+ cells count below 200 copies/ml and increased quantities of the virus. The number of CD8+ cytotoxic lymphocytes also decreases and lymphoid cells and tissues are damaged.

CD4+T cell depletion

The hypothesis that CD4+ cell depletion is caused the lysis of infected cells during viral replication has been supported by the observation of an immediate and large increase of CD4+ count after the initiation of antiretroviral therapy that blocks viral replication (Ho, et al., 1995; Wei, et al., 1995). This hypothesis has not withstood more detailed analyses of T cell dynamics (Roederer, 1998). In fact, it has been turned out that in HIV-infected patients all T cell subset are progressively destroyed, irrespective of CD4+ expression, and AIDS appear to be a disease of perturbed homeostasis. Many pathogenetic mechanisms have been proposed, including viral gene products, syncitium formation, direct virus killing of cell, apoptosis, autoimmunity, cytokine and chemokines expression, superantigens, virus directed cell mediated cytolysis and disruption of lymphoid architecture.

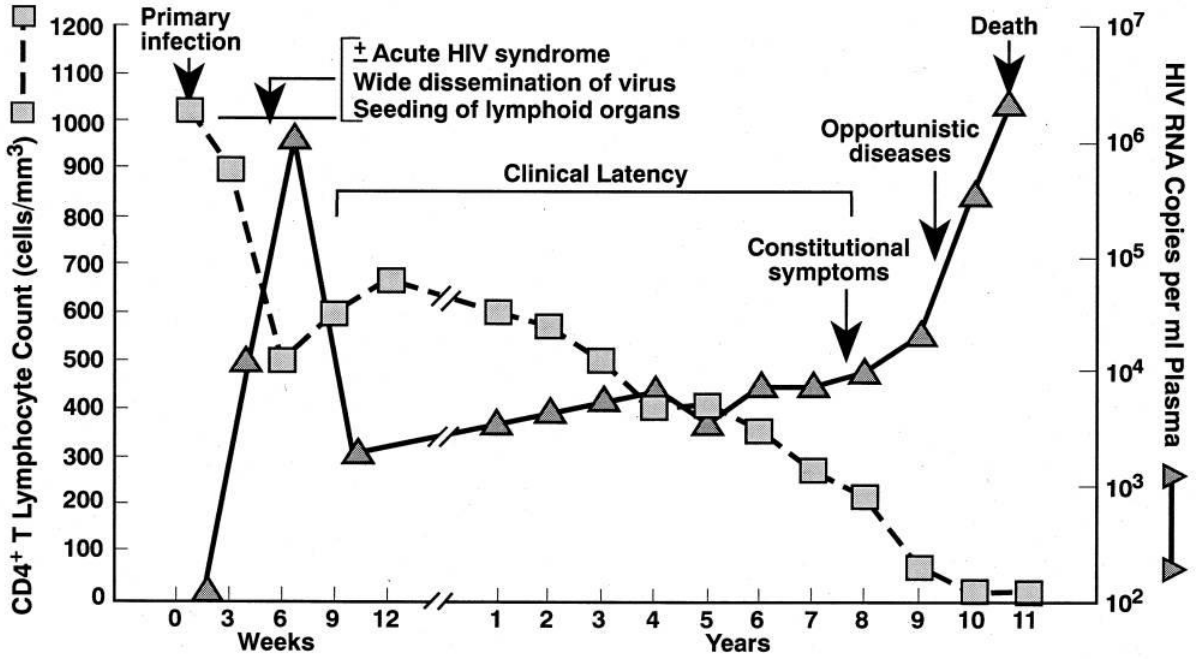


Figure 1.4. Schematic representation of the course of HIV infection

1.1.5 Epidemiology

Several African primates harbour lentiviruses and HIV is believed to be entered the human population in Africa by zoonotic transmission of SIV_{cpz} from chimpanzee population. The first cross species transfer has been estimated to have occurred between 1915 and 1941(Korber, et al., 2000). Two types of HIV are known: the most common HIV-1, which is responsible to the world-wide AIDS epidemic and the immunologically distinct HIV-2 (Clavel, et al., 1986), which is much less common and less virulent (Ariyoshi, et al., 1999; Ariyoshi, et al., 2000), but produces clinical findings similar to HIV-1 (Wilkins, et al., 1993). The HIV-1 type itself includes four groups M, N, O, P which have different geographic distributions but all produce similar clinical symptoms (Fig. 1.5). The M group is further divided into 9 pure subtypes (A, B, C, D, F, G, H, J, K), 4 sub-subtype (A1, A2, F1, F2) and 45 circulating recombinant forms on the basis of phylogenetic analysis. Almost all subtypes are present in Africa, while in Europe, North America, and Australia subtype B is more dominant, and subtype C is more common in Asia (Robertson, et al., 2000; Mc Cutchan, 2000; Plantier, et al., 2009).

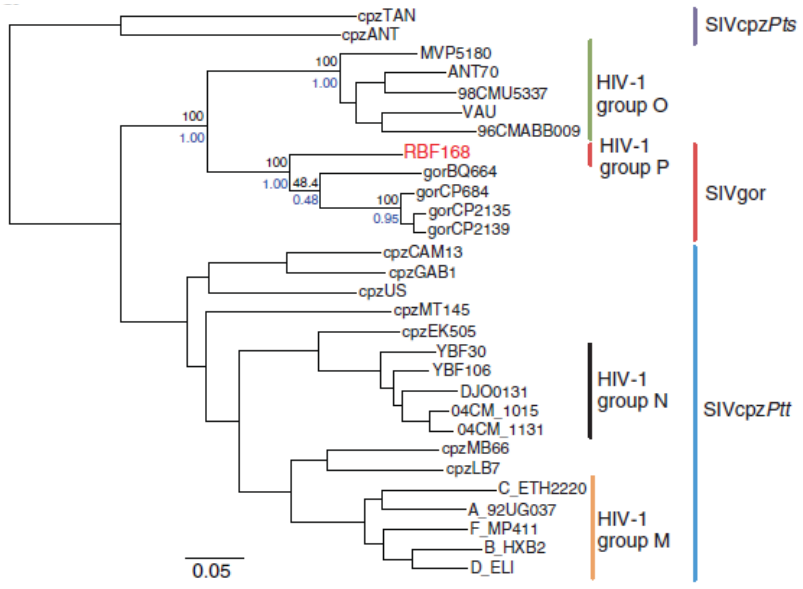


Figure 1.5. Phylogenetic relationship of primate lentiviruses. Phylogenetic tree derived from the alignment of *pol* gene sequences of HIV-1 and SIV strain (SIVcpz and SIVgor). Reproduced from *Plantier 2009*

At the end of 2009, 33.4 million adults and children have been estimated to live with HIV/AIDS, most of them in Sub-Saharan Africa and South East Asia (Fig. 1.6). Only a minority of HIV-infected individuals live in the industrialized countries and has access to the anti-HIV drugs and professional health care.

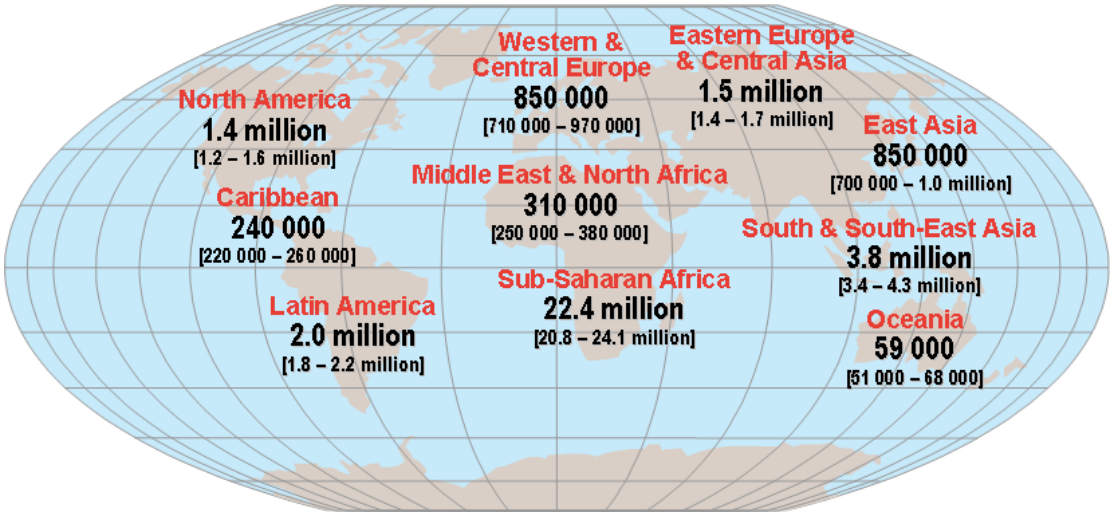


Figure 1.6. Geografical distribution of HIV/AIDS cases. From UNAIDS 2009

1.2 Antiretroviral therapy

The drugs currently used to treat HIV-1 infection are directed against the four viral enzymes, an envelope glycoprotein and a human receptor: protease (PR), reverse transcriptase (RT), the transmembrane glycoprotein gp41 and more recently, also against Integrase (IN) and human CCR5 receptors. In table 1.2 all anti-HIV compounds currently approved for clinical use by the U.S. Food and Drug Administration (FDA) [Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health] are listed. In figure 1.7 the available drugs in clinic by today, according with the viral life cycle steps impaired are shown.

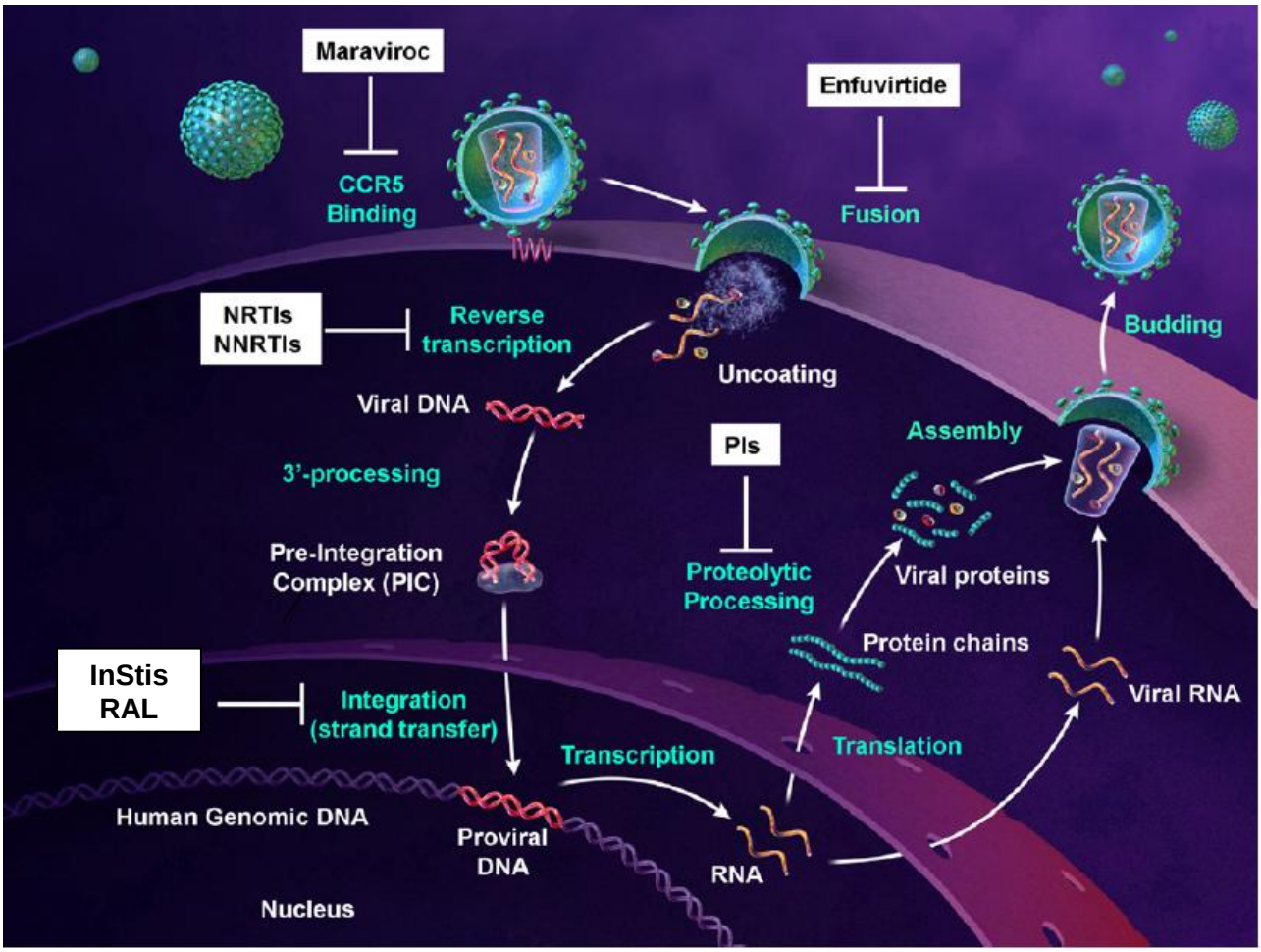


Fig. 1.7 HIV replication cycle according with ARV's available by today.

Initial entry of HIV into a target cell can be blocked by use of the entry inhibitor maraviroc, which prevents viral interaction with the CCR5 coreceptor. Fusion of the viral membrane with the target cell membrane can be blocked by the peptidic inhibitor enfuvirtide, which prevents a conformational change in the viral Env protein needed to bring the two membranes into close proximity. Reverse transcription of the viral RNA into DNA can be blocked by nucleoside/tide reverse transcriptase inhibitors (NRTIs) which are incorporated into the viral DNA and act to chain terminate DNA synthesis. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are non-competitive inhibitors of reverse transcriptase. Integrase strand transfer inhibitors (INSTIs), such as raltegravir, are active site inhibitors of the viral integrase enzyme and prevent the strand transfer reaction, the final ligation of the 3_-processed viral DNA into the host genome. Protease inhibitors (PIs) prevent the proteolytic processing of translated viral proteins by the viral protease enzyme, resulting in defective virions. Combinations of drugs from two or more of these classes when combined together form the basis of highly active antiretroviral therapy (HAART).(From:D.J. McColl, X. Chen. 2008. Antiviral Research)

Table 1.2. Antiretroviral drugs in clinical use.

Multi-class combination products				
Brand Name	**Generic Name**	**Manufacturer Name**	**Approval Date**	**Time to Approval**
Atripla	efavirenz, emtricitabine and tenofovir disoproxil fumarate	Bristol-Myers Squibb and Gilead Sciences	12-July-06	2.5 months
Nucleoside(tide) Reverse Transcriptase inhibitors (NRTIs)				
Brand Name	**Generic Name**	**Manufacturer Name**	**Approval Date**	**Time to Approval**
Combivir	lamivudine and zidovudine	GlaxoSmithKline	27-Sep-97	3.9 months
Emtriva	emtricitabine, FTC	Gilead Sciences	02-Jul-03	10 months
Epivir	lamivudine, 3TC	GlaxoSmithKline	17-nov-95	4.4 months
Epzicom	abacavir and lamivudine	GlaxoSmithKline	02-Aug-04	10 months
Hivid	zalcitabine, dideoxycytidine, ddC (no longer marketed)	Hoffmann-La Roche	19-Jun-92	7.6 months
Retrovir	zidovudine, azidothymidine, AZT, ZDV	GlaxoSmithKline	19-mar-87	3.5 months
Trizivir	abacavir, zidovudine, and lamivudine	GlaxoSmithKline	14-nov-00	10.9 months
Truvada	tenofovir disoproxil fumarate and emtricitabine	Gilead Sciences, Inc.	02-Aug-04	5 months
Videx EC	enteric coated didanosine, ddI EC	Bristol Myers-Squibb	31-Oct-00	9 months
Videx	didanosine, dideoxyinosine, ddI	Bristol Myers-Squibb	9-Oct-91	6 months
Viread	tenofovir disoproxil fumarate, TDF	Gilead	26-Oct-01	5.9 months
Zerit	stavudine, d4T	Bristol Myers-Squibb	24-Jun-94	5.9 months
Ziagen	abacavir sulfate, ABC	GlaxoSmithKline	17-Dec-98	5.8 months
Non-nucleoside Reverse Transcriptase inhibitors (NNRTIs)				
Brand Name	**Generic Name**	**Manufacturer Name**	**Approval Date**	**Time to Approval**
Intelence	etravirine	Tibotec Therapeutics	18-Jan-08	6 months
Rescriptor	delavirdine, DLV	Pfizer	04-apr-97	8.7 months
Sustiva	efavirenz, EFV	Bristol Myers-Squibb	17-Sep-98	3.2 months
Viramune	nevirapine, NVP	Boehringer Ingelheim	21-Jun-96	3.9 months

Protease inhibitors (PIs)				
Brand Name	**Generic Name**	**Manufacturer Name**	**Approval Date**	**Time to Approval**
Agenerase	amprenavir, APV	GlaxoSmithKline	15-apr-99	6 months
Aptivus	tipranavir, TPV	Boehringer Ingelheim	22-Jun-05	6 months
Crixivan	indinavir, IDV,	Merck	13-mar-96	1.4 months
Fortovase	saquinavir (no longer marketed)	Hoffmann-La Roche	07-nov-97	5.9 months
Invirase	saquinavir mesylate, SQV	Hoffmann-La Roche	6-Dec-95	3.2 months
Kaletra	lopinavir and ritonavir, LPV/RTV	Abbott Laboratories	15-Sep-00	3.5 months
Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03	10 months
Norvir	ritonavir, RTV	Abbott Laboratories	01-mar-96	2.3 months
Prezista	darunavir	Tibotec, Inc.	23-Jun-06	6 months
Reyataz	atazanavir sulfate, ATV	Bristol-Myers Squibb	20-Jun-03	6 months
Viracept	nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14-mar-97	2.6 months
Fusion inhibitors				
Brand Name	**Generic Name**	**Manufacturer Name**	**Approval Date**	**Time to Approval**
Fuzeon	enfuvirtide, T-20	Hoffmann-La Roche & Trimeris	13-mar-03	6 months
Entry inhibitors - CCR5 co-receptor antagonist				
Brand Name	**Generic Name**	**Manufacturer Name**	**Approval Date**	**Time to Approval**
Selzentry	maraviroc	Pfizer	06-August-07	8 months
HIV integrase strand transfer inhibitors				
Brand Name	**Generic Name**	**Manufacturer Name**	**Approval Date**	**Time to Approval**
Isentress	raltegravir	Merck & Co., Inc.	12--Oct-07	6 months

Approval dates are taken from the FDA web site (http://www.fda.gov/forconsumers/byaudience/forpatientadvocates/hivandaidsactivities/ucm118915.htm)

1.2.1. Protease inhibitors

Structure and function of protease

The HIV protease (PR) (HIV-1 and HIV-2) is a homodymeric aspartyl protease consisting of 99 amino acids per monomer. Three domains of the PR are frequently referred to in the scientific literature: the active site cavity, the dimerization domain, and the flaps (see Fig. 1.8). The main contribution of the HIV PR to the viral life cycle is in the maturation of the assembled viral particle. The PR recognizes a series of heptamers in the Gag (p55) and Gag-Pol (p160) polyproteins and cleaves them at 9 distinct sites releasing the constitutive components of the viral matrix (MA/p17) capsid (CA/p24) and nucleocapsid (NC/p7) as well as the functional enzymes reverse transcriptase (RT), PR and integrase (IN) (Kohl, et al., 1988; Jacks, et al., 1988) . At the core of the HIV PR, two aspartic acid residues (one in each monomer) stabilize the addition of water across the amide bond of a susceptible polypeptide to create a tetrahedral transition state intermediate. This intermediate form is then broken generating the C-terminal carboxylic acid and N-terminal amine, thereby resulting in cleavage of the substrate(Navia, et al., 1989; Wlodawer, et al., 1989).

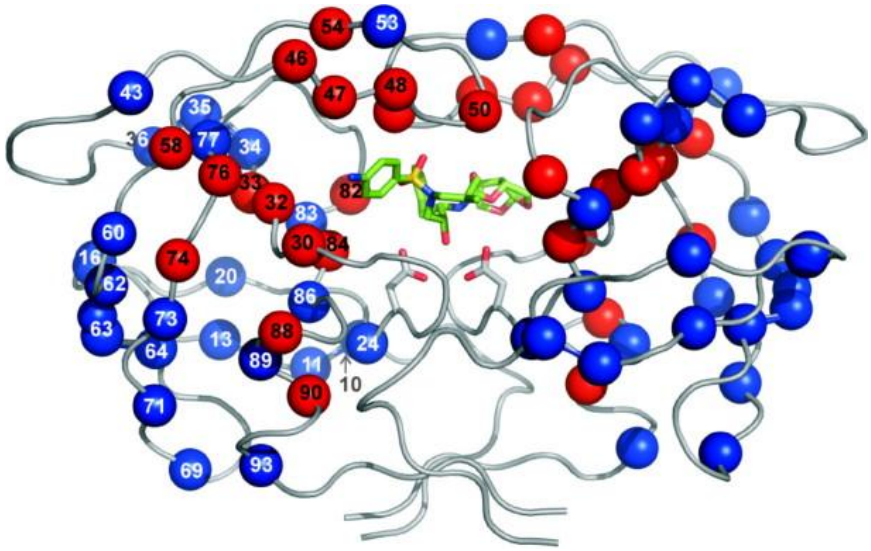


Fig. 1.8 Three-dimensional structure of HIV PR dimer depicting the primary (major) and secondary (minor) mutations associated with resistance to protease inhibitors (Johnson et al., 2009). Mutated residues are represented with their Cα atoms (spheres) and colored red and blue for major and minor mutations, respectively. Active site aspartates and darunavir bound to the active site are represented in sticks. The figure was generated using the structure of highly mutated patient derived HIV PR (Saskova et al., 2009) [PDB code 3GGU, doi:10.1128/JVI.00451-09] and program PyMol [DeLano Scientific LLC, San Carlos, CA, USA.; http://www.pymol.org].

Protease inibitors

Detailed knowledge of the structure of HIV protease and its substrate has led to the development of specific protease inhibitors (PIs). They have been designed to bind the viral protease with high affinity but tend to occupy more space than the natural substrates. Currently, there are nine PIs approved for clinical use: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, tipranavir and darunavir (Fig. 1.9, Table 1.2). Most of them are prescribed with a concomitant low dose of ritonavir as boosting agent. All of them, with the exception of tipranavir, are competitive peptidomimetic inhibitors, mimicking the natural substrate of the viral protease. The peptidomimetic inhibitors contain a hydroxyethylene core, which prohibits cleavage of the protease inhibitor by the HIV-1 protease (Craig, et al., 1991; Kempf, et al., 1995; Koh, et al., 2003; Partaledis, et al., 1995; Patick, et al., 1996; Robinson, et al., 2000; Sham, et al., 1998; Vacca, et al., 1994) (Fig.1.9). Instead of a peptidomimetic hydroxyethylene core, tipranavir contains a dihydropyrone ring as a central scaffold (Turner, et al., 1998) (Fig. 1.9).

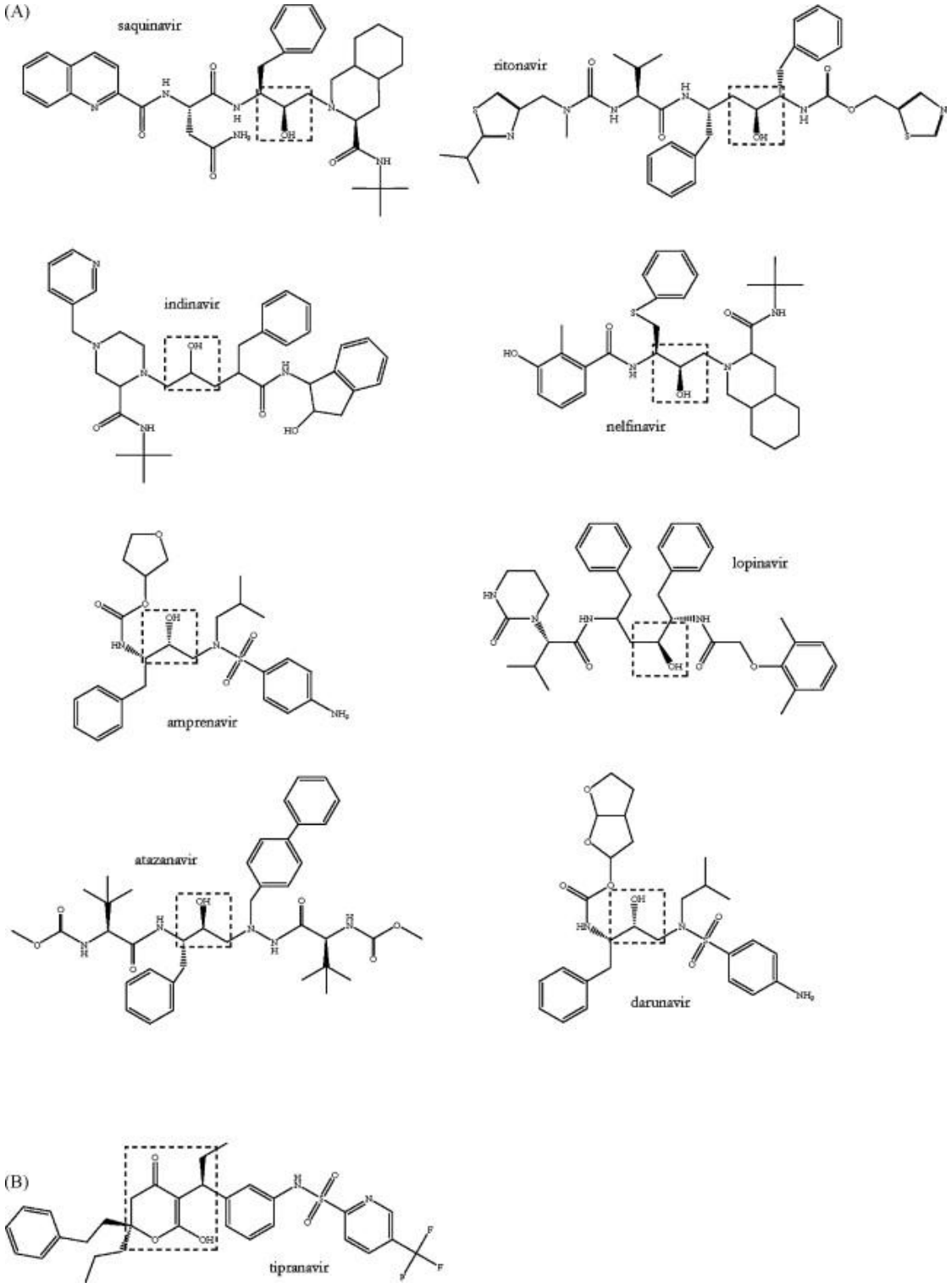


Fig. 1.9 Chemical structures of the nine HIV-1 protease inhibitors approved for clinical use. (A) Peptidomimetic protease inhibitors, characterized by a hydroxyethylene core, indicated with dashed-line boxes. (B) Non-peptidomimetic protease inhibitor characterized by a dihydropyrone ring, as indicated with a dashed-line box.

1.2.2 Reverse transcriptase inhibitors

Structure and function of reverse transcriptase

Reverse transcriptase is the replicative enzyme of HIV and other retroviruses (Fig. 1.10). Reverse transcriptase copies the single-stranded viral genomic RNA into double-stranded DNA, which is consequently integrated into host cell genome. Reverse transcriptase has two enzymatic activities: a polymerase that can copy either RNA or DNA and an RNase H that degrades the RNA strand of RNA–DNA intermediates formed during viral DNA synthesis. HIV-1 reverse transcriptase is composed of two subunits, p66 and p51; p51 and p66 have the same N terminus. p66 has 560 amino acid residues; p51 has 440 residues (Telesnitsky, et al., 1997). Crystallographic studies of HIV-1 reverse transcriptase revealed important features of the enzyme's structure and function (Kohlstaedt, et al., 1992; Jacobo-Molina, et al., 1993). p66 contains two domains: polymerase and RNase H. p51 lacks the RNase H domain. The polymerase domain of p66 and p51 contains four common subdomains, termed 'fingers', 'palm', 'thumb' and 'connection'. The folding of the individual subdomains is similar in p66 and p51, but the spatial arrangement of the subdomains differs markedly. p66 contains the active sites for both polymerase and RNase H; p51 primarily plays a structural role. Highly conserved regions in the fingers and palm subdomains of p66, together with two helices of the thumb subdomain, act as a clamp that helps position the template–primer. One of these regions (part of the palm subdomain) is the DNA 'primer grip'. The primer grip is responsible for the appropriate placement of the primer terminus at the polymerase active site and is involved in translocation of the template–primer following nucleotide incorporation (Jacobo-Molina, et al., 1993; Ding, et al., 1998; Ghosh, et al., 1996). Appropriate binding/positioning of the template–primer is also important for appropriate cleavage of the RNA–DNA substrate by the RNase H activity of reverse transcriptase (Sarafianos, et al., 2001; Julias, et al., 2002; Julias, et al., 2003). HIV-1 reverse transcriptase inhibitors currently available as anti-AIDS drugs target the polymerase activity of the enzyme (Table 1.2).

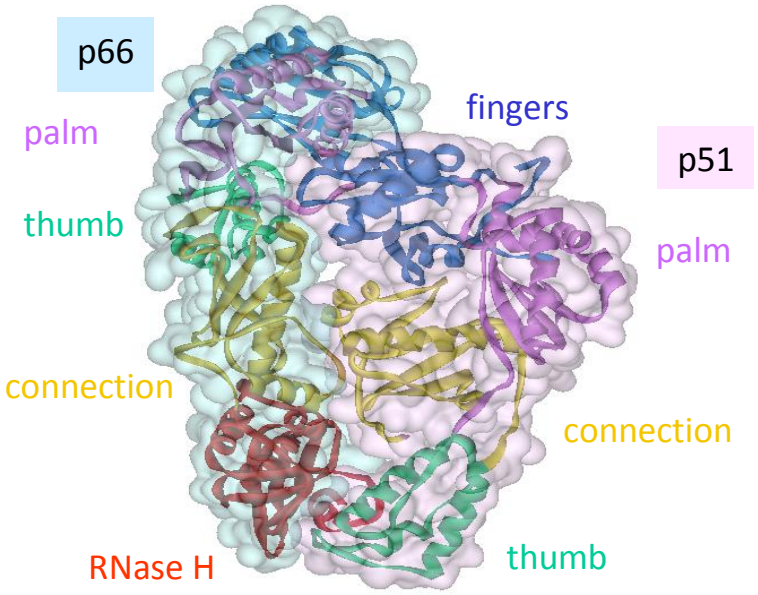
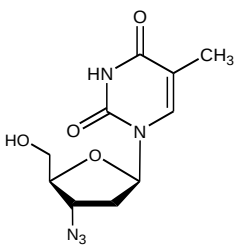


Figure 1.10. Reverse transcriptase structure. The representation is based on a crystal structure with PDB code 1rtd.

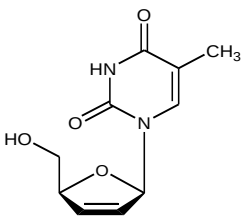
Nucleoside/tide reverse transcriptase inhibitors (NRTIs)

The mechanism of action of NRTIs is based on competitive inhibition of reverse transcription. After tryphosphorylation by cellular kinases, NRTIs compete with the natural deoxynucleoside triphosphates (dNTPs) for the incorporation into the nascent chain of viral DNA thus acting as chain terminator of the DNA chain elongation during reverse transcription. To date 8 NRTIs are in clinical use (Table 1.2, Figure 1.11).

Analog of T

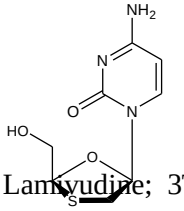


Zidovudine; ZDV

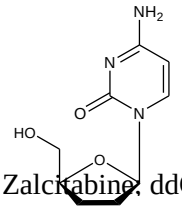


Stavudine; d4T

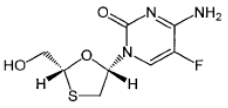
Analog of C



Lamivudine; 3TC

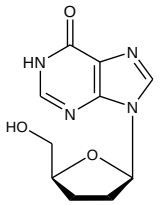


Zalcitabine; ddC

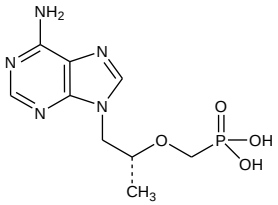


Emcitrabine; FTC

Analog of A

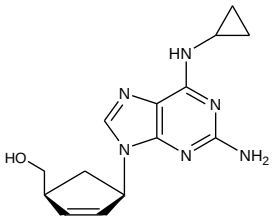


Didanosine; ddI



Tenofovir;TDF*

Analog of G



Abacavir; ABC

Figure 1.11. Nucleoside/tide reverse transcriptase inhibitors (NRTIs)
*Nucleotide Reverse transcriptase inhibitors (NRTIs)

Non-Nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs (Fig. 1.12) bind at the NNRTI-binding pocket (NNIBP), a hydrophobic pocket adjacent to the polymerase active site (~10 Å) (Fig. 1.13).

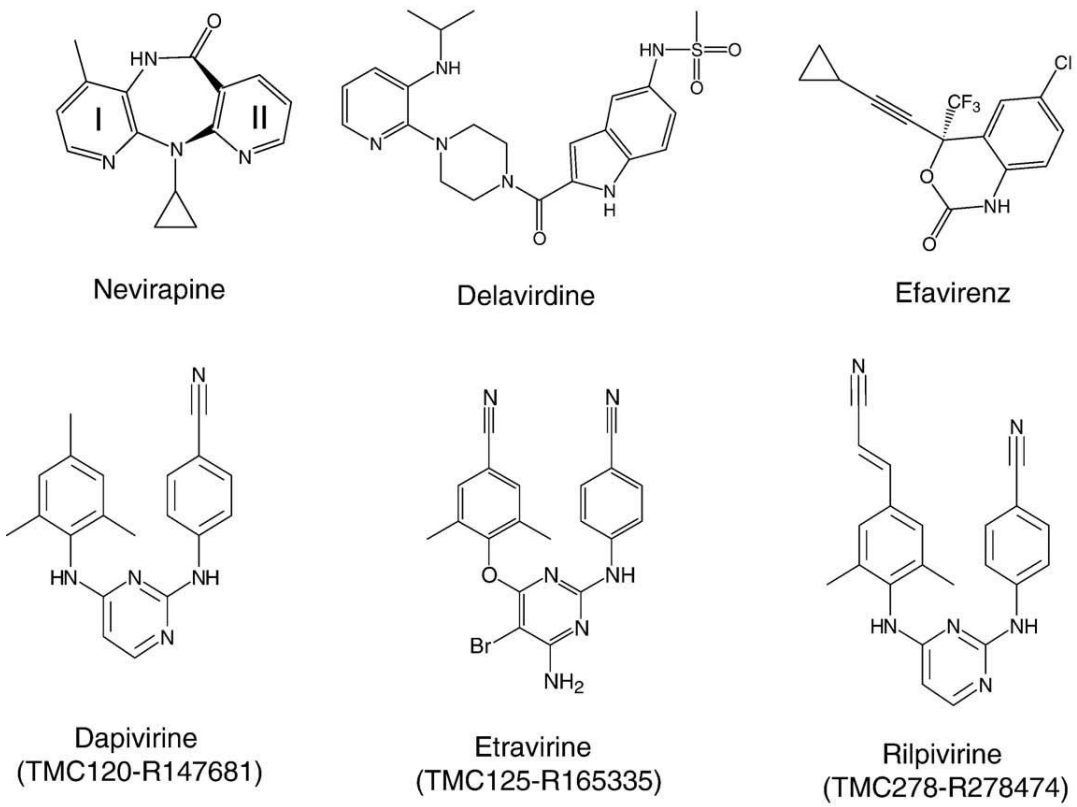


Figure 1.12. Non Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The NNRTI-binding pocket consists of residues L100, K101, K103, V106, T107, V108, V179, Y181, Y188, V189, G190, F227, W229, L234, and Y318 of p66 and E138 of p51.

Biochemical data have shown that NNRTIs are noncompetitive inhibitors and do not interfere directly with the binding of either the dNTP or the nucleic acid substrates of RT. Pre-steady state kinetic analysis of single nucleotide addition in the presence of NNRTIs has shown that binding of NNRTI interferes with the chemical step of DNA synthesis.29,30 (Kati, et al., 1992; Zang, et al., 2005)

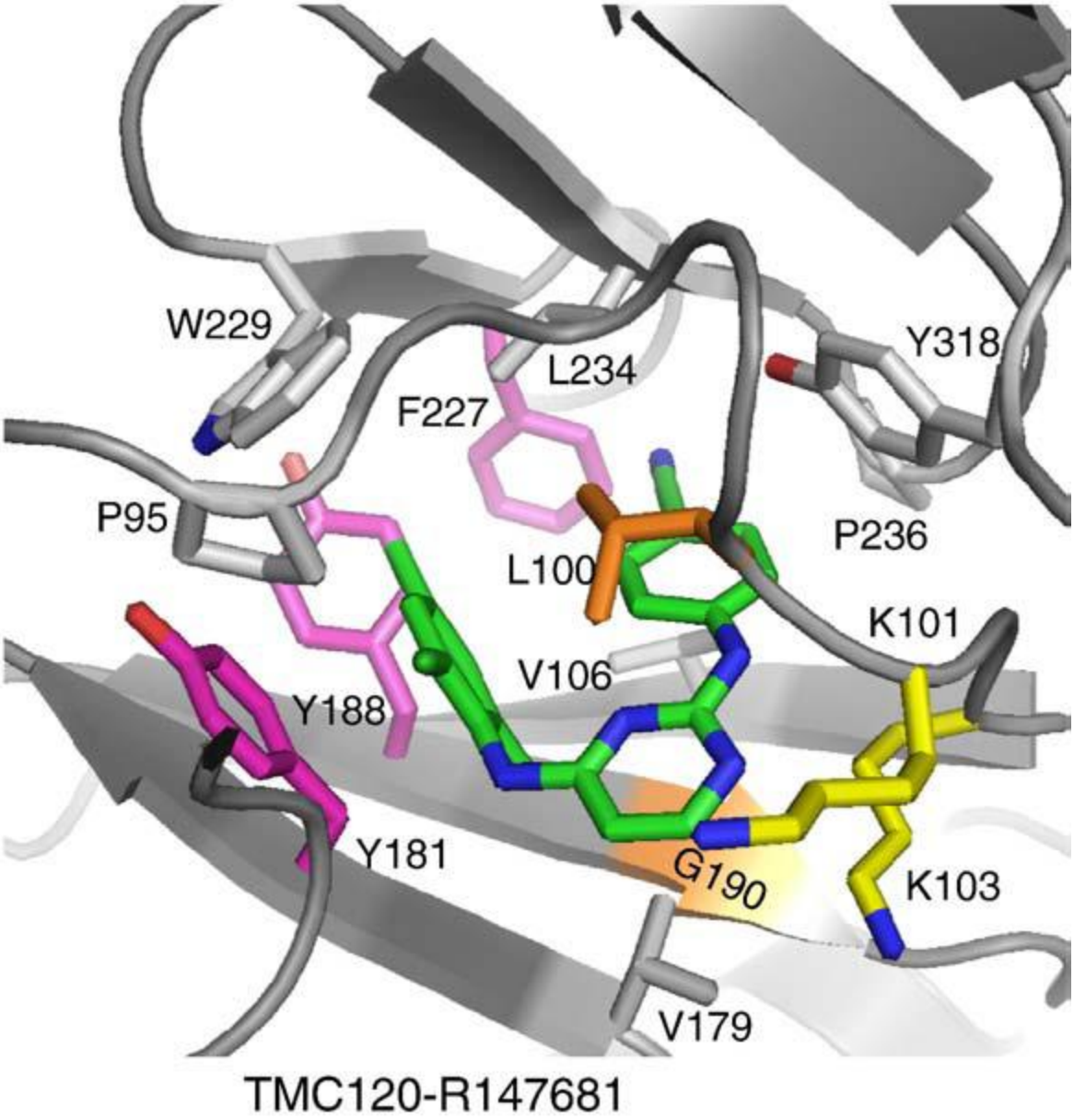


Fig. 1.13. Ribbon representation of the NNRTI-binding pocket, showing the residues where NNRTI-resistance mutations occur.

1.2.3. Integrase inhibitors

Structure and function of Integrase

HIV-1 integrase is a 32 kDa protein of 288 amino acids, comprising three functional domains: the N-terminal domain (amino acids 1-49), the catalytic core domain (amino acids 50-212), and the C-terminal domain (amino acids 213-288) 23(Engelman, et al., 1992) (Fig 1.14-1.15). The N-terminal domain contains a highly conserved zinc-binding $H_{12}H_{16}C_{40}C_{43}$ motif 22,24 (Rice, et al., 1996; Polard, et al., 1995) involved in the stabilization folding and proper multimerization of the integrase subunits 25-27 (Burke, et al., 1992; Zheng, et al., 1996). The catalytic core domain, which plays a critical role in integrase enzymatic activity, contains the catalytic $D_{64}D_{116}E_{152}$ motif that is conserved in all retroviral integrase, as well as in retro-transposons from plants, animals and fungi and in some bacterial transposases (Rice, et al., 1996; Polard, et al., 1995; Avidan, et al., 2008; Kulkosky, et al., 1995). It also contains other functional domains and residues such as the nuclear localization signal, a critical sequence mediating the nuclear import of the integrase in the context of the preintegration complex (Bouyac-Bertoia, et al., 2001); the K_{186}-R_{187}-K_{188} multimerization motif at the dimer:dimer interface ,(Wang, et al., 2001; Berthoux, et al., 2007); and several important residues (H12, L102, A128, A129, C130, W131, W132, I161, R166, Q168, E170, H171, T174, M178, Q214L) involved in the chemical bond and hydrophobic contacts with the human lens epithelium-derived growth factor (LEDGF/p75), which is an essential cellular cofactor for HIV integration, linking the integrase to chromatin (Busschots, et al., 2007; Cherepanov, et al., 2005; Hombrouck, et al., 2007; Maertens, et al., 2003; Rahman, et al., 2007). The C-terminal domain has strong but nonspecific DNA-binding activity and is involved in the binding with viral and cellular DNA with the minimal nonspecific DNA binding region (MDBD 220-270 aa)(Engelman, et al., 1994; Lutzke, et al., 1998; Lutzke, et al., 1994) . This domain, required for the integration reaction, is involved also in protein oligomerization and interactions with the reverse transcriptase (Lutzke, et al., 1998). Following reverse transcription, a multimer form of the integrase enzyme catalyzes two

reactions: the first is a cleavage of two conserved nucleotides from the 3’ ends of both long terminal repeat (LTR) strands of the viral cDNA (3’processing)(Engelman, et al., 1991). This reaction takes place in the cytoplasm within a nucleoprotein complex, referred to as the pre-integration complex (Miller, et al., 1997). The pre-integration complex is transported through the nuclear pore to the nucleus where the second step (strand transfer) occurs. This consists of the insertion and covalent ligation of the viral cDNA into the host genome (Engelman, et al., 1991). Gap filling of the interfaces between the viral and host genomic DNA is then completed using the host DNA repair machinery via a mechanism that is not yet fully understood (Yoder, et al., 2000). Since there is no human homolog of this enzyme, the HIV integrase represents a rational and important target for treating HIV infection and preventing AIDS (Fig 1.16). All integration steps can potentially be inhibited and each step can be considered a possible drug target. Multiple integrase inhibitors have been in different phases of development and can be divided into five classes: (i) DNA-binding inhibitors, (ii) 3’ processing inhibitors, (iii) nuclear translocation/import inhibitors, (iv) strand transfer inhibitors, and (v) gap repair inhibitors (Fig 1.16)(Lataillade, et al., 2006; Pommier, et al., 2005; Semenova, et al., 2008).

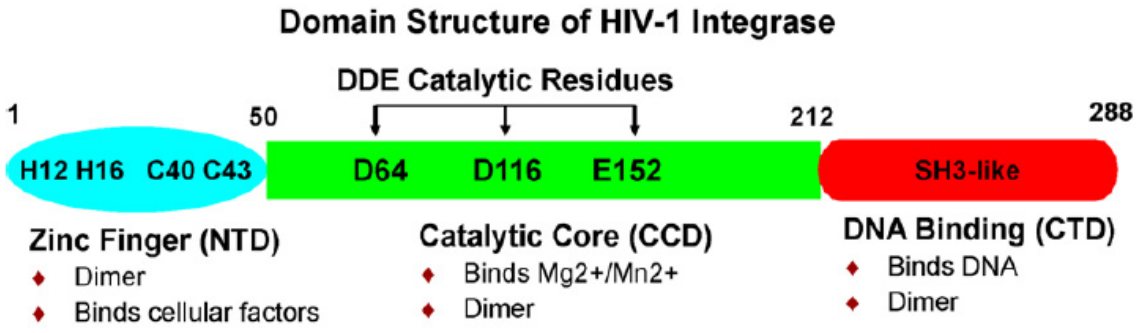


Fig.1.14.Structural domains of HIV integrase.

Schematic of the domain structure of HIV integrase. Three structural and functional domains have been identified. The N-terminal domain (residues 1–50, NTD) contains a HH-CC zinc finger motif and is required for dimerization and binding of cellular factors. The catalytic core domain (residues 51–212, CCD) contains the conserved residues forming a catalytic triad (Asp64, Asp116, and Glu152) that are required to coordinate essential divalent metal ions (Mn2+ or Mg2+). The C-terminal domain (residues 213–288, CTD) shares homology with the SH3 DNA-binding domains and binds DNA non-specifically. (From: D.J. McColl, X. Chen. 2010. Antiviral Research)

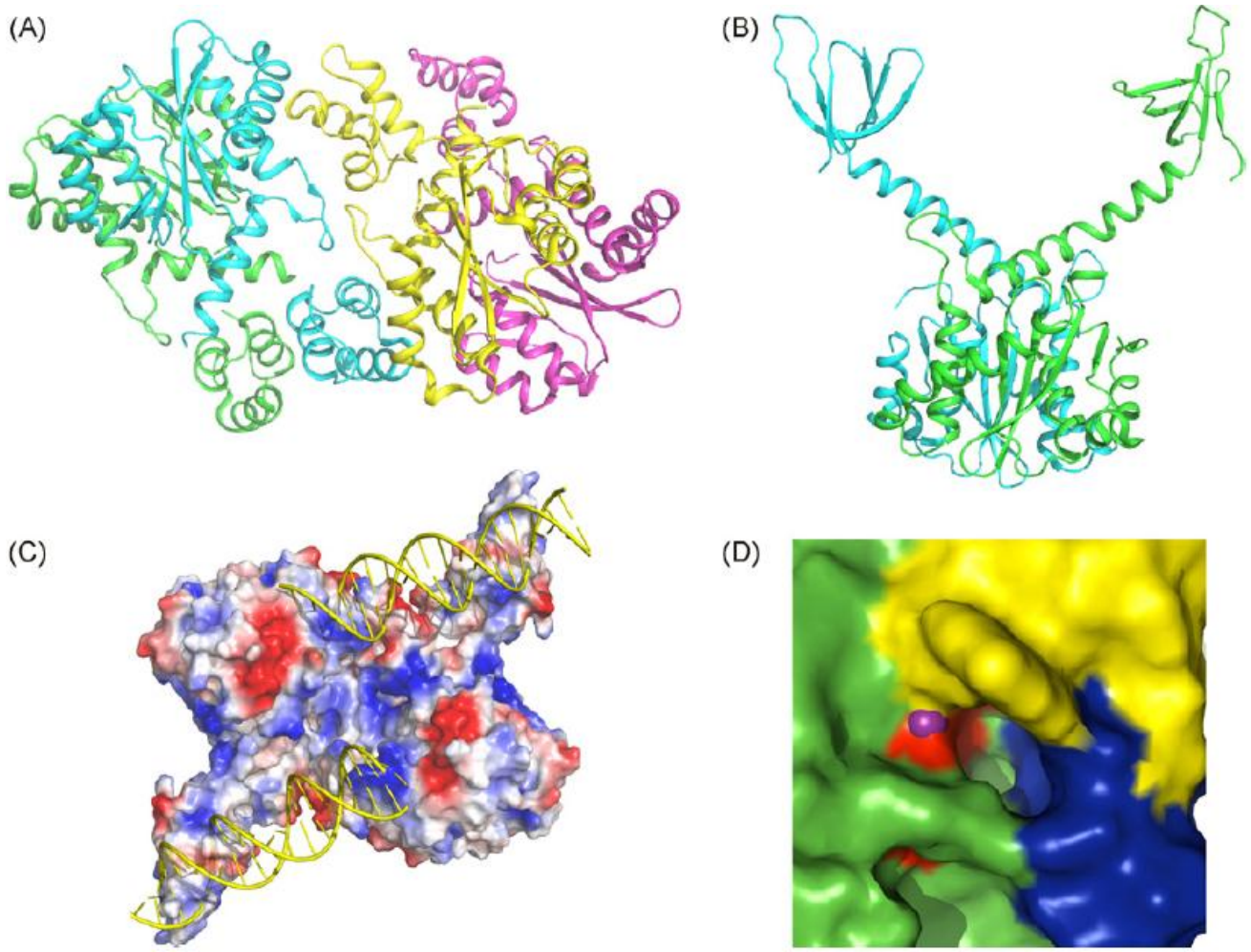


Fig. 1.15. Crystal structure of HIV integrase (A and B) and the DNA binding domain.

(A) Crystal structure of the NTD and CCD domains of integrase showing amodel of an IN tetramer composed of a dimer of integrase dimers. Each individual integrase monomer (composed of a NTD and CCD) is shown in a distinct color. (B) Crystal structure of an integrase dimer composed of the CCD and CTD domains. (C) Hypothetical space filling model of an integrase tetramer in which each monomer contains all three domains of integrase. Electrostatic surface potential is also shown (positive in blue; negative in red). A ribbon model of the viral DNA bound to the tetrameric integrase complex (in trans binding mode) is also shown. The viral DNA ends are coordinated together in close proximity. Host chromosomal DNA (not shown) most likely lies in the central groove (D) Close up view a model of an integrase active site showing how an induced hydrophobic pocket is formed upon viral DNA binding (taken from the paper of Chen et al., 2008).

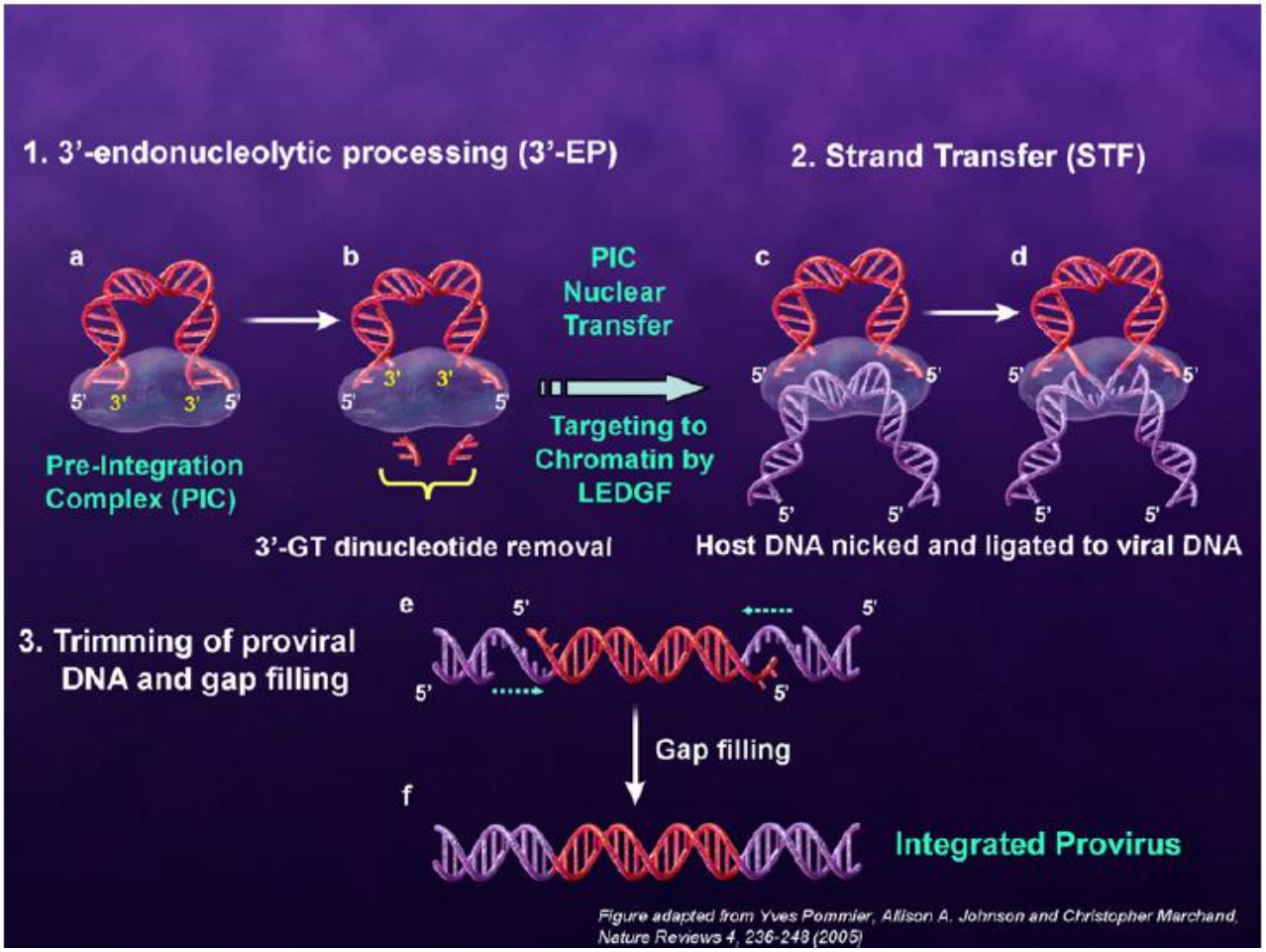


Fig. 1.16. Mechanism of proviral DNA integration.

The integration step of the HIV lifecycle requires two steps mediated by the integrase enzyme, 3'-end processing (3_-EP) and strand transfer reaction. The 3'-EP reaction occurs in the cytoplasm following completion of viral DNA synthesis by reverse transcriptase. The 3'-EP reaction is an endonucleolytic cleavage of the viral DNA and occurs immediately 3' of a conserved CA dinucleotide motif. This produces a reactive 3' hydroxyl at each end of the viral DNA. Integrase remains bound to the ends of the viral DNA which remain in close proximity to one another. This complex of viral DNA, integrase multimers and associated cellular factors form the preintegration complex (PIC). The PIC is transported across the nuclear membrane and is then targeted to chromatinized host genomic DNA via LEDGF. The second reaction catalyzed by integrase, the STF reaction then takes place. STF is 3'-end joining. Each of the 3'hydroxyl ends of the viral DNA are coordinated to attack the phosphodiester bond on the host chromosomal DNA and then ligated to the ends of the nicked chromosomal DNA. The 3' ends of the viral DNA are positioned such that they attack the host chromosomal DNA across a span of 5 base pair along the major groove. The STF reaction results in a 5 base pair, single stranded gap at the join between the viral and chromosomal DNA and a 2 base pair "flap" at the end of the 5'end of the viral DNA. Cellular repair enzymes then fill the gap, resulting in production of the mature integrated provirus from which viral transcription can be initiated. (From: D.J. McColl, X. Chen. 2010. Antiviral Research)

Strand transfer inhibitors of integrase

Several criteria define an INSTI or any other inhibitor of integrase (Pommier, et al., 2005). The candidate INSTI must be active at the appropriate point in the viral lifecycle, after reverse transcription and before maturation, as defined by time-of addition experiments. The window of activity of an INSTI is therefore about 4–16 h post infection. Secondly, treatment of infected cells with a candidate INSTI should lead to accumulation of 2-long terminal repeat circles (2-LTR circles). The 2-LTR circles occur due to the accumulation of the viral DNA and its subsequent circularization by cellular enzymes. Successful integration and production of the proviral DNA should concomitantly be decreased, a process that can be measured by Alu-PCR. Finally, treatment of HIV-1 with a putative INSTI should lead to the selection of mutations in the integrase gene in the selected viruses and these viruses should show reduced susceptibility to the selecting compound. Transfer of these mutations to recombinant integrase should also show reduced susceptibility of the resulting mutant enzyme to the inhibitor in STF assays, in vitro. INSTIs are active site inhibitors in that they bind tightly and specifically to IN and chelate the divalent metal ions coordinated by the catalytic triad, i.e. the DDE motif, in the CCD of IN (Fig. 1.15 (Marchand, et al., 2003; Marchand, et al., 2002; Grobler, et al., 2009). Structure activity studies have identified that INSTIs bind to integrase following a DNA-induced conformational change, indeed, viral DNA may well form part of the inhibitor binding site (Chen, et al., 2008; Alian, et al., 2009). INSTIs bind to a conformation of integrase that is present only after processing of the 3'viral DNA ends, in effect a form of allosteric inhibition as it implies blockage of a specific integrase-viral DNA complex.

INSTIs have been observed to be structurally diverse (Fig. 1.17) and encompass a variety of pharmacophores, however, all appear to have features in common, reflecting a likely common mode of action involving binding to divalent metal ions.

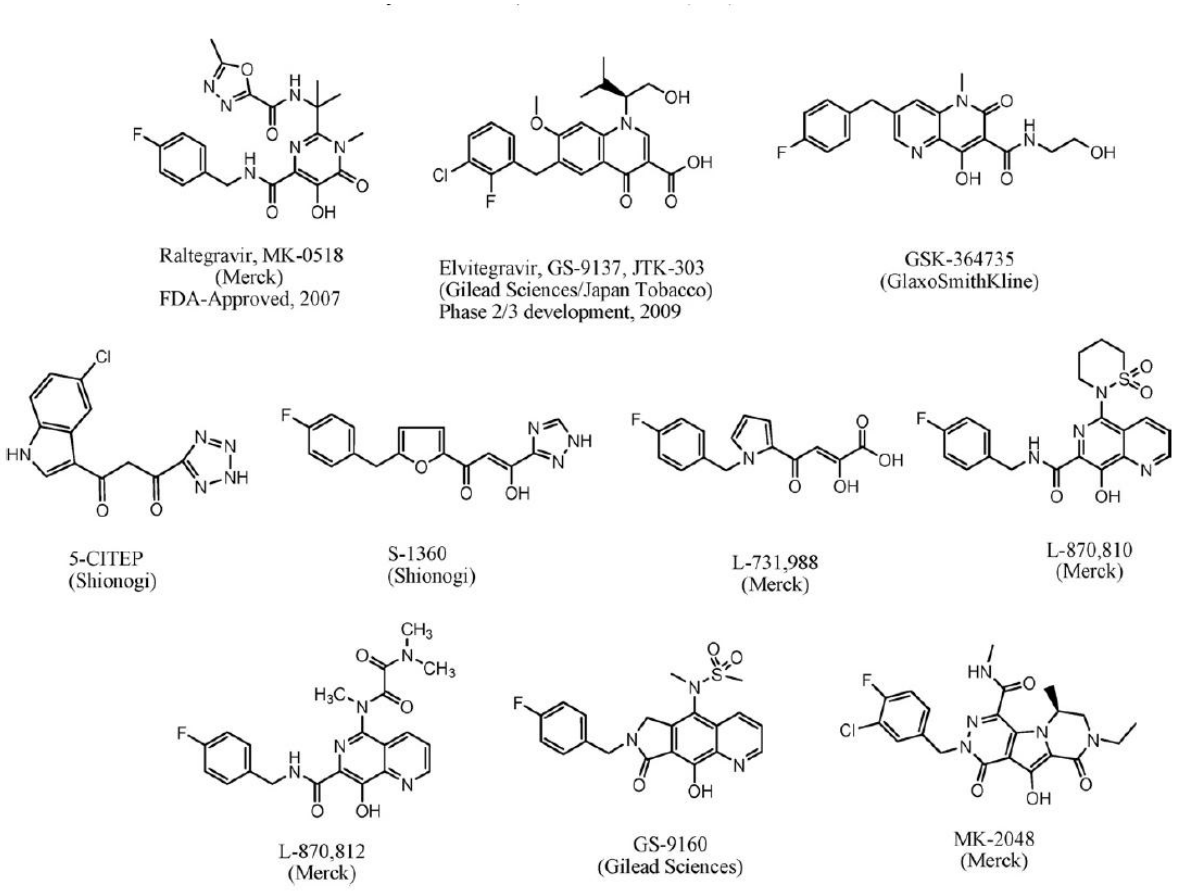


Fig. 1.17. Chemical structures of INSTIs. The evolution of INSTIs from discovery to clinical trials to approval, including raltegravir (RAL, MK-0518, Isentress®, Merck Research Laboratories, approved for use in HIV infected patients in 2007); elvitegravir (in development by Gilead Sciences and discovered by Japan Tobacco, currently in phase 2/3 development); the naphthyridinone GSK-364735 (GSK/Shionogi); early diketo acid INSTIs including 5-CITEP and S-1360 (Shionogi) and L-731,988 (Merck Research Laboratories); the naphthyridines L-870,810 and L-870,812 (Merck Research Laboratories); a tricylic INSTI GS-9160 (Gilead Sciences) and a "second generation" INSTI with an enhanced resistance profile, MK-2048 (Merck Research Laboratories) (From: D.J. McColl, X. Chen. 2010. Antiviral Research).

Raltegravir

RAL is an oxadiazole with three heteroatoms and is one of the most potent compounds investigated in cell based assays with an IC95 of 31nM in the presence of 50% human serum. RAL was highly selective against other enzymes working through Mg2+-based mechanisms being inactive against HCV polymerase, HIV RT and RNaseH, and human α, β and γ polymerases, and showed no activity up to 10μM concentration on a panel of 150 enzymes, channels and receptors. RAL also did not have activity against the major cytochrome P450 isoforms including 1A2, 2C9, 2D6, 3A4 and 2C9 nor did it show time dependent inhibition of 3A4. Binding affinity on cardiac HERG channels was >50μM, suggestive of a good cardiac safety profile. Pharmacokinetic studies in animals

showed good bioavailability with a multiphasic elimination, including a relatively short α-phase and a prolonged β-phase (Summa, et al., 2008; Markowitz, et al., 2006). Studies of metabolism demonstrated that RAL was primarily eliminated as a glucuronidated metabolite through bile and urine with the glucoronidation occurring on the 5-hydroxyl group of the pyrimidinone ring. Based on the need to keep the plasma concentration above the cell culture 95% inhibitory concentration (CIC_{95}) and considering a variety of key factors including the biphasic elimination, the metabolic stability, protein binding and plasma clearance, the human dosing regimen of RAL was predicted to be twice daily (BID). Metabolism of RAL occurs primarily through glucoronidation and not via Cyp3A4, therefore RAL cannot be boosted with ritonavir as is the case for EVG (Summa, et al., 2008). Subsequent clinical development of this compound in clinical trials and its approval focused on BID dosing in both ARV experienced and naïve subjects ((Cooper, et al., 2008; Steigbigel, et al., 2010; Lennox, et al., 2009). Raltegravir has been approved for BID dosing in both patient populations; an ongoing phase 3 trial is investigating once daily dosing (800mgQD) of raltegravir in ARV naïve subjects.

Elvitegravir

Elvitegravir (EVG JTK-303/GS-9137, Fig. 1.17) is a second INSTI in phase 3 clinical development in ARV treatment-experienced subjects and is also undergoing phase 2 development in ARV naïve subjects as part of a fixed dose combination regimen. EVG was discovered by researchers at Japan Tobacco who described a new pharmacophore, specifically 4-quinolone-3-glycoxylic acid, which maintained the coplanarity observed in DKA INSTIs (Sato, et al., 2006). Compounds containing the 4-quinolone-3-carboxylic acid motif, but not the 4-quinolone-3-glycoxylic acid, were inhibitors of IN, with the coplanar monoketo acid motif in 4-quinolone- 3-carboxylic acids providing an alternative to the DKA motif. Elvitegravir showed potent anti-HIV activity in vitro against HIV-1 of multiple subtypes (EC50 ranging from 0.1 to 1.26 nM) as well as against HIV-2 strains (EC50 1.4–2.8 nM) (Shimura, et al., 2008), and both SIV and murine leukemia viruses. Elvitegravir, like

raltegravir has also shown potent antiviral activity in vivo against HIV-1 carrying resistance mutations to multiple antiretroviral drug classes (DeJesus, et al., 2006; Zolopa, et al., 2007). Development of EVG, has focused on boosting it to QD dosing via inhibition of CyP3A4 metabolism.

Others InSti

More recently, Shionogi and GlaxoSmithKline described a series of two-metal binding INSTIs based on a naphthyridinone scaffold (Garvey, et al., 2008). One of these, S/GSK1349572 has recently shown impressive activity in a phase 2A study, in which it was studied as a once-daily unboosted INSTI dosed at 2, 10 or 50 mg once daily (Lalezari, et al., 2009). The 50mg dose produced a −2.46 log10 decline in HIV RNA after a 10-day monotherapy. Resistance data suggested that this compound may have an improved resistance profile on RAL and EVG selected resistance mutations, which may allow it to be used for salvage of patients with virologic failures on the other INSTIs (Underwood, et al., 2009). The structure of S/GSK1349572 has not been disclosed at this time.

1.2.4 Fusion inhibitors

These drugs interfere with viral entry into the host cell by disrupting the interaction between the viral glycoprotein gp41 and and the target cell membrane (Kilby, et al., 2003). Enfuvirtide (T-20) is the first fusion inhibitor approved in 2003 (Fig. 1.18). Unlike PIs and RTIs, enfuvirtide is a 36-amino-acid-peptide that needs to be injected. Despite these differences the development of drug resistance seems to be comparable

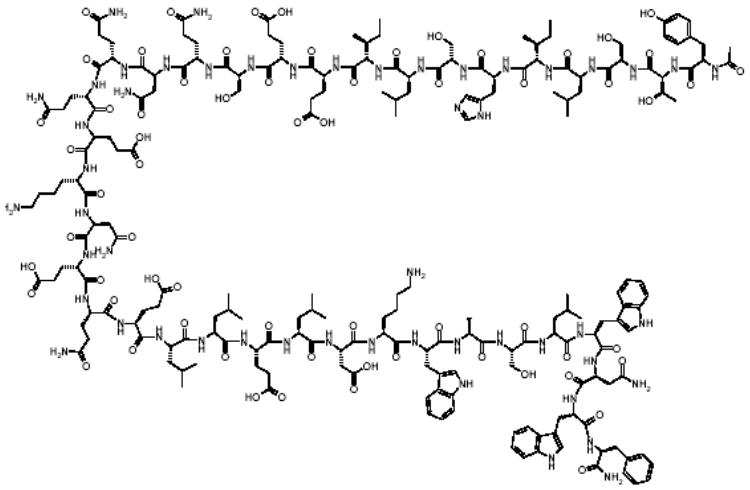


Figure 1.18. Enfuvirtide (ENF/Fuzeon/T-20)

1.2.5 CCR5 Inihibitors.

Maraviroc is the first and only currently approved antagonist of the CCR5 coreceptor (Fig.1.19). The mechanism of action of maraviroc was discovered by use of cell-based assays (Dorr, et al., 2005). Maraviroc is a noncompetitive, specific, slowly reversible CCR5 coreceptor antagonist that selectively binds to the human chemokine receptor CCR5 present on the host cell membrane, preventing the interaction and binding of HIV-1 gp120 and CCR5, and the subsequent membrane fusion events required for the entry of CCR5- tropic HIV-1 into the host cell (Palani, et al., 2006; Carter, et al., 2007; Dorr, et al., 2005; Brown, et al., 2009; Castagna, et al., 2005). Neither CCR5 cell surface levels nor associated intracellular signalling were affected by maraviroc, indicating that it is a functional CCR5 antagonist (Dorr, et al., 2005). The entry into the host cell of both CXCR4-tropic and CXCR4/CCR5 (dual)-tropic HIV-1 strains is not inhibited by maraviroc, although ongoing investigations have shown that maraviroc may have activity against dual-R5- tropic strains of HIV-1 (Vandekerckhove, et al., 2009; Lewis, et al., 2008)

Maraviroc, administered orally twice daily in combination with other antiretroviral agents, is approved in the EU for treatment-experienced patients infected with only CCR5-tropic HIV-1 detectable. In the US, maraviroc is approved for the combination treatment of treatment experienced or treatment-naive adult patients infected with only CCR5-tropic HIV-1. Maraviroc is not indicated for the combination treatment of patients infected with CXCR4-tropic strains of HIV-

1 (which use the CXCR4 receptor for entry into the host cell) or dual/ mixed populations of CXCR4- and CCR5-tropic HIV-1. Dual/mixed isolates of HIV-1 may include viruses that use either CCR5 or CXCR4 (Poveda, et al., 2009).

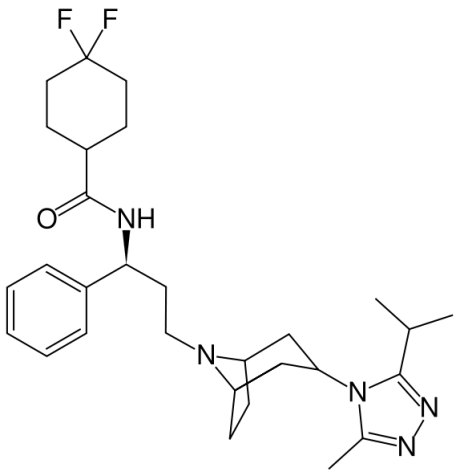


Fig. 1.19. Maraviroc (Celsentri; Selzentry)

1.3. Drug resistance

Resistance is the cause and/or the consequence of treatment failure. HIV infection is characterized by a very high replication rate, with the production of 1 to 10 billion new virus particles per day in an untreated infected individual (Perelson, et al., 1996). Moreover, HIV-1 RT lacks exonucleolytic proof-reading functionality, and this results in an average error rate per detectable nucleotide incorporated of 1/1700(Roberts, et al., 1988). Combining these two factors with the length of the viral genome (~10,000 nucleotides), it can be calculated that a mutant at each nucleotide position in the viral genome is produced every day. As a consequence, suboptimal treatments, like monotherapy regimens, will readily select the mutants in the replicating population that are resistant to the administered drug(s). Moreover, the selected drug resistant viruses will compromise the efficacy of subsequent HAART regimens, as extensive cross-resistance was rapidly observed within each class of antiretroviral drugs (Hertogs, et al., 2000; Shulman, et al., 2003; Miller, et al., 1998).

1.3.1. Mechanism of Resistance

1.3.1a PI

The development of protease inhibitor resistance is a stepwise process in which a substitution in the substrate-binding cleft of the viral protease is usually observed first. These resistance mutations in the viral protease result in an overall enlargement of the catalytic site of the enzyme. This leads to decreased binding to the inhibitor (causing a decrease in drug sensitivity) and, in parallel, to some decrease in binding to the natural substrate and thus to decreased viral replication (Croteau, et al., 1997; Mammano, et al., 2000; Nijhuis, et al., 1999; Quinones-Mateu, et al., 2001). These mutations, which are selected first and individually reduce the susceptibility to a protease inhibitor, are called primary or "major" resistance mutations (Fig. 1.8)(Johnson, et al., 2009). Secondary or "minor" mutations generally emerge later and by themselves do not have a substantial effect on the resistance phenotype but improve replication of viruses containing major mutations (Fig. 1.8) (Mammano, et al., 2000; Mammano, et al., 1998; Nijhuis, et al., 1999; Quinones-Mateu, et al., 2001). Some minor mutations are present as common polymorphic changes in HIV-1 non-subtype-B clades. The International AIDS Society-USA group reviews data on HIV-1 drug resistance that have been published or presented at recent scientific meetings to maintain a current list of mutations associated with PI resistance. At the moment major PI-resistance mutations at 15 protease codons and minor mutations at an additional 19 codons have been included in the update (Johnson, et al., 2009).

Occasionally, amino acid insertions are selected during PI-based antiretroviral therapy. Insertions ranging from 1 to 6 amino acids have been detected at various sites in the viral PR sequence, e.g., in regions between codons 17 and 18, 22 and 25, 31 and 32, 35 and 38, 40 and 41, 70 and 71, and 95 and 96 (Kim, et al., 2001; Winters, et al., 2005; Kozìsek, et al., 2008; Jordan, et al., 2009). It is shown that protease insertions, particularly those between residues 32 and 42, have become more prevalent since 1999. Presence of these insertions is positively correlated with protease mutations

associated with resistance to PIs whose usage has increased in recent years, including atazanavir, lopinavir, amprenavir and tipranavir (Kozìsek, et al., 2008). While the insertions lead to a decrease in PI susceptibility and modestly improve viral replication (Kim, et al., 2001; KozÃsek, et al., 2008), they seem to contribute to PI resistance only in combination with other mutations either in the PR or in Gag (Kim, et al., 2001). Protease inhibitor associated mutations have not only been observed in the viral protease itself, but also in the substrate of the viral protease, the Gag protein. They are commonly found in, or closely to, the protease cleavage sites and are thought to adapt the virus to the altered substrate-binding cleft of the mutant drug resistant viral protease (Doyon, et al., 1996; Maguire, et al., 2002; Mammano, et al., 1998; Zhang, et al., 1997).

1.3.1b NRTIs

Nucleoside reverse transcriptase inhibitors are prodrugs that only become effective after being converted to triphosphates. Nucleotide analogs require only two instead of three phosphorylation steps. Phosphorylated NRTIs compete with naturally occurring dNTPs (deoxynucleotide triphosphates). The incorporation of a phosphorylated NRTI into the proviral DNA blocks further elongation of the proviral DNA and leads to interruption of the chain. There are two main biochemical mechanisms that lead to NRTI resistance(Soriano, et al., 2002). *Sterical inhibition* is caused by mutations enabling the reverse transcriptase to recognize structural differences between NRTIs and dNTPs. Incorporation of NRTIs is then prevented in favor of dNTPs (e.g. in the presence of the mutations M184V, Q151M, 74V, or K65R; (Naeger, et al., 2001; Clavel, et al., 2004) (fig. 1.20A). *Phosphorylysis* via ATP (adenosine triphosphate) or pyrophosphate leads to the removal of the NRTIs already incorporated in the growing DNA chain. This is the case with the following mutations: M41L, D67N, K70R, L210W, T215Y and K219Q (Meyer, et al., 1999) (fig. 1.20 B). Phosphorylysis leads to cross-resistance between NRTIs, the degree of which may differ between substances (AZT, d4T >ABC > ddC, ddI > 3TC).

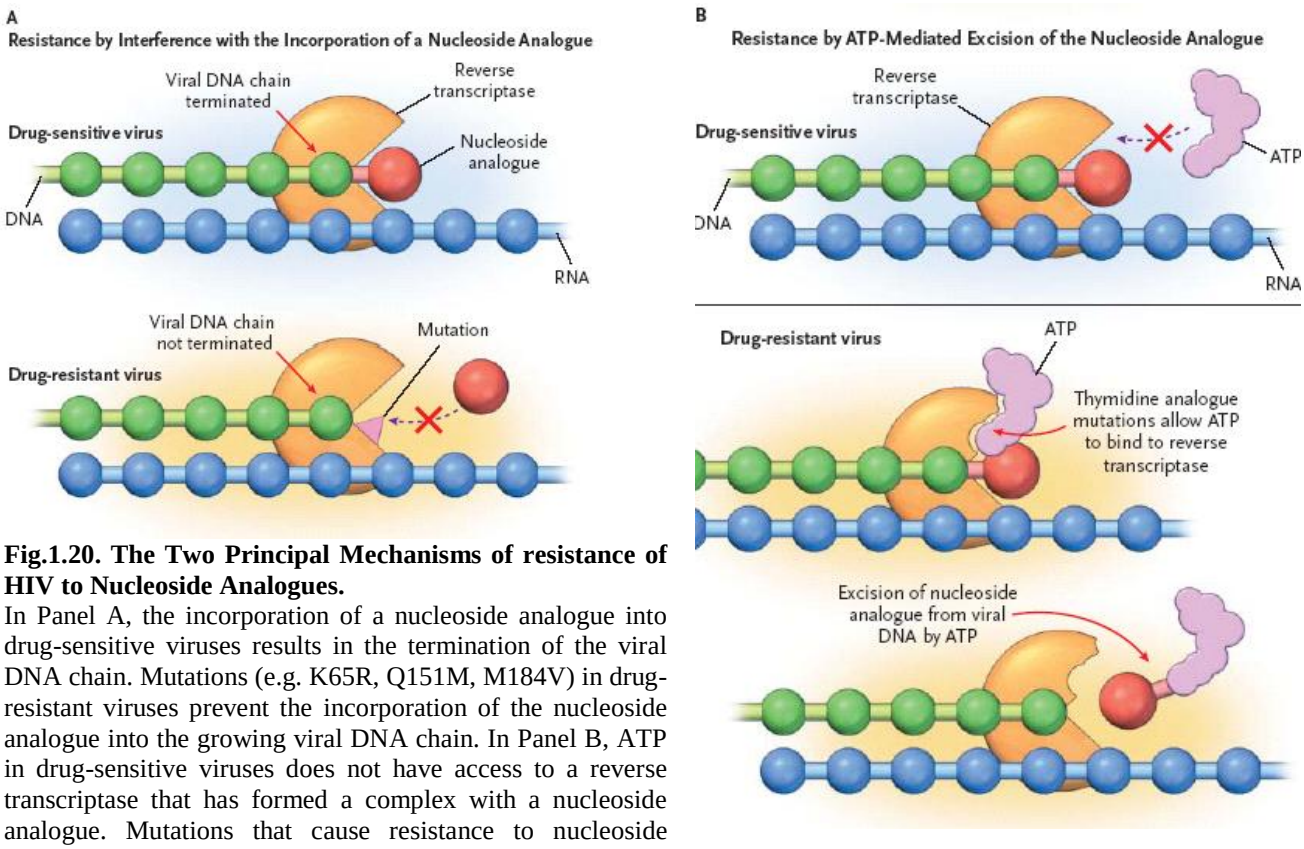


Fig.1.20. The Two Principal Mechanisms of resistance of HIV to Nucleoside Analogues.
In Panel A, the incorporation of a nucleoside analogue into drug-sensitive viruses results in the termination of the viral DNA chain. Mutations (e.g. K65R, Q151M, M184V) in drug-resistant viruses prevent the incorporation of the nucleoside analogue into the growing viral DNA chain. In Panel B, ATP in drug-sensitive viruses does not have access to a reverse transcriptase that has formed a complex with a nucleoside analogue. Mutations that cause resistance to nucleoside analogues, referred to as thymidine analogue mutations (TAM1: M41L, L210W, T215Y; TAM2: D67N,K70R, T215F) allow ATP to bind reverse transcriptase near the 3' end of viral DNA terminated by the incorporation of a nucleoside analogue. ATP then excises the analogue from viral DNA, allowing reverse transcription to proceed normally. (From Clavel et al., N Engl J Med 2004;350:1023-1035)

1.3.1c NNRTIs

There are four NNRTI drugs (nevirapine, delavirdine (first generation), efavirenz (second generation), and etravirine (third generation) that are currently approved for treating HIV-1 infections and several other potent NNRTIs that inhibit HIV-1 at nanomolar concentrations (EC50) are in clinical trials. However, there are mutations in RT that can cause resistance to all of the approved NNRTIs. Most of the NNRTI resistance mutations are found in and around the NNIBP (Fig1.11).

K103N and Y181C are the most frequently observed resistance mutations in patients treated with the approved NNRTIs. Other NNRTI resistance mutations observed in patients include L100I, K101E, V106A, V179D, Y188L, G190A, and P236L; the NNRTI resistance mutations can occur

singly, or in combinations. Unfortunately, resistance to first and second generation NNRTIs can evolve relatively quickly.

The most promising of the third generation NNRTIs are effective against HIV strains that carry most common single and double mutations; however, viral strains carrying multiple NNRTI-resistance mutations can exhibit significant levels of drug resistance. Extensive crystallographic, molecular modeling, and biochemical studies have contributed towards understanding NNRTI drug resistance and the development of better NNRTIs, which is an ongoingeffort. Our current understanding suggests that there are at least three broad classes of NNRTI-resistance mechanisms.

a)Loss/change of key hydrophobic interactions. Amino acid residues Y181, Y188, and F227 are located in the hydrophobic core of the NNIBP (Fig. 1.11).(Kohlstaedt, et al., 1992; Ding, et al., 1995; Hsiou, et al., 1998). Specific residues in this core have extensive interactions with NNRTIs. Mutations in some of the key residues (Y181C, Y188L, and F227L) cause significant resistance through the loss of the aromatic ring interactions with NNRTIs, which are generally hydrophobic (Kohlstaedt, et al., 1992; Das, et al., 1996; Ren, et al., 1995; Ren, et al., 2004; Ren, et al., 2001). This causes high levels of resistance to the first generation NNRTIs, which are relatively rigid. More advanced NNRTIs, however, are designed with so-called strategic flexibility. This intrinsic flexibility makes it possible for the newer drugs to have compensatory interactions with RTs that have mutations causing resistance to the first-generation NNRTIs (Das, et al., 2004).

This flexibility in the binding has been called wiggling and jiggling, and its structural basis has been described in recent structural studies of wild-type, K103N/Y181C, and L100I/K103N HIV-1 RT complexes with TMC278/ rilpivirine (Das, et al., 2008; Hsiou, et al., 2001). Wiggling and jiggling allow NNRTIs to adapt to changes in the NNIBP caused by resistance mutations; the side chains of the pocket residues adjust to accommodate inhibitor binding in a “shrink-wrap” mode.

b)Steric hindrance. Amino acid residues L100 and G190 are in the central region of the NNIBP. Mutations in either of these residues cause high levels of resistance to many NNRTIs. The L100I mutation confers resistance by changing the shape of the pocket (the amino acid is β-branched instead of γ-branched)(Ren, et al., 2004), whereas G190A introduces a bulge (Sarafianos, et al., 2004).

c)pocket entrance mutations. The K103N and K101E mutations are NNRTI resistance mutations that frequently cause resistance to first generation NNRTIs. Amino acid residues K101 and K103 are located at the rim of the entrance to the NNIBP with their side chains pointing out. These mutations apparently cause resistance by interfering with the entry of NNRTIs into the pocket (Hsiou, et al., 2001; Ren, et al., 2007). Second generation NNRTIs were designed to overcome this problem. However, new drug-resistance mutations mutations, or combinations of mutations, will be selected when the more advanced NNRTIs are used to treat HIV-1-infected patients. Recently, it has been reported that a number of mutations in the connection, or RNase H subdomains of RT, can enhance resistance to both NRTI and NNRTI inhibitors of RT(Yap, et al., 2007; Hachiya, et al., 2008).

1.3.1d INIs

raltegravir resistance is associated with three genetic pathways defined by the N155H, Q148H/R/K or Y143RC primary mutations (Hazuda, et al., 2009; Ceccherini-Silberstein, et al., 2009; Malet, et al., 2008; Canducci, et al., 2009; Reigadas, et al., 2010).

Secondary G140S/A mutation in the Q148H/R/K background compensates for replication defects associated with primary mutations and increases resistance. These substitutions significantly decrease the binding affinity of the integrase inhibitor and allow IN to retain its activity.

By a molecular dynamics model, it was supposed that the three characteristic pharmacophores of raltegravir —two ‘‘adenine-like’’ fragments and a chelating center — can be specifically

recognized by either Q148 or N155 in the catalytic site. This model indicates that IN is blocked by the adenine bioisoster competing with the terminal bases of the viral DNA recognized by IN. If they are to block this inhibitory effect, the selected mutations must allow alternative possibilities for DNA recognition, while maintaining the integrity of the IN structure and impairing inhibitor binding (Fig. 1.20) (Mouscadet, et al., 2009).

In another study, analyzing the spatial position of primary resistance mutations for raltegravir in SIVmac251 integrase structure complexed with raltegravir, proviral DNA and Mg^{2+}, it was supposed that mutations in position Q148 could influence the binding of raltegravir to enzyme while mutation in position N155 or Y143 could affect the interaction with ions and proviral DNA respectively in the enzyme (Fig. 1.21 (Lewis, et al., 2010).

Thus, the presence of primary resistance mutations could induce conformational change in IN that either impair the affinity of raltegravir for the enzyme or allow alternative possibilities to catalyze integration.

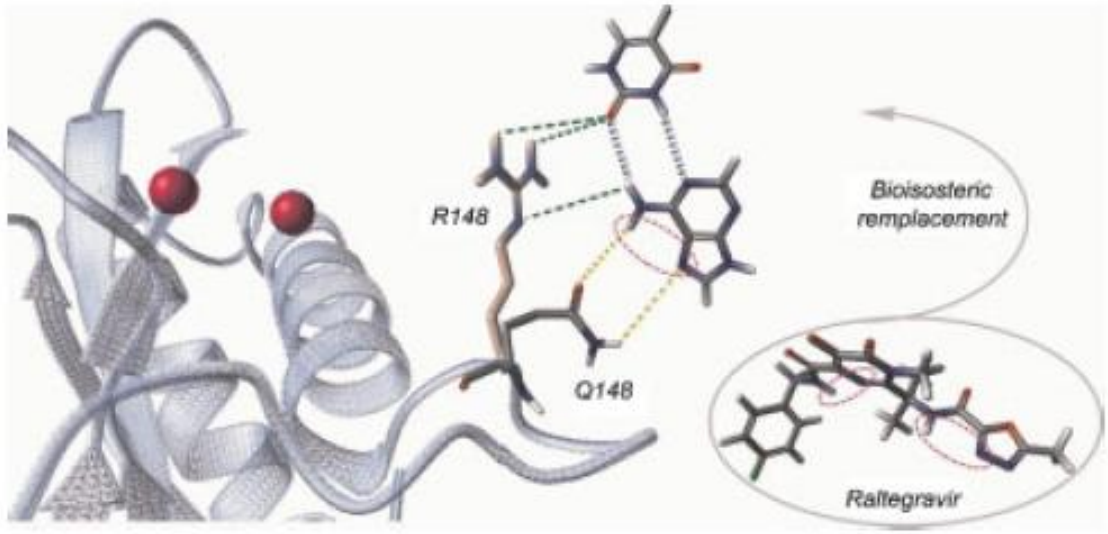


Fig. 1.20 . Model representing the alternative molecular recognition of the A–T base pair by HIV-1 IN induced by raltegravir resistance mutations. Molecular display style: the enzyme structure is shown by the ribbon, Mg2þ cations are shown as balls; the wild-type Q148 and mutated R148 residues, the A–T base pair of DNA and raltegravir are shown as sticks; H-bonds are shown as green dashed lines and Watson–Crick bonds are shown as blue dashed cylinders.(From: Reigadas et al, 2009, J Mol Recognit)

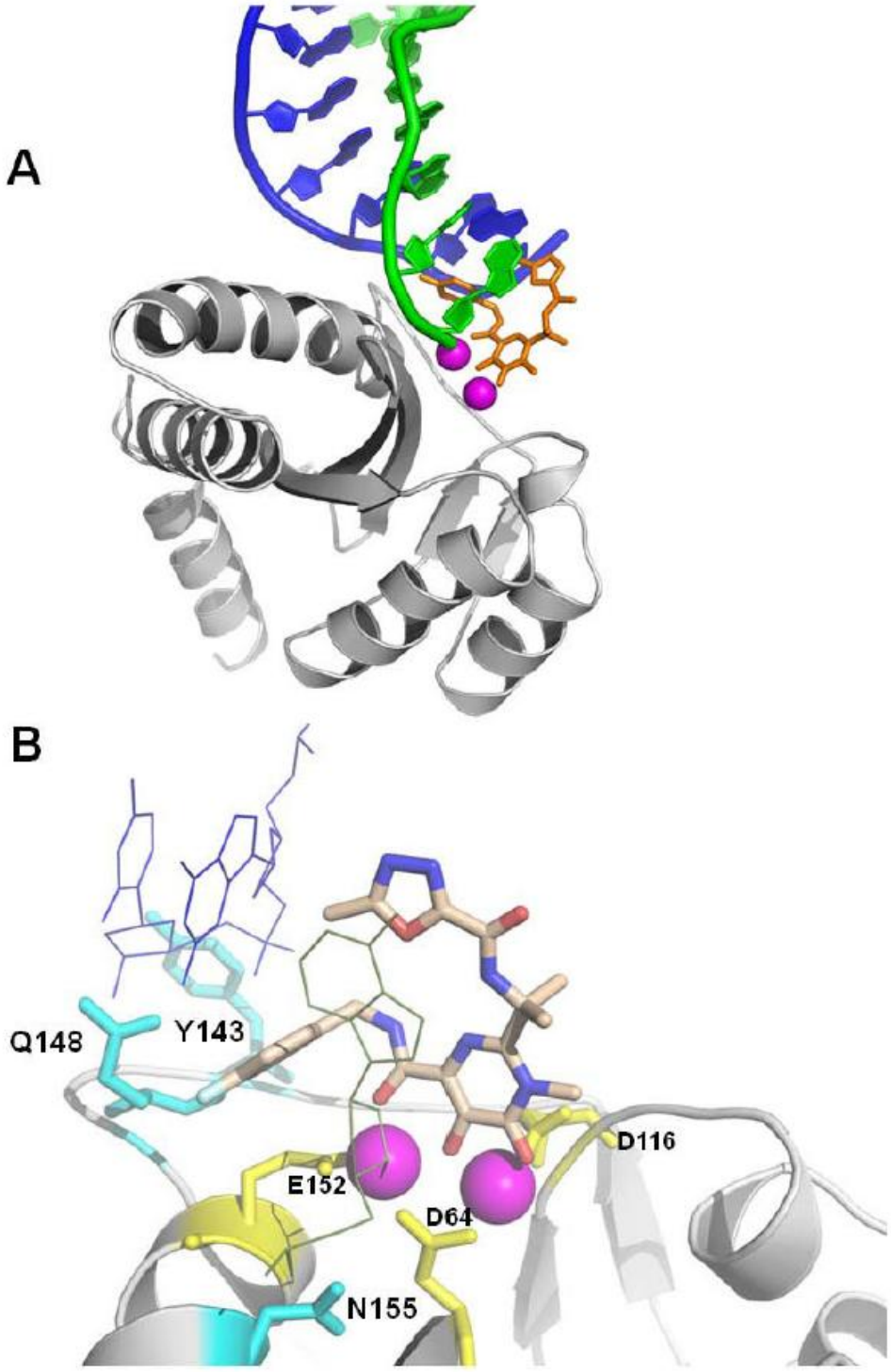


Fig. 1.21. In silico docking of raltegravir at the SIVmac251 integrase (IN) active site. Panel A: An overview of the interaction between SIVmac251 integrase (in grey), 3' processed proviral DNA (green and blue cartoons) and raltegravir (in orange). The three terminal nucleotides of the 5' DNA strand (in blue) have been removed for better clarity. Metal (Mg^{2+}) ions are shown in magenta. Panel B: Interaction of Raltegravir (shown in CPK) and the integrase amino acids susceptible to primary drug resistance mutations (cyan sticks). The protein backbone is shown by cartoons. Metal ions are presented in magenta. The catalytic triad (D64, D116 and E152) is shown in yellow. Ligand-interacting nucleotides, dC25 and dA20, are shown as thin lines. A full three-dimensional view of the complex can be obtained using the 3D coordinates provided as additional material [see Additional file 4]. Image obtained using PyMOL [73].
(From: Lewis et al., 2009, Retrovirology)

1.3.1e FIs

T20 is being prescribed in patients having failed most, if not all, of the other classes of antiretroviral drugs. In these patients, T20 exerts a strong antiviral activity, but, because the antiretrovirals that are part of same combination have frequently lost much of their activity (Kilby, et al., 1998), T20 response is often transient. Rebound of plasma virus is then accompanied by the emergence of resistant variants, which then carry characteristic mutations (Wei, et al., 2002; Bean, 2002). T20 resistance mutations emerge in the gp41 subunit of the envelope glycoprotein. They are located within a domain termed the "proximal helix", also known as HR1, whose normal function is to do fold together with the "distal helix" (HR2). Since T20 mimics the structure of HR2, it interferes with folding of the two helices, thereby preventing membrane fusion. Mutations in HR1 reduce recognition of T20 and promote resistance. A single amino acid substitution in gp41 is enough to reduce the efficacy of T20 (Fig.1.22)

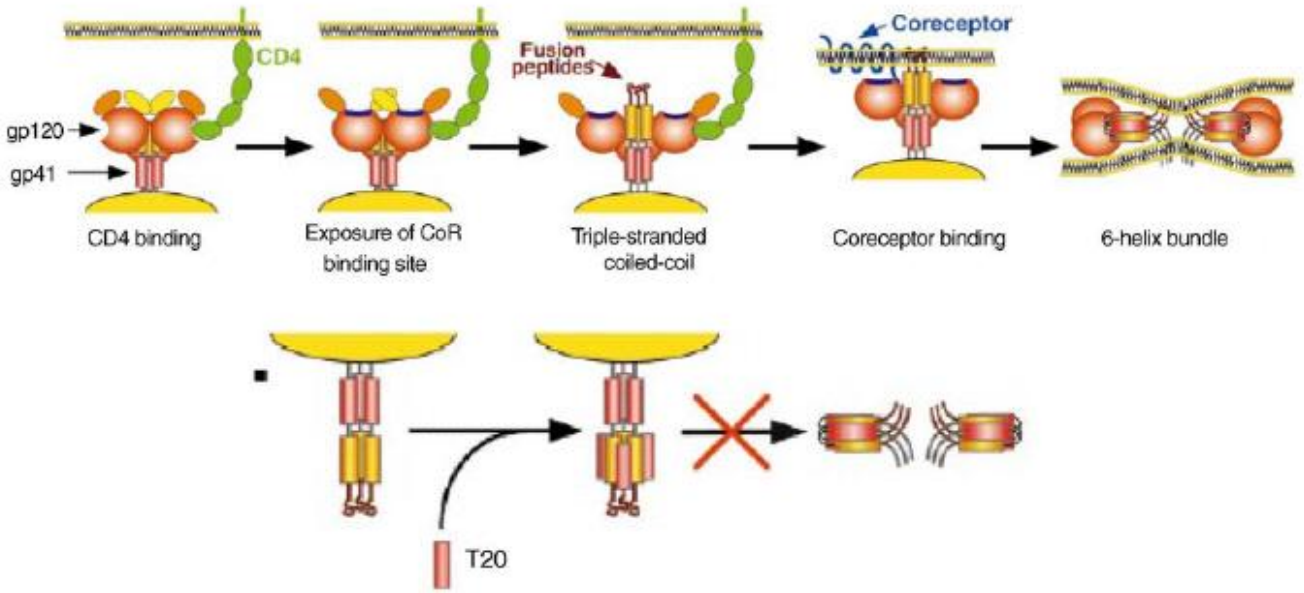


Figure 1.22. Model for HIV-1 Envelope fusion with the cell membrane and inhibition of fusion by enfuvirtide (T20). Binding of CD4 to the gp120 subunit induces exposure of a conserved region in gp120 implicated in coreceptor binding. Binding to coreceptor could bring the viral envelope in closer proximity to the target membrane, enabling the fusion peptide to insert in the bilayer, or it could impact formation of the six-helix bundle, the transition to which leads to membrane fusion. Enfuvirtide binds to the grooves on the outside of the triple-stranded coiled-coil formed by the NH2-terminal helices. Therefore, it prevents transition to the six-helix bundle and membrane fusion (after Doms and Moore, 2000).

1.3.1f CCR5 inhibitors

Resistance of HIV-1 to CCR5 antagonist drugs can be the result of mutations within gp120, particularly in the V3 loop (associated with coreceptor binding), which is structurally flexible (SouliÃ©, et al., 2008) Indeed, V3 loop changes play a key role in the development of drug resistance in CC1/85 isolates, which is to be expected given the importance of this region in coreceptor recognition and subsequent binding (Westby, et al., 2007).

There are two mechanisms by which resistance to CCR5 antagonists emerges. First, viruses that use X4 or are dual tropic emerge during therapy, and these are thought to grow out from a pre-existing reservoir. This occurs in about two-thirds of failures and has not been associated with a rapid fall in CD4 count. The second mechanism is via alterations in the amino acid sequence of the V3 loop which then allow the virus to bind to the CCR5 with the inhibitor bound in place (Mendoza, et al., 2008; Raymond, et al., 2008; Garrido, et al., 2008; Moncunill, et al., 2008). A possible mechanism which has not been observed is the evolution of the V3 loop such that it becomes CXCR4 tropic, a parallel situation to that observed during the normal course of disease.

1.4. Resistance test

1.4.1. Clinical importance of performing resistance tests

The goal of resistance testing is to identify, in clinical samples, viral variants harbouring mutations causing or contributing to drug-resistance, and thus to provide information to assist in the selection of active antiretroviral regimen(s) more likely to achieve and to maintain viral suppression. Genotypic resistance testing (GRT) is generally preferred because of the faster turnaround time, lower cost, and enhanced sensitivity for detecting mixtures of wild-type and resistant-virus. The test is based on traditional population (Sanger) sequencing of HIV, and is able to detect quasi-species representing on average at least 20% of a viral population. However, for patients with a complex treatment-history, results derived from both genotypic and phenotypic resistance tests might provide

critical and complementary information to guide regimen changes (Hirsch, et al., 2008). Once obtained the viral gene target sequence, several interpretation algorithms are freely-available to analyze the resistance for all approved NRTIs, NNRTIs, PIs and INIs: – ANRS drug-resistance interpretation algorithm [http://www.hivfrenchresistance.org/], HIVdb drug-resistance interpretation algorithm [http://hivdb.stanford.edu/], or Rega Institute Drug-Resistance Interpretation Algorithm [http://www.rega.kuleuven.be].

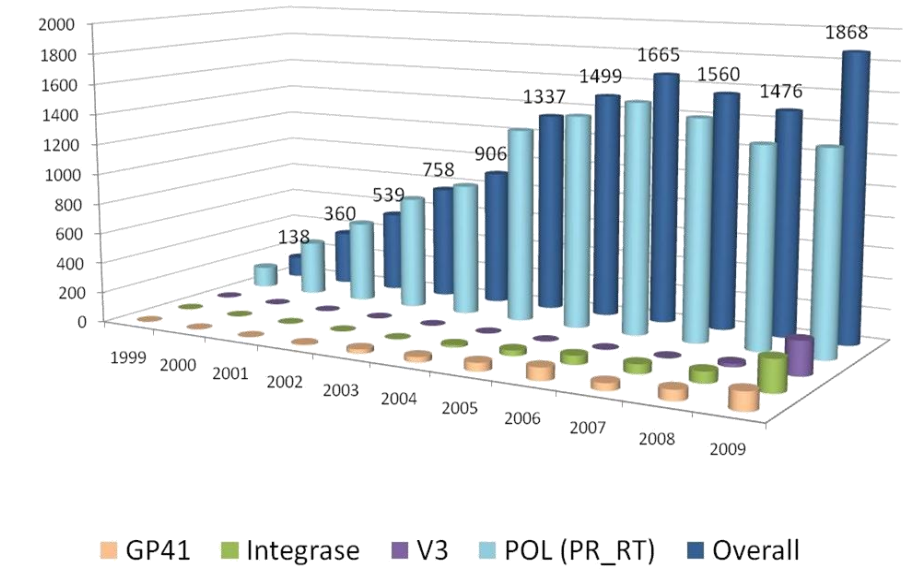
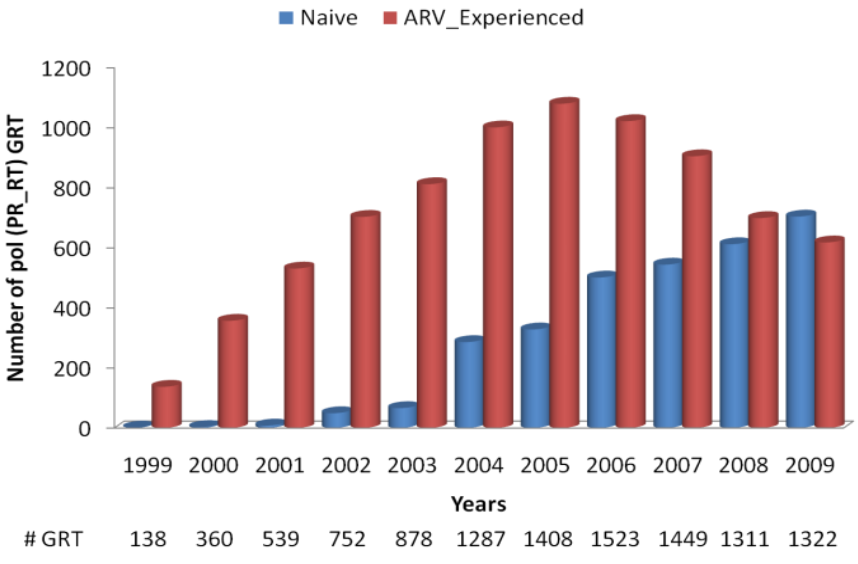


Fig. 1.23. Histograms representing the number of GRTs performed in virology laboratory of University of Rome "Tor Vergata", in the last 10 years.

A)Number of *pol* (PR/RT) GRTs performed over the years. Red bars represent treatment-experienced patients, while blue bars represent drug-naïve patients. The numbers reported at the bottom indicate the total number of GRTs per year.



B) Number of GRTs of PR/RT (light-blue bars), GP41 (pink bars), integrase (green bars), and V3-Gp120 (purple bars) performed over the years. The numbers reported at the top of the "overall" bar (dark- blue) indicate the total number of GRTs per year.

In Figure 1.23 is reported an example of 'real-life' working-routine in a virology center. Histograms represent the number of GRTs performed in the last ten years in our laboratory, the largest reference centre in Rome and its surrounding area for HIV genotypic resistance testing. Not surprisingly, and in line with the increased efficacy of antiviral regimens, the number of GRTs of HAART-treated patients decreased in the last years (>600 performed in 2009 vs $\geq$1000 in the years 2004-2006), though the number remains now stable, thus confirming that failures continue to occur in clinical practice. Following guidelines recommendation, an impressive increase of GRTs requested also for drug-naive patients was observed, starting from 2004 (with > 600 performed in 2009).

1.4.2 Phenotypic test

Phenotypic resistance tests involve direct quantification of drug sensitivity. Viral replication is measured in cell cultures under the selective pressure of increasing concentrations of antiretroviral drugs and is compared to viral replication of wild-type virus. Drug concentrations are expressed as IC_{50} values (50% inhibitory concentration). The IC_{50} is the concentration of drug required to inhibit viral replication by 50%. The sensitivity of the virus is expressed as the IC_{50} compared to the so-called cut-off value. The cut-off value indicates by which factor the IC_{50} of an HIV isolate can be increased in comparison to that of the wild-type, whilst still being classified as sensitive. The determination of the cut-off is crucial for the interpretation of the results. Three different cut-offs are currently being used. The *technical cut-off* is a measure of the methodological variability of the assay and is approximately 2.5 fold more than the IC_{50}. The *biological cut-off*, for example the comparative value on an antivirogram, involves the interindividual variability of virus isolates from ART-naive HIV patients and is slightly higher than the technical cut-off. The biological cut-off does not, however, allow prediction of the clinical response to a drug. The *clinical cut-off* indicates up to which levels of IC_{50} virological success can still be expected.

By the introduction of anti-CCR5 drugs in the battery of antiretrovirals available in clinic, phenotypic assays to evaluate the coreceptor use of viral population in HIV-1 infected patients have become mandatory.

The Trofile assay evaluates the coreceptor use of recombinant luciferase-reporter viruses pseudotyped with a population (or clones) of patient-derived HIV envelopes in a single-cycle infection assay. The original version of the assay was validated to detect low-level X4 or R5 variants with 100% sensitivity when those variants comprised 10% of a mixed HIV envelope population and with 85% sensitivity when those variants comprised 5% of a population (Whitcomb, et al., 2007). Clinical trial data indicated that an improved sensitivity for the detection of low-level CXCR4- using variants might improve the selection of patients for CCR5 antagonist therapy (Gulick, et al., 2007; Gulick, et al., 2008). Therefore, an enhanced version of the Trofile assay with an approximately 30-fold increased sensitivity for the detection of low-level X4 variants was developed and validated to detect low-level X4 variants with 100% sensitivity when the variants comprised as little as 0.3% of a mixed envelope population(Vandekerckhove, et al., 2009).

Disadvantages of phenotypic testing include the lengthy procedure and high expense of the assay.

1.4.3 Genotypic test

Genotyping resistance testing (GRT) is the common method used for detecting drug-resistant strains of HIV. It measures indirectly the drug-resistance by detecting mutations in the HIV genome known to be associated with drug resistance either in the laboratory and/or in clinical situations (Shafer, 2002). These are determined by the direct sequencing of the amplified HIV genome or by specific hybridization techniques with wild type or mutant oligonucleotides. Mutations that are associated with reduced sensitivity have been well-described for most HIV drugs, but the high number of different resistance patterns, which may also contain compensatory mutations, make the determination of the degree of resistance to particular drugs difficult.

The analysis of genotypic resistance patterns is based on the correlation between the geno- and the phenotype. There is data available from *in vitro* studies, clinical observations and duplicate testing, in which genotypically localized mutations were investigated for phenotypic resistance.

Another novel application of genotypic test is prediction of coreceptor usage based on the interpretation of V3 sequences using bioinformatics tools. By today the most known algorithms to infer coreceptor usage are "geno2pheno" (http://coreceptor.bioinf.mpi-inf.mpg.de/) and PSSM (http://indra.mullins.microbiol.washington.edu/webpssm/) (Sing, et al., 2007; Jensen, et al., 2003).

The conventional genotypic methods for HIV drug resistance and tropism testing employs bulk population genotyping (Sanger, 1981; Sanger, 1988) of the plasma virus swarm in an infected patient. However, the sensitivity limitation of the method is such that variants present in an individual at levels, 20%–30% are masked in this analysis (Fig.1.24) (Halvas, et al., 2006).

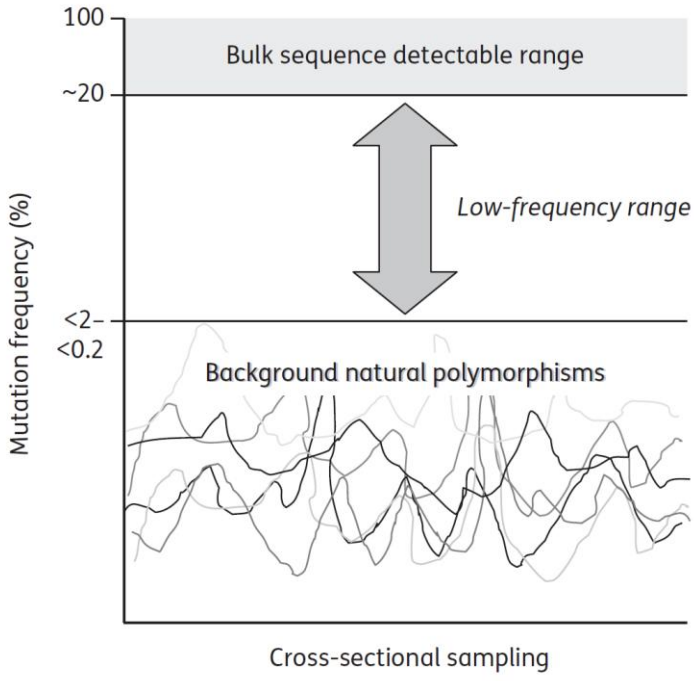


Fig. 1.24 Low-frequency HIV-1 variants containing mutations associated with drug resistance. The low-frequency range is defined at the upper end by mutations that are present at high frequency and can be detected by bulk population sequencing, and at the lower end by the natural background of mutations that emerge spontaneously within the viral quasispecies. The background reactivity was determined using samples collected from HIV-1-infected patients in the years preceding the introduction of ART. (From: Johnson & Geretti, 2010, J Antimicrob Chemother)

Several studies, regarding NNRTI and NRTI drugs, have demonstrated that minor drug resistant HIV populations that are not detectable in the standard assays, can impair the response to therapy (Palmer, et al., 2005; Johnson, et al., 2008; Kuritzkes, et al., 2008; Mellors, et al., 2004) and limited data is currently available about the prevalence and role of InSTI-resistance minor variants in HIV-infected people (Ceccherini-Silberstein, et al., 2008; Ceccherini-Silberstein, et al., 2009; Charpentier, et al., 2009; Liu, et al., 2009).

Different ultra-sensible assays were developed to detect the presence of HIV-1 minor quasispecies. Indeed, the clonal methods, based on cloning the coding regions from patient samples into a standard HIV plasmid backbone allow generation of viral stocks, representing all the viral variants, and functional analysis of viral drug sensitivity in short-term culture (Clavel, et al., 2004; Van Baelen, et al., 2008; Ceccherini-Silberstein, et al., 2010).

However, to outperform this plodding technique, different genotyping methods were developed to allow characterization of minor HIV drug-resistant populations (Palmer, et al., 2005; Halvas, et al., 2006; Cai, et al., 2007). Of note, techniques as the olioligase assay (OLA) (Villahermosa, et al., 2001), the LigAMP (Shi, et al., 2004), the allele-specific, the mutation-specific real-time PCR (Palmer, et al., 2006; Johnson, et al., 2007) or parallel allele-specific sequencing (PASS) (Cai, et al., 2007) allow to query individual base pairs at a time .

Differently, pyrosequencing provides massive parallel sequencing that can be used to produce complete genome coverage from a conserved sequence or create an array of reads from mixed sequences (Fig 1.25-27) (Margulies, et al., 2005; Shendure, et al., 2008). Among heterogeneous collections of sequences, the current applications of pyrosequencing have been to, either resolve the sequences from different organisms in the sample (Andersson, et al., 2008; Zhang, et al., 2009) or be used to probe extremely low frequency variants existing within a single target (Simen, et al., 2009; Hoffmann, et al., 2007; Rozera, et al., 2009; Wang, et al., 2007), as the HIV-1 minor quasispecies.

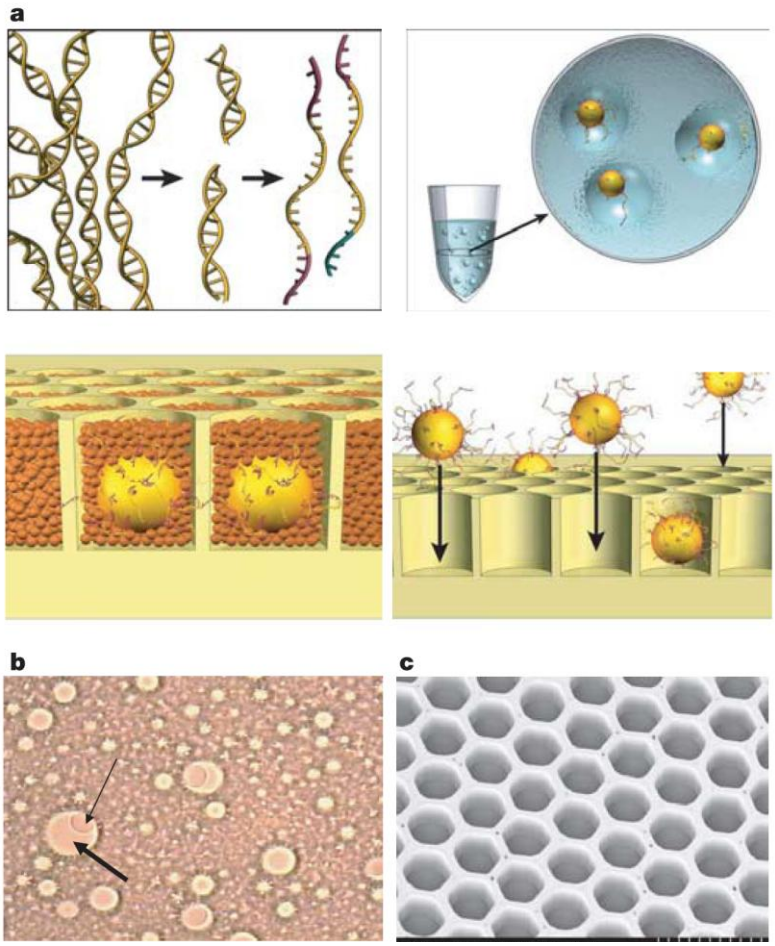


Fig.1.25. 454-Pyrosequencing: sample preparation(A) Clockwise from top left: (i) genomic DNA is isolated, fragmented, ligated to adapters and separated into single strands; (ii) fragments are bound to beads under conditions which favor one fragment per bead, the beads are captured in the droplets of a PCR-reaction-mixture-in-oil emulsion and PCR amplification occurs within each droplet, resulting in beads each carrying ten million copies of a unique DNA template; (iii) the emulsion is broken, the DNA strands are denatured, and beads carrying single-stranded DNA clones are deposited into wells of a fibre optic slide; (iv) smaller beads carrying immobilized enzymes required for pyrophosphate sequencing are deposited into each well. **(B)** Microscope photograph of emulsion showing both droplets containing a bead and empty droplets. The thin arrow points to a 28 µm bead, the thick arrow points to an approximately 100 µm droplet. **(C)** SEM photograph of portion of a fibre optic slide, showing fibre optic cladding and wells prior to bead deposition (from:Margulies et al., Nature, 2005).

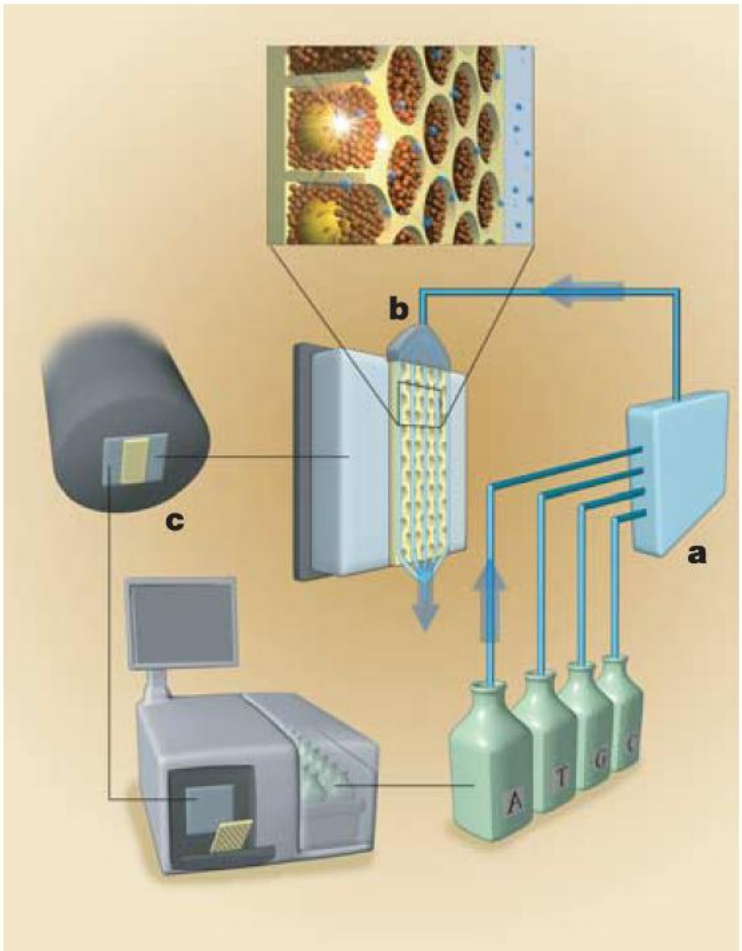


Fig.1.26.454-pyrosequencing: sequencing instrument. The sequencing instrument consists of the following major subsystems: a fluidic assembly (a), a flow chamber that includes the well-containing fibre-optic slide (b), a CCD camera-based imaging assembly (c), and a computer that provides the necessary user interface and instrument control (from:Margulies et al., Nature, 2005).

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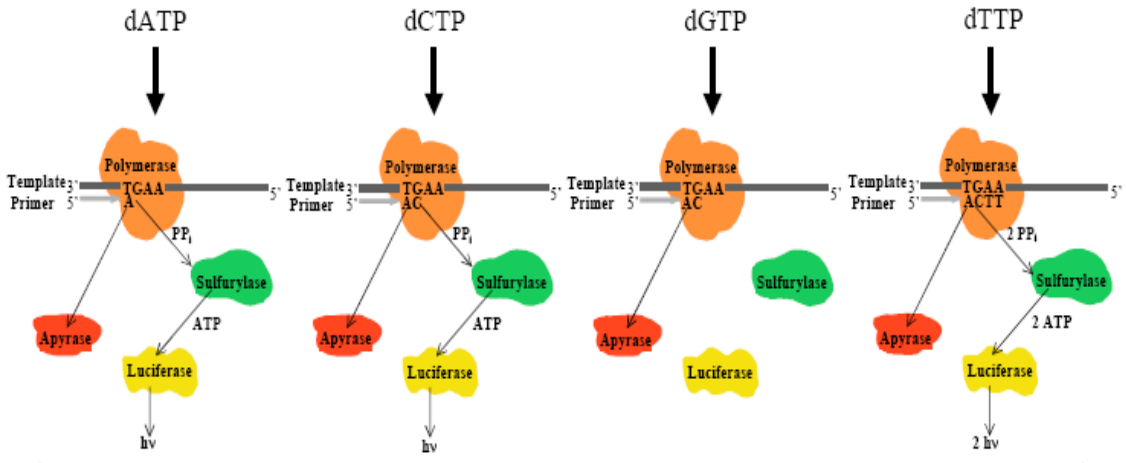
A)



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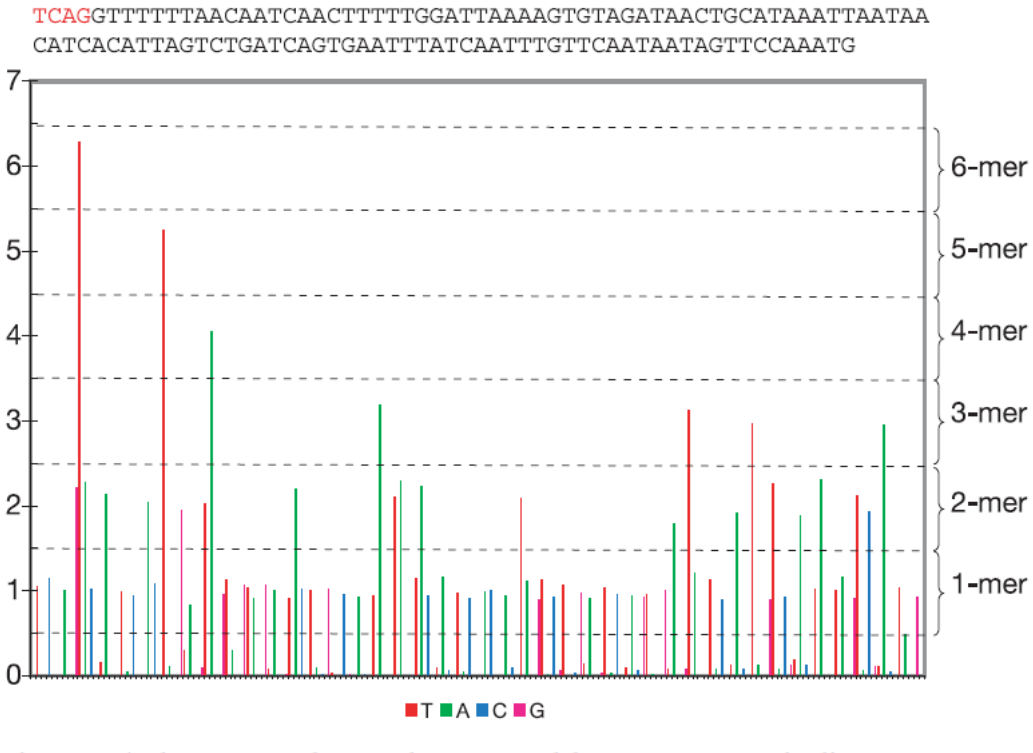

Fig.1.27. 454-Pyrosequencing: pyrophosphate sequencing reaction and flowgram of a 113-bases read from an M. genitalium run. A) Nucleotide incorporation is detected by the associated release of inorganic pyrophosphate (PPi) and the generation of photons. B)Nucleotides are flowed in the order T, A, C, G. The sequence is shown above the flowgram. The signal value intervals corresponding to the various homopolymers are indicated on the right. The first four bases (in red, above the flowgram) constitute the 'key' sequence, used to identify wells containing a DNA-carrying bead (from:Margulies et al., Nature, 2005).

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1.4.4. Interpretation of genotypic resistance profiles

1.4.4a PIs

More mutations are selected by the PI than by any other ARV class. The effect of PI resistance mutations on individual PI may be difficult to quantify when many mutations are present in the same virus isolate or when mutations occur in unusual patterns. The effect of PI resistance mutations on drug susceptibility can also be modulated by *gag* cleavage site mutations and possibly other parts of *gag* that influence Gag-Pol processing. Although multiple protease mutations are often required for HIV-1 to develop clinically significant resistance to a ritonavir-boosted PI (Condra, et al., 1996; Molla, et al., 1996; Kempf, et al., 2001), some mutations indicate that a particular PI, even when boosted, may not be effective. Many protease mutations are accessory, compensating for the replication impairment of other PI resistance mutations or reducing PI susceptibility only in combination with other PI resistance mutations.

Indeed, 17 largely non-polymorphic positions are associated with resistance with clinical significance. Mutations at 13 of these 17 positions have been shown to reduce susceptibility to one or more PI, including mutations at the substrate cleft positions 23, 30, 32, 47, 48, 50, 82, and 84, the flap positions 46 and 54, and interior enzyme positions 76, 88, and 90. Mutations at four of these 17 positions (Yahi, et al., 2000; Svicher, et al., 2006; Patick, et al., 1996; Bennett, et al., 2005) are included because they are non-polymorphic, occur commonly, and have disparate effects on different PI (Rhee, et al., 2006). Whereas many mutations reduce nelfinavir susceptibility, L23I, D30N, M46I/L, G48V/M, I84V, N88D/S, and L90M are relative contraindications to the use of nelfinavir in that an inferior virologic response to therapy relative to that obtainable with most other PI would be expected (Vray, et al., 2003; Patick, et al., 1996; Lawrence, et al., 1999; Walmsley, et al., 2001; Casado, et al., 2001; Johnston, et al., 2004; Winters, et al., 2008). I50L and N88S and possibly I84V, are relative contraindications for the use of atazanavir/r 23,61, (Vermeiren, et al., 2007; Rhee, et al., 2006; Colonno, et al., 2004). G48V/M, I84V, and L90M are relative

contraindications to the use of saquinavir/r (Zolopa, et al., 1999; Marcelin, et al., 2007; Marcelin, et al., 2004). V32I, I47V/A, I54L/M, and I84V are relative contraindications to the use of fosamprenavir/r (Winters, et al., 2007; Dandache, et al., 2007; Pellegrin, et al., 2007; Masquelier, et al., 2008). Mutations at position 82 as well as I84V may be relative contraindications to the use of indinavir/r. There are few known contraindications to the salvage therapy PI (lopinavir/r, tipranavir/r, darunavir/r), except V47A for lopinavir/r (Dandache, et al., 2007; Friend, et al., 2004; Kagan, et al., 2005) and V82L/T for tipranavir/r (Baxter, et al., 2006). At six of the 17 PI resistance mutations, only a single mutation has been shown to be associated with PI resistance – L23I, L24I, D30N, V32I, L76V, and L90M. At 11 positions, different mutations are associated with PI resistance, and at positions 50, 54, 82, and 88 these differences can be responsible for dramatically different effects on PI susceptibility. Additional, uncommon, include L33I, M46V, F53Y, I54S, G73C/A, V82M/C, and N88T/G23,41,185. V82I, which does not contribute to PI resistance, is a polymorphism that is the consensus residue for subtype G isolates. L33V is another polymorphism that is not associated with PI therapy or resistance. L33F and M46I/L, although non-polymorphic in most subtypes, occur at a prevalence of about 0.5-1% in subtype A and CRF01_AE isolates (http://hivdb.stanford.edu/cgi-bin/MutPrevBySubtypeRx.cgi)148.

Several resistance mutations are associated with increased susceptibility to one or more PI, including I50L which increases susceptibility to all PI other than atazanavir (Colonno, et al., 2004), I50V and I54L which increase tipranavir susceptibility (Elston, et al., 2006), N88S which increases fosamprenavir susceptibility (Ziermann, et al., 2000), and L76V which increases susceptibility to atazanavir, saquinavir, and tipranavir (Vermeiren, et al., 2007; Braun, et al., 2007)

Mutations at positions 10, 20, 36, 63, and 71 upregulate protease processivity to compensate for the decreased fitness associated with the major PI resistance mutations (Mammano, et al., 1998; Nijhuis, et al., 1999; Martinez-Picado, et al., 1999; Hoffman, et al., 2003). Positions 20, 36, and 63 are highly polymorphic. In contrast, L10I/V and A71V/T occur in 5 and 10%, respectively, of PI-

naive patients, and in a much higher proportion of PI-treated patients, while L10F/R and A71I/L do not occur in the absence of PI therapy (Rhee, et al., 2003). In one retrospective study, baseline mutations at positions 10 and 36 were associated with an increased risk of virologic failure in patients receiving older PI-based regimens containing nelfinavir or an unboosted PI 194,195 (Perno, et al., 2001; Perno, et al., 2004). Additional PI-selected accessory mutations include the highly polymorphic mutations I13V, D60E, I62V, V77I and I93L, and many uncommon non-polymorphic mutations including V11I, E34Q, E35G, K43T, K45I, K55R, Q58E, T74P/A/S, V75I, N83D, P79A/S, I85V, L89V, T91S, Q92K and C95F (Vermeiren, et al., 2007; Rhee, et al., 2005; Ceccherini-Silberstein, et al., 2004; Parkin, et al., 2003; Svicher, et al., 2005). Several of the nonpolymorphic mutations have become part of the genotypic susceptibility scores for tipranavir/r (E35G, K43T, Q58E, T74P, and N83D) and darunavir (V11I, T74P, and L89V), based on analyses of the RESIST (Baxter, et al., 2006; Scherer, et al., 2007) and POWER and DUET (de Meyer, et al., 2008; de Meyer, et al., 2008) clinical trials. These mutations, however, have not been evaluated for their effects on other PI, but their presence at baseline in these two clinical trials for heavily PI-experienced patients suggests that they are also associated with decreased susceptibility to the older PI.

The *gag* gene codes for the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins, a protein of uncertain function, p6, and two spacer peptides: p2 (between CA and NC) and p1 (between NC and p6). The gag polypeptide is cleaved at the MA/CA, CA/p2, p2/NC, NC/p1, and p1/6 junctions. A stem-loop structure between p1 and p6 stimulates the frame shifting necessary to create the Gag-Pol polypeptide. The residues surrounding each protease cleavage site are designated 5'-P4, P3, P2, P1/P1', P2', P3', P4'-3'.

Mutations that improve the kinetics of PI-resistant proteases emerge at several protease cleavage sites during PI treatment (Dauber, et al., 2002; Prabu-Jeyabalan, et al., 2004; Coté, et al., 2001). Most *gag* cleavage site mutations occur at NC/p1 and p1/p6– sites (Coté, et al., 2001; Malet, et al.,

2007) at which cleavage may be rate limiting for gag and Gag-Pol polyprotein processing (Pettit, et al., 2005). A431V, at the P2 position of NC/p1, is associated with mutations at protease positions 24, 46, and 82 (Zhang, et al., 1997; Verheyen, et al., 2006). L449F, at the P1' position of p1/p6, is associated with the protease mutation pair D30N/ N88D and with I84V (Kolli, et al., 2006; Verheyen, et al., 2006)209,210. P453L, at the P5' position of the p1/p6 site, is associated with protease mutations at positions 32 (Carrillo, et al., 1998), 47 (Carrillo, et al., 1998), 50 (Maguire, et al., 2002), 84, and 90 (Bally, et al., 2000; Verheyen, et al., 2006).

Naturally occurring polymorphisms in the different protease subtypes often occur at sites of accessory PI resistance mutations in subtype B isolates (Parkin, et al., 2004). For example, the accessory PI resistance mutations I13V, K20I, M36I, and I93L represent the consensus variant in one or more non-B subtypes (Rhee, et al., 2006). Although these mutations may result in subtle structural and biochemical differences among subtypes (Velazquez-Campoy, et al., 2001; Holguin, et al., 2004; Sanches, et al., 2007), the vast majority of *in vitro* and *in vivo* studies suggest that the licensed PI are as active against wild-type non-B viruses as they are against wild-type subtype B viruses (Parkin, et al., 2004; Geretti, 2006). With several notable exceptions, the genetic mechanisms of PI resistance are also highly similar among the different subtypes (Kantor, et al., 2005). Although both D30N and L90M occur in non-B viruses during nelfinavir therapy, D30N occurs more commonly in subtype B viruses and L90M occurs more commonly in subtype C, F, G, and CRF01_AE viruses (Abecasis, et al., 2005; Calazans, et al., 2005; Cane, et al., 2001; Grossman, et al., 2004; Sugiura, et al., 2002). The increased predilection for certain subtypes to develop L90M may relate to the presence of variants other than L (the subtype B consensus) at position 89 (Abecasis, et al., 2005; Calazans, et al., 2005; Gonzales, et al., 2003). Similarly, T74S, a polymorphism that occurs in 8% of subtype C sequences, but rarely in other subtypes, is associated with reduced susceptibility to nelfinavir (Rhee, et al., 2006; Deforche, et al., 2006). The fact that V82I is the consensus amino acid for subtype G affects the spectrum of mutations observed at this

position in PI-resistant subtype G isolates: V82T and the rare mutation V82M occur more frequently than V82A in subtype G isolates because T and M require a single base pair change, whereas A requires two base pair changes (Camacho, et al., 2005). However, for nearly all other subtypes and protease mutations, a similar number of nucleotide changes is required to convert a wild-type residue into one associated with drug resistance (Van De Vijver et al., 2006).

Table 1.3. Mutation in the protease gene associated with resistance to protease inhibitors (Johnson, et al., 2009)**.**

Drug	Position	Wild type	Mutations
Atazanavir +/– ritonavir[r]	10	L	I, F, V, C
	16	G	E
	20	K	R, M, I, T, V
	24	L	I
	32	V	I
	33	L	I, F, V
	34	E	Q
	36	M	I, L, V
	46	M	I, L
	48	G	V
	50	I	L
	53	F	L, Y
	54	I	L, V, M, T, A
	60	D	E
	62	I	V
	64	I	L, M, V
	71	A	V, I, T, L
	73	G	C, S, T, A
	82	V	A, T, F, I
	84	I	V
	85	I	V
	88	N	S
	90	L	M
	93	I	L, M
Darunavir/ ritonavir[s]	11	V	I
	32	V	I
	33	L	F
	47	I	V
	50	I	V
	54	I	M, L
	74	T	P
	76	L	V
	84	I	V
	89	L	V
Fosamprenavir/ ritonavir	10	L	F, I, R, V
	32	V	I
	46	M	I, L
	47	I	V
	50	I	V
	54	I	L, V, M
	73	G	S
	76	L	V
	82	V	A, F, S, T
	84	I	V
	90	L	M
Indinavir/ ritonavir[t]	10	L	I, R, V
	20	K	M, R
	24	L	I
	32	V	I
	36	M	I
	46	M	I, L
	54	I	V
	71	A	V, T
	73	G	S, A
	76	L	V
	77	V	I
	82	V	A, F, T
	84	I	V
	90	L	M
Lopinavir/ ritonavir[u]	10	L	F, I, R, V
	20	K	M, R
	24	L	I
	32	V	I
	33	L	F
	46	M	I, L
	47	I	V, A
	50	I	V
	53	F	L
	54	I	V, L, A, M, T, S
	63	L	P
	71	A	V, T
	73	G	S
	76	L	V
	82	V	A, F, T, S
	84	I	V
	90	L	M
Nelfinavir[t,v]	10	L	F, I
	30	D	N
	36	M	I
	46	M	I, L
	71	A	V, T
	77	V	I
	82	V	A, F, T, S
	84	I	V
	88	N	D, S
	90	L	M
Saquinavir/ ritonavir[t]	10	L	I, R, V
	24	L	I
	48	G	V
	54	I	V, L
	62	I	V
	71	A	V, T
	73	G	S
	77	V	I
	82	V	A, F, T, S
	84	I	V
	90	L	M
Tipranavir/ ritonavir[w]	10	L	V
	13	I	V
	20	K	M, R
	33	L	F
	35	E	G
	36	M	I
	43	K	T
	46	M	L
	47	I	V
	54	I	A, M, V
	58	Q	E
	69	H	K
	74	T	P
	82	V	L, T
	83	N	D
	84	I	V
	90	L	M

1.4.4b NRTIs

M184V

M184V is the most commonly occurring NRTI resistance mutation. In vitro, it causes high-level resistance to lamivudine (3TC) and emtricitabine (FTC), low-level resistance to didanosine (ddI) and abacavir, (ABC) and increased susceptibility to zidovudine (ZDV), stavudine (d4T), and tenofovir (TDF) (Whitcomb, et al., 2002). The possibility that isolates with M184V are compromised was suggested by the initial 3TC monotherapy studies showing that plasma HIV-1 RNA levels remained about 0.5 log10 copies below baseline in patients receiving lamivudine for 6-12 months, despite the development of M184V and a high level of phenotypic resistance to 3TC (Eron et al., 1996; Ingrand, et al., 1995; Pluda, et al., 1995). Data from multiple 3TC-containing dual-NRTI regimens also suggest that 3TC continues to exert an antiviral effect even in patients whose virus isolates contain M184V (Miller, et al., 2002; Diallo, et al., 2003; Vray, et al., 2003). M184V causes a median 1.5-fold and 3.0-fold reduction in susceptibility to ddI and ABC, respectively, in the PhenoSenseGT™ assay (Monogram Biosciences) (Petropoulos, et al., 2000; Rhee, et al., 2004). These are levels of reduction that are above the wildtype range but below the level at which these NRTI are inactive (Petropoulos, et al., 2000). Several clinical trials have also shown that ABC and ddI retain clinical activity in the presence of M184V (Brun-Vézinet, et al., 2003; Winters, et al., 2003; Lanier, et al., 2004; Marcelin, et al., 2004; Eron, et al., 2007; Molina, et al., 2005). For example, the addition of ddI or ABC to the regimen of a patient with virologic failure has been associated to plasma HIV-1 RNA reductions of 0.6 and 0.7 log10, respectively, in patients harboring viruses with M184V and no other drug resistance mutations M184V (Lanier, et al., 2004; Molina, et al., 2005). The phenotypic and clinical significance of M184V is influenced by the presence or absence of other NRTI resistance mutations. For example, the presence of K65R or L74V in combination with M184V is sufficient for high-level resistance to both ABC and ddI16. In contrast, three or more TAM plus M184V are required for high-level ABC and ddI resistance

(Whitcomb, et al., 2003; Rhee, et al., 2004; Lanier, et al., 2004; Marcelin, et al., 2005; Vermeiren, et al., 2007).

Thymidine analog mutations

Thymidine analog mutations are selected by the thymidine analogs ZDV and d4T. Thymidine analog mutations decrease susceptibility to these NRTI and to a lesser extent to ABC, ddI, and TDF8. Thymidine analog mutations are common in low-income countries in which fixed-dose combinations containing thymidine analogs are the mainstays of therapy. Thymidine analog mutations are also common in viruses from persons who began therapy in the pre-HAART era with incompletely suppressive thymidine analog-containing regimens, but are becoming less common in areas in which the fixed-dose combinations of TDF/FTC and ABC/3TC have become the most common NRTI backbones. However, even in these areas, TAM and in particular the partial T215 revertants remain the most common type of transmitted NRTI resistance mutation (Bennett et al., 2005; Fessel et al., 2008). Thymidine analog mutations accumulate in two distinct but overlapping patterns (Yahi, et al., 1999; Gonzales, et al., 2003; Marcelin, et al., 2004; Miller, et al., 2004; Cozzi-Lepri, et al., 2005; De Luca, et al., 2007). The type I pattern includes the mutations M41L, L210W, and T215Y. The type II pattern includes D67N, K70R, T215F, and K219Q/E. Mutation D67N also occurs commonly with type I TAM (Cozzi-Lepri, et al., 2005; Rhee, et al., 2007). However, K70R and L210W rarely occur together (Yahi, et al., 2000). Type I TAM cause higher levels of phenotypic and clinical resistance to the thymidine analogs and cross-resistance to ABC, ddI, and TDF than do the type II TAM. Indeed, the presence of all three type I TAM markedly reduces the clinical response to ABC, ddI, and TDF (Lanier, et al., 2004; Miller, et al., 2004; Cozzi-Lepri, et al., 2005; Marcelin, et al., 2006; De Luca, et al., 2006). The clinical significance of the type II TAM is not as well characterized. Other mutations at several of the TAM positions are common. The most common of these are the partial T215 revertants T215C/D/E/I/S/V (Yerly, et al., 1998; Garcia-Lerma, et al., 2001). These mutations arise from the drug resistance mutations T215Y/F to increase

HIV-1 fitness in the absence of selective drug pressure. They occur more commonly than reversion to the wild-type T because most of the partial T215 revertants require only a single nucleotide mutation rather than the double nucleotide mutation required for Y or F to revert to T. The partial T215 revertants do not reduce drug susceptibility by themselves, but their presence in a previously untreated patient suggests that the patient may have been infected originally with a virus containing T215Y or F. Both K219N/R are two variants that unlike K219Q/E usually occur with type I rather than type II TAM (Rhee, et al., 2007). Interestingly, two variants at position 70, K70E/G, are not selected by thymidine analogs and have phenotypic effects diametrically opposite to those of K70R, decreasing ABC, ddI, TDF, 3TC, and FTC susceptibility, and increasing ZDV susceptibility (Delaugerre, et al., 2001; Sluis-Cremer, et al., 2007; Bradshaw, et al., 2007). Both D67G and D67E are selected by NRTI therapy, but their phenotypic and clinical significance are not well characterized (Rhee, et al., 2005). E44D/A and V118I are accessory mutations that generally occur with type I TAM. These mutations occur in about 1% of viruses from untreated patients and in a significantly higher proportion of viruses from patients receiving NRTI (Gonzales, et al., 2003; Delaugerre, et al., 2001; Montes, et al., 2002). Although E44D plus V118I were first shown to cause low-level 3TC resistance when they occur in combination (Hertogs, et al., 2000), subsequent studies have suggested that in combination with TAM, these mutations reduce the susceptibility and clinical activity of most NRTI (Marcelin, et al., 2005; Montes, et al., 2002; Perno, et al., 2001; Walter, et al., 2002; Romano, et al., 2002; Stoeckli, et al., 2002; Girouard, et al., 2003; Säberg, et al., 2004; Gianotti, et al., 2006; Zaccarelli, et al., 2007). F214L is a common polymorphism that is negatively associated with type I TAM, and as a consequence may raise the genetic barrier to resistance in viruses developing type I TAM (Svicher, et al., 2006; Ceccherini-Silberstein, et al., 2007).

Mutations occurring in the absence of thymidine analogs

The most common mutations in patients developing virologic failure while receiving a non thymidine analogcontaining NRTI backbone include M184V alone or M184V in combination with K65R or L74V (Moyle, et al., 2005; Gallant, et al., 2006; Eron, et al., 2006). K65R causes intermediate resistance to TDF, ABC, ddI, 3TC, and FTC, low-level resistance to d4T, and increased susceptibility to ZDV (Lanier, et al., 2004; Antinori, et al., 2007; Staszewski et al.,2006). L74V causes intermediate resistance to ddI and ABC, and a slight increase in susceptibility to ZDV and TDF (Rhee, et al., 2006). L74I has similar phenotypic properties to L74V, but is found primarily in viruses with multiple TAM, possibly because it increases ZDV and TDF susceptibility less than L74V (Wirden, et al., 2006; Berkhout, et al., 2006). Mutations M184V plus K65R have been reported primarily in patients receiving the NRTI backbone TDF/3TC (Gallant, et al., 2004; Margot, et al., 2006) and less commonly ABC/3TC (Moyle, et al., 2005; Sosa, et al., 2005) or TDF/ FTC 56,67. M184V plus L74V occurs primarily in persons receiving ABC/3TC or ddI/3TC/FTC backbones (Moyle, et al., 2005; Sosa, et al., 2005; Descamps, et al., 2006). K65R and L74V rarely occur in the same viruses; however, several patients developing virologic failure with L74V while receiving an ABC or ddI-containing regimen have been found to have minor variants containing K65R (Descamps, et al., 2006; Svarovskaia, et al., 2007). There is a bidirectional antagonism between K65R and the TAM. K65R interferes with TAM-mediated primer unblocking and the TAM interfere with K65R mediated NRTI discrimination (Parikh, et al., 2006; Parikh, et al., 2007). As a result, viruses containing K65R in combination with TAM are uncommon (Parikh, et al., 2006). The emergence of K65R is suppressed to a greater extent in regimens containing ZDV compared with d4T (Staszewski et al., 2006; Shafer, et al., 1994; Røge, et al., 2003; Shafer, et al., 2003; Rey, et al., 2006; Masquelier, et al., 2006; Elion, et al., 2006; Stürmer, et al., 2007). Less common mutations occurring during virologic failure with non thymidine analog regimens include K65N, K70E/G, and Y115F (Delaugerre, et al., 2005; Bradshaw, et al., 2007; Moyle, et al., 2005;

Ross, et al., 2006; Bartlett, et al., 2006). K65N and K70E/G have a resistance profile similar to K65R, but appear to cause less resistance than K65R to ABC, ddI, TDF, 3TC, and FTC (Delaugerre, et al., 2005; Sluis-Cremer, et al., 2007; Bradshaw, et al., 2007; Ross, et al., 2006; Ross, et al., 2006; Van Huotte et al. 2006). Y115F reduces ABC susceptibility (Tisdale, et al., 1997) and causes low-level cross-resistance to TDF (Vermeiren, et al., 2007; Lanier, et al., 2004; Rhee, et al., 2006; Margot, et al., 2005). Although T69D and V75T were originally identified as causing resistance to ddC (Fitzgibbon, et al., 1992) and d4T (Lacey, et al., 1994), respectively, a range of mutations at these positions (e.g. T69N/S/I/G and V75M/A) have been associated with reduced susceptibility to other NRTI, including ddI and d4T (Vermeiren, et al., 2007; Lacey, et al., 1994; Winters, et al., 2001; Selmi, et al., 2001; Lennerstrand, et al., 2001). Two lines of evidence suggest that K65R may occur more commonly in non subtype B compared with subtype B viruses. K65R has emerged more rapidly during the in vitro passage of subtype C compared with subtype B isolates in the presence of increasing TDF concentrations (Brenner, et al., 2006). Anecdotal reports have also suggested that K65R may occur more commonly in low-income countries when patients with non-B subtype viruses are treated with d4T/ddI and d4T/3TC (Doualla-Bell, et al., 2006) (Hawkins et al. 2007) or TDF/3TC (Rey et al., 2007).

Multi-nucleoside resistance mutations

Amino acid insertions at codon 69 generally occur in the presence of multiple TAM, and in this setting are associated with intermediate resistance to 3TC and FTC and high-level resistance to each of the remaining NRTI (Winters, et al., 1998; Van Vaerenbergh, et al., 2000; Masquelier, et al., 2001; McColl, et al., 2004; Prado, et al., 2004). Q151M is a 2-bp mutation (CAG→ATG) that is usually accompanied by two or more of the following mutations: A62V, V75I, F77L, and F116Y. The Q151M complex causes high-level resistance to ZDV, d4T, ddI, and ABC, and intermediate resistance to TDF, 3TC, and FTC (Rhee, et al., 2006; Shirasaka, et al., 1995; Iversen, et al., 1996). This complex developed in 5% of patients who received ddI in combination with ZDV or d4T (Van

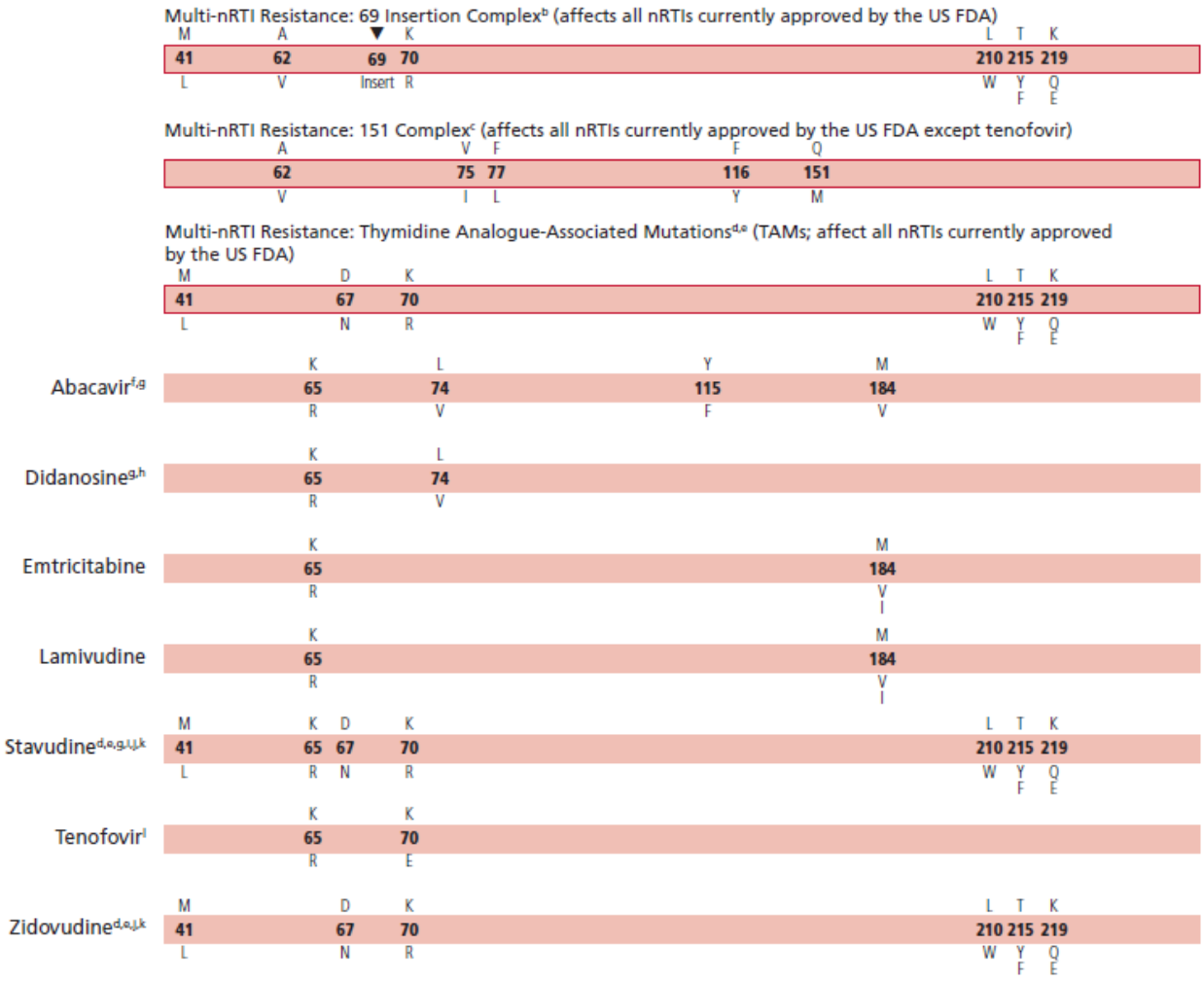
Vaerenbergh, et al., 2000; Shafer, et al., 1995) but is rarely selected by 3TC- or FTC-containing regimens. Q151M may be uncommon because the two intermediate amino acids Q151L (CAG→CTG) and Q151K (CAG→AAG) are poorly replicating and rarely observed (Kosalaraksa, et al., 1999; García-Lerma, et al., 2000; Dykes, et al., 2007). Q151M is a common genetic mechanism of NRTI resistance in HIV-2-infected persons (Rodés, et al., 2000; Brandin, et al., 2003; Gallego, et al., 2003). The optimal NRTI combination to use in patients with codon 69 insertions or Q151M is not known (Gallego, et al., 2003; Zaccarelli, et al., 2004).

Miscellaneous mutations Mutations

K43E/Q/N, E203D/K, H208Y, D218E, H221Y, K223Q, and L228H/R are non-polymorphic NRTIselected mutations which generally follow TAM and which have subtle effects on HIV-1 NRTI susceptibility and replication (Gonzales, et al., 2003; Rhee, et al., 2006; Saracino, et al., 2006). Q145M is a rare mutation that has been reported by one group to reduce susceptibility to multiple NRTI and NNRTI (Paolucci, et al., 2004; Paolucci, et al., 2003). P157S, which is homologous to the mutation causing 3TC resistance in FIV, has been reported once in an HIV-1 isolate (Smith, et al., 1999; Picard, et al., 2001). Several mutations in the connection and RNaseH domains of HIV-1 RT play an accessory role in reducing HIV-1 susceptibility in combination with TAM, most likely by slowing the activity of RNaseH and thereby allowing more time for TAM-mediated primer unblocking (Nikolenko, et al., 2005). The single most important of these mutations may be N348I, a non-polymorphic mutation that occurs in about 10% of NRTI-treated patients (Yap, et al., 2007). N348I causes a twofold reduction in ZDV susceptibility when it occurs in combination with multiple TAM (Yap, et al., 2007) G333E/D, A360T, and A371V, mutations with similar phenotypic effects, occur in about 5% of NRTI-naive and 10% of NRTI-treated patients (Kemp, et al., 1998; Nikolenko, et al., 2007; Brehm, et al., 2007; Zelina, et al., 2008). Although several RNaseH mutations may potentially reduce ZDV susceptibility in combination with TAM

(Delviks-Frankenberry, et al., 2007), few have been observed in clinical isolates (Roquebert, et al., 2007; Ntemgwa, et al., 2007).

Table 1.4. Mutation in the RT gene associated with resistance to NRTIs (Johnson, et al., 2009)**.**



1.4.4c NNRTIs

The NNRTI resistance mutations can be classified into the following categories: (i) primary NNRTI resistance mutations that cause high-level resistance to one or more NNRTI and that are among the first to develop during NNRTI therapy; (ii) secondary NNRTI resistance mutations that usually occur in combination with primary NNRTI resistance mutations, but that also have clinically significant implications for choosing an NNRTI, particularly etravirine; (iii) minor non-polymorphic mutations that may occur alone or in combination with other NNRTI resistance mutations and that cause consistent but low-level reductions in NNRTI susceptibility; and (iv) polymorphic accessory mutations that modulate the effects of other NNRTI resistance mutations(Table 1.4).

Each of the primary NNRTI resistance mutations – K103N/S, V106A/M, Y181C/I/V, Y188L/C/H, and G190A/ S/E – cause high-level resistance to nevirapine and variable resistance to efavirenz, ranging from about twofold for V106A and Y181C, sixfold for G190A, 20-fold for K103N, and more than 50-fold for Y188L and G190S (Rhee, et al., 2006; Bacheler, et al., 2001; Vingerhoets, et al., 2005). Although transient virologic responses to an efavirenz-based salvage therapy regimen occur in some NNRTI-experienced patients, a sustained response has been uncommon (Shulman, et al., 2003; Antinori, et al., 2002; Delaugerre, et al., 2001). In contrast, patients with any single one of the primary NNRTI resistance mutations may benefit from etravirine salvage therapy, although the mutations at position 181 and to a lesser extent 190 compromise etravirine response and may provide the foundation for the development of high-level etravirine resistance (Madruga, et al., 2007; Lazzarin, et al., 2007; Vingerhoets, et al., 2007). L100I, K101P, P225H, F227L, M230L, and K238T are secondary mutations that usually occur in combination with one of the primary NNRTI resistance mutations. L100I and K101P, which occur in combination with K103N, further decrease nevirapine and efavirenz susceptibility from 20-fold with K103N alone to more than 100-fold

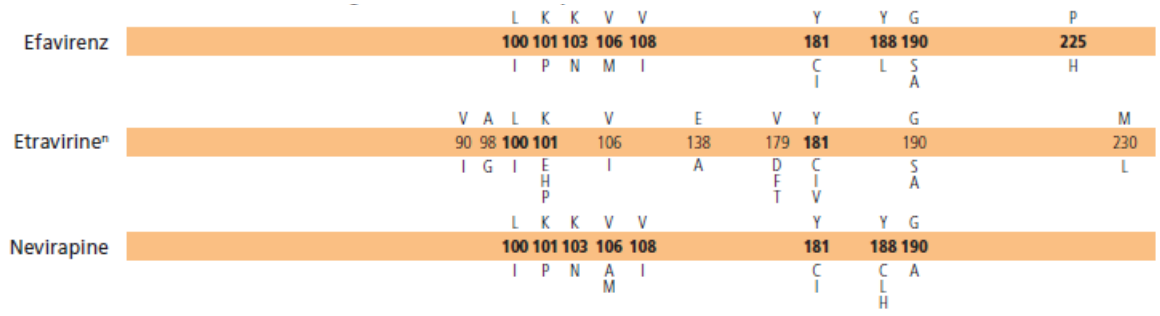
(Rhee, et al., 2006). Although viruses with K103N are fully susceptible to etravirine, viruses with L100I plus K103N display about 10-fold decreased susceptibility (Vingerhoets, et al., 2005).

P225H and K238T/N usually occur in combination with K103N and synergistically reduce nevirapine and efavirenz susceptibility (Bacheler, et al., 2001; Pelemans, et al., 1997; Parkin, et al., 2006). F227L nearly always occurs in combination with V106A, leading to synergistic reductions in nevirapine susceptibility (Balzarini, et al., 1998). M230L, which may occur alone, decreases the susceptibility of all NNRTI including etravirine by 20-fold or more (Vingerhoets, et al., 2005; Huang, et al., 2000). V179F, F227C, L234I, and L318F are rare mutations that are of increased importance now that etravirine is licensed. V179F occurs solely in combination with Y181C/I/V and acts synergistically to increase etravirine resistance from fivefold to 10-fold with Y181C/I/V alone to more than 100-fold (Vingerhoets, et al., 2005) . F227C, an exceedingly rare mutation, reduces etravirine susceptibility 10-fold to 20-fold (Vingerhoets, et al., 2005; Su, et al., 2007). L234I, which has been selected *in vitro* by etravirine, acts synergistically with Y181C to reduce etravirine susceptibility (Vingerhoets, et al., 2005). L318F, which was first reported to reduce delavirdine and nevirapine susceptibility by 15-fold and threefold, respectively (Harrigan, et al., 2002), has also been selected *in vitro* by etravirine and found to reduce etravirine susceptibility synergistically with Y181C (Vingerhoets, et al., 2005).

A98G, K101E, V108I, and V179D/E are common NNRTI resistance mutations that reduce susceptibility to nevirapine and efavirenz about twofold to fivefold (Rhee, et al., 2003). Although K103R alone, which occurs in about 1% of untreated persons, has no effect on NNRTI susceptibility, the combination of K103R plus V179D reduces nevirapine and efavirenz susceptibility by 15-fold (Parkin, et al., 2006). Data are not available on the effect of these mutations on etravirine susceptibility. V179D, and rarely A98G and V108I, are observed in patients who have never been treated with NNRTI (Shafer, et al., 2007). The optimal management of patients with viruses containing these mutations is not known. Although low-level baseline

resistance has not been shown to decrease the virologic responses to first-line NNRTI-containing regimens (Harrigan, et al., 2003), efavirenz and etravirine may be preferable to nevirapine because these NNRTI have generally been more active than nevirapine against these and other NNRTI-resistant variants (Bannister, et al., 2008; Ren, et al., 2001).

Table 1.4. Mutation in the RT gene associated with resistance to NNRTIs (Johnson, et al., 2009)**.**



1.4.4d INIs

As a result of clinical trial data, 3 distinct resistance pathways associated with raltegravir virologic failure have been identified: Y143R/C, Q148K/R/H, and N155H (Cooper, et al., 2008; Hazuda, et al., 2007) .52,61 In a retrospective analysis of the BENCHMRK trials, mutations in the integrase gene were found in 68% of patients receiving raltegravir who experienced virologic failure (Cooper, et al., 2008). A total of 105 patients were identified (n = 462), 94 of whom had baseline and failure samples available for analysis. No genetic changes in the integrase enzyme were found in 30 patients, while the remaining 64 had genotypic evidence of resistance. Most (48/64) patients had multiple mutations present at virologic failure, and longitudinal analyses of samples indicated an accumulation of mutations over time. Moreover, there appeared to be a shift toward mutations that conferred high-level resistance (ie, Q148R/C). At virologic failure, the proportion of patients with Q148 mutations was 27%, whereas 53% showed these mutations on post-virologic failure analysis. The opposite was true for N155H expression: the proportion of patients with N155H mutations decreased from 45% at virologic failure to 18% at postvirologic failure. A lower baseline viral load (<100,000 copies/mL), higher genotypic sensitivity scores, and the presence of other

active agents decreased the likelihood of developing raltegravir resistance. In 35 isolates from patients experiencing virologic failure in the Phase 2 Protocol 005 study, the presence of N155H (14/35) and Q148H/R/K (20/35) mutations predominated (Grinsztejn, et al., 2007). N155H decreased viral susceptibility to raltegravir by 10- fold, while Q148H/R/K decreased susceptibility 25-fold (Hazuda, et al., 2007). The presence of additional mutations including L74M, E92Q, and G163R combined with N155H or the presence of E138K and G140S/A combined with Q148H/R/K led to high levels of resistance. Again, lower baseline viral loads (<100,000 copies/mL), or 1 or more active agent in the OBR decreased the likelihood of accumulating resistance.

The existence of distinct integrase resistance profiles is similar to what has been described for other antiretroviral classes. However, it is unknown what the determinants of the evolution toward these different profiles are.

The potential role of natural occurring polymorphisms in HIV-1 integrase may have clinical and virologic implications for integrase inhibitors, and in clinical practice has yet to be established. In this context, HIV-1 group and subtype differences may also have an impact on the evolution of resistance to integrase inhibitors, as has been described for protease inhibitors, nucleoside reverse transcriptase inhibitors, and nonnucleoside reverse transcriptase inhibitors (Brenner, et al., 2006; Calazans, et al., 2005; Doualla-Bell, et al., 2006; Grossman, et al., 2004). Hackett, et al., by analyzing 1,304 sequences from group M, N, and O viruses, have recently reported that some of the mutations associated with resistance to raltegravir and/or elvitegravir, such as L74M, T97A, E157Q, as well as other integrase inhibitor resistance mutations (V165I, V201I, T206S) occurred as natural polymorphisms (≥ 1%) and occurred differently according to different HIV-1 subtype/circulating recombinant form/group (Hackett, et al., 2008). Similarly, Rhee, et al., by analyzing more than 1,500 published integrase sequences of group M, showed that some secondary mutations associated with resistance to raltegravir and/or elvitegravir, such as L74M, T97A, V151I, E157Q, G163K/R, and S230N, occurred differently according to different HIV-1

subtypes/circulating recombinant form (Rhee, et al., 2008). In some cases, the prevalence was > 10% in specific subtypes (T97A only in subtype A; V151I and S230N only in subtype B; G163K/R only in subtype F). The mutation E157Q occurred in about 2-4% of integrase inhibitor-naive patients with subtype B, AG, and D. In addition, the comparison of integrase amino acid sequences between subtype B and CRF02-AG showed that 13 positions (K/R14, V/I31, L/I101, T/V112, T/A124, T/A125, G/N134, I/V135, K/T136, V/I201, T/S206, L/I234, S/G283) differed between the HIV-1 integrase of these two subtypes (Malet, et al., 2008).

In addition, analyses in small cohorts of HAART multi-experienced patients suggest that some integrase polymorphisms at baseline (K156N, M50I, S17N, D256E, T206S, E157Q) would be associated with different virologic response to raltegravir (Ceccherini-Silberstein, et al., 2008; Da Silva, et al., 2008; Ceccherini-Silberstein, et al., 2009; Miller, et al., 2009; Low, et al., 2009) but the role of integrase polymorphisms in IntSti naïve patients is still unclear.

Analysis of treatment-emergent elvitegravir resistance is currently limited to 28 of 30 patients with documented virologic failure at 24 weeks of a Phase 2 randomized, dose finding study in ART-experienced patients (Da Silva, et al., 2008). Substitutions at E92Q, E138K, Q148R/K/H, or N155H were the most common mutations identified, and at least 1 of these was present in 39% of failure samples. Additionally, cross resistance to raltegravir was demonstrated, as an analysis of all virologic failures revealed that mean susceptibility declined by more than 151-fold for elvitegravir and more than 28-fold for raltegravir. Subsequent case reports and in vitro studies have also demonstrated that cross-resistance between elvitegravir and raltegravir is likely to occur (Ceccherini-Silberstein, et al., 2008; Hackett, et al., 2008).

1.4.4e FIs

Mutations in gp41 codons 36 to 45, the region to which enfuvirtide binds, are primarily responsible for enfuvirtide resistance (Melby, et al., 2006; Sista, et al., 2004; Menzo, et al., 2004; Marcelin, et

al., 2004; Mink, et al., 2005; Su, et al., 2006). A single mutation is generally associated with about 10-fold decreased susceptibility, whereas double mutations can decrease susceptibility more than 100-fold. Several accessory mutations in the HR2 region corresponding to the peptide sequence of enfuvirtide including N126K, N137K, and S138A appear to improve fitness in combination with specific mutations at positions 36-45 (Xu, et al., 2005; Baldwin, et al., 2004; Tolstrup, et al., 2007). Similar enfuvirtide resistance mutations appear to emerge in subtype B and non-B isolates (Cilliers, et al., 2005; D'Arrigo, et al., 2007). Enfuvirtide-resistant HIV-1 isolates replicate less well than enfuvirtide-susceptible isolates, as evidenced by *in vitro* competition studies (Lu, et al., 2004) and by the rapid reversion to wild-type that occurs in patients who discontinue enfuvirtide (Deeks, et al., 2007). There are some conflicting data on the clinical benefit of continued therapy in the presence of incomplete virologic suppression. One study showed that interruption of therapy was associated with a mean increase in plasma HIV-1 RNA levels of just 0.2 log10 and no decrease in CD4 count281. However, other studies have suggested that some enfuvirtide resistance mutations, particularly those at position 38, may be associated with CD4 count increases (Aquaro, et al., 2006), possibly because mutations at this position may decrease virus replication or render the virus more susceptible to neutralizing antibodies that target fusion intermediates (Reeves, et al., 2005).

1.4.5f CCR5 Inibitors

Viruses with high levels of CCR5 inhibitor resistance (> 1,000-fold reductions in IC50 as well as an MPI plateau) have been identified during *in vitro* passage experiments with most CCR5 inhibitors (Marozsan, et al., 2005; Westby, et al., 2007; Kuhmann, et al., 2004; Baba, et al., 2007; Ogert, et al., 2008). The amino acid changes responsible for resistance may be entirely within the V3 loop (Westby, et al., 2007; Kuhmann, et al., 2004), entirely outside of the V3 loop (Marozsan, et al., 2005), or may result from synergistic interactions between substitutions in the V3 loop and other parts of *env*302. These amino acid changes may include known polymorphisms as well as novel substitutions, insertions, and deletions. Further complicating the genetic basis of CCR5 inhibitor

resistance is the observation that the same inhibitor may select for different mutations in different virus isolates (Marozsan, et al., 2005; Westby, et al., 2007; Ogert, et al., 2008). The mechanisms of CCR5 inhibitor resistance *in vivo* may be even more complicated than those that have been observed to emerge *in vitro*. First, virus isolates from the majority of patients developing virologic failure while receiving maraviroc (Mori, et al., 2007) or vicriviroc (Tsibris, et al., 2007) have not demonstrated phenotypic resistance. Second, the few viruses with phenotypic resistance (four of 37 for maraviroc and one of seven for vicriviroc) have demonstrated only subtle MPI reductions rather than the MPI reductions and large increases in IC50 that have been observed during the emergence of resistance *in vitro*. Finally, the mutations that have been observed *in vivo* have been highly variable, differing for each virus isolate (Tsibris, et al., 2007; Mori, et al., 2007).

1.5 Rational of the work

As described in the previous paragraphs, novel class of ARVs, as Integrase inhibitors and CCR5 antagonists, were introduced in the clinical practice. The enriched battery of ARV's available by today and the increased clinical experience have substantially improved the clinical management of HIV-1 infection in terms of delaying disease progression, prolonging survival, and improving quality of life.

However, the role and clinical relevance either of the natural polymorphisms of HIV-1 integrase (IN), according to different subtype and recombinant form, or the pre-existing resistant minor quasispecies in InSti naive patients, are still unclear.

Therefore this study is focalized to explore the presence of InSti resistance mutations in HIV-1 quasispecies present in InSti-naïve patients and to evaluate their impact on in vitro phenotypic susceptibility to InSTIs, on replication capacities and on virologic response to raltegravir by 3 different approaches:

1) the **clonal methods**, based on cloning the RT-IN regions from patient samples into a HIV plasmid backbone RT-IN deleted, allowing generation of viral stocks, representing all the viral variants, and functional analysis of viral drug sensitivity in short-term culture (Clavel, et al., 2004; Van Baelen, et al., 2008). This approach was used to determinate the genotypic and phenotypic natural resistance to InSti.
2) the **population sequencing**, based on Sanger-sequencing method (Sanger, 1981) that although allows to detect mutations at 20-30% of viral specimens, it is the most standardized in large scale and cost-effective genotyping method (Hirsch, et al., 2008). By this approach the prevalence of all baseline integrase mutations (natural polymorphisms and known resistance mutations) according with virologic response at 24 weeks were evaluated in InSti naïve patients who started a raltegravir containing regimen.

3) the **Ultra-Deep-454-pyrosequencing (UDPS)**, that provides massive parallel sequencing producing complete genome coverage from a conserved sequence or an array of reads from mixed sequences (Simen, et al., 2009; Hoffmann, et al., 2007; Rozera, et al., 2009; Wang, et al., 2007). By this approach, the prevalence of mutations in HIV-1 quasispecies was explored and their impact on virologic response and development of mutations at failure were evaluated in InSti naïve patient who started a raltegravir containing regimen.

2. Methods

2.1. Clonal analysis

2.1.1 RNA isolation, cDNA synthesis and PCR.

Plasma samples were obtained from 49 HIV-1 subtype B-infected InSTI-naïve individuals; 21 were drug-naïve and 28 failed HAART (not containing InSTI). Viral RNA was extracted and cDNA encoding the complete RT, RNAseH and IN genes (referred to as RT-IN, 2898 bp in HXB2, Genbank accession number K03455) was amplified by nested PCR using forward primers PR_F1 (positions 2252-2277 in HXB2) and PR_F3 (2316-2340) and reverse primers VIF_R3 (5243-5266) and VIF_R5 (5193-5213). RT-Outer PCR was performed using One-Step SuperScript™III RT/Platinum® *Taq* High Fidelity (Invitrogen) and inner PCR was done using the Expand High Fidelity Polymerase (Roche). Thermal cycling consisted of reverse transcription for 30 minutes at 56 °C, followed by outer amplification comprising 2 min at 94 °C; 30 cycles of 15 s at 92 °C, 30 s at 62 °C and 3 min 30 s at 68 °C; and a final elongation for 10 min at 68 °C. Subsequently, inner amplification consisted of 2 min at 94 °C; 35 cycles of 15 s at 94 °C, 30 s at 60 °C and 3 min at 68 °C; and a final elongation for 10 min at 68 °C. Starting from site-directed mutant plasmids, RT-IN was amplified using only the inner amplification reaction and protocol.

2.1.2 Cloning of the RT-IN amplicons into an HXB2D-based RT-IN deleted backbone

RT-IN amplicons were cloned into an HXB2D-based RT-IN-deleted backbone (Van Baelen, et al., 2008; Van Baelen, et al., 2009) using the In-Fusion™ Dry-Down PCR cloning technology (Clontech-Westburg) following the manufacturer's protocol. Cloning mixes were transformed into MAX Efficiency® Stbl2™ cells (Invitrogen) using the manufacturer's procedure. A total of 970 clones (20 for each patient except for 1 patient 10 clones) were randomly picked and cultured to prepare DNA. Plasmid DNA was prepared using the QiaPrep Spin Miniprep system (Qiagen).

2.1.3 IN site-directed mutant (SDM) plasmids

Mutations known to be associated with InSTI resistance were introduced into selected original recombinant clones by overlap extension PCR on the original recombinant plasmids, followed by recloning of the mutated PCR fragment into the HXB2D-based, RT-IN-deleted backbone as described above. The following site-directed mutants **(**SDMs) were constructed: T97A+Y143R, Y143R, and G140S+Q148H in patient-derived recombinant viral clones and E92G in HXB2D backbone.

2.1.4 Production of replication-competent recombinant viruses

Clonal replication-competent recombinant viruses were generated by Amaxa nucleofection (Amaxa Biosystems) of recombinant plasmids into MT4 cells following the manufacturer's recommendations. The cytopathic effect (CPE) was monitored during the course of infection. When full CPE was reached, recombinant viruses were harvested by centrifugation.

2.1.5 Drug susceptibility testing of recombinant viruses (phenotyping)

Recombinant viruses were titrated and drug susceptibility determined essentially as described by Van Baelen et al., 2009. Recombinant viruses derived from site-directed mutants E92Q and T66I (known InSTI resistance mutations; (Hazuda, et al., 2007; Ceccherini-Silberstein, et al., 2009)) were used as positive controls.

Biological cutoff (BCO) values (taken from Van Baelen et al., 2009) were 2.1 for raltegravir and 2.0 for elvitegravir. Raltegravir and elvitegravir were obtained from Merck and Co (New Jersey, USA), and Gilead Sciences (California, USA), respectively.

Recombinant viruses were also subjected to antiviral experiments using Nucleoside Reverse Transcriptase inhibitors (NRTIs) and Non-Nucleoside Reverse Transcriptase inhibitors (NNRTIs) (zidovudine, tenofovir, emtricitabine, efavirenz and nevirapine). Resistance to these drugs was determined using the Antivirogram BCO values.

2.1.6 IN sequencing

Clonal recombinant viruses were subjected to RT-IN sequencing as previously described (56). The population IN genotype analysis on plasma samples was performed as follows: the RNA was extracted from plasma using the QIAamp Viral RNA kit (Qiagen, Heiden, Germany), reverse transcribed and PCR amplified with SuperScript One-Step RT-PCR for Long Templates (Invitrogen), employing the primers *3 IN sense* (positions 3505-3526 in HXB2) and *3 IN antisense* (positions 5963-5982). Conditions for the reaction were: one cycle of RT to 50° C for 30 min, one cycle at 94°C for 2 min, 40 cycles (95°C 30 sec, 51°C 30 sec, 72 °C 2 min and 30 sec) and a final cycle at 72°C 10 min. Amplification products were sequenced, in sense and antisense orientations by using 4 different overlapping sequence-specific primers, using a BigDye terminator v. 3.1 cycle sequencing kit (Applied Biosystems) and an automated sequencer (ABI-3100). The sequencing primers were the following: primer 1 (positions 4149-4168), primer 2 (positions 4654-4673), primer 3 (positions 5050-5068) and primer (positions 4652-4671). The sequences were analyzed using SeqScape-v.2.0 software. The nucleotide sequences of the clones are in process to be submitted to GenBank.

2.1.7 Mutations

Consensus B (http://hivdb.stanford.edu/) was used as a reference strain for the definition of mutations. The following IN mutations were analyzed: 1) mutations associated (by *in vitro* or *in vivo* studies) with resistance to InSTIs: primary mutations for raltegravir and/or elvitegravir (T66I, E92Q, Y143R/C, S147G, Q148H/K/R, N155H); secondary mutations for raltegravir and/or elvitegravir (H51Y, T66A/K, L68I/V, L74M, E92A, T97A, S119G/R, E138A/K, G140A/C/S, Y143H, V151I, E157Q, K160N, G163K/R, R166S, E170A, S230R, D232N); additional mutations (L74A/I, I72V, E92G, Q95K, T112I, H114Y, F121Y, T125K, A128T, Q146K/P, S153A/Y, M154I, N155S, K156N, K160D, V165I, V201I, I203M, T206S, S230N, V249I, R263K, C280Y) (6, 44, 53), and 2) all other IN mutations, by screening of all 288 IN amino acids.

2.1.8 Replication capacity assays

Human T-lymphocytic C8166 cells (obtained from the American Type Culture Collection (Manassas, VA)) were suspended at 20,000 cells/well in 200 µl of culture medium and infected with 1,000 or 10,000 pg/ml of p24 gag Ag of each viral recombinant stock, in triplicate. After incubation for 2 hours at 37ºC, cells were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (BioWittaker Europe, Verviers, Belgium), 2 mM L-glutamine and 0.075 M NaHCO3.

Starting from 3 days after HIV-1 infection, cellular cytopathic effect and syncytium formation were evaluated. At days 5 and 7, viral replication was quantified by measuring HIV-1 p24 gag Ag production in the culture supernatants by using a commercially available kit (Bio-Rad, Marnes La Coquette, France). The geometric mean of p24 gag Ag production of replicates in each experiment was used to determine the difference in virus production in C8166 obtained by using HIV-1 HXB2 as a control.

2.1.9 Statistical analysis

To assess the association between IN mutations and susceptibility to raltegravir and elvitegravir, the frequency of each mutation was calculated in recombinant viruses with fold change > or < than the BCO. Statistically significant differences between mutation frequencies were calculated using the Fisher's exact test. The Benjamini-Hochberg method was used to correct for multiple testing at a FDR of 0.05 (28). T-Test for unpaired samples was used to evaluate the differences in p24 antigen production in C8166 cells infected with the different recombinant viruses. Poisson distribution was performed to evaluate the sensitivity for detecting minority variants in the dataset, analyzing the total number of clones successfully analyzed per person.

2.2. Population sequencing.

2.2.1 Patients

In this study, 206 adult HIV-1 treatment experienced infected patients who started a raltegravir containing regimen within 8 different centers in Italy and France ("INMI Spallanzani" Hospital; Policlinico Tor vergata, Rome; "L.Sacco" Hospital, Milan; "Pitié-Salpetrière" Hospital, Paris; CHU de Bordeaux, Bordeaux; "San Martino" Hospital, Genoa; S.Annunziata Hospital, Florence; "Amedeo di Savoia" Hospital, Turin) were analyzed. The 78.6% of patients were male with a mean age of 46.2 year. Patients with a baseline HIV-RNA > 400 copies/mL were selected. The median baseline HIV-1 RNA (IQR) was 4.5 (3-7-5.2) log_{10}copies/mL, CD4 cell count (IQR) was 197 (95-315) cell/mm^3 and GSS (IQR) was 1(0.6-1.7) (Tab.2.2.1). The 90.3% (N=186) of patients were infected by HIV-1 subtype B while the 9.7% (N=20) by non-B subtypes (A 1.9% [N=4]; C 0.5% [N=1]; CRF02AG 2.4% [N=5]; CRF12BF 0.5% [N=1]; D 1.0% [N=4]; F 2.4% [N=5]; G 1% [N=2])(Fig.2.2.1). The mean number ± standard deviation of NRTIs, NNRTI's and PI's mutations were 4.25±1.5, 1.8±0.8 and 4.0±1.8 respectively. In the majority of patients raltegravir were used as salvage drug in the regimen. The antiretroviral drugs co-administered with Raltegravir was: regarding RTI's, 3TC in 38.8%, TDF in 47.1%, AZT in 6.8%, DDI in 5.3%, D4T in 1.9 % , ABC in 17.7% , DDC in 0.5%, FTC in 34.0%, EFV in 1.9%, NPV in 0% and ETR in 37.4% of patients respectively; regarding the PIs, IDV in 0 %, LPV/rtv in 3.4% , NFV in 0 %, SQV/rtv in 2.4 %, APV in 0.5%, ATV in 8.3% (of which 64.7% with RTV boosting), TPV/rtv in 4.4% and DRV/rtv in 66.5% in patients respectively; regarding FI and CCR5 Inhibitors, T20 in 23.8 % and MVC in 6.8% of patients respectively.

Table.2.2.1 Patients Characteristic

Mean Age, Years (N=206)	46.2
% Male	78.2
Baseline Median HIV RNA (IQR) (log_{10} copies/mL)	4.5 (3.7-5.2)
Median CD4 Cell Count (IQR) (cells/mm^3)	197 (95-315)
GSS (Rega 8.02)IQR (N=152)	1(0.5-1.7)
Mean No. NRTI Mutations	4.25±1.5
Mean No. NNRTI Mutations	1.8±0.8
Mean No. PI Major Mutations	4.0±1.8

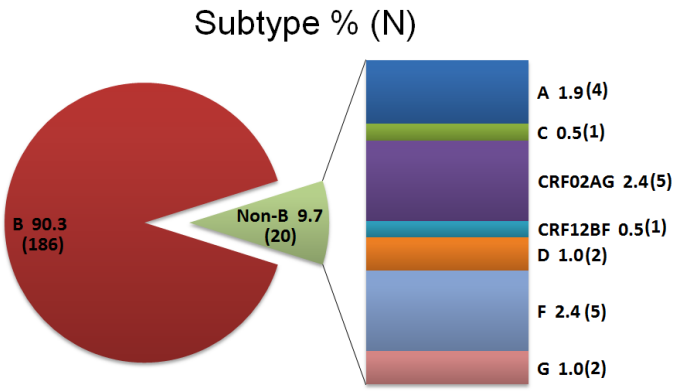


Fig 2.2.1. Subtypes and CRFs prevalence

2.2.2 HIV IN sequencing

The IN genotype analysis was performed on plasma samples by using home-made genotype methods, based on commercially available RNA-extraction, reverse transcription (RT)-PCR amplification and genotyping kits. In brief, the RNA was extracted from plasma using the QIAamp Viral RNA kit (Qiagen, Heiden, Germany), reverse transcribed and PCR amplified with SuperScript One-Step RT-PCR for Long Templates (Invitrogen), employing the primers 3 IN sense (3520, pol) [5' GAC CCA TCA AAA GAC TTA ATA 3'] and 3 IN antisense (5960, tat) [GCT TCT TCC TGC CAT AGG A 3'].

Conditions for the reaction were: one cycle of RT to 50° C for 30 min, one cycle at 94°C for 2 min, 40 cycles (95°C 30 sec, 51°C 30 sec, 72 °C 2 min and 30 sec) and a final cycle at

72°C 10 min. Pol amplified products (containing the entire IN) were full-length sequenced in sense and antisense orientations by using 4 different overlapping sequence-specific primers, a BigDye terminator v. 3.1 cycle sequencing kit (Applied Biosystems) and an automated sequencer (ABI-3100). The sequencing primers were the following: primer 1 [5' CAT GGG TAC CAG CAC ACA AA 3'], primer 2 [5' CCT ACA ATC CCC AAA GTC AA 3'], primer 3 [5' CAC AAT CAT CAC CTG CCA T 3'], and primer 4 [5' GGA TTA TGG AAA ACA GAT GGC A 3']. The sequences

were analyzed using SeqScape-v.2.0 software. The quality endpoint for each individual, was ensured by a coverage of the entire IN sequence by at least two sequence segments.

Sequences having a mixture of wild-type and mutant residues at single positions were considered to have the mutant(s) at that position. When the mixture was between two different mutations, both mutations were considered and reported.

2.2.3. Phylogenetic analyses

Sequence data were obtained and complete sequences encompassing the IN gene were assembled and manually edited using Seqscape version 2.5. All HIV-1 IN sequences (1 to 867≈nt) were aligned in BioEdit version 5.0.6 using CLUSTAL W (Hall, 1999) and compared with reference sequences for the major HIV-1 subtypes and Circular Recombinant Forms (CRFs), available at Los Alamos database (http://www.hiv.lanl.gov). The sequences were then manually edited with Seqscape v.2.5 and gaps were removed from the final alignment. Maximum likelihood analysis for phylogenetic tree inference was performed using PAUP* package (Swofford, 2002). The transvertion model (GTR+I+G) of nucleotide substitution was chosen using Modeltest v.3.7 implemented in PAUP* (Posada, et al., 2004) as the best fitting evolution model for tree reconstruction. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed through a bootstrap analysis using 1000 replicates on a maximum likelihood tree obtained by PhyML (Guindon, et al., 2003). Phylogenetic trees were inferred to ensure there was no cross-contamination of samples and to assign the subtype. Recombination among HIV-1 subtypes was assessed by SCUEAL (Kosakovsky Pond et al., 2009), COMET (Struck et al., 2010; available at http://comet.retrovirology.lu) and SimPlot (Lole et al., 1999) software's.

2.2.4 Mutations

As described in 3.1.7 paragraph.

We considered as polymorphisms mutations with a frequency >5% in overall population and the codon usage were considered when the prevalence of not-synonimous codon were more than 5% for at least 2 degenerated codons in overall population.

2.2.5 Genotypic sensisitive score

To investigate about the level of efficacy of other drugs contained in the first line raltegravir regimen and their predictive role in raltegravir virologic response, GSS by Rega V 8.02 (algorithm was performed for the 152 patients with genotypic resistance test for protease and RT available, by submission of FASTA sequences to Stanford Hiv Drug Resistance database website (http://hivdb.stanford.edu/index.html) and consequent correlation with baseline antiretroviral regimens. The usage of T20 and Maraviroc in therapeutic regimen was not considered in the GSS scoring.

2.3.6 Statistical Analysis

The different prevalence of all integrase baseline mutations among patients (N=177) which achieve or not achieve HIV RNA < 50 copies/mL at 24 week of raltegravir containing regimen, was evaluated by Fisher Test with Benjamini-Hockberg correction for multiple comparison with FDR=0.1.

Univariate and multivariate logistic regression were performed to investigate about predictor role in virologic success for the baseline variables: plasma HIV-1 RNA, ARV's in co-usage with raltegravir and all mutations associated with virologic response ($p<0.05$ by Fisher Test, Benjamini-Hockberg correction for multiple comparison with FDR=0.1)

Calculation of not-synonymous codon frequency was performed by software MutationFinder 2.0 (University of Catania). Statistical analysis was performed by SPSS 17.0 and R 2.9.0 (Hornik, 2010) software's.

2.3. Ultra Deep-454 Pyrosequencing

2.3.1 Patients

The study included a subgroup of 27 HIV-1 infected individuals treated with raltegravir from the population of patients previously described in the paragraph 2.2.1.

IN-Ultra-Deep 454-pyrosequencing (UDPS) were performed at baseline and during treatment with a total of 56 samples analyzed, of which 23 at baseline and 32 during treatment at different time point (Tab.2.3.1).

Tab.2.3.1 Patient Characteristic.

Patient	Baseline CD4 cell count (cell/mm^3)	Baseline HIV RNA (copies/ml)	Subtype	Genotype Sensisitive Score	Therapy with raltegravir	Virologic response at 24 weeks[a]	Genotypic Tests (N)					
							Baseline		Follow_up		Overall	
							UDPS	Popul[b]	UDPS	Popul[b]	UDPS	Popul[b]
12	24	5.1	B	0	3TC, DRV/r, T20	F	1	1	5	4	6	5
18	200	2.6	B	1	3TC, ABC, AZT, DRV/r	F	-	-	2	-	2	-
27	231	3.6	B	1.75	DRV/r, TDF, TMC125	F	1	1	1	-	2	1
49	21	4.5	B	2.5	DRV/r, FTC, TDF	F	1	1	-	-	1	1
56	1	5.5	B	0	3TC, DDI, DRV/r, T20	F	1	1	-	-	1	1
69	328	4.4	B	0	3TC, DRV, RTV	F	1	1	2	2	3	3
78	65	4.3	B	2.5	3TC, ABC, TDF, TPV/r	F	-	-	2	2	2	2
80	2	4.7	F	Nd	3TC, AZT, DRV/r	F	1	1	1	1	2	2
81	83	5.3	B	Nd	3TC, ETR	F	1	1	1	1	2	2
84[c]	14	5.5	B	0.5	3TC, MCV, TDF	F	2	2	7	4	9	5
141	276	4.9	B	1	3TC, AZT, DRV/r, ETR	F	1	1	1	1	2	2
142	7	5.7	B	Nd	DRV/r, ETR, FTC, T20, TDF	F	1	1	-	-	1	1
145	480	5	B	0	3TC, DRV/r	F	1	1	1	1	2	2
156	320	5.4	B	1.5	EFV, LPV/r	F	1	1	1	-	2	1
162	192	5.1	B	2.5	ETR, SQV/R	F	1	1	2	-	3	1
229	8	4.7	B	1.5	DRV/r, ETR, FTC, TDF	F	1	1	3	2	4	3
230	53	5.3	B	0	FTC, LPV/r, TDF	F	-	-	2	2	2	2
5	315	5.3	F	2.5	DRV/r, ETR	R	1	1	-	-	1	1
15	178	5.2	B	0	DRV/r, FTC, MCV, TDF	R	1	1	-	-	1	1
16	132	4.6	B	0.5	3TC, DRV/r, TDF	R	1	1	-	-	1	1
44	9	5.6	B	0.75	DRV, RTV, T20	R	-	-	1	1	1	1
45	106	5.7	B	Nd	3TC, DRV/r, EFV	R	1	1	-	-	1	1
57	105	5.3	B	Nd	DRV/r, FTC, TDF	R	1	1	-	-	1	1
58	563	4	B	0.75	DRV/r, FTC, TDF	R	1	1	-	-	1	1
63	170	5.6	B	1.75	DRV/r, FTC, TDF	R	1	1	-	-	1	1
151	207	4.7	B	Nd	DRV/r, FTC, TDF	R	1	1	-	-	1	1
155	11	5.7	B	1	ETR, T20	R	1	1	-	-	1	1
Total	**27**						**23**	**23**	**32**	**21**	**56**	**44**
Median (IQR)	132 (14-231)	5.1 (4.6-5.4)										

a) F indicates the failing patients; R the responding patients; b) Popul, population sequencing; c) Patient 84 had two available plasma samples at time 0 and 3 months before the raltegravir containing regimen.

The patients ID, the baseline HIV-RNA and CD4 cell count, the subtype, the genotype sensitive score (Rega 8.02), the drugs co-admistered with raltegravir, the virologic response at 24 weeks and the overview of genotypic test performed are reported. The totals and the median (IQR) values for CD4 cell count, HIV-RNA and GSS are indicated on the bottom of table.

2.3.2 Massively parallel sequencing

Viral RNA was extracted from 30 µl of plasma (QIAamp Viral RNA kit, Qiagen, Heiden, Germany), reverse transcribed to cDNA and the integrase region spanning amino acids 66 to 163 loop was amplified (HXB2 positions: forward primer 4400→4423, reverse primer 4743→4719). Amplicon primer pairs were tailed at their 5'end with the 454 specific sequencing primers followed by a barcode. Addition of barcode sequences to the primers allowed the simultaneous processing of amplicons originating from multiple individuals in a single experiment (Parameswaran, et al., 2007). To maximize the number of input templates and to minimize variation due to PCR drift, 7 parallel RT-PCR reactions were performed per patient sample and pooled (Polz, et al., 1998; Vandenbroucke, et al., 2008). Barcoded amplicons were equimolarly pooled and sequenced on the GS-FLX instrument according to the manufacturer's amplicon sequencing protocol (454 Life Sciences, Roche, Branford, CT, USA). Sequences were analysed using the AVA (Amplicon Variant Analyzer) software (454 Life Sciences, Roche)

2.3.3 Mutations

As previously described in 3.1.7 paragraph. By UDPS were analyzed the mutations present within the integrase region covered from IN position 66 to 163 (see 3.3.3 paragraph). The cut-off limit of rielable mutations detection for UDPS was considered as >0.1%(≥50 reads).

2.3.4 Phylogenetic analyses

The phylogenetic analysis was performed for each patient on unique overlapping sequences obtained with quantitative deep sequencing. Only sequences represented by >10 reads were included in the alignments. The only exception was for viral strains harboring Q148R mutation in baseline population of patients 84 (prevalence <0.1%). Sequences from each data set were aligned using CLUSTAL X software (Thompson, et al., 1997); then manually edited with Bioedit software (Hall, 1999). For all patients, bulk T_0 sequences were added to aligned pyrosequencing-derived

sequences. Phylogenetic trees were estimated using PAUP* package [Swofford DL.(2002). PAUP phylogenetic analysis using parsimony (*and other methods): Version 4.0. Sunderland. Massachussets: Sinauer Associates]. The transvertion model (GTR+I+G) of nucleotide substitution was chosen using Modeltest v.3.7 implemented in PAUP* (Posada, et al., 2004), and then manually modified to optimize parameter settings for each dataset. Maximum Likelihood trees were inferred under selected models using tree bisection-reconnection (TBR) branch swapping. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed through a bootstrap analysis using 1000 replicates on a Maximum Likelihood tree by PhyML 3.0 algorithm (Guindon, et al., 2003), and through the Zero Branch Length Test.

2.3.6 Cloning of the IN amplicons into an HXB2D-based, IN deleted backbone

IN amplicons were cloned into an HXB2D-based, IN-deleted backbone (Van Baelen, et al., 2009; Van Baelen, et al., 2008)using the In-Fusion™ Dry-Down PCR cloning technology (Clontech-Westburg) following the manufacturer's protocol. Cloning mixes were transformed into MAX Efficiency® Stbl2™ cells (Invitrogen) using the manufacturer's procedure. Recombinant bacteria colonies populations from 34 patients' samples were washed and cultured to prepare DNA. Plasmid DNA was prepared using the QiaPrep Spin Miniprep system (Qiagen).

2.3.7 IN site-directed mutant (SDM) plasmids

As described in 2.1.3 paragraph. The following site-directed mutants **(**SDMs) were constructed: Y143C, N155H, Y143C+ N155H, Y143R in HXB2D backbone and Y143 wild type / N155H, Y143C / N155 wild type and Y143 wild type / N155 wild type in derived recombinant viral clones from patient 229.

2.3.8 Statistical analyses

Median test and fisher-exact Test was used to compare the median intra-patients percentage and the frequencies of all baseline mutations among patients who responded or not at 24 weeks of raltegravir treatment. Wilcoxon Matched-Pairs Signed-Ranks Test was used to compare the median

number of mutations for patients detected by UDPSor bulk-sequencing both among baseline and and follow-up genotypes. Kruskal-Wallis test were used to evaluate the differences in number of mutations over time according with the genotyping techniques.

3. Results

3.1. Secondary HIV-1 Integrase Resistance Mutations, Found as Minority Quasispecies in Integrase Therapy Naive Patients, Have Little or no Effect on Susceptibility to Integrase Inhibitors

This work has been recently published in Antimicrobial Agents Chemotherapy (Ceccherini-Silberstein, et al., 2010)

3.1.1 Production of clonal recombinant virus stocks

Transformation of the HIV-1 recombinant plasmids in E.coli resulted in populations represented by 10 to approximately 1000 colonies per sample. Twenty clones per sample were randomly picked (for one sample only 10 clones) and DNA was extracted, resulting in a total of 970 clonal DNA preparations. After transfection of the recombinant plasmids (n= 970) into MT4-cells, infectious replication-competent recombinant viruses were produced from 427 clones (44%, 1-17 clones/sample). The remaining 543 clones failed to produce replication competent virus, probably due to the presence of empty vectors or smaller fragments into the backbone after cloning (secondary PCR fragments are cloned preferentially because they are smaller than the RT-IN fragment), and/or bad infectivity of the created virus stocks (mutations in the RT-IN fragment both introduced by PCR or present in the plasma viral RNA itself).

Among clones the successfully production infectious replication-competent recombinant viruses is associated with the treatment experience of patients and viral

3.1.2 Determination of raltegravir and elvitegravir susceptibility of clonal RT-IN recombinant viruses

Susceptibility testing against the InSTIs raltegravir and elvitegravir was successful for 345 out of the 427 clonal recombinant viruses analyzed (80.8%). Indeed, some clones failed the susceptibility

test, even if able to infect MT4 cells, because the viral titer was not high enough to generate reliable fluorescent signals in a 3-days antiviral experiment, or did not reach the internal quality criteria for the dose response curves. Overall, a median number of 7 samples per patient were analyzed, providing a sensitivity of the analysis to detect variants at the level below 20% in at least 18 patients, based on Poisson distribution.

The EC50-values obtained for the IIIB reference strain were 6.1 ± 2.3 nM for raltegravir and 11.6 ± 3.5 nM for elvitegravir. FC-values of the recombinant viruses ranged from 0.1 to 2.6 (average 0.9 ± 0.4) for raltegravir and from 0.1 to 13 (average 0.9 ± 0.8) for elvitegravir. Positive controls with the mutation E92Q (known to confer some resistance to both raltegravir and elvitegravir) showed an average FC of 3.8 ± 0.5 for raltegravir and 20.8 ± 2.3 for elvitegravir, whereas the average FC obtained for T66I mutant (known to confer resistance to elvitegravir) was 0.8 ± 0.3 for raltegravir and 10.5 ± 3.4 for elvitegravir.

Applying the BCOs to fold change values obtained from repeat testing in quadruplicate, the majority (>99%) of clones were not phenotypically resistant to InSTIs: only 3 clones out of 345 tested (0.9%) showed decreased susceptibility to elvitegravir (with mean FC ± SD > BCO) and none for raltegravir (with mean FC ± SD > BCO) (Fig.3.1.1). Overall, 1 out of 21 drug-naïve patients (4.8%) and 2 out of 28 HAART-treated patients (7.1%) carried singular clones (<20% of total variants/patient) with mean FC + SD > BCO for elvitegravir. So, phenotypic resistance to InSTI is very limited in InSTI naïve patients, and confirmed when present, to rare clones whose resistance to raltegravir is minimal (2 clones with FC near BCO, but + SD < BCO) and very limited to elvitegravir (only 2 clones with FC >3).

Differently, 38.4% of clones showed mean FC ± SD > BCO for at least one NRTI or NNRTI tested (AZT, TDF, FTC, EFV and NVP). As expected, a higher phenotypic resistance, was observed in clones obtained from HAART-treated patients (at least 1 FC>BCO for one RTI in 120 out 163

clones, 73.6%), in comparison of what observed in clones obtained from drug-naïve patients (13 out of 183, 7.1%; P<0.0001, Fisher's Exact test) (data not shown).

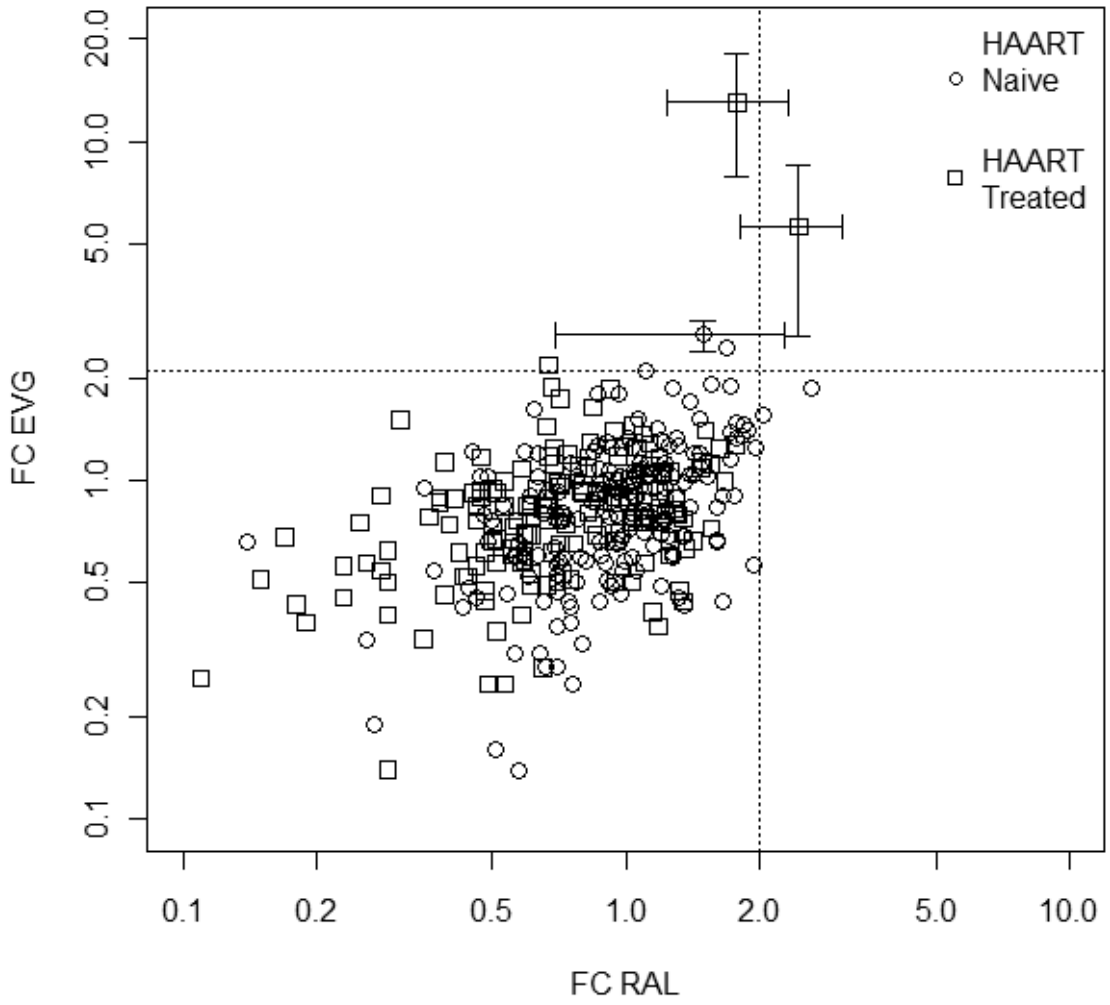


Figure 3.1.1 Raltegravir and elvitegravir susceptibility of clonal RT-IN recombinant viruses.

Scatter Plot of mean fold changes (FC) values for raltegravir (RAL) and elvitegravir (EVG) obtained from 345 recombinant clones, analyzed in 1 to 2 experiments, each performed with four replicate determinations in duplicate plates. Standard deviation (SD) bars are showed only for clones with mean FC > BCO. Square dots indicate clones obtained from HAART-experienced patients and circle dots indicate clones obtained from HAART-naive patients; all patients are naïve to integrase inhibitors.

3.1.3 Genotypic characterization of mutations associated with resistance to raltegravir and elvitegravir

All 427 clonal recombinant viruses were genotypically analyzed, and a full-length RT-IN sequence was successfully obtained for 409 clones (96% efficacy), with a median number of 9 samples per patient analyzed, providing a sensitivity of the analysis to detect variants at the level below 20% in at least 15 patients, based on Poisson distribution.

None of the IN sequences showed primary resistance mutations for raltegravir (Y143R/C, Q148H/R/K, N155H) or elvitegravir (T66I, E92Q, S147G, Q148H/R/K, N155H). Similarly, secondary resistance associated mutations to raltegravir and/or elvitegravir such as H51Y, T66A/K, L68I, S119R, E138A/K, G140A, Y143H, S153Y, K160N, G163K/R, R166S, E170A, D232N, were also completely absent among all IN sequences analyzed. Other secondary mutations, such as L68V, L74M, T97A, G140S, and S230R were rarely found as minor quasispecies (<20% of total variants/patient, in only 2/49 patients) (Table 3.1.1). Secondary mutations, such as V151I and E157Q, were also rarely found, but, when present, they were as major variants (>65% of the clonal sequences, in 2/49 patients). Other natural polymorphisms such as I72V, K156N, V165I, V201I, T206S and S230N were found as major variants with a prevalence of >10% of patients (6 to 26 patients out of 49). Interestingly, a novel mutation (E92G) recently reported as potentially associated with resistance (1, 8) was rarely found, and only in minor quasispecies (<20% of total variants/patient, in only 2/49 patients) (Table 3.1.1).

Table 3.1.1 Genotypic and phenotypic characterization of HIV-1 integrase mutations in HIV-1 infected patients naïve to integrase inhibitors.

INI Known Mutations	Genotype analysis		Genotype and phenotype analyses			
	Clones (N=409) N (%)	Patients (N=49)	Clones (N=344)			
			N	Mean RAL-FC ± SD	N	Mean EVG-FC ± SD
L68V	1 (0.2)	1	1	0.4 **	1	1.1**
I72V	199 (48.5)	26	166	0.9 ± 0.4	166	0.8 ± 0.4
L74I	25 (6.1)	3	25	1.2 ± 0.4	25	1.2 ± 0.6
L74M	7 (1.7)	2	7	0.9 ± 0.3	7	0.7 ± 0.2
E92G	2 (0.5)	2	1	1.8 ± 0.5	1	**13.0 ± 5.1**
T97A	3 (0.7)	2	2	1.6 ± 0.6	2	**2.6 ± 0.5**
S119G	62 (15.1)	8	54	1.1 ± 0.4	54	1.0 ± 0.5
G140S	1 (0.2)	1	1	2.4 ± 0.6	1	**5.6 ± 2.9**
V151I	14 (3.4)	1	11	1 ± 0.4	11	0.6 ± 0.2
M154I	81 (19.8)	10	63	0.8 ± 0.4	63	1 ± 0.7
K156N	31 (7.6)	6	26	0.4 ± 0.2	26	0.7 ± 0.2
E157Q	12 (2.9)	2	9	1.2 ± 0.4	9	1.2 ± 0.4
V165I	70 (17.1)	8	55	0.7 ± 0.3	54	0.8 ± 0.3
V201I	179 (43.7)	22	153	1 ± 0.4	153	0.9 ± 0.9
T206S	71 (17.3)	12	54	1 ± 0.4	55	1.1 ± 1.5
S230N	61 (14.9)	8	53	1 ± 0.4	53	0.8 ± 0.3
S230R	1 (0.2)	1	0	-	0	-

** Standard deviation (SD) not determined.

The prevalence and susceptibility of HIV-1 integrase mutations associated with resistance to integrase-inhibitors (INI) in INI-naive patients are reported. Consensus B was used as a reference strain for the definition of mutations. The mutations have been selected according to previously published data (Ceccherini-Silberstein, et al., 2009; Semenova, et al., 2008; van Han, et al., 2009). Fold-changes (FC) are mean values from 1 to 2 experiments, each performed with four replicate determinations in duplicate plates. In bold are shown the mutations found associated with mean FC values ± SD > BCO for raltegravir (RAL) and/or elvitegravir (EVG).

3.1.4 Phenotypic characterization of mutations associated with resistance to raltegravir and elvitegravir

In order to analyze the association between IN mutations and the susceptibility to raltegravir and elvitegravir, the frequency of each mutation was calculated in all recombinant viruses. By comparing the prevalence of all IN mutations between the resistant clones (i.e. having a FC > BCO) and the non-resistant clones (FC <BCO), we observed only E92G, T97A, and G140S mutations positively associated with resistance to elvitegravir and only G140S mutation associated with resistance to raltegravir. However, none of these mutations was statistically associated with an increased phenotypic FC after multiple comparison test corrections, by using Benjamini-Hochberg method (data not shown). Indeed, the few clones with FC> BCOs showed specifically the presence of the following raltegravir/elvitegravir secondary mutations: T97A, S119G and G140S, together with other natural polymorphisms associated with INI-resistance (such as L74I, M154I, V201I and T206S) and other not yet described mutations (Table 3.1.1, Table 3.1.2). Interestingly, all clones harbouring mutations proposed as potentially contributing to resistance to raltegravir and/or elvitegravir such as L68V, L74M, V151I, K156N, E157Q, showed all FC<BCO for both raltegravir and elvitegravir (Table 3.1.1).

The secondary raltegravir-associated mutation T97A was observed in 2 recombinant clonal viruses from a single drug-naïve patient (pt15), showing a limited FC>BCO only for elvitegravir (2.7+0.3 and 2.5+0.6, respectively) (Table 3.1.2, Fig.3.1.2). Similarly, the secondary resistance mutation to raltegravir and elvitegravir, G140S, was observed in a single recombinant clonal virus in a single HAART-treated patient (pt18), and showed mean FC + SD > BCO for elvitegravir (5.6+2.9) and an increased mean FC also for raltegravir (2.4+0.6) (Table 3.1.2, Fig 3.1.2). Finally, a novel mutation, E92G, was observed in a single recombinant virus in a single HAART-treated patient (pt50) showing high-level resistance only to elvitegravir (mean FC value 13.0±5.1) but no resistance to raltegravir (mean FC value 1.8±0.5) (Table 3.1.2, Fig 3.1.2).

Table 3.1.2 Integrase amino acid substitutions found in recombinant viral clones with decreased susceptibility to raltegravir and /or elvitegravir. Fold-changes (FC) are mean values from two experiments, each performed with four replicate determinations in duplicate plates. In bold are shown the mutations found associated with in vitro resistance to raltegravir (RAL) and/or elvitegravir (EVG) with mean FC values + SD > biological cutoffs. All the patients are naïve to integrase inhibitors. Patient 15 is drug-naive; 18 and 50 are HAART-treated patients. Consensus B was used as a reference strain for the definition of mutations.

Sample	**Integrase mutations**	**RAL FC** ±StDev	**EVG FC** ±StDev
Clone 15_7	E11D,S17T,L45V,M50T,I72V,L74I,**T97A**,K111T,S119G**,**T125A	1.5±0.8	**2.7±0.3**
Clone 15_19	E11D,S17T,L45V,M50T,I72V,L74I,**T97A**,K111T,S119G**,**T125A	1.7±0.5	2.5±0.6
Clone 18_13	S17N,T124N,T125A,**G140S**,M154I**,**S195C	2.4±0.6	**5.6±2.9**
Clone 50_2	R20K,A23V,**E92G**,T124N,I141V,G193E,V201I,T206S,I220V,D279G	1.8±0.5	**13.0±5.1**
Controls	T66I	**3.8±0.5**	**20.8±2.3**
	E92Q	0.8±0.3	**10.5±3.4**
	HXB2	1.0±0.2	1.2±0.3

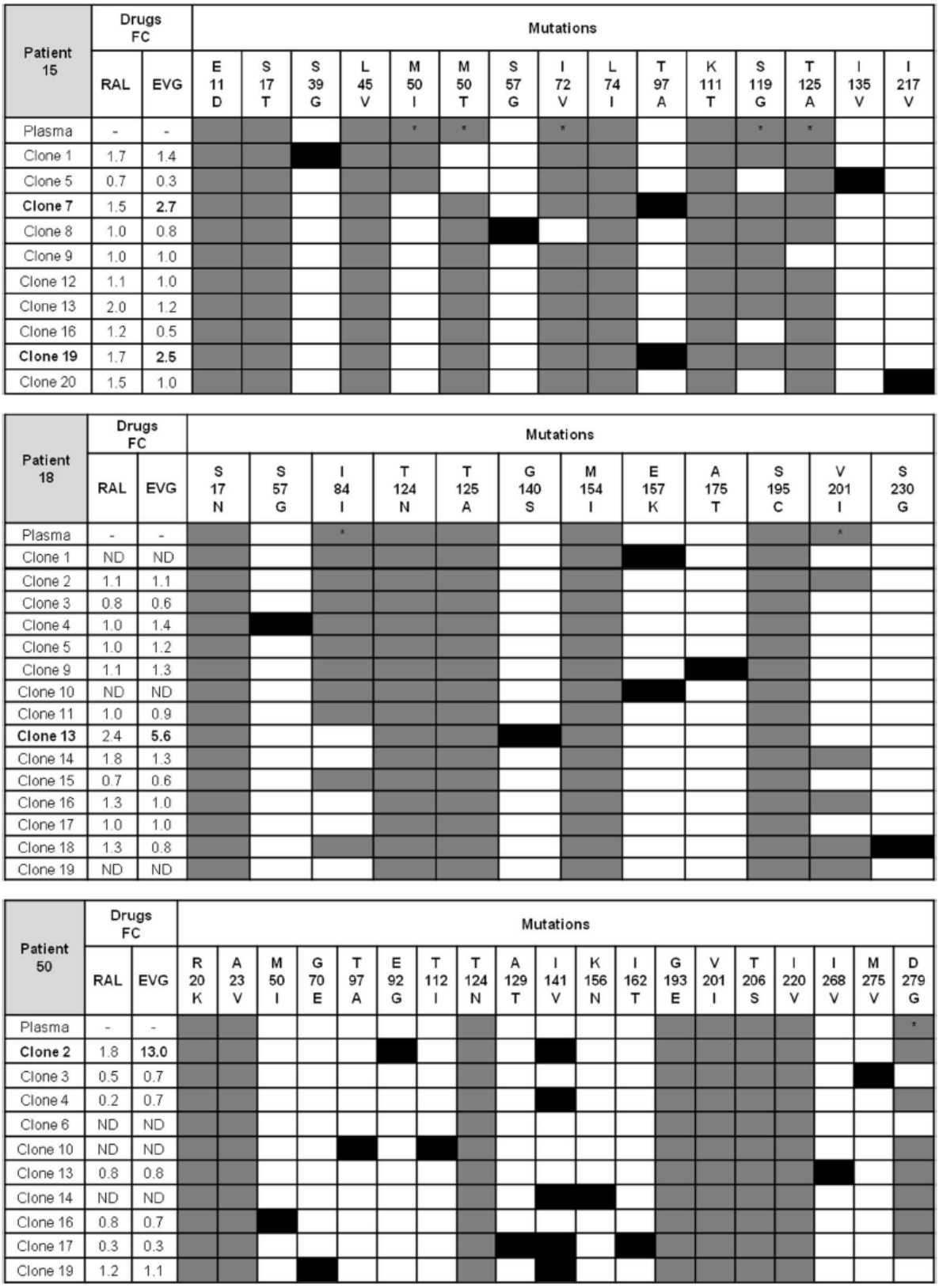


Fig 3.1.2. Integrase amino acid substitutions found in recombinant viral clones of patients naïve to integrase inhibitors with resistance to elvitegravir. Patient 15 is drug-naive; patients 18 and 50 are HAART-treated. Consensus B was used as a reference strain for the definition of mutations. Gray cells represent mutations detected both in plasma population and in specific recombinant viral clones; * indicates quasispecies in population sequences. Red cells represent mutations detected only in specific recombinant viral clones; in bold fold changes (FC) values $\pm$ SD > biological cutoffs. ND = FC not determined.

3.1.5 Susceptibility to raltegravir and elvitegravir of site direct mutant viruses carrying primary and secondary resistance IN mutations

To evaluate whether the negligible effect on raltegravir and elvitegravir resistance observed in clones with secondary mutations T97A and G140S could be further increased by the presence of primary related resistance mutations (Y143R and Q148H, respectively; 6, 30), site direct mutagenesis experiments were also performed. Primary Y143R mutation was added in clones 15_7 and 15_19 (carrying T97A mutation) and in clone 15_12 (with T97Twt), while Q148H mutation was added for clone 18_13 (carrying G140S mutation) (Table 3.1.3). All clones obtained showed highly increased levels of resistance to both raltegravir and elvitegravir. In particular, the addition of Y143R in clones 15_7, 15_19 was associated with mean FC=91.6+21.5 and FC=88.2+7.8 for raltegravir, and mean FC=14.6+1.6, FC=9.4+1.0 for elvitegravir, compared to mean FC=13.1+0.2 and FC=4.2+0.4 for raltegravir and elvitegravir respectively, in clone 15_12 (not carrying T97A). Thus, these data show that the primary raltegravir resistance mutation Y143R may reduce susceptibility to both raltegravir (to a greater extent) and elvitegravir (to a lesser extent), especially if the mutation is combined with T97A secondary mutation (clones 15_7, 15_19).

Similarly, as expected, the addition of Q148H in clone 18_13 containing the G140S mutation was associated with high resistance to both drugs, with mean FC=768+17.0 for raltegravir and mean FC>633.7 for elvitegravir (Table 3.1.3). Finally, to confirm the association of E92G mutation with a decreased susceptibility to elvitegravir, a mutant of HIV-1 HXB2 carrying E92G mutation was also generated by mutagenesis. The phenotypic test confirmed that the presence of E92G, as it was observed in clone 50_2 obtained from an HAART-experienced patient, is associated with resistance for elvitegravir, with a mean FC=9.9+2.7 (FC=1.5+0.2 for raltegravir) (Table 3.1.3).

Table 3.1.3. Susceptibilities of site-directed mutant viruses carrying primary and secondary integrase resistance mutations.

Clone	Original INI-resistance profile	SDM final profile	RAL-FC ±StDev	EVG-FC ±StDev
Clone 15_7	**T97A**	**T97A+Y143R**	**91.6 ± 21.5**	**14. 6 ± 1.6**
Clone 15_19	**T97A**	**T97A+Y143R**	**88.2 ± 7.8**	**9.4 ± 1.0**
Clone 15_12	T97Twt	**Y143R**	**13.1 ± 0.2**	**4.2 ± 0.4**
Clone 18_13	**G140S**	**G140S+Q148H**	**768 ± 17.0**	**> 633.7***
HXB2	-	**E92G**	1.5 ± 0.2	**9.9 ± 2.7**
Controls	**HXB2**		1.1±0.2	1.2±0.3

*SD not available.

Five different site-directed mutants (SDM) were generated and evaluated for their susceptibility in vitro. Fold-changes (FC) are mean values from two experiments, each performed with four replicate determinations in duplicate plates. In bold are shown the integrase mutations found associated with resistance to integrase strand transfer inhibitors (InSTI) raltegravir (RAL) and/or elvitegravir (EVG), with mean FC values + SD > biological cutoffs. Consensus B was used as a reference strain for the definition of mutations.

3.1.6 Genotypic characterization of RT mutations in RT-IN recombinant viruses

Analyzing the full-length RT-RNAseH sequences available of the recombinant viruses obtained from these InSTI-naive patients, we observed that, overall, resistance mutations for at least one NRTI or one NNRTI, according to the IAS list of primary resistance mutations (30), was found in 194 and 109 out of 411 sequences (47% and 26.5%), respectively. As expected, higher prevalence of resistance was observed in clones obtained from HAART-treated patients (at least 1 NRTI mutation in 155 out 207 clones, 74.9%; at least 1 NNRTI mutation in 92 out 207 clones, 44.4%), in comparison to what was observed in clones obtained from drug-naïve patients (at least 1 NRTI mutation in 39 out of 204, 19.1%; at least 1 NNRTI mutation in 17 out of 204, 8.3%; $P<0.0001$, Fisher's Exact test) (data not shown). Interestingly, all clones with genotypic and/or phenotypic

resistance to elvitegravir and/or raltegravir, showed also RT resistance mutations (Table 3.1.4). Of substantial interest, the drug-naïve patient (pt15) with the IN T97A mutation as minor variant, showed in plasma and in all the recombinant clones the RT mutations T69N and K70R (thus carrying NRTI-resistance mutations despite being drug naïve). The other two clones originated from HAART-treated patients all showed several NRTI and NNRTI resistant mutations (Table 3.1.4).

Table 3.1.4. PR and RT resistance mutations in INI-naive patients harbouring minor species with decreased susceptibility to InSti[a].

Sample	Enzyme	Resistance Mutation(s)
Clone[b] 4_8	**IN**	S17N, I72V, **E92G**, K156N, V201I
	RT	**M184V, N348I[c] (+ M41L, T69N, K70R,K103N** in plasma**)**
	PR	**No resistance mutations** (plasma)
Clone 15_7	**IN**	I72V, L74I, **T97A**, S119G
	RT	**T69N, K70R**
	PR	**No resistance mutations** (plasma)
Clone 15_19	**IN**	I72V, L74I, **T97A**, S119G
	RT	**T69N, K70R**
	PR	**No resistance mutations** (plasma)
Clone 18_13	**IN**	S17N, **G140S**, M154I
	RT	**M41L, D67N, L74V, V118I, M184V, L210W, T215Y, K219N K101E, V108I, Y181C, G190A, F227FL**
	PR	**L10I, V11I, K43T, M46I, I54V, A71V, V82T, I84V, L90M, I85V** (plasma)
Clone 50_2	**IN**	**E92G,** V201I, T206S
	RT	**M41L, D67N, T69N, M184V, L210W, T215Y, K219E K101E, E138K, Y181C, G190A**
	PR	**A71V** (plasma)

a Consensus B was used as a reference strain for the definition of mutations. The mutations associated with resistance to RTIs, PIs and INIs are shown in boldface. All the patients were INI naive. Patient 15 was drug naive; patients 4, 18, and 50 were HAART treated. IN and RT mutations were found in the recombinant viral clones. Protease (PR) mutations were found in plasma of the INI-naive patients. All RT resistance mutations were also found in plasma. b Clone 4_8 did not grow well enough for phenotyping. c RT sequence not complete, RT mutations of plasma sample are indicated

3.1.7 Replication capacity of recombinant clonal viruses

In order to analyze the replication capacity of each recombinant virus that showed INI-resistance mutations and/or FC>BCO, p24 gag antigen production in human C8166 T-lymphocytes was analyzed. Virus replication of HXB2 molecular clone and viruses without specific InSTI resistance mutations (18_14 and 47_19 clones, randomly selected among clones with FC values < BCOs) was at very similar levels. Differently, p24 production of recombinant viruses containing the mutation G140S (clone 18_13), or T97A (clones 15_7 and 15_19), or the known InSTI resistance mutations T66I and E92Q (positive controls) was much lower than that observed for the HXB2 molecular clone, but the differences were not statistically significative. Significant difference on p24 production with HXB2 was observed only for the recombinant virus containing the mutation E92G (clone 50_2) (P=0.037, T Test, Fig.3.1.3A).

The addition by SDM of primary resistance mutations Q148H and Y143R to recombinant patient-derived viruses was associated with an additional significantly diminished replication capacity compared to clones carrying only secondary mutations (Fig. 3.1.3A and 3B). Differently, the HXB2 molecular clone containing only the E92G mutation showed a small reduction of viral p24 production in comparison to the control HXB2. This reduction was not so dramatic as it was observed with the patient-derived virus (clones 50_2) containing the E92G mutation (Fig. 3.1.3A and 3B), suggesting that the overall combination of RT mutations (as M184V, TAMS, N348I) plus the E92G IN mutation could be the explanation for the impaired replication capacity of viral clones containing such mutation (Table 3.1.4).

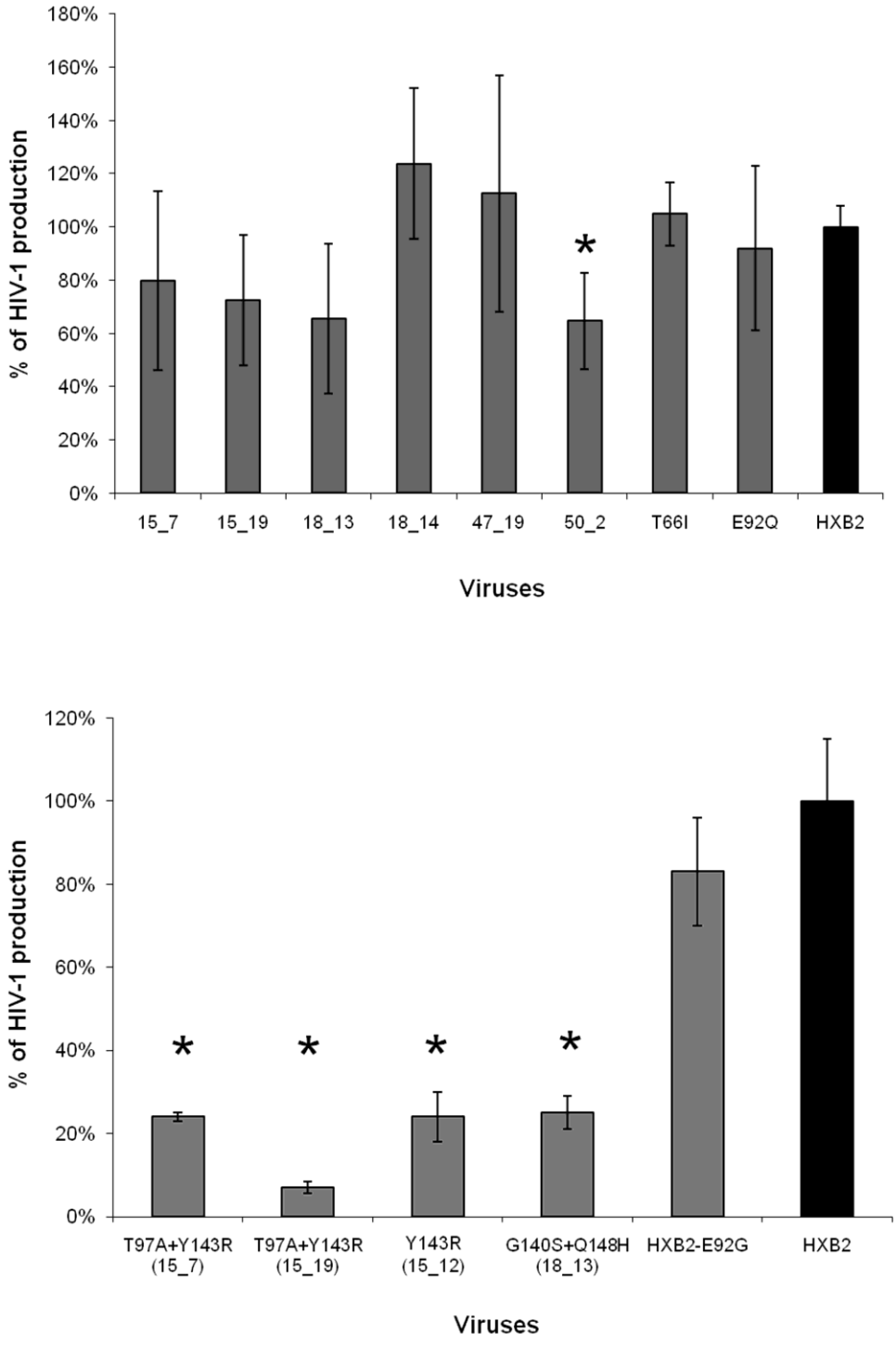


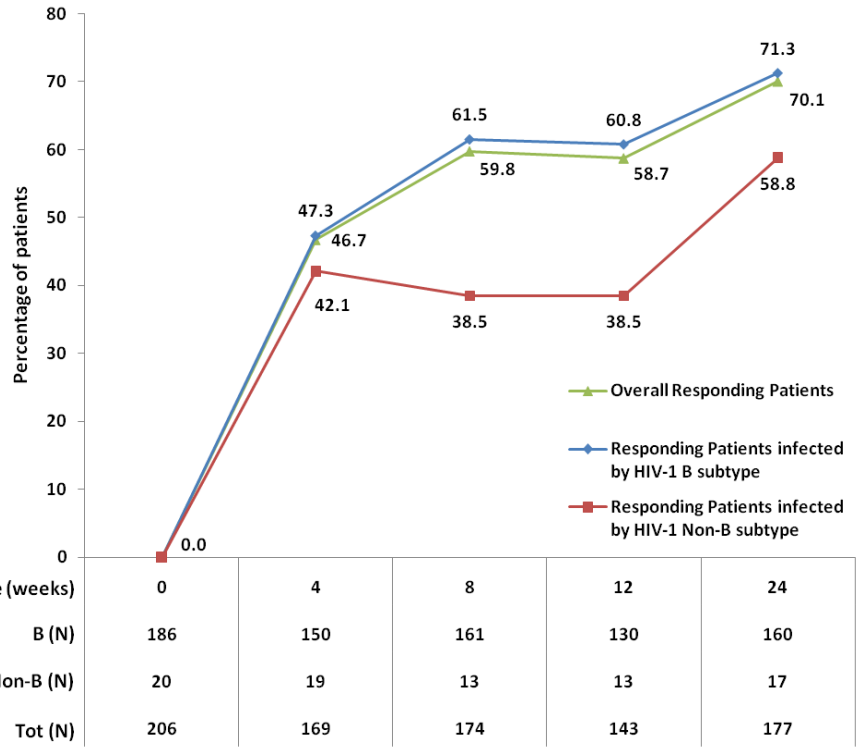
Fig. 3.1.3. Replication capacities of recombinant clonal viruses. (A) Bar graph of percentages (average and standard deviation) of virus produced in cell-free supernatant after 5 days of infection with 10,000 pg/ml of p24 gag Ag for each recombinant and control virus in C8166 T cells. The infectivities of recombinant viruses carrying INI resistance mutations (15_7 and 15_19, T97A; 18_13, G140S; 50_2, E92G), recombinant viruses without specific INI resistance mutations (47_19 and 18_14), two control mutant viruses (T66I and E92Q), and the molecular clone HXB2 were analyzed. Experiments were done in triplicate. (B) Bar graph of percentages (average and standard deviation) of virus produced in cell-free supernatant after 5 days of infection with 10,000 pg/ml of p24 gag Ag for each site-directed mutant and control virus in C8166 T cells. One experiment, representative of three done in triplicate, is shown.
*, $P<0.05$; t test.

3.2 Impact of baseline HIV-1 integrase polymorphisms with virological outcome in patients starting a Raltegravir-containing regimen

This work has been presented orally at the 8th European HIV Drug Resistance Workshop, 17-19 March 2010, Sorrento, Italy (Armenia et al, 2010a).

3.2.1 Efficacy of raltegravir containing regimen at 24 weeks of treatment

Among the 206 patients who started raltegravir, 169, 174, 143, 177 had an available HIV-RNA measurement at week 4, 8, 12, 24 respectively (Fig.3.2.1). At week 4 the percentage of patients who achieved HIV-RNA < 50 copies was 47.3, 46.7 and 42.1 in overall, B and non-B groups respectively. At week 8 the percentage of patients who achieved HIV-RNA < 50 copies was 61.5, 59.8 and 38.5 in overall, B and non-B groups respectively. At week 12 the percentage of patients who achieved HIV-RNA < 50 copies was 60.6, 58.7 and 38.5 in overall, B and non-B groups respectively.



Time (weeks)	0	4	8	12	24
B (N)	186	150	161	130	160
Non-B (N)	20	19	13	13	17
Tot (N)	206	169	174	143	177

Fig.3.2.1 Virologic response during raltegravir treatment. Green, blu and red lines indicates overall patients, patients infected by B subtype viruses and non-B viruses respectively.

Finally, at week 24 the percentage of patients who achieved HIV-RNA < 50 copies was 71.3, 70.1 and 58.8 in overall, B and non-B groups respectively. At each time point, no differences among patients infected by B or non-B viruses in percentage of virologic success were found (Fisher test, P>0.05) and the efficacy of raltegravir in the two groups of patients was confirmed (Chi Square for trend P<0.05)

3.2.2 Prevalence of integrase baseline polymorphisms according with subtype

Among all integrase mutations 45, were polymorphisms of which E11D, D25E,L101I and T125A showed a specific codon usage with a prevalence>5% in overall patients (Tab 3.2.1).

Of note only the secondary resistance mutations S230N was polymorphic with a prevalence of 8.7% in overall population.

The mutations L101I did not show any association with subtype (B vs not-B), however stratifying its prevalence for the codon usage, L101I(ATA) had higher prevalence in not-B infected patients (1.6% in B vs 65.0% non-B; p<0.001) while L101I(ATC) had higher prevalence in B infected patients (40% in B vs 5.0% non-B; p<0.001) (Fisher Exact Test with Benjamini hoeckberg correction FPR=0.1) (Tab 3.2.1).

Similarly, the codon usage influenced the prevalence of T125A polymorphism that showed higher prevalence in non -B infected patients (33.3% in B vs 80.0% non-B; p<0.001), but stratifying its prevalence for the codon usage, only T125(GCA) was significantly associated with non-B subtype (18.8% in B vs 65.0% non-B; p<0.001) (Fisher Exact Test with Benjamini hoeckberg correction FPR=0.1).

In addition, K14R(11.3% in B vs 35.0% non-B; p=0.041), T112V (7.0% in B vs 65.0% non-B; p=0.0006), T124A(25.3% in B vs 80.0% non-B; p<0.001), S119T (4.0% in B vs 20.0% non-B; p=0.026), I135V (8.1% in B vs 40.0% non-B; p=0.002), V201I (41.4% in B vs 90.0% non-B; p<0.001), T206S (16.7% in B vs 50.0% non-B; p=0.007), L234I (5.4% in B vs 65.0% non-B;

p<0.001) and S283G (9.1% in B vs 30.0% non-B; p<0.014) showed higher prevalence in non-B subtype (Fisher Exact Test with Benjamini hoeckberg correction FPR=0.1).

3.2.3 Prevalence of integrase polymorphisms and Raltegravir resistance mutations according to virologic response

In order to evaluate the clinical impact of Integrase mutations at baseline of raltegravir containing regimen, the prevalence either of known raltegravir resistance mutations (Stanford resistance note; http://hivdb.stanford.edu/cgi-bin/INIResiNote.cgi) or the IN polymorphisms were calculated according with virologic response at 24 weeks.

No primary resistance mutations at position Y143, N155 or Q148 were detected while some secondary mutations were found among the 124 responding and the 53 failing patients analyzed.

Indeed, L74M, T97A, G140A, N155S, E157Q, G163R were indifferently found either in responding or failing patients (Tab 3.2.1A) below the 2% of overall popululation, confirming that these mutations could be found at low frequency.

The secondary V151I were detected only in 3 failing patients (5.7% in failing group; 1,7% in overall population).

However, even if this mutation showed a different prevalence between the 2 groups of patients the statistic significance was not confirmed after multiple comparison test correction.

Therefore in this population of InSti naive patients, the presence of secondary raltegravir resistance mutations at baseline did not correlate with different virologic response at 24 weeks.

Tab.3.2.1. Prevalence of Integrase polymorphisms in 206 InSti naive patients according with subtype (B versus Non-B).

Mutation	Subtype				Overall		P Value
	B (N=186)		Non-B (N=20)				
	N	%	N	%	N	%	
K7R	12	6.5	0	0.0	12	5.8	0.612
E10D	21	11.3	1	5.0	22	10.7	0.703
E11D	39	21.0	0	0.0	39	18.9	0.226
E11D(GAT)	14	7.5	0	0.0	14	6.8	0.370
E11D(GAC)	26	14.0	1	5.0	27	13.1	0.483
K14R	**21**	**11.3**	**7**	**35.0**	**28**	**13.6**	**0.009**
S17N	55	29.6	5	25.0	60	29.1	0.799
R20K	17	9.1	3	15.0	20	9.7	0.421
A23V	16	8.6	1	5.0	17	8.3	1.000
S24N	13	7.0	0	0.0	13	6.3	0.620
D25E	19	10.2	2	10.0	21	10.1	1.000
D25E(GAG)	9	4.8	2	10.0	11	5.3	0.290
D25E(GAA)	11	5.9	0	0.0	11	5.3	0.605
L28I	31	16.7	1	5.0	32	15.5	0.325
V31I	47	25.3	5	25.0	52	25.2	1.000
S39C	27	14.5	1	5.0	28	13.6	0.323
M50I	20	10.8	1	5.0	21	10.2	0.700
I72V	73	39.2	4	20.0	77	37.4	0.143
L101I	91	48.9	14	70.0	105	60.0	0.099
L101I(ATA)	**3**	**1.6**	**13**	**65.0**	**16**	**7.8**	**<0.001**
L101I(ATC)	**75**	**40.3**	**1**	**5.0**	**76**	**36.9**	**<0.001**
L101I(ATT)	15	8.1	0	0.0	15	7.3	0.371
L101I(GTC)	2	1.1	0	0.0	2	1.0	1.000
K111T	13	7.0	1	5.0	14	6.8	1.000
T112V	**13**	**7.0**	**13**	**65.0**	**26**	**12.6**	**<0.001**
T112I	13	7.0	3	15.0	16	7.8	0.192
I113V	22	11.8	2	10.0	24	11.7	1.000
S119T	**9**	**4.8**	**4**	**20.0**	**13**	**6.3**	**0.026**
S119P	30	16.1	0	0.0	30	14.6	0.050
T122I	37	19.9	1	5.0	38	18.4	0.133
T124A	**47**	**25.3**	**16**	**80.0**	**63**	**30.6**	**<0.001**
T124N	17	9.1	2	10.0	19	9.2	1.000
T125A	**62**	**33.3**	**16**	**80.0**	**78**	**37.9**	**0.007**
T125A(GCA)	**35**	**18.8**	**13**	**65.0**	**48**	**23.3**	**<0.001**
T125A(GCG)	30	16.1	4	20.0	34	16.5	0.750
T125A(GCT)	6	3.2	0	0.0	6	2.9	1.000
I135V	**15**	**8.1**	**8**	**40.0**	**23**	**11.2**	**<0.001**
M154I	18	9.7	1	5.0	19	9.2	0.701
K156N	18	9.7	2	10.0	20	9.7	1.000
V165I	25	13.4	2	10.0	27	13.1	1.000
V201I	**77**	**41.4**	**18**	**90.0**	**95**	**46.1**	**<0.001**
T206S	**31**	**16.7**	**10**	**50.0**	**41**	**19.9**	**0.001**
I208L	11	5.9	2	10.0	13	6.3	0.366
K211R	14	7.5	1	5.0	15	7.3	1.000
T218S	23	12.4	2	10.0	25	12.1	1.000
S230N	18	9.7	0	0.0	18	8.7	0.227
L234I	**10**	**5.4**	**13**	**65.0**	**23**	**11.2**	**<0.001**
D256E	37	19.9	6	30.0	43	20.9	0.383
S283G	**17**	**9.1**	**6**	**30.0**	**23**	**11.2**	**0.014**

Integrase Position, Frequency and percentage in B, non-B and overall infected patients are indicated. Fisher exact test with Benjamini-Hockberg correction (FPR=0.1) were used to evaluate the association of mutations with subtype (B vs Non-B). In bold blue and black mutations associated with B or Non-B subtypes respectively, underlined the mutations associated with raltegravir resistance.

Among the 45 IN polymorphisms described before, T122I, K156N and E11D (GAT) mutations showed higher prevalence in failing patients, but the multiple comparison correction did not confirmed the significance. In addition, mutations S17N, M50I, V201I, T206S, D256S, already described in some preliminary study raltegravir (Ceccherini-Silberstein, et al., 2008; Da Silva, et al., 2008; Ceccherini-Silberstein, et al., 2009; Miller, et al., 2009), were not associated with virologic response (Table.3.2.2B)

Interestingly, among the polymorphisms tested, T125A showed higher prevalence in failing patients without statistical significance (47.2% in failing patiens vs 32.2% in responding patients; p<0.068), but stratifying its prevalence for the codon usage, only T125A with the specific GCA codon showed higher prevalence in failing patients (37.7%) than in responding patients (15.3%) with statistical significance (P<0.002; Fisher exact test confirmed with multiple comparison Benjamini-Hoeckberg method). As described before, this mutations is associated with non-B subtype, and the divergence of prevalence among B and non-B infected failing patients is more consistent than in overall patients (6/7 [86%] non-B subtype vs 14/46 [30%] B subtype, OR=0.07 [CI:0.01-0.52],p=0.009).

3.2.4 Independent predictors of virologic success at 24 weeks of raltegravir treatment by univariate and multivariate logistic regression.

In order to investigate if baseline integrase polymorphisms associated with virologic response and other variables such as baseline HIV-1-RNA, co-administrered drugs and subtype (B vs non-B) were independent predictors of virological success, univariate and mutivariate logistic regression were performed. The GSS was not considered for the low number of genotypic tests for PR and RT available.

By univariate logistic regression the predictors of worse virological success were Baseline HIV-1 RNA (p<0.001; Odd Ratio 0.5 C.I. 95% [0.3-0.7] and the presence of T125A(GCA) polymorphism (p=0.001; Odd Ratio 0.3; C.I. 95% [0.1-0.6]) (Table3.2.3A). In the multivariate analysis the independent predictors of worse virologic success were: Baseline HIV-1 RNA (p<0.001, Odd Ratio

0.4, C.I. 95% [0.3-0.7]); T125A(GCA) (p<0.006, Odd Ratio 0.3, C.I. 95% [0.1-0.7]) and AZT or D4T Co-administration (p<0.04, Odd Ratio 0.3, C.I. 95% [0.1-0.9]) (Table3.2.3B).

All the drugs contained in the regimens with raltegravir , with the exception of Thymidine analogues, and the subtype did not influence the virologic response at 24weeks.

Table.3.2.2 Prevalence of polymorphism and raltegravir resistance mutations in InSti naive patients at raltegravir baseline according with virologic response.
The prevalence of IN mutations stratified for virologic response at 24 weeks of raltegravir treatment is indicated

Mutation	Virologic Response						P value
	Responding Patients (N=124)		Failing Patients (N=53)		Overall Patients (N=177)		
	N	%	N	%	N	%	
L74M	2	1.6	0	0.0	2	1.1	1
E92Q	0	0	0	0.0	0	0	-
T97A	1	0.8	1	1.9	2	1.1	1
F121Y	0	0	0	0.0	0	0	-
E138K	0	0	0	0.0	0	0	-
G140A	1	0.8	0	0.0	1	0.6	1
G140S	0	0	0	0.0	0	0	-
Y143H	0	0	0	0.0	0	0	-
Y143C	0	0	0	0.0	0	0	-
Y143R	0	0	0	0.0	0	0	-
S147G	0	0	0	0.0	0	0	-
Q148H	0	0	0	0.0	0	0	-
Q148R	0	0	0	0.0	0	0	-
N155H	0	0	0	0.0	0	0	-
N155S	1	0.8	0	0.0	1	0.6	1
V151I	0	0	3	5.7	3	1.7	0.026*
E157Q	2	1.6	0	0.0	2	1.1	-
G163R	0	0.0	1	1.9	1	0.6	1
I203M	5	4.0	3	5.7	8	4.5	0.705
S230N	13	10.5	1	1.9	14	7.9	0.067

A) Prevalence of raltegravir resistance associated mutations according with virologic response.

In bold underlined, the primary resistance mutations.
*Statistical significance not confirmed after multiple comparison correction

Mutation	Virologic Response						P value
	Responding Patients (N=124)		Failing Patients (N=53)		Overall Patients (N=177)		
	N	%	N	%	N	%	
E11D(GAT)	13	10.5	0	0.0	13	7.3	0.011*
E11D(GAC)	13	10.5	11	20.8	24	13.6	0.092
S17N[a]	33	26.6	18	34.0	51	28.8	0.366
M50I[a]	11	8.9	7	13.2	18	10.2	0.420
T122I	19	15.3	16	30.2	35	19.8	0.038*
T125A	40	32.2	25	47.2	65	40.1	0.068
T125A(GCA)	**19**	**15.3**	**20**	**37.7**	**39**	**22.0**	**0.002**
T125A(GCG)	20	16.1	6	11.3	26	14.7	0.492
T125A(GCT)	4	3.2	2	3.8	6	3.4	1.000
K156N[a]	7	5.6	9	17.0	16	9.0	0.022*
V201I[a]	53	42.7	29	54.7	82	46.3	0.188
T206S[a]	25	20.2	10	18.9	35	19.8	1.000
D256E[a]	24	19.4	15	28.3	39	22.0	0.235

B) Prevalence of IN polymorphisms according with virologic response. In bold are indicated the mutation significantly associated with worse virologic response;

[a] polymorphisms already associated with VS in other studies (Ceccherini-Silberstein, et al., 2008; Da Silva, et al., 2008; Ceccherini-Silberstein, et al., 2009; Miller, et al., 2009)

*Statistical significance not confirmed after multiple comparison correction

Table 3.2.3. Univariate and multivariate logisitic regression models evaluating the predictors of virologic response to raltegravir at 24 weeks.

The variables included in the models with the statistic significance and the relative Odd Ratio (C.I. 95%) are indicated.

A)Univariate model. In bold the predictors. B)Multivariate model. In Bold the indipendent predictors.

A)

Variables	P value	Odd Ratio	95.0% C.I. for Odd Ratio	
			L	U
Baseline HIV-1 RNA	**<0.000**	**0.5**	**0.3**	**0.7**
SubType (B vs NotB)	0.292	1.7	0.6	4.8
DRV Co-administered	0.550	1.2	0.6	2.4
T20 Co-administered	0.278	0.7	0.3	1.4
MVC Co-administered	0.632	0.7	0.2	2.6
ETR Co-administered	0.652	0.9	0.4	1.7
TDF Co-administered	0.658	1.2	0.6	2.2
AZT or D4T Co-administered	0.056	0.4	0.1	1.0
DDI or DDC or ABC Co-administered	0.667	0.9	0.4	1.8
FTC or 3TC Co-administered	0.738	0.9	0.4	1.9
T125A(GCA)	**0.001**	**0.3**	**0.1**	**0.6**

B)

Variables	P value	Odd Ratio	95.0% C.I. for Odd Ratio	
			L	U
Baseline HIV-1 RNA	0.000	0.4	0.3	0.7
SubType (B vs NotB)	0.995	1.0	0.3	3.4
DRV Co-administered	0.938	1.0	0.5	2.3
T20 Co-administered	0.900	1.1	0.5	2.4
MVC Co-administered	0.505	0.6	0.1	2.9
ETR Co-administered	0.896	1.0	0.4	2.0
TDF Co-administered	0.536	1.3	0.6	2.7
AZT or D4T Co-administered	**0.040**	**0.3**	**0.1**	**0.9**
DDI or DDC or ABC Co-administered	0.853	0.9	0.4	2.2
FTC or 3TC Co-administered	0.704	0.8	0.3	2.3
T125A(GCA)	**0.006**	**0.3**	**0.1**	**0.7**

3.2.5. Prevalence of T125A mutations according with raltegravir treatment

In order to evaluate if T125A mutation were associated with raltegravir treatment, its prevalence was explored in a large group of InSti naive patients (N=482) (data retrieved by our resistance database) and in a group of patients at raltegravir failure (N=66) (data retrieved by both our resistance database and Stanford resistance database [http://hivdb.stanford.edu/cgi-bin/II_Form.cgi]). T125A showed higher prevalence in raltegravir treated patients (47% [N=31]) than in raltegravir naive patient (33% [N=159]) with statistical significance (p=0.028 Fisher test) (Fig 3.2.2). Interestingly, stratifying the prevalence of this mutation for codon usage, only the specific GCA codon showed higher prevalence in raltegravir treated patients (34.8%[N=23] treated vs naive 19.3%[N=93]; p=0.006, Fisher test) with statistical significance (Fig 3.2.2)

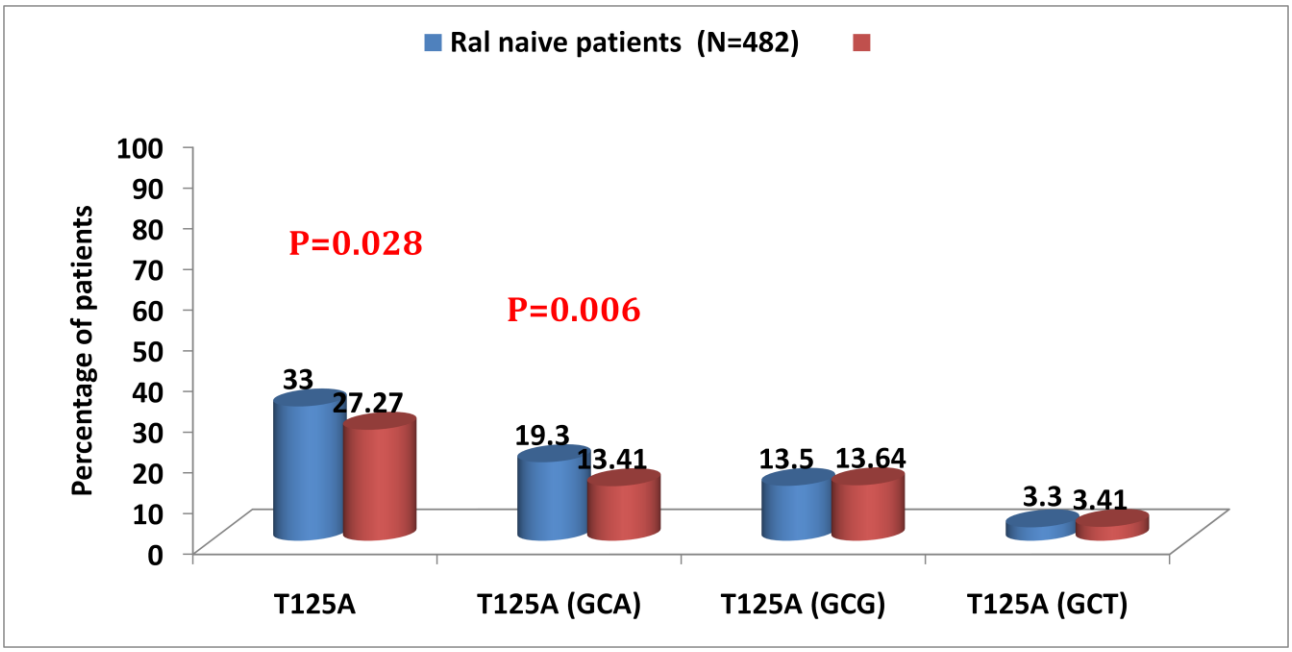


Fig 3.2.2. Prevalence of T125A stratified for codon usage among raltegravir naive and failing patients. Blu and red bars indicate the percentage of raltegravir naive and failing patients respectively; for statistical comparison were used fisher test.

3.3. Primary Mutations Associated with Resistance to raltegravir are not Detectable by Pyrosequencing in Integrase Inhibitors Naïve Patients

This work has been in part presented as posters at 15th and 16th Conference on Retroviruses and Opportunistic Infections (CROI) (Ceccherini-Silberstein, et al., 2008; Ceccherini-Silberstein, et al., 2009) and as oral presentation at 2nd Italian Conference on AIDS and Retrovirus (ICAR) (Armenia et al.,2010b)

3.3.1 UDPS coverage and sample size results

More than 500000 (range: 20,075 – 328) reads were obtained by the 56 samples analyzed. Due to read length limits of ±250 bp, double stranded sequencing was obtained for a smaller part of the amplicon (around 90 to 135 IN positions) (Fig 3.3.1).

3.3.2 Prevalence of baseline raltegravir resistance associated mutation detected by Population-sequencing

As previously described in 3.2.3 paragraph and table 3.2.2.

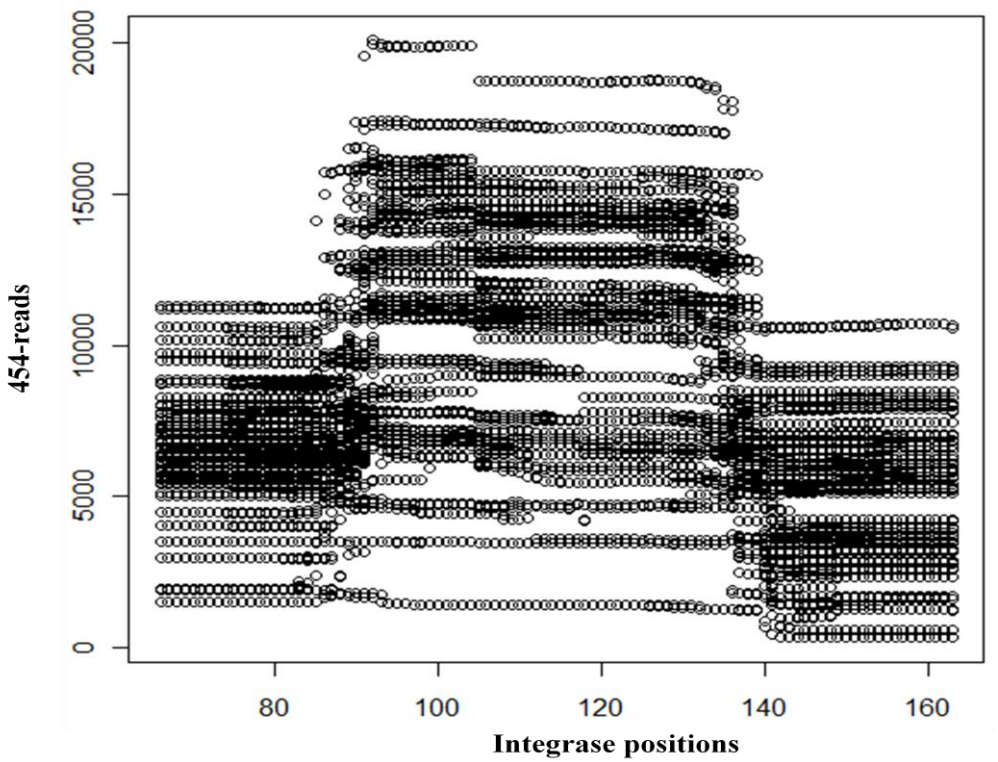


Fig.3.3.1 Integrase Coverage and number of sequences obtained by UDPS. Dots indicate the number of sequences obtained per each integrase positions for the 56 samples genotyped by 454-pyrosequencing.

3.3.3 Comparison of mutation detectability with Population sequencing and UDPS

Regarding UDPS, only mutations with ≥50 reads were considered, to exclude the possible systematic errors as recommended by Roche. We calculated the median number of mutations, considering the overall or the resistance mutations, detected for patients by both sequencing assays at baseline and at failure (Fig 3.3.2). At baseline 23 patients were analyzed with both techniques and the median (IQR) number of overall mutations was 9 (6-12) vs 3 (2-4) for UDPS and Population-sequequecing respectively ($p<2.8 \times 10^{-5}$ Wilcoxon Matched-Pairs Signed-Ranks Test; Fig 3.3.2) while for raltegravir-resistance mutations was 0 (0-1) vs 0 (0-0) respectively ($p<0.125$ Wilcoxon Matched-Pairs Signed-Ranks Test; Fig 3.3.2). At failure 10 patients were analyzed with both techniques and for 6 more than 1 genotypic tests were available. Thus, for 10 patients, at first failure, the median (IQR) number of overall mutations was 11 (11-14) vs 4 (3-5) for UDPS and bulk-sequequecing respectively ($p<0.002$ Wilcoxon Matched-Pairs Signed-Ranks Test; Fig 3.3.2) while for raltegravir-resistance mutations was 3 (1.25-4) vs 1 (1.25-2) respectively ($p<0.02$ Wilcoxon Matched-Pairs Signed-Ranks Test; Fig 3.3.2). In addiction, for 6 patients, at last failure, the median (IQR) number of overall mutations was 14 (10-15) vs 5 (5-6) for and Population-sequequecing respectively ($p<0.001$ Wilcoxon Matched-Pairs Signed-Ranks Test; Fig 3.3.2) while for raltegravir-resistance mutations was 4 (3-4) vs 2 (2-3) respectively ($p<0.12$ Wilcoxon Matched-Pairs Signed-Ranks Test; Fig 3.3.2).

The number of mutations detected for each genotyping technique, considering either overall mutations or INI resistance mutations, changed over time with statistical significance for Population-sequencing (Overall mutations, $P=0.032$; INI-resistance mutations $P<0.001$; Kruskal-Wallis Test) while only the number of INI-resistance mutations for UDPS (Overall mutations, $P=0.072$; INI-resistance mutations $P<0.001$; Kruskal-Wallis Test).

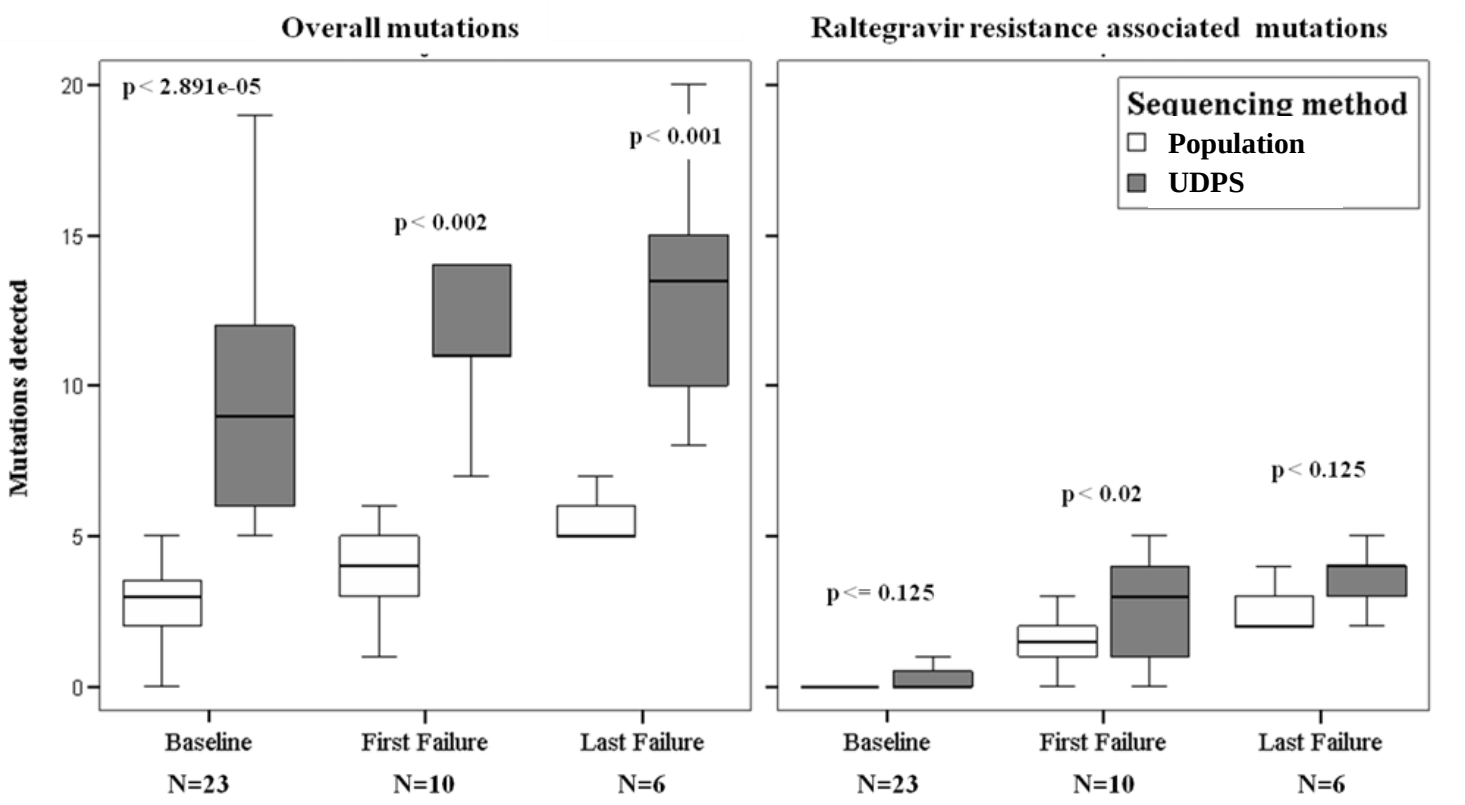


Fig.3.3.2 Comparison of mutation detectability with Population-sequencing and UDPS.
The box plots represent the distribution of number of mutations detected per patient according to the genotypic assays used at baseline genotypes (N=23) and at first (N=11) and last (N=6) genotypes at failure available. In the left panel were plotted the distribution of all mutations detected. In the right panel were plotted the distribution of raltegravir-resistance mutations detected. White box and grey box indicates the mutations detected by Population-sequencing and UDPS respectively. Wilcoxon Matched-Pairs Signed-Ranks Test were used to evaluate the differences in median number of mutations detected according with the 2 genotyping approach used.

3.3.4 Baseline prevalence of raltegravir resistance mutations detected by UDPS according to virologic response at 24 weeks of raltegravir containing regimen.

The primary resistance mutations (Y143C/H/R, Q148H/K/R, N155H) were detected neither by Population-Sequencing nor by UDPS. Of note, among > 200000 sequences analyzed by UDPS, the mutations N155H and Y143R had never been detected, while the mutation Q148H/R and Y143CH were been detected with ≤10 reads and not considered in analyses. Among the 23 patients analyzed at baseline, 14 did not achieved the virologic success at 24 weeks of raltegravir treatment. For each baseline mutations detected, the frequency and the median percentage of reads among patients who responded or not at 24 weeks of raltegravir treatment were evaluated and no mutations were found associated with virologic response (Median Test, Fisher exact test, data not show) (Table 3.3.1)

Specifically, T97A were detected in 1 failing patients with a frequency of 99% in viral variants while in 1 responding patient it were detected as minor quasispecies with a prevalence of 0.3%. V151I mutation were found only in 2 failing patients with a prevalence of 1.5% and 98.3% respectively among viral species. F121Y mutation was found with a low frequency (0.6% of sequences) in 1 failing patient. Differently, G163R were detected in 1 responding patients with a prevalence of 8.6% (Table 3.3.1).

Independently of the sequencing method, the presence of secondary-resistant species at baseline was not associated, at failure, with evolution at the same amino acid position or to specific primary raltegravir resistance mutations.

Table 3.3.1 Baseline prevalence of Stanford raltegravir resistance mutations by UDPS according to virologic response at 24 weeks. The prevalence of IN mutations associated with resistance to INIs (http://hivdb.stanford.edu/cgi-bin/INIResiNote.cgi) at baseline of the raltegravir containing regimen, in overall patients and among failing and responding patients at 24 weeks of treatment is reported. The maximum and minimum number and the percentage of UDPS-reads is reported according overall, failing and responding patients too.

Mutation	HIV RNA >50 cps/mL at 24 weeks				HIV RNA <50 cps/mL at 24 weeks				Overall Frequency (N=23)		Sign.
	Frequency (N=14)		Reads Range (Min-Max)		Frequency (N=9)		Reads Range (Min-Max)				
	N	%	N	%	N	%	N	%	N	%	
L74M	0	0	-	-	0	0	-	-	0	0	-
E92Q	0	0	-	-	0	0	-	-	0	0	-
T97A	1	7.1	6491	99	1	11.1	54	0.3	2	8.6	NS
F121Y	1	7.1	75	0.6	0	0	0	0	1	4.3	NS
E138K	0	0	-	-	-	-	-	-	0	0	-
G140A	0	0	-	-	0	0	-	-	0	0	-
G140S	0	0	-	-	0	0	-	-	0	0	-
Y143H	0	0	-	-	0	0	-	-	0	0	-
Y143C	0	0	-	-	0	0	-	-	0	0	-
Y143R	0	0	-	-	0	0	-	-	0	0	-
S147G	0	0	-	-	0	0	-	-	0	0	-
Q148H	0	0	-	-	0	0	-	-	0	0	-
Q148R	0	0	-	-	0	0	-	-	0	0	-
N155H	0	0	-	-	0	0	-	-	0	0	-
N155S	0	0	-	-	0	0	-	-	0	0	-
V151I	2	14.3	156-6106	1.5-98.3	0	0	-	-	2	8.6	NS
E157Q	0	0	-	-	0	0	-	-	0	0	-
G163R	0	0	-	-	1	11.1	500	8.6	1	4.3	NS

3.3.5 Prevalence of raltegravir resistance mutations over time by UDPS

Among 23 patients analyzed by UDPS at baseline, for 10 out 14 patients which failed regimen or showed a virologic rebound, we genotyped at least 1 sample at different time point during raltegravir treatment. The primary resistance mutations N155H, Q148H/R and Y143R appeared in 5, 2 and 1 failing patients respectively (Table 3.3.2). Differently, 2 patients failed without any primary resistance mutation (data not show).

Regarding the patient with resistance patterns including Y143R mutation (Table 3.3.2A), one patient (ID 12), carrying the secondary mutation T97A at baseline (99.0% of variants), developed the mutations Y143R, detected in 95%, 99.8%, 99.7, 99.7% and 99.6 % of variants among 1, 3, 7, 9, 12 months with raltegravir therapy respectively. Of note the mutation E92Q were also detected in 1.9% of variants at 1 month, but after disappeared. After 6 months the secondary mutations L74M, E157Q and the novel T112A gained their prevalence over time among variants.

Regarding the 2 patients with resistance patterns including Q148H/R mutations (Table 3.3.2B, patient 184, showed Q148R at months 1 to 5 in 1.5%, 15.8%, 63.3%, 70.7% and 76.6% of variants respectively. Interestingly, the mutation Q148R were present at baseline in 2 viral variants (0.08%), under reliability cut-off established. G140S mutation were detected at 3 to 5 months the raltegravir starting regimen in 34.0%, 66.7% and 68.1% of variants respectively (Table 3.3.2B). Three and 9 months after raltegravir treatment interruption, viruses carrying G140S and Q148R mutations were not cleared (month 3: G140S [1.6%], Q148R [1.8%]; month 9: G140S [0.9%], Q148R [1.2%]) (Table 3.3.2B). V151I and G140A were detected, at 2 month and from 3 to 5 months respectively, at low frequency (<4.3%) among variants too. Patient 27, at month 10, developed Q148H and G140S in 99.4% and 99.7% of variants, at the same time point F121Y were detected with frequency <1% too (Table 3.3.2B).

Regarding the 5 patients with resistance patterns including N155H mutation (Table 3.3.2C), the secondary mutations L74M, E92Q, T97A, Y143C, V151I, E157Q and G163R were detected in 1, 2, 1, 2, 4, 2, 2 and 1 patients respectively out 5, with a prevalence presently above the 1% of variants over time. In addition, in one patients the mutation N155H were present in 65.3% of variants without any other secondary resistance mutations.

Regarding the patients without any primary resistance mutations at failure, 2 out 6 had an available sample genotyped during raltegravir treatment, in which any raltegravir associated mutations were detected (data not showed).

Tab.3.3.2. Prevalence of raltegravir resistance mutations using UDPS. The percentage of reads of IN mutations associated with resistance to INIs for patients who developed primary resistance mutations is reported. In bold is reported the intra-patients percentage of each primary resistance mutations

A) Patients who developed resistance pattern with Y143R mutation

Patient	Time (M)	HIV RNA	L 74 M	E 92 Q	T 97 A	T 112 A*	F 121 Y	E 138 K	G 140 A	G 140 S	Y 143 C	Y 143 H	**Y 143 R**	S 147 G	Q 148 H	Q 148 R	Q 148 K	V 151 I	N 155 H	E 157 Q	G 163 R
12	0	4.9			99																
	1	5		1.9	99.8								**95**								
	3	5.2			99.6	0.5							**99.8**								
	7	5.1	1.7		99.4	31	0.6						**99.7**								
	9	4.8	3.3		99.4	40.1	0.4						**99.7**							1.1	
	12	4.8	9		99.7	66.9							**99.6**							6.9	

B) Patients who developed resistance pattern with Q148H/R mutation

Patient	Time (M)	HIV RNA	L 74 M	E 92 Q	T 97 A	T 112 A*	F 121 Y	E 138 K	G 140 A	G 140 S	Y 143 C	Y 143 H	Y 143 R	S 147 G	**Q 148 H**	**Q 148 R**	**Q 148 K**	V 151 I	**N 155 H**	E 157 Q	G 163 R
27	0	3.5					0.6														
	10	3.6								99.4					**99.4**						
84	-3	5.9																			
	0	5.7														0.08*					
	1	3.2														**1.5**					
	2	2.7														**15.8**		1.6			
	3	4.7							4.3	34						**63.3**					
	4	5.4							1	66.7						**70.7**					
	5	5.1							3.7	68.1						**76.3**					
	3**	5.5								1.6						**1.8**					
	9**	5.8								0.9						**1.2**					

*in patient 84 at time 0, Q148R mutation was detected in 2 reads (0.08%).
** Time after raltegravir interruption.

C) Patients who developed resistance pattern with N155H mutation.

Patient	Time (M)	HIV RNA	L 74 M	E 92 Q	T 97 A	T 112 A*	F 121 Y	E 138 K	G 140 A	G 140 S	Y 143 C	Y 143 H	Y 143 R	S 147 G	Q 148 H	Q 148 R	Q 148 K	V 151 I	**N 155 H**	E 157 Q	G 163 R
141	0	4.9																98.3			
	6	4.3					0.8											99.8	98.7	2	37.2
145	0	5																			
	10	3.2		0.5	96.5						0.5							14.9	99.6		
229	0	4.7																			
	7	5.7	15								88.9							10.2	98		
	9	5.7	15.1								88.3							10.6	98.3		
	11	4.1									99.2							10.3	99.4	10	
69	0	4.4																			
	5	3.8		4.1														6.5	98.1		2.2
	7	3.6		21.2														41.6	97.4		4.3
81	0	5.3																			
	3	5.1																	65.3		

3.3.6 Phylogenetic analyses of haplotypes evolution during raltegravir treatment of 4 representative failing patients

The phylogenetic analysis was performed for patients 12, 69, 84 and 27 on unique overlapping sequences obtained with UDPS, forming four datasets. T_0 population sequences were always found to be highly similar to the contemporaneous predominant viral strains as determined by pyrosequencing analysis.

Patient 12 (Fig 3.3.3A): The baseline viral population consisted of different T_0 strains, all carrying T97A mutation. From the one with higher prevalence, three different strains harboring specific resistance mutations (E92Q, N155H or Y143R) developed. Viral strains expressing E92Q or N155H mutations did not further evolve and they were never found at later times. On the other hand, viral strains harboring Y143R mutation took rapidly the advantage over all other T1

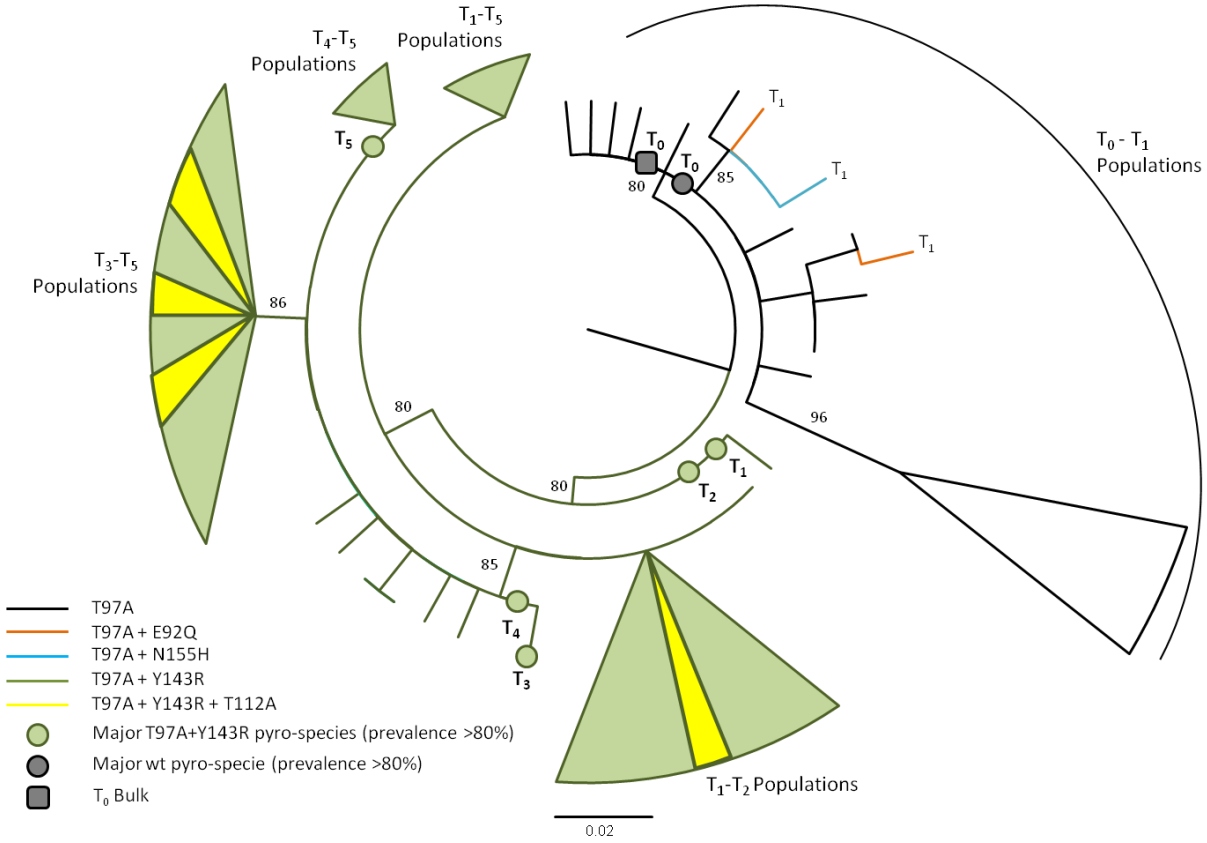
population. Viral evolution since T2 was thus exclusively based on viral strains expressing Y143R. Several strains in T2, T3, T4 and T5 populations also developed T112A mutation in addition to pre-existing Y143R.

Patient 27 (Fig 3.3.3B): Only two samples were available for this patient. Viral evolution simply followed the pathway of resistance development over a wide baseline population of wt viruses. In fact, all viral strains at T12 sampling already had the Q148H and G140S resistance mutations.

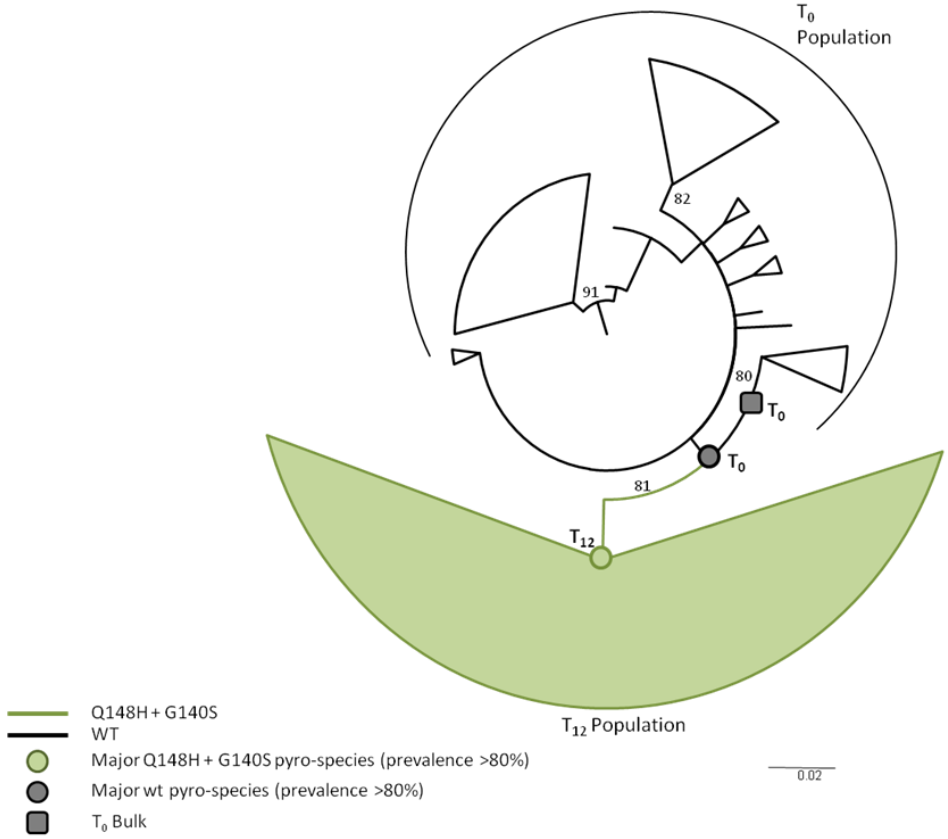
Patient 69 (Fig 3.3.3C): The baseline viral population consisted of different T_0 *wt s*trains, from whom subsequently developed (T3 time) two different strains harboring Y143S or N155H resistance mutations. Viral strains expressing Y143S mutation did not further evolve, while from viral strains harboring N155H mutation were generated all viruses of T4 generation. Upon N155H viral population, mutations E92A and E92Q were further developed through independent evolutionary pathways.

Patient 84 (Fig 3.3.3D): The baseline viral population consisted of different T_0 *wt s*trains and a minority population harboring Q148R resistance mutation (prevalence <0.1%). Major viral population at T1 and T2 sampling times were always mainly constituted by *wt* strains, which not had yet developed resistance. However, from one of the baseline mutated viral strains, some minority populations expressing Q148R became to develop. After the further addition of G140S mutation on pre-existing Q148R, resistant strains became predominant over wild types one. These resistant viruses were identified as the major viral populations of T3, T4 and T5 sampling times. However, after drug discontinuing, wild type viruses took the advantage once again, becoming predominant in later T6 and T7 samplings.

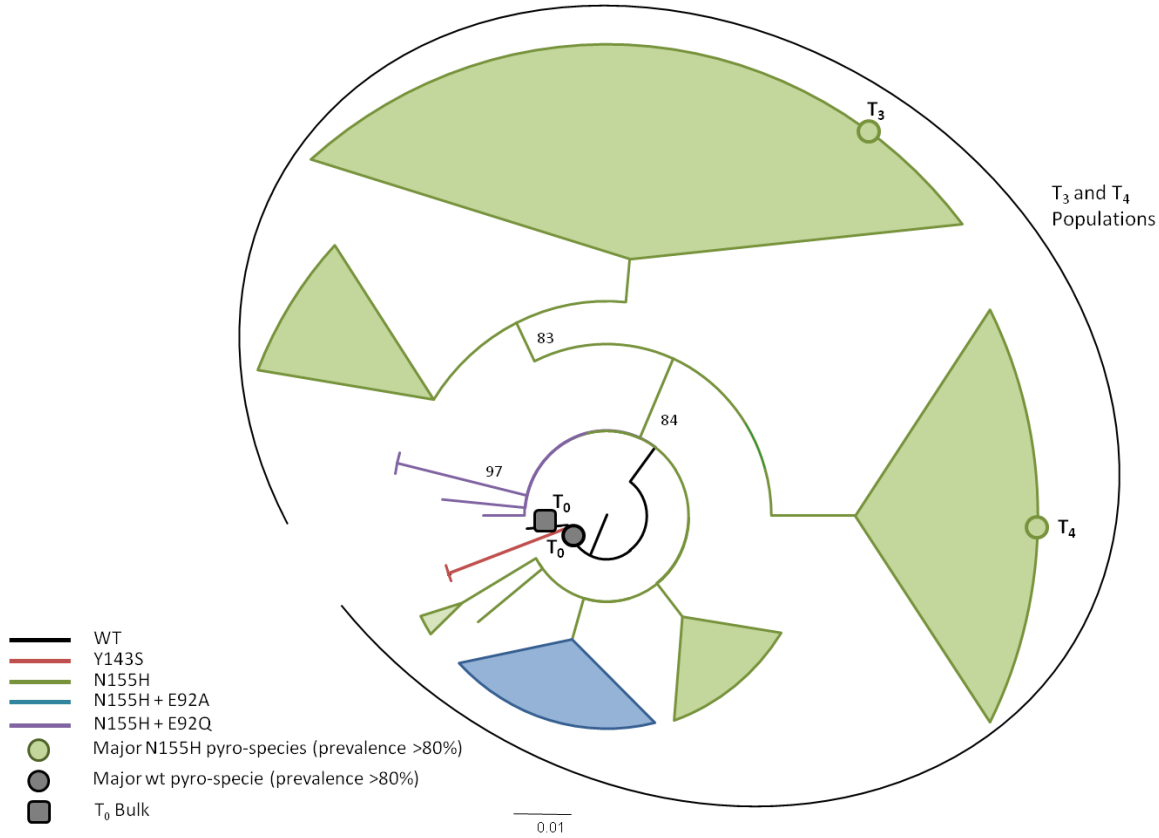
A)Patient 12



B)Patient **27**



C)Patient69



D) Patient 84

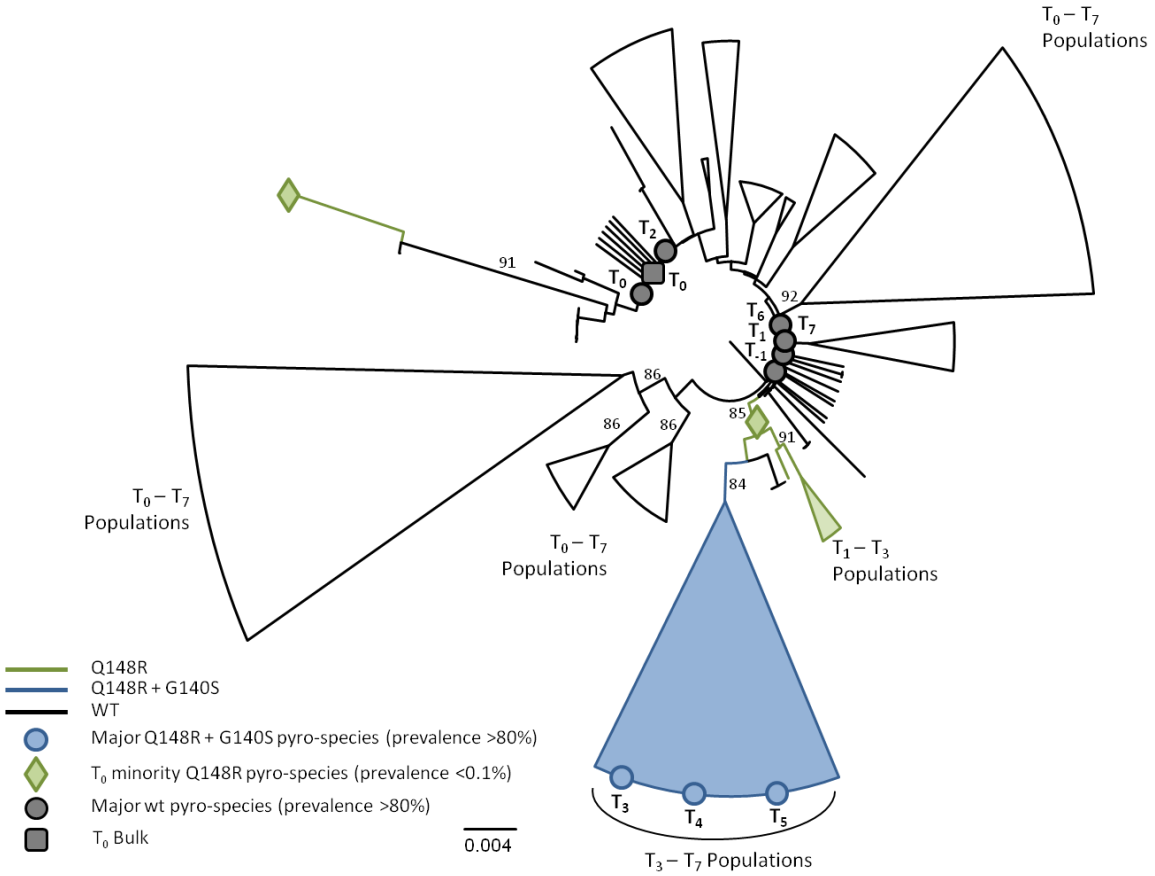


Fig.

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Fig 3.3.3. Evolution of viral haplotypes over time during raltegravir treatment in 4 different failing patients.
ML trees inferred for the haplotypes (90 to 163 IN positions) from patients 12, 27, 69, 84. Dots indicates major variant detected by UDPS, square indicates specimens by population sequencing, rhombus indicates UDPS haplotype carrying Q148R mutations detected at baseline under technical cut-off (2 variants at 0.04% respectively). **A) Patient 12.** The initial viral population consists of different T0 wt strains. From the one with higher prevalence, three different strains harboring specific resistance mutations develop. Viral strains expressing E92Q (orange line) or N155H (cyan line) mutations do not further evolve and they're not found at later times. On the other hand, viral strains harboring Y143R (green line) mutation take rapidly the advantage over all other T1 population. Viral evolution since T2 is exclusively based on viral strains expressing Y143R. The mutation T112A (yellow line) harbored among some variants at T3 to T5. **B) Patient 27.** Viral evolution simply followed the pathway of resistance development over a wide baseline population of wt viruses. In fact, all viral strains at T12 sampling already had the Q148H and G140S resistance mutations (green line). **C)Patient 69.** The initial viral population consists of different T0 wt strains. Viral strains expressing Y143S mutation (red line) do not further evolve and they're not found at later times. On the other hand, viral strains harboring N155H mutation (green line) take rapidly the advantage over all other T1 population. Viral evolution since T2 is exclusively based on viral strains expressing N155H. Upon these viral populations, mutations E92A (cyan line) and E92Q (purple line) are further developed through independent evolutionary pathways. **D)Patient 84**. From one of the baseline mutated viral strains, some minority populations expressing Q148R became to develop (green rhombus). After the further addition of G140S mutation on pre-existing Q148R, resistant strains became predominant over wild types one. These resistant viruses were identified as the major viral populations of T3, T4 and T5 sampling times. However, after drug discontinuing, wild type viruses took the advantage once again, becoming predominant in later T6 and T7 samplings.

3.3.7 Phenotypic resistance associated with resistance mutations harbored at failure

In order to analyze the association between raltegravir resistance mutations and the susceptibility to raltegravir, phenotyping assays were performed on samples from patients who developed raltegravir resistance mutations at failure.

For patients 12, as expected, no phenotypic resistance was observed at baseline (FC RAL=1.2, FC EVG=1.2), although the presence of T97A mutation. The fold change gained over time (months 1 to 12: FC RAL: 33.1 to 205.5; FC EVG: 5.2 to 14.2; Table 3.3.3) with the accumulation of mutations T112A and E157Q, already known as associated with InSti resistance *in vivo* and *in vitro* (Jones et al. 2007; Shimura et al. 2007, Malet et al. 2008).

For patient 69 , as expected for the absence of resistance mutations, no phenotypic resistance was observed at baseline (FC RAL=1.2, FC EVG=0.8; Table 3.3.3). The presence of N155H was associated with high (>100) and moderate (<30) FC for elvitegravir and raltegravir at failure respectively. Of note, the combination of N155H with the novel mutation E92A, as major quasispecies, was associated with higher resistance to both drugs tested (months 2 and 5: FC RAL=

10.4 and 31.6; FC EVG= 43.32 and 117.5; Table 3.3.3) than the E92Q mutation found only as minor quasispecies at month 7.

Patient 81 and 229 harbored the N155H mutation at failure without other mutations and with Y143C and S230R respectively. The uncommon combination of Y143C and S230R with N155H showed an impressive phenotypic resistance (FC RAL= 1255.3 ± 297.1; FC EVG=625.3 ± 382.9; Table 3.3.3) despite the N155H alone in patient 81 (FC RAL = 4.5; FC EVG = 29.4; Table 3.3.3).

The mutations G163R and T97A/T, harbored at failure in patient 78, were associate with moderate phenotypic resistance for both drugs tested (FC RAL= 4.0; FC=3.6; Table 3.3.3).

As expected, the replication capacity, as the p24 antigen production, of viruses carrying resistance mutations, especially for N155H, were lower than the HXB2 control strain.

Table 3.3.3. Phenotype effect of raltegravir resistance mutations on raltegravir and elvitegravir susceptibility and on replication capacity. The patients ID, the time of sample collection according with raltegravir starting regimen, the mutations detected by Population-sequencing in recombinant virus harvested after antiviral experiments, the replication capacity as percentage of p24 antigen respect control virus (HXB2D or baseline sample of patient), the mean Fold change resistance ± standard deviation for raltegravir and Elvitegravir respectively are indicated.

Patient	Time (months)	Mutations in virus harvested after antiviral experiments	Replication Capacity*	Fold Change Elvitegravir	Fold Change Raltegravir
12	0	**T97A**	100	1.2 ± 0.1	1.2±0.4
	1	**T97A, Y143R**	**47**	**28.0 ± 7.3**	**4.6 ± 0.9**
	3	**T97A, Y143R**	**38**	**33.5 ± 7.8**	**6.6 ± 2.9**
	7	**T97A, Y143R,** T112A/T	-	**59.4**	**4.8**
	9	**T97A, Y143R,** T112A/T	-	**96.1**	**7.5**
	12	**T97A, Y143R,** T112A, **E157E/Q**	-	**205.5**	**14.2**
69	0	No resistance mutations	90	1.3	0.8
	2	E92A, **N155H**	**36**	**10.4**	**45.3**
	4	E92E/A**, N155H**	**16**	**7.8**	**27.7**
	5	E92A**, N155H,** D232D/N	**37**	**31.6 ± 7.4**	**117.5 ± 34.4**
	7	**E92E/Q/A/P, N155H, E138E/K, V151I/V**	**22**	**5.4 ± 1.9**	**7.5 ± 2.3**
78	9	**G163R, T97T/A**	100	**4.0**	**3.6**
81	0	No resistance mutations	100	1.05	0.77
	3	**N155H**	55	**29.49**	**4.52**
27	0	No resistance mutations	100 ± **2.8**	0.4	0.5
	10	**G140S, Q148H**	**89.7 ± 2.5**	**248.0**	**456.0**
84	-3	No resistance mutations	-	0.7 ± 0.1	1.1 ± 0.0
	0	No resistance mutations	38	0.9 ± 0.0	0.7 ± 0.1
	4	**G140S, Q148R**	**43**	**34.4**	**50.6**
229	0	No resistance mutations	100 ± **8.0**	1.2	0.8
	11	**N155H, Y143C, S230R**	**62.4 ±19.3**	**1255.3 ± 297.1**	**625.3 ± 382.9**

In bold underlined primary raltegravir resistance mutations;
in bold secondary Raltegravir resistance mutations.

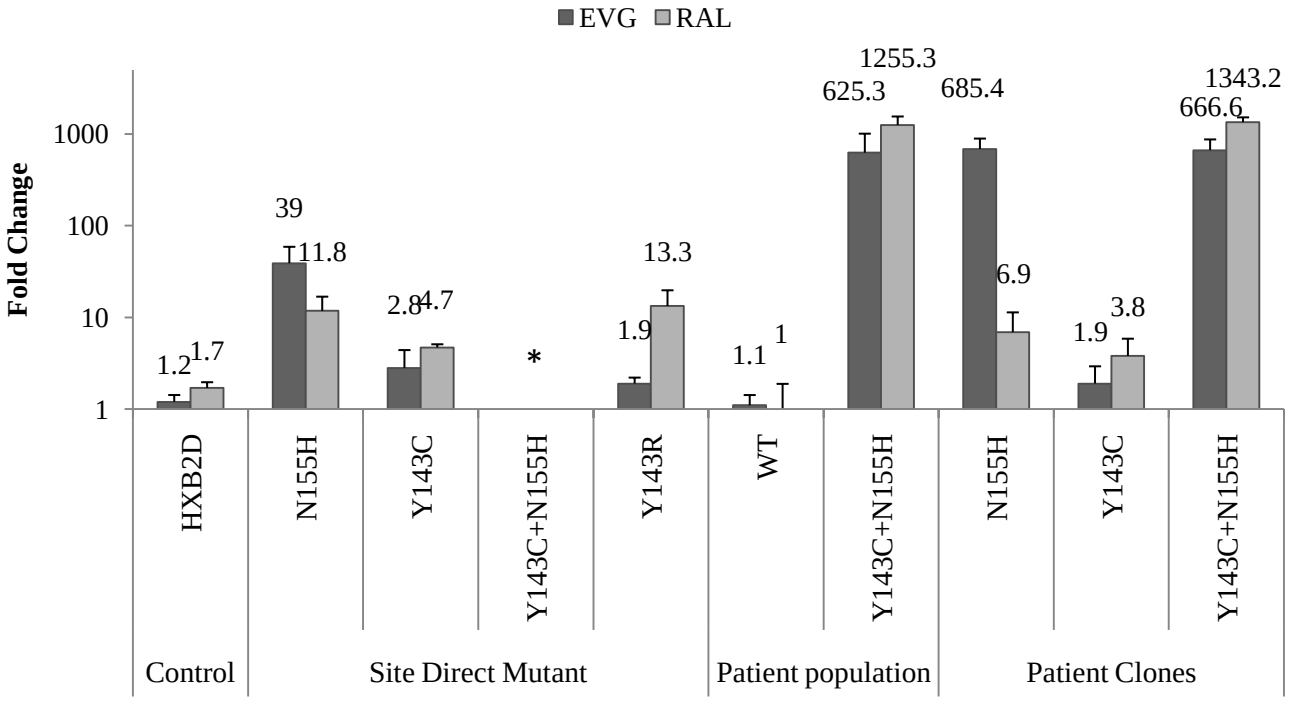
3.3.8 Phenotypic resistance profile and replication capacity of viruses carrying Y143C/R and/or N155H mutations.

Antiviral experiments on HXB2D site direct mutants with Y143C or N155H, showed FC>BCO for RAL and EVG (Y143C: FC RAL=4.7 ± 0.3, FC EVG=2.8 ± 1.6; N155H: FC RAL=11.8± 5.0, FC EVG=39.0±19.8;Fig.3.3.4 panel A).

However antiviral experiment for HXB2D mutation the combination of 2 mutations are still inprogress (Fig 3.3.4A). Interestingly, as corroboration, the antiviral experiments for single patient derived clones with N155H or Y143C or N155H+Y143C, confirmed a moderate and very high level of resistance for single and combined mutants, respectively (Y143C: FC RAL=6.9 ± 4.4, FC EVG=1.9 ± 1.0; N155H: FC RAL=6.9 ± 4.4, FC EVG= 685.0 ± 206.3; Y143C+N155H: FC RAL=1343.2 ± 175.2; FC EVG= 666.6 ± 205.9; Fig 3.3.4A)

As expected, the replication capacity, as the p24 antigen production, of viruses carrying resistance mutations, especially for N155H, were lower than the HXB2D control strain. Viruses carrying both N155H and Y143C mutations did not show different replication capacity than viruses with the N155H mutation alone.

A)



B)

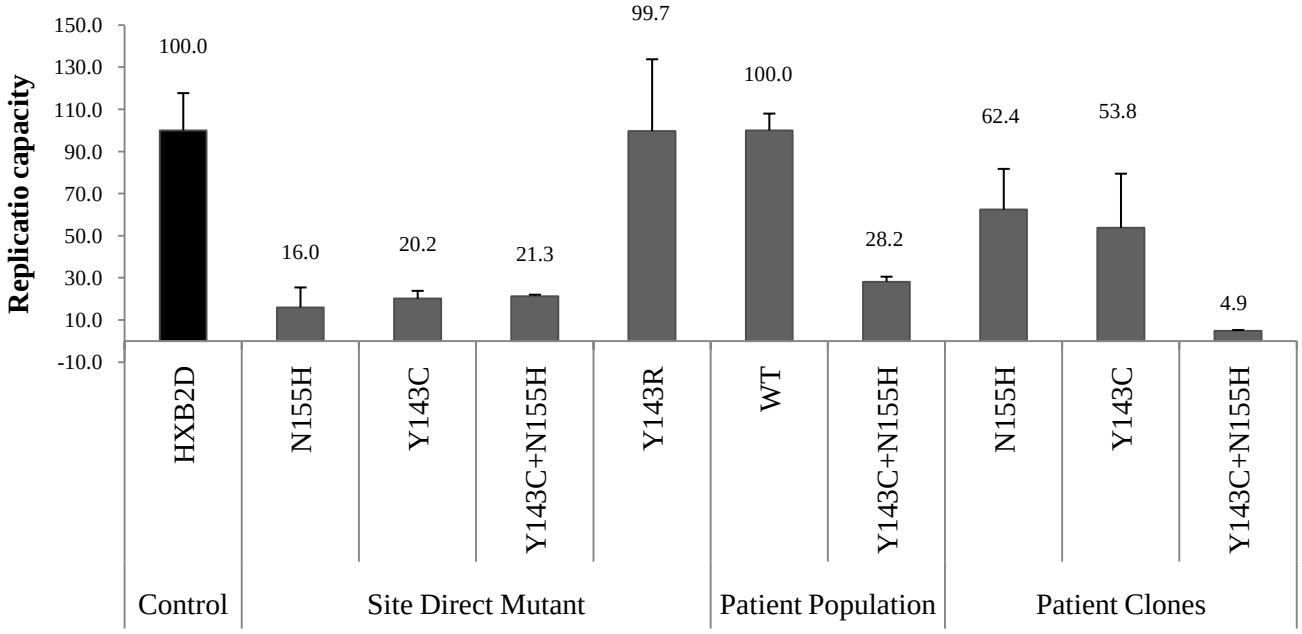


Fig 3.3.4. Fold change resistance and replication capacity of viruses carrying resistance pattern with N155H and Y143C mutations. A) Bar graf of RAL (light grey) and EVG (dark grey) fold change for different HXB2D site direct mutants (SDM) and recombinant population (RPV) or clonal (RCV) viruses from patients 229 is represented. From the left to right: HXB2D (negative control); N155H SDM; Y143C SDM; Y143C+N155H SDM; Y143R SDM; RPV WT baseline; N155H+Y143C RPV; N155H RCV; Y143C RCV; Y143C+N155H RCV. B) Bar graph of percentages (average and standard deviation) of virus produced in cell-free supernatant after 5 days of infection with 10,000 pg/ml of p24 gag Ag for virus, previously decribed for panel A, in C8166 T cells. One experiment, representative of three done in triplicate, is shown. *FC in progress.

4. Discussion

In this study, using three different genotyping approachs, the natural HIV-1 variability of IN was analyzed to explore the presence of InSti-resistant quasispecies and evaluate their effect on *in vitro* phenotypic susceptibility to raltegravir and elvitegravir, on replication capacity, on virologic response and on the evolution of resistance during treatment, in InSti naïve patients.

First of all, by a clonal genotyping and phenotyping approach, no primary resistance mutations for raltegravir (Y143R/C, Q148H/K/R, N155H) and elvitegravir (T66I, E92Q, S147G, Q148R/H/K, N155H) were found as major or minor species in 344 clones from 49 InSTI-naïve patients. Similarly, the majority of secondary resistance associated mutations to raltegravir and/or elvitegravir were also completely absent among all IN sequences analyzed. Few secondary mutations, such as L68V, L74M, T97A, G140S and V151I, were found in very few patients and only at level below 20% of viral population. Almost no phenotypic resistance viruses were present, with the exception of 3 clones (out of 344) showing decreased susceptibility only to elvitegravir (but none to raltegravir). In addition, a novel mutation, E92G (in the known primary elvitegravir-resistance associated position 92), was also rarely found in minor species and was specifically associated *in vitro* with decreased susceptibility to elvitegravir.

The E92G mutation has been never detected among more than 2000 integrase sequences, from raltegravir-naïve and -treated patients, with the exception for two HIV-1 isolates from HAART-naïve patients (Stanford DB) and for some patients where the mutation was detected at low frequency (<1%) by UDPS in this work (data not show). Therefore, particular attention should be dedicated in further studies to evaluate the impact of this rare mutation, present only at low frequency, on virological outcome in patients starting InSTI.

Several recent studies based on Sanger population sequencing, which generally does not allow reliable identification of resistant variants below 20%–30% of the virus population, have shown that all mutations clinically relevant for resistance to raltegravir and/or elvitegravir are absent or highly

infrequent in InSTI-naïve patients, either infected with HIV-1 B subtype or non-B subtypes (Ceccherini-Silberstein, et al., 2007; Ceccherini-Silberstein, et al., 2009; Lataillade, et al., 2007; Low, et al., 2009; Myers, et al., 2008; Passaes, et al., 2009; Rhee, et al., 2008; Sichtig, et al., 2009; Van Baelen, et al., 2008; van Han, et al., 2009).

There is a clear evidence in the literature that the utilization of methods able to detect minority quasispecies, increases the number of patients carrying viral strains resistant to NRTI, NNRTI, PI, and that, at least for NNRTI, there is also a correlation with an impaired virological outcome in patients treated with these drugs (Simen, et al., 2009). This is mostly due to the rapid selection, by initiation of antiretroviral treatment, of minor resistant-quasispecies, which become the major virus population and subsequently lead to early failure (Simen, et al., 2009; Johnson, et al., 2008; Metzner, et al., 2009). For this reason, knowing that minority species potentially resistance to InSTIs (a drug class characterized by a medium-low genetic barrier) are nearly absent in drug naïve patients, may have substantial relevance also from the practical point of view.

As a confirmation that there is no selection in patients randomly chosen for this study, we found a consistent number of RTI-resistant clones in drug-naïve patients, with a specific prevalence of phenotypic resistance around 7.1% (13 resistant clones for at least one NRTI or NNRTI out of 183 clones from drug-naïve patients), and of 23.8% of genotypical resistance (with at least 1 IAS NRTI or NNRTI mutation in 5 out 21 drug-naïve patients, very frequently only as minor variants <10%). Very similar results have been recently found using ultra-deep sequencing (at least 1 NRTI or NNRTI resistance mutation from the IAS list in 55 patients out of 258 (21.3%) (Simen, et al., 2009). Transmission of drug-resistant HIV-1 has been observed in most countries where antiretroviral treatment is available, and generally leads to a delay in virologic suppression and to an increased risk of earlier virologic failure, particularly (but not only) for drugs with low genetic barrier (Günthard, et al., 1998; Hirsch, et al., 2008; Kuritzkes, et al., 2008).

The near absence of InSTI-resistant strains in our population suggests that the circulation of strains naturally resistant to InSTI in drug-naïve patients will be limited until the widespread use of InSTI

will increase the chances of being infected with a strain from a patients treated with, and resistant against, such drugs.

We could not exclude that the approach used was sensitive enough to detect such resistance at levels below 10% of viral population. Indeed, a relative weakness of our clonal study could be that despite the impressive number of clones analyzed (near 400), relatively few samples (7 phenotypically and 9 genotypically) per patient were available, thus providing a sensitivity of the analysis to detect variants below the Sanger population-sequencing level of 20-30% in near half of the patients, with 265 clones analyzed.

In the second part of the study, analyzing a larger population of patients naïve to InSti (N=206) who started raltegravir, the prevalence and the impact of natural polymorphisms and known raltegravir resistance mutations, according with subtype, virologic response and codon usage were evaluated by population sequencing.

By these analyses, the absence of primary resistance mutations was confirmed, and the non-polymorphic secondary resistance mutations detected L74M, T97A, G140A, N155S, V151I, E157Q, G163R and I203M did not correlate with different virologic response at 24 weeks of raltegravir treatment, both in B and non-B infected patients.

Of note mutation E11D (GAT,GAC), D25E (GAG,GAA), L101I (ATA,ATC,ATT,GTC) and T125A (GCA,GCG,GCT) showed different prevalence in the usage of degenerated codons and each codons were considered as different polymorphism.

Nowadays, variables such as the codon usage and genetic barrier, as the number of nucleotidic substitutions allowing a change of aminoacid often related with subtype, have started to be considered in the analyses exploring the HIV-1 drug resistance. In a recent study on integrase, Maiga et al. assessed that InSti resistance mutations E92Q, Q148K/R/H, N155H and E157Q are highly conserved between subtypes B and CRF02_AG displaying a similar genetic barrier while G140S, G140C and V151I showed a higher genetic barrier to development in CRF02_AG.

In addition, the influence of codon usage, due by non-B HIV-1 subtypes, on virologic response for NRTIs drugs is already confirmed. The K65R mutation in HIV type-1 reverse transcriptase is selected more rapidly in subtype C than subtype B HIV-1 in cell culture and clinical studies (Brenner, et al., 2006; Doualla-Bell, et al., 2006). By template-usage experiments it was confirmed that subtype C nucleotide coding sequences caused RT to preferentially pause, leading to K65R acquisition (Coutsinos, et al., 2010). In another recent study it was assessed that by to codon usage variation, there is a significantly lower incidence of the substitutions L210W, Q151M, and F116Y in subtype F1 isolates than in the subtype B counterparts (Walária-Aleixo, et al., 2008).

Among the 45 IN natural polymorphisms found, mutations K14R, T112V, T124A, S119T, I135V, V201I, T206S and L234I showed higher prevalence in non-B subtypes, confirming other prevalence data of IN polymorphisms according with subtypes and CRFs (Lataillade, et al., 2007; Malet, et al., 2008; Garrido, et al., 2010).

Interestingly, we observed tahat the codon usage of mutations such as L101I and T125A had different prevalence according with HIV-1 subtypes. Indeed, L101I(ATA) and L101I(ATC) showed higher prevalence in B and non-B infected patients respectively, although, without codon usage stratifications, the prevalence of L101I did not correlate with subtype. Similarly, the prevalence of specific GCA codon for 125A showed a more consistent association with non-B subtypes than the overall codons for 125A. These findings may be explained observing the consensus sequences of the different subtypes and CRFs of HIV-1. Indeed, ATA and GCA are the consensus codons for many HIV-1 non-B subtypes and CRFs (CRF_02AG, CRF_06CPX, CRF_12BF, CRF_04CPX, CRF_11CPX, CRF_14BG, A2, C, F, H) at 101 and 125 IN positions respectively.

Overall, by the prevalence analysis according with virologic response at 24 weeks of treatment, and univariate and multivariate logistic regression, the T125A(GCA) mutation, the baseline HIV-1 RNA and the co-usage of AZT/D4T with raltegravir, were independent predictors of worse virologic response.

By these results, it's attainable that the codon usage and the subtype might have an impact on virologic response to raltegravir (and/or other InSti) and further investigation is needed, as previously described for other ARV's (Coutsinos, et al., 2010). The clinical implications and relevance of this polymorphism should be confirmed by other studies. Of note, in 58 HXB2-clonal recombinant viruses with RT-IN region from INI naïve patients, carrying T125 (GCA) mutation, no phenotypic resistance to raltegravir was observed (all FC values < Biological cutoff).

Preliminary genotypic data, based on ultra-sensible genotype assays (454-pyrosequencing, or parallel allele-specific sequencing, or allele-specific polymerase chain reaction, that allow to detect minor variants present at the level of 0.1-1% of the viral population), recently presented at CROI 2009 and confirmed in in this work (Table 3.3.1), showed a complete absence of all primary resistance mutations, with the exception of a rare presence of Q148H/R and of some secondary mutations, that, when present, were all confined to a restricted minority of variants <1% (Ceccherini-Silberstein, et al., 2009; Charpentier, et al., 2009; Liu, et al., 2009), and did not correlate with subsequent virological failure to raltegravir (Ceccherini-Silberstein, et al., 2009; Liu, et al., 2009).

Indeed, in the third part of the study, for a sub-group of 27 multi-experienced patients that started raltegravir, the UDPS allowed to analyze more than 500,000 sequences covering the IN region from position 66 to 163, with a mean number for sample ranging among 1,189 to 16,300. Using the cut-off of mutations detected with ≥50 reads among variants, the UDPS allowed to detect much more mutations, at raltegravir baseline, and at first and last failure genotyping , than the population-sequencing, according with other studies that compared the detectability of protease and reverse-transcriptase mutations by UDPS vs population sequencing (Le, et al., 2009; Simen, et al., 2009). Interestingly, in this cohort, we found that UDPS allowed to detect more raltegravir resistance mutations than those by population sequencing only at failure, confirming the absence of pre-existing resistance to raltegravir in INI naïve patients .

Moreover, we have never observed the primary resistance mutations N155H and Y143R at baseline while mutations Q148HR and Y143CH were rarely detected at very low level (<10 reads in <1% of variants) below the cut-off established, according with other studies (Charpentier, et al., 2009; Liu, et al., 2009). Differently, the secondary resistance mutations, were detected at baseline but, as expected by results of detectability, we observed neither association with the virologic response nor with the evolution at failure. The median intra-patient percentage of mutations and their frequency did not show any difference among responding and failing patient groups at baseline. Of note, the mutations T97A and V151I, were detected as major variants only in failing patients both at baseline and at failure .

T97A is a polymorphic accessory INI-resistance mutation selected *in vivo* by raltegravir (Malet et al. 2008; Miller et al. 2008; Canducci et al. 2009; Fransen et al. 2009; Ceccherini-Silberstein et al., 2008; Ceccherini-Silberstein et al., 2009), data confirmed in our analyses (Table 3.3.3), but its role as predictor of virological failure is still unclear. However, we found, by the clonal analyses, that in InSti naïve patients, T97A mutation decreases elvitegravir susceptibility and the raltegravir susceptibility near to biological resistance cut-off (Table 3.1.2). V151I is a polymorphic mutation which has been selected in vitro by multiple INIs, it has no effect on RAL or EVG susceptibility (Hazuda et al. 2004; Markowitz et al. 2007; McColl et al. 2007; Rowley 2008; Low et al. 2009)(Table 3.1.1). By population sequencing , we found this mutation at baseline only in 3 patients that failed raltegravir regimen, although without statistical significance (Table 3.2.2). By UDPS results, at failure, this mutation was often present togheter with N155H as minor or major quasispecies. Thus, evidence that T97A and V151I are selected *in vivo* by raltegravir pressure is clear, however we still haven't a robust confirmation about the clinical impact of these mutations and further investigation is needed.

By phenotypic analyses performed from viral samples collected over time from failing patients, the decreased susceptibility for raltegravir in virus carrying primary resistance mutations (Y143C/R,

Q148H/R, N155H) were confirmed. The secondary mutations L74M, E92Q, T97A, V151I, E157Q and the novel E92A and T112A were often found in combinations with primary resistance mutations at failure, contributing to increase the phenotypic resistance. In addition the combination of N155H and Y143C mutations, confirmed by haplotypes evolution analyses, was associated with very high phenotypic resistance for both raltegravir (FC>1200) and elvitegravir (FC>600) both in clonal and population viruses from patients and site direct mutant.

Although the results obtained by UDPS agree with the majority of data available in literature by today (Charpentier, et al., 2009; Liu, et al., 2009), in a clinical case, recently presented at 8th European HIV Resistance Workshop, an HAART treated patient who received raltegravir in salvage regimen, harbored the mutations N155H and Q148R at very low level (4 reads 0.12%; 1 read 0.03%) at baseline and developed the same mutations at failure (Codoner et al., 2010).

Interestingly, in our analyses, we found that in a single multi-resistant patient (pt 84), rapidly experiencing virologic-failure to raltegravir and maraviroc salvage-treatment, primary Q148R mutation at low-frequency (1 read, 0.08%) was observed before starting treatment, and it increased over time (Table 3.3.2C). In addition, three different CXCR4-using viruses were also detected at baseline at low-frequencies (2.5%, overall) which rapidly increased over time (4.9%-36.7%-48.7%, at month-2-3-4, respectively) (Ceccherini et al.,2009b).

Therefore, these data, according with the Spanish colleagues, suggest that mutations detected below the reliable cut-off of the UDPS technique should be considered with particular attention, especially in patients with high viral load and multi-experience historical failures. In both clinical cases, the 2 patients showed a nadir of CD4 cell count < 10 cell/mm^3, and had never achieved virologic success in their therapeutic history, showing very high level of viremia and carrying resistant viruses for all class of ARV's available at the starting of the salvage regimen.

Therefore at today the key unresolved questions regarding minority variants could be :

- Better define a threshold below which minority species do not affect virological response

- Is the threshold the same for all types of mutations?
- Is the threshold the same for the different drugs?
- Is the threshold the same for naive patients vs experienced?
- Is linkage of mutations on the same genome necessary for failure?

5. Summary and Conclusions

In this work, the impact of the HIV-1 integrase polymorphisms and minor quasispecies on virologic response and resistance development were characterized by three different genotyping methods (Cloning, population sequencing and Ultra-Deep-454 Pyrosequencing (UDPS) on in InSti naive patients who started a raltegravir containing regimen.

- The added-value of ultra sensible genotyping assays were confirmed both for cloning and UDPS, allowing to detect more HIV-1 integrase specimens and resistant variants than population sequencing.
- The primary resistance mutations (Y143CHR, Q148HKR, N155H) were never been detected by both cloning, population sequencing and UDPS (>0.1% of variants) methods at baseline in InSti naive patients.
- The secondary resistance mutations rarely found at baseline, did not show any significant association neither with virologic response at 24 weeks of treatment nor with the development of the same mutations at failure.
- No phenotypic resistance to InSti among 344 clones tested, from 49 InSti naive patients, were found, with the exceptions of 2 and 1 clones carrying T97A and G140S mutations respectively, showing low level resistance only for elvitegravir.
- The novel E92G mutation, found in a single clone from a multi-experienced treated patient, was associated with resistance *in vitro* to only for elvitergravir.

- Among the 45 polymorphisms, with a prevalence >5%, found in 206 InSti naive patients, only the mutations T125A with the specific GCA codon, higher prevalent in HIV-1 non-B subtypes infected patients, showed higher prevalence in patients who did not achieve virologic success at 24 week of raltegravir treatment.
- By statistical analysis, the independent predictors of worse virologic response at 24 weeks of raltegravir treatment were: baseline HIV-1 RNA, the Co-usage of thymidine analogues and the presence of T125A(GCA) mutation.
- At failure the uncommon combination of Y143C and N155H primary resistance mutations was associated with high phenotypic resistance to both raltegravir (FC>1200) and Elvitegravir (FC=653).

Therefore, based on this evidence, the pre-existing RAL resistance is a rare event in InSti-naïve patients, and when present, is confined only into a restricted minority of secondary variants. In addition, at baseline, only T125A (specific GCA codon) polymorphism, higher prevalent in non-b subtype viruses, at baseline was associated with poorer virologic response to raltegravir. This finding in non-B subtypes is intriguing and further research is warranted. The clinical implications and relevance of this polymorphism is still to be determined.

In conclusion, this study, suggest that at this point IN genotyping, in all patients before RAL treatment may not be cost effective and should not be recommended today, at least until evidence of transmitted drug resistance to INIs, or the clinical relevance of IN minor-variants/polymorphisms will be determined.

List of abbreviations used

3TC: lamivudine

ABC: abacavir

APV: amprenavir

ARV: antiretroviral drug

ATV: atazanavir

AZT: zidovudine

D4T: stavudine

DDC: zalcitabine

DDI: didanosine

DRV: darunavir

EFV: efavirenz

ETR: etravirine

FI: fusion inhibitor

T20: enfuvirtide

GRT: genotypic resistance test

HAART: highly active antiretroviral therapy

HIV-1: human immunodeficiency virus type 1

IC_{50}: 50% inhibitory drug concentration

IDV: indinavir

IN : integrase

INI : integrase inhibitor

InSti : integrase stand-transfer inhibitor

IQR: inter-quartile range

LPV: lopinavir

MCV: maraviroc

NAMs: NRTI-associated mutations

NFV: nelfinavir

NNRTI: non-nucleoside RT inhibitor

NRTI: nucleoside reverse-transcriptase inhibitor

NVP: nevirapine

PI: protease-inhibitor

PR: protease

RT: reverse transcriptase

RTI: reverse transcriptase inhibitor

SQV: saquinavir

RTV: ritonavir

TDF (TFV): tenofovir

UNAIDS: joint united nations program on HIV/AIDS

UDPS: Ultra-Deep-454-Pyrosequencing

VR: virological response

VS: virological success

WHO: World healt organization

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