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XXII CICLO

CHARACTERIZATION OF HIV-1 POL SEQUENCES IN NEWLY HIV-1 DIAGNOSED PATIENTS: IDENTIFICATION OF DRUG RESISTANCE MARKERS AND THEIR INVOLVEMENT IN THE TRANSMISSION EVENTS

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Italian Abstract

Ad oggi sono disponibili 25 farmaci per il trattamento dell'infezione da HIV. L'uso combinato di tali farmaci, la cosiddetta Highly Active Antiretroviral Therapy (HAART), ha consentito di migliorare le prospettive e la qualità di vita dei pazienti, rendendo possibile la soppressione della replicazione virale nella maggior parte dei soggetti infetti.
Tuttavia, se la terapia non riesce a sopprimere adeguatamente il virus, si osserva l'insorgenza di ceppi virali farmaco-resistenti in grado di compromettere inesorabilmente l'efficacia dei farmaci stessi. Attualmente, la presenza di virus farmaco-resistenti è messa in evidenza attraverso il test di resistenza genotipica che prevede il sequenziamento dei geni bersaglio dei farmaci antiretrovirali. Recentemente, sono state inoltre messe a punto delle metodiche in grado di rilevare ceppi resistenti anche in forma minoritaria, presenti cioè con una prevalenza inferiore al 20% nell'intera popolazione virale, il cui utilizzo nella pratica clinica è però ancora compromesso dai costi elevati. La più nota tra tutte è sicuramente il pyrosequencing.
Un problema strettamente connesso all'insorgenza di ceppi resistenti è rappresentato dalla loro trasmissione (sia come specie predominante che in forma minoritaria) a soggetti HIV-infetti che non hanno mai assunto alcun farmaco antiretrovirale (definiti drug-naïve). Attualmente, nei paesi occidentali, la prevalenza di virus resistente nei pazienti drug-naïve è stimata intorno al 10%, e diversi studi hanno evidenziato come tali ceppi (anche quando presenti in specie minoritarie) possano compromettere il successo della prima linea terapeutica.
In base a quanto riportato, l'obiettivo di questa tesi è stato quindi quello di caratterizzare la distribuzione e la diffusione della resistenza trasmessa in Italia, con particolare attenzione alle dinamiche di trasmissione della stessa e al ruolo che i soggetti drug-naïve rivestono in questo fenomeno.
Un ulteriore obiettivo è stato quello di definire nuovi marcatori genetici di HIV rilevabili attraverso metodiche standard di sequenziamento in grado di predire la presenza di varianti minoritarie resistenti nella popolazione virale. L'individuazione di mutazioni "sentinella" in grado di predire la presenza di quasispecie minoritarie resistenti riveste un ruolo centrale nel miglioramento dell'interpretazione genotipica e nella selezione di pazienti potenzialmente portatori di farmaco-resistenza.
Analizzando 255 pazienti di nuova diagnosi e naïve alla terapia antiretrovirale, abbiamo riscontrato che il 5,9% di questi mostrava resistenza trasmessa; in particolare il 3,9% era resistente alla classe farmacologica degli analoghi nucleosidici della trascrittasi inversa (NRTI), il 3,5% alla classe dei non-NRTI e lo 0,4% alla classe degli inibitori della proteasi (PI). Il 3,5% dei pazienti era inoltre portatore di un virus resistente a più classi farmacologiche. Comparando pazienti con infezione recente a pazienti con infezione cronica, abbiamo riscontrato che la prevalenza di virus farmaco-resistente era leggermente più bassa nella prima categoria rispetto alla seconda (3,4% versus 6,6%, anche se accompagnata da un P=0,09 non significativo), riflettendo probabilmente l'uso negli ultimi anni di farmaci sempre più potenti ed ad alta barriera genetica. Infine, la via di trasmissione che più delle altre sembrava incidere nella trasmissione della farmaco-resistenza era l'omosessualità (OR 7,7; 95% CI: 1,7–35,0, P=0,008).
Nella seconda parte di questa tesi, mediante metodiche filogenetiche avanzate, abbiamo caratterizzato i clusters epidemiologici di infezione in una coorte di 884 pazienti HIV-positivi, di cui 306 naïve alla terapia e 578 trattati. Da questa analisi è emerso come

benché la fonte principale di trasmissione di virus farmaco-resistente fosse rappresentata dai pazienti in fallimento terapeutico, ben il 38,5% dei virus resistenti figurava in clusters composti esclusivamente da individui drug-naïve con infezione sia recente che cronica, sostenendo il ruolo di tali pazienti quale ulteriore fonte di farmaco-resistenza.
Nella terza parte di questa tesi abbiamo identificato alcune mutazioni nella trascrittasi inversa di HIV, la cui presenza nel test di resistenza genotipico correlava in modo significativo con specie minoritarie farmaco resistenti. In particolare, l'analisi di di 40 pazienti drug-naïve ha mostrato come il 70% di pazienti con la mutazioni L210M presentasse specie minoritarie farmaco-resistenti (P=0.03), supportando il ruolo di tale mutazione come "sentinella" di farmaco-resistenza nascosta.
In conclusione, questa tesi ha consentito di caratterizzare la resistenza trasmessa e le dinamiche di trasmissione di ceppi HIV-farmacoresistenti in Italia. Queste informazioni sono di fondamentale importanza per impostare programmi di sorveglianza e prevenzione dell'infezione da HIV. Tale tesi ha portato inoltre all'identificazione di mutazioni "sentinella" in grado di predire la presenza di specie minoritarie resistenti non rilevabili dai test genotipici utilizzati comunemente nella pratica clinica. Ciò è di fondamentale importanza per ottimizzare la gestione clinica del pazienti infetto da HIV.

English Abstract

To date 25 drugs are available for the HIV-1 treatment. The combined use of these drugs, known as Highly Active Antiretroviral Therapy (HAART) has successfully suppressed the HIV-1 replication and has dramatically improved the prognosis of HIV-1 infected patients.

However, if viral rebound occurs during therapy, viruses with mutations conferring drug resistance can be selected. To date, drug resistant viruses can be detected by the genotypic resistance test, based on the sequencing of the genes target of the antiretroviral therapy. Recently, new assays (as ultra deep pyrosequencing) have allowed to assess antiretroviral drug resistance even when present as minority species (with a prevalence <20% of the entire viral population). However, their use is still limited in clinical practice due to their high cost.

The frequently selection of drug resistant strains in treatment failing patients can in turn increase the risk of their transmission (both in predominant and both in minority species) to HIV-1 infected individuals, naïve to the antiretroviral drugs (drug-naïve). To date, in countries with a wide access to HAART, the prevalence of drug resistant strains in drug-naïve patients is around 10%, and many studies have demonstrated that these drug resistance strains (also in minority species) are associated with an increased probability of virological failure to the first-line antiretroviral therapy.

In this light, the aim of this thesis was to characterize the distribution and the spread of HIV-1 drug resistance in Italy, with particular attention to the population dynamics of transmitted resistance and the role of untreated patients in the spread of drug-resistance. The last objective of this thesis was to define new genetic markers of HIV that can predict the presence of transmitted drug resistant minority species. The identification of these “sentinel” mutations could improve the genotypic interpretation and could help the selection of patients potentially reservoir of drug-resistance.

Among 255 newly diagnosed and untreated individuals, the 5.9% of patients showed signs of transmitted resistance; in particular, 3.9% of patients was infected with nucleoside reverse-transcriptase inhibitors (NRTI)-resistant viruses, 3.5% with non-NRTI-resistant viruses and 0.4% with protease inhibitors-resistant viruses. In addition, the 3.5% of patients carried HIV-1 resistant strains with more than one major drug resistance mutation. Comparing chronic infections with recent infections, we also found a decreased rate of resistance in recent infections (P=0.09), reflecting the increasing use of potent drugs and highly active antiretroviral regimens in recent years. Homosexual individuals were also more likely to harbour a virus with at least one primary resistance mutation (OR 7.7; 95% CI: 1.7–35.0, P=0.008).

In the second part of this thesis, we have investigated the epidemiological networks characterizing the HIV-1 infection by using a phylogenetic approach in a cohort of 884 patients. Of them, 306 were drug-naïve and 578 were HAART-treated individuals. Even if patients failing HAART remained the principal source of transmitted drug resistance, the 38.5% of drug resistant viruses was involved in clusters composed only by drug-naïve individuals, supporting the role of these patients as drug-resistance transmitters.

In the third part of this thesis, we have characterized specific mutations in the reverse-transcriptase of HIV, those presence significantly correlated with minority drug resistance variants. In particular, among the 40 drug naïve patients analyzed, we found that the 70.0% of patients with L210M carried drug resistance minority species

(P=0.03), supporting the role of this mutation as “sentinel” of hidden drug resistance mutations.
In conclusion, this thesis was able to characterize the HIV-1 transmitted drug resistance and the transmission dynamics of HIV-1 drug resistant strains in Italy. These data were essential to improve the surveillance and the preventive programmes on HIV infections. This thesis has also characterized “sentinel” mutations able to predict minority drug resistance variants, undetectable by standard genotypic tests. Taking into account these genetic markers right from diagnosis can help clinicians to improve the management of HIV-1 infected patients.

Keywords: HIV-1; drug naïve patients; transmission of drug resistance; drug resistance markers

Contents

1 Introduction

1.1 HIV and the retroviruses

The human immunodeficiency virus (HIV), identified in 1983, is a member of the Lentiviruses genus which are exogenous, non-oncogenic retroviruses 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. 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.

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 Cullen, 1998; Emerman and Malim, 1998; Kjems and Askjaer, 2000; Piguet and Trono, 1999; Pollard and Malim, 1998; Trono, 1995).

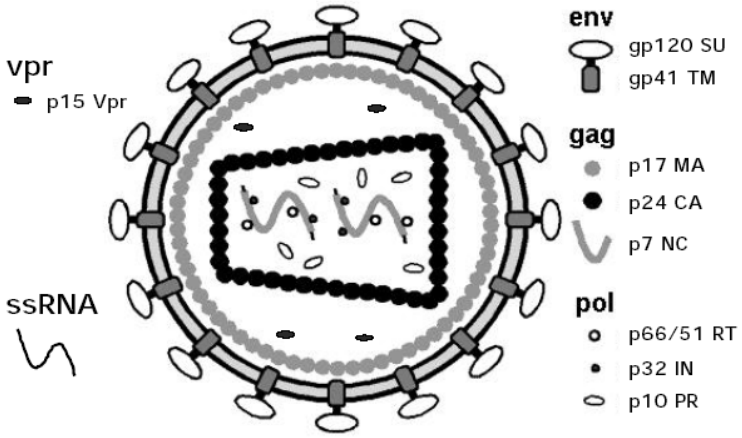


Figure 1.1. HIV-1 mature virion structure. Modified from WebPath resource collection (Klatt, 2000). Typical lentivirus particles are spherical, about 80-110 nm in diameter, and consist of a lipid bilayer membrane surrounding a conical core. The two identical singlestranded RNA (ssRNA) molecules, of about 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 (IN), and protease (PR). 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). Among the accessory proteins encoded by HIV-1, certainly Vpr and perhaps Nef and Vif are packaged into virions, although the precise location have not yet been elucidated. Neither the other accessory protein Vpu nor the regulatory proteins Tat and Rev have been detected in virion particles.

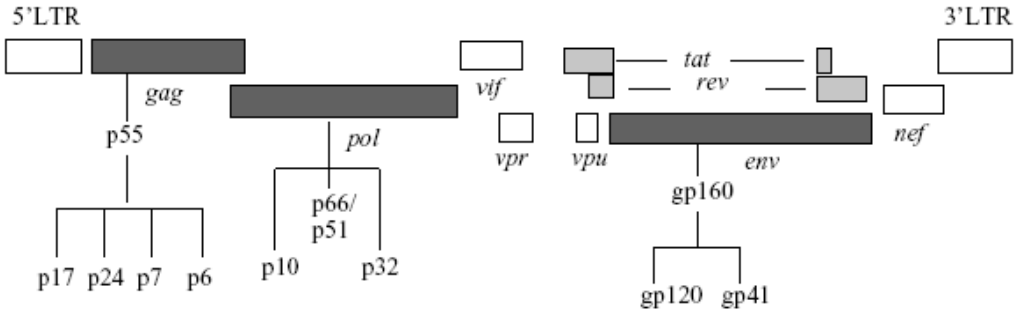


Figure 1.2. HIV genomic organization. Like all other retroviruses, HIV has three structural genes gag, pol and env (heavily shaded), which are flanked by the long terminal repeats (LTR's). In addition it has six more genes, including two regulatory genes tat and rev (stippled) and four accessory genes 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; Doranz 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; Kozlowski et al., 1991) 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 (von Schwedler et al., 1993) and the nucleocapsid protein NC (Darlix et al., 1993). 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). Nuclear import of the PIC seems to be directed by the accessory protein Vpr (Fouchier et al., 1998; Heinzinger et al., 1994), the Gag matrix protein p17 (Bukrinsky et al., 1993; von 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., 1998a; Popov et al., 1998b). 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 and Peterlin, 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 and Malim, 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, while 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; Hadzopoulou-Cladaras 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 and Sodroski, 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., 1992a, 1992b). 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 (Hoglund et al., 1994; Lamb and Pinto, 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.

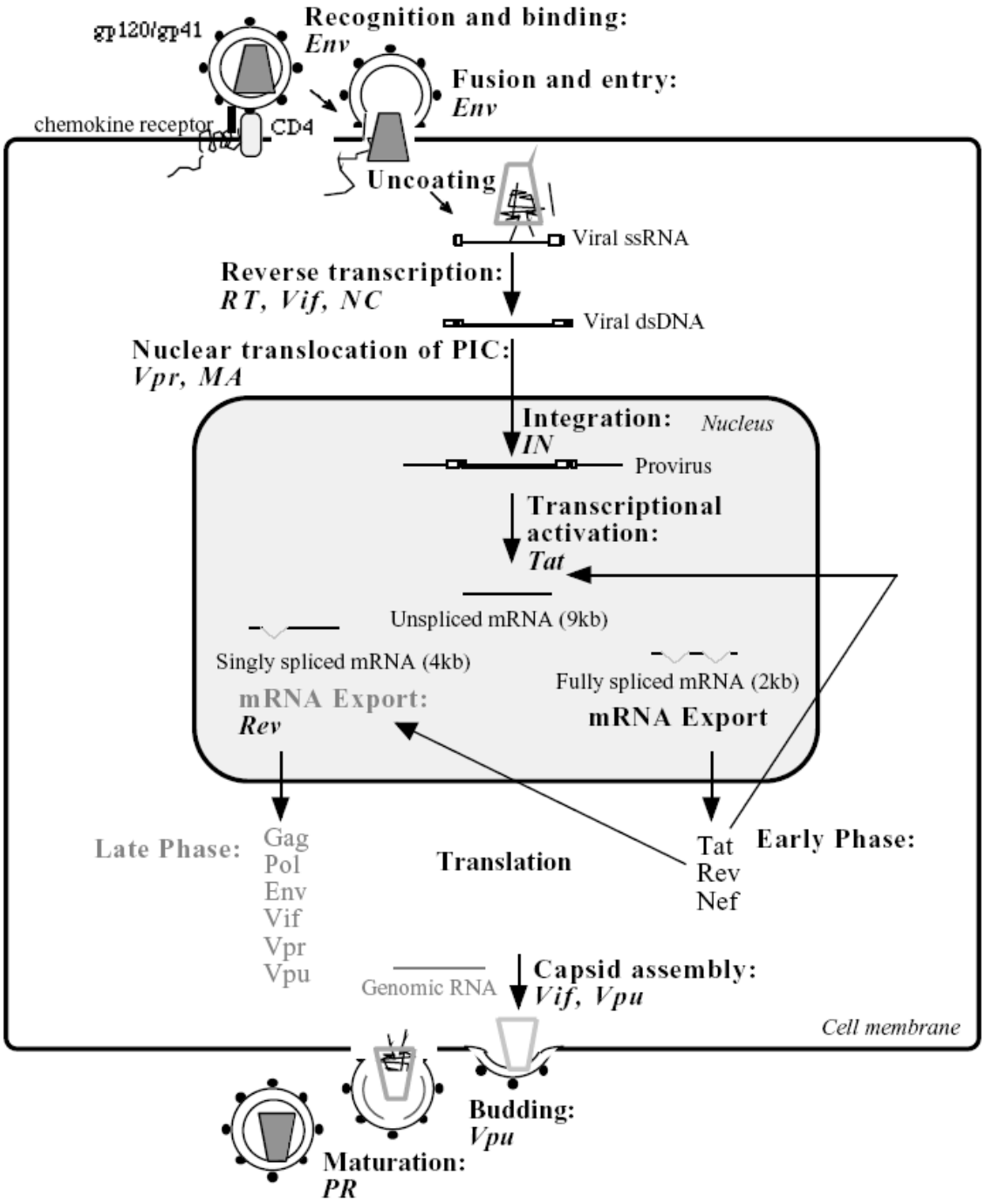


Figure 1.3. Replication cycle of HIV-1. Modified from Ceccherini-Silberstein, 2001 (http://edoc.ub.Muenchen.de/archive/00000533/01/Ceccherini-Silberstein_Francesca.pdf). Each fundamental step is presented in bold. Names in *italic* refer to viral gene products involved in the specific steps. HIV-1 gene 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.

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 et al., 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.

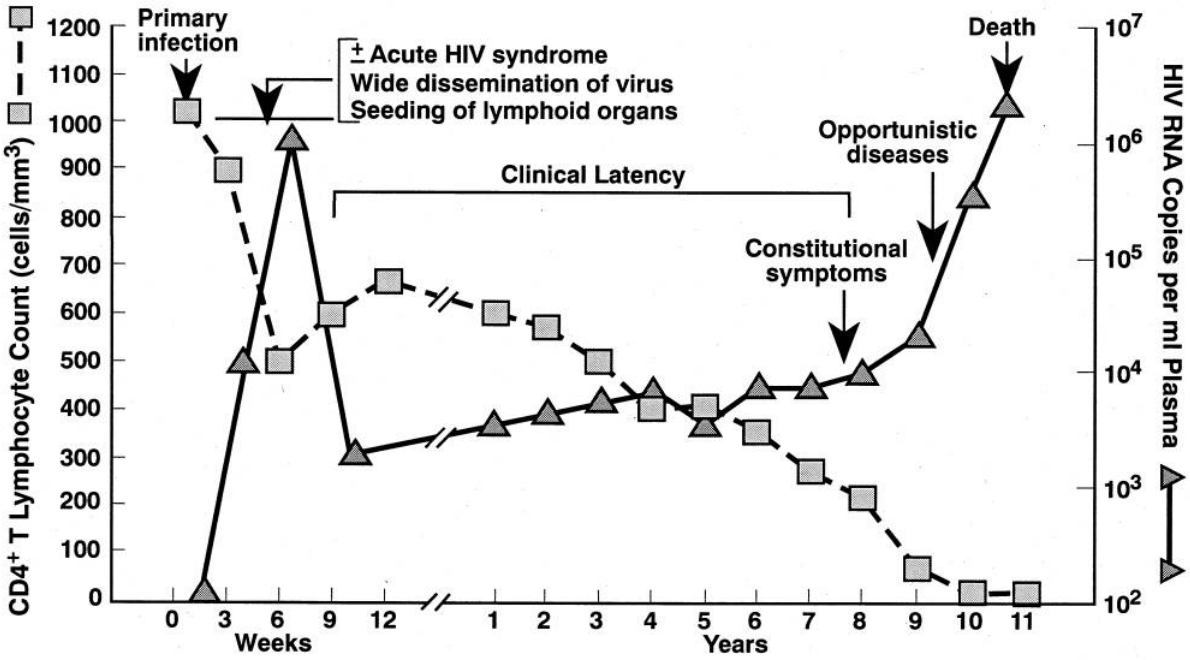


Figure 1.4. Schematic representation of the course of HIV infection

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., 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 (McCutchan, 2000; Robertson et al., 2000).

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.

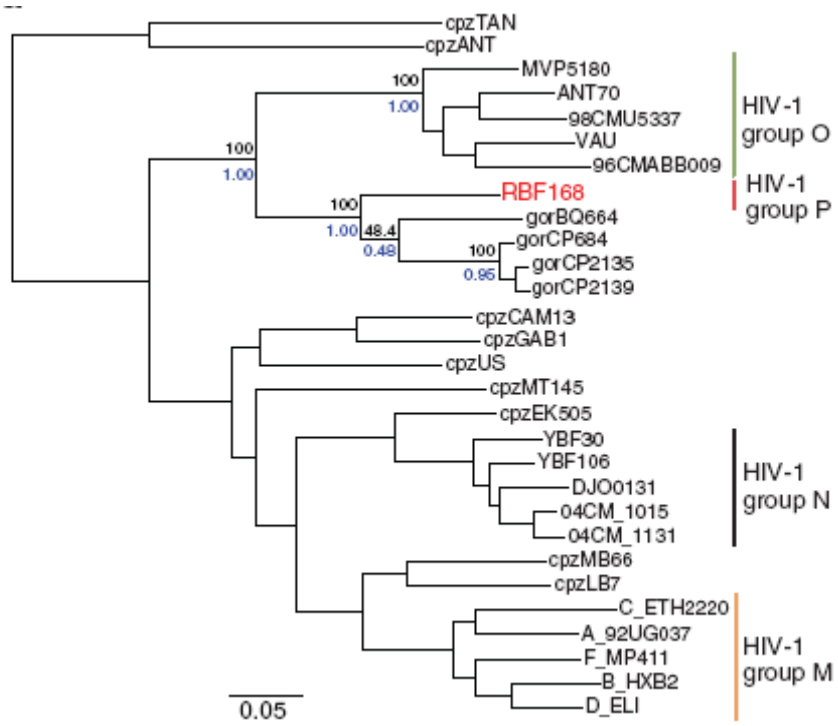


Figure 1.5. Phylogenetic relationship of primate lentiviruses. Phylogenetic tree derived from the alignment of *pol* gene sequences of HIV-1 and SIV strain (SIV_{cpz} and SIV_{gor}).Reproduced from Plantier *et al.*, 2009.

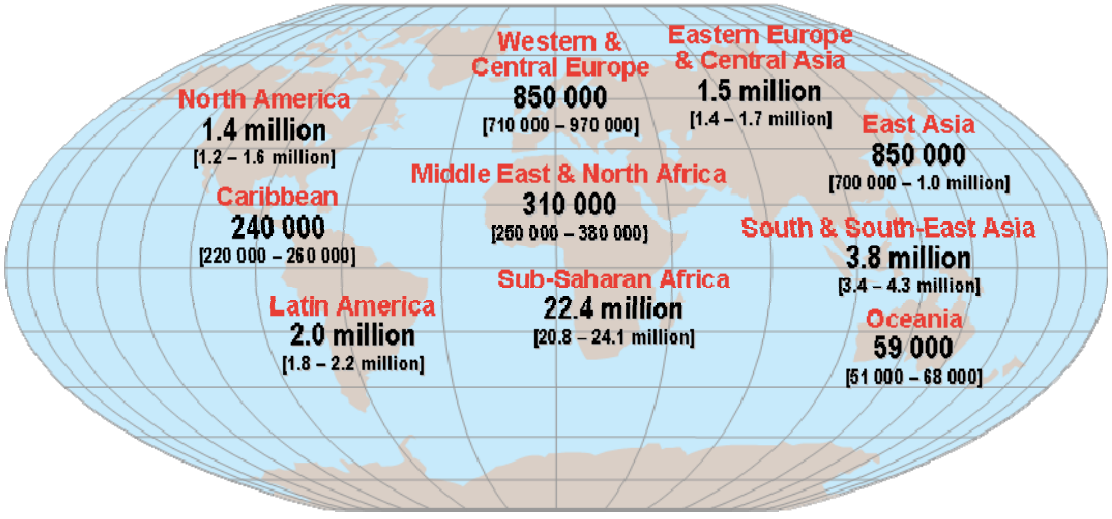


Figure 1.6. Geografical distribution of HIV/AIDS cases. From UNAIDS 2009

1.2 Drug resistance development

1.2.1 Antiretroviral treatment

Anti-retroviral (ARV) agents target different stages in the life cycle of HIV, the binding to cells expressing CD4 receptor, fusion (viral and cellular membrane fusion), reverse transcription of viral RNA to DNA, integration of viral DNA into cellular DNA, transcription of proviral DNA into viral RNA, protein synthesis from the HIV RNA, post translational modification of proteins through proteolytic cleavage, virus assembly and budding from cells. The reverse transcriptase and subsequently the protease enzymes have been the original targets of anti-HIV therapy with fusion inhibitors introduced as a reserve agent for highly treatment- experienced patients (enfuvirtide or T20 approved in March 2003). These drugs were followed by the introduction of integrase inhibitors such as raltegravir, that inhibits the strand transfer process, and the chemokine receptor antagonists, such as maraviroc, that disrupt binding of HIV-1 to either CCR5 or CXCR4 co-receptors, both approved in 2007.

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.

Table 1.1. Antiretroviral drugs in clinical use.

Drug	Abbreviation	FDA Approval
NRTI		
Zidovudine	ZDV	1987
Didanosine	ddI	1991
Zalcitabine	ddC	1992
Stavudine	d4T	1994
Lamivudine	3TC	1995
Abacavir	ABC	1998
Tenofovir	TDF	2001
Emcitrabine	FTC	2004
NNRTI		
Nevirapine	NVP	1996
Delevirdine	DLV	1997
Efavirenz	EFV	1998
Etravirine	ETV	2008
PI		
Saquinavir	SQV	1995
Ritonavir	RTV	1996
Indinavir	IDV	1996
Nelfinavir	NFV	1997
Amprenavir	APV	1999
Lopinavir	LPV	2000
Atazanavir	ATV	2003
Fosamprenavir	FSV	2003
Tipranavir	TPV	2005
Darunavir	DRV	2006
INI		
Raltegravir	RAL	2007
FI		
Enfuvirtide	ENF	2003
EI		
Maraviroc	MRV	2007

Approval dates are taken from the FDA web site (http://www.fda.gov/aoshi/aids/hiv.html)

1.2.2 Drug resistance

The development of resistance to the currently available drugs derived mainly by the interplay of three factors:

Replication. Due to the lack of proof-reading mechanism, reverse transcription is a highly error-prone process. It has been estimated that the mutations rate of HIV is in the range of 10^{-4} to 10^{-6} substitution per base pair per replication cycle, and recombination rates are also estimated to be high with 4% per kilobase per replication cycle (Mansky et al., 1998). In blood plasma of drug-naïve patients, HIV may reach a titer ranging 10^{-3} to 10^{-6} copies/ml, in lymphonodes this concentration may be 2 to 3 order higher. Moreover, HIV is characterized by a short generation time (1-3 days). Thus, this high and erroneous turn over represents the driving force of viral evolution and variation within a single patients (Coffin, 1995).

Diversity. HIV exist in a single individual as a mixture of genetically different variants, described as "quasispecies", whose distribution reflects the relative fitness of the single virus (Holland et al., 1992). Thus, it is believed that drug resistant viruses pre-exist in the population especially if the genetic distance to the dominate wild-type virus is short.

Selection. In presence of antiretroviral drugs, drug resistant variants may replicate better than the wild-type. Thus, if therapy failed to completely suppress viral replication, drug resistant variants may compete with the wild-type and become dominant in the viral population (Fig. 1.20), thus leading to viral rebound. In addition to mutations that directly confer resistance, additional

mutations are selected during suboptimal therapy in order to rescue losses in fitness due to the presence of resistance-conferring mutations.

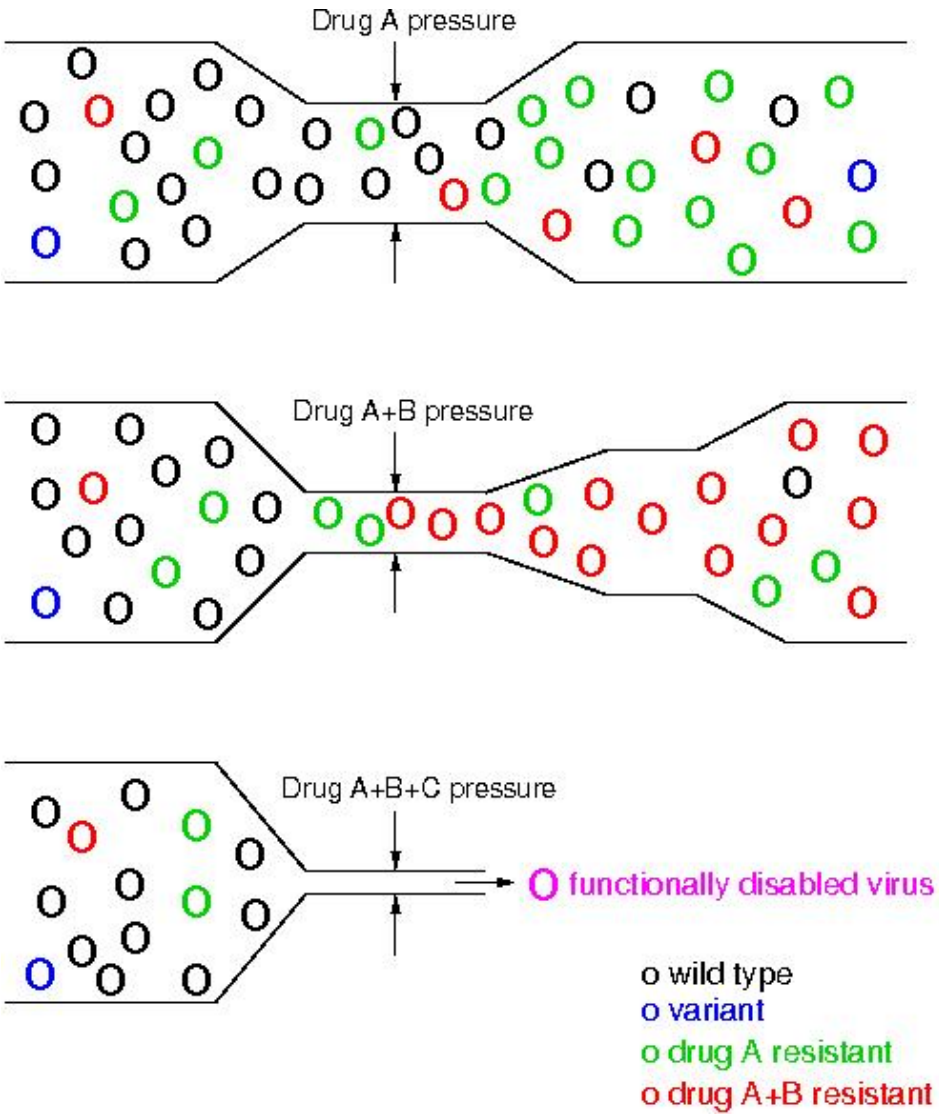


Figure 1.8. Schematic representation of selection of resistant virus under drug pressure

1.2.3 Mechanism of drug resistance

PIs

Protease inhibitors (PI) prevent cleavage of the viral gag-pol polyprotein resulting in production of immature, non-infectious viral particles (Debouk et al., 1992 and Kaplan et al., 1993). Unfortunately, the early protease inhibitors had poor oral bio-availability and short half-lives due to limited oral absorption and rapid hepatic clearance through cytocrome P450 enzymes (Flexner, 1998). Consequently, patients were required to take a large number of pills at frequent dosing intervals, compromising therapy adherence. Due to these limitations, a viral replication persisted in presence of drug selection pressure and variants with decreased susceptibility to the drugs in HAART regimen were frequently selected. From 1997 onwards it became evident that a therapeutically suboptimal dose of ritonavir enhances the concentration of a administrated PI by inhibition of its hepatic cytochrome metabolism (Kempf et al., 1997). This approach effectively raises the genetic barrier to resistance.

Resistance to protease inhibitors is the consequence of amino acid substitutions that emerge either inside the substrate-binding domain of the enzyme or at distant sites (Condra et al., 1995; Kaplan et al, 1994; Molla et al., 1996). Directly or indirectly, these amino acid changes modify the number and the nature of the points of contact between the inhibitors and the protease, thereby reducing their affinity for the enzyme. As an example, the common resistance mutation V82A reduces the size of an amino acid residue in the protease that is more important for binding most inhibitors than for binding the natural viral protein substrate (Prabu-Jeyabalan et al., 2002). Protease inhibitors have been designed to bind the protease with maximal affinity and tend to occupy more space inside the active site cavity than do natural substrates. Unlike the

inhibitors, the natural substrates of the protease have a variable, but generally less tight, interaction with the catalytic site, a phenomenon that promotes the ordered sequential cleavage of the polyproteins required for proper assembly of the viral particle. Resistance mutations in the protease, which result in an overall enlargement of the catalytic site of the enzyme, would thus be predicted to have a greater effect on the binding of inhibitors than the natural templates. Some mutations are selected for only by certain PIs (Fig. 1.9), reflecting particularities in the chemical structure of the inhibitors that influence their interaction with the substrate-binding domain of the enzyme.

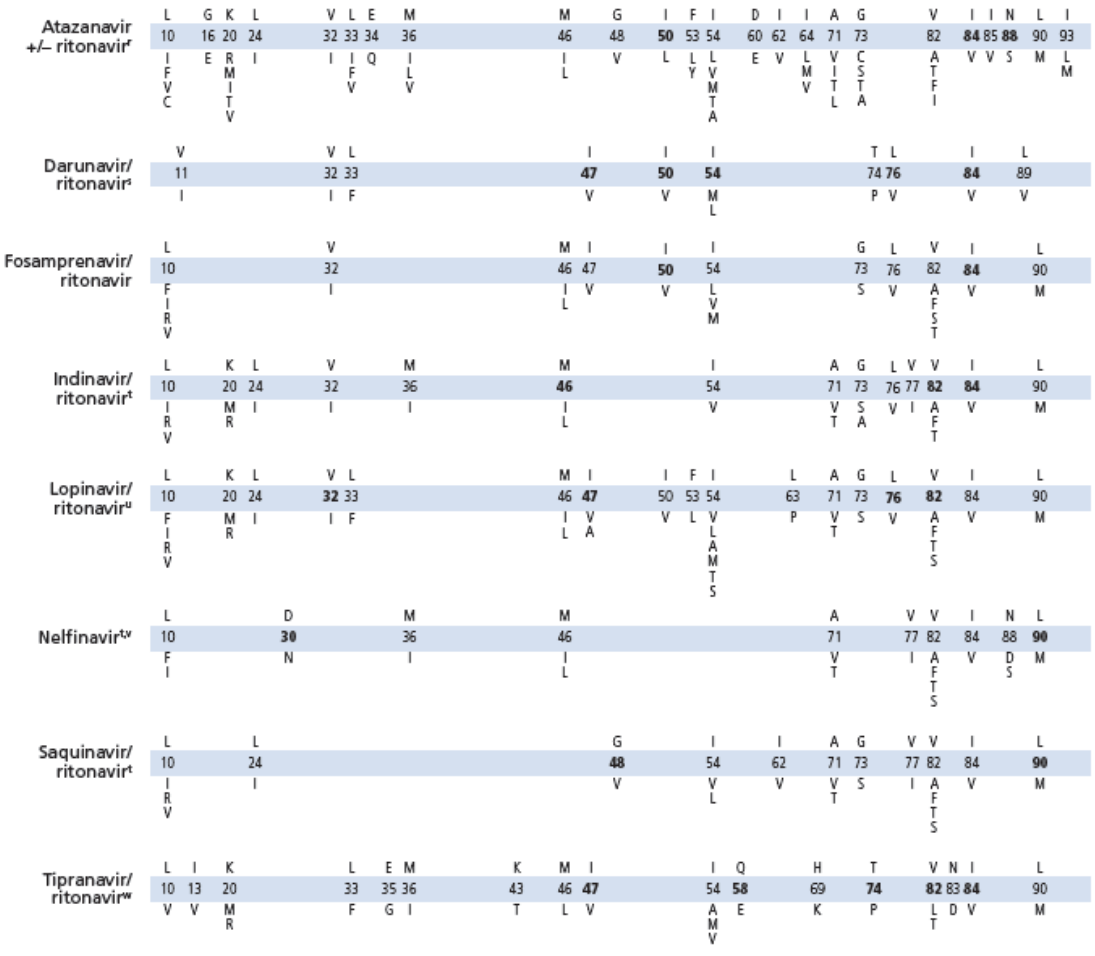


Figure 1.9. Mutations associated with PI resistance (Johnson et al., 2009)

NRTIs

Nucleoside reverse trasnscriptase inhibitors (NRTI) were the first antiretroviral drugs that became available. The viral enzyme reverse transcriptase (RT) is responsible for copying a single-stranded viral RNA genome into double-stranded DNA. NRTIs compete with natural nucleosides for incorporation into pro-viral genome by RT. Since NRTIs lack the 3'-hydroxyl group, no additional nucleotides can be attached after incorporation of NRTIs in the proviral genome resulting in DNA-chian termination and interruption of HIV-replication (Mitsuya et al., 1985). Soon after the introduction of the thymidine analogue zidovudine in 1987 it became evident that, when used as monotherapy, the antiviral effect was transient and rapidly blunted by the selection of viral variants with decreased susceptibility (De Jong at al., 1996).

Resistance to NRTIs may be mediated by al least three different mechanisms:

Impairment of Analogue Incorporation. Several mutations or groups of mutations in reverse transcriptase can promote resistance by selectively impairing the ability of reverse transcriptase to incorporate an analogue into DNA. These mutations include M184V, K65R, L74V, Q151M, Y115F, and V75I (Fig 1.10).

Removal of the Analogue from the Terminated DNA Chain. Removal of the nucleoside analogue from the terminated DNA chain is associated with a group of mutations commonly termed "thymidine analogue mutations" (Figure 1.10), most frequently selected for after the failure of drug combinations that include thymidine analogues, such as zidovudine and stavudine. These mutations promote resistance by fostering ATP- or pyrophosphate-mediated removal of nucleoside analogues from the 3' end of the terminated DNA strand (Arion et al., 1998; Meyer et al., 1999). ATP and pyrophosphate, which are abundant in normal lymphocytes, do not participate in the

DNA-polymerization reaction, but the structure of a reverse transcriptase expressing thymidine analogue mutations facilitates their entry into a site adjacent to the incorporated analogue (Boyer et al., 2001; Chamberlain et al., 2002). In this position, ATP or pyrophosphate can attack the phosphodiester bond that links the analogue to DNA, resulting in removal of the analogue. Interestingly, the efficiency of this process, also known as "primer rescue," can be significantly decreased by the presence of other mutations in reverse transcriptase, a phenomenon that has been best described in the case of the M184V mutation (Larder et al., 1995), that slows the selection of thymidine analogue mutations by thymidine analogues (Picard et al., 2001) and may slightly increase the residual antiviral activity of some nucleoside analogues in spite of the presence of thymidine analogue mutations.

Increased reverse transcriptase packaging. Mutations in the p1-p6 region of HIV gag have been described to increase the number of reverse transcriptase molecules per virion (Peters et al., 2001).

NNRTIs

The introduction of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in 1998 provided the opportunity for simpler and more tolerable drug-regimens. NNRTIs bind in a non-competitive manner to a hydrophobic pocket in close proximity to the active site of RT, resulting in inhibition of the catalytic step (De Clercq, 1994). In contrast to unboosted PIs, NNRTIs have an excellent bioavailability; unfortunately, they have a low threshold of resistance.

Mutations responsible for NNRTI resistance are located in the pocket targeted by these compounds, and they reduce the affinity of the drug.

Some mutations associated with NNRTI resistance may also compromise virus replication by changing the conformation of the dNTP binding pocket or altering RNAse H activity. Moreover, mutations in the NNRTI binding pocket may interfere with NRTI resistance. The NNRTI resistance conferring mutations Y181C and L100I hypersensitize HIV-1 to Zidovudine (Larder et al., 1994), and the multi-nucleoside resistance phenotype can be partially reversed by NNRTI mutations (Van Leathem et al., 2001). See Figure 1.11.

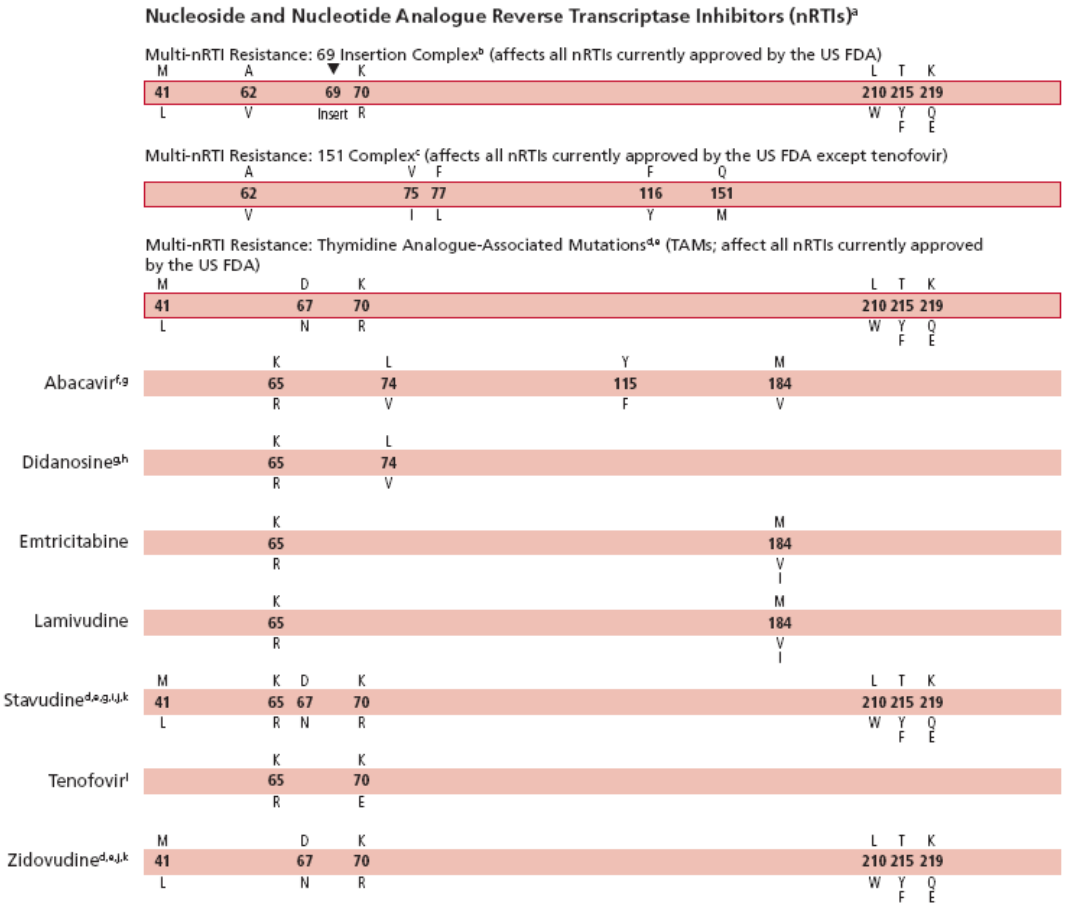


Figure 1.10. Mutations associated with NRTI resistance (Johnson et al., 2009)

INIs

Integrase catalyses the insertion of HIV-DNA into the genome of the host cell. The integrase strand transfer inhibitor Raltegravir inhibits the linkage of the viral DNA 3' ends to the cellular target DNA. Unfortunately, this potent drug appears to have a low genetic barrier: just one mutation in the integrase gene can result in considerable resistance. Raltegravir failure is associated with integrase mutations in at least 3 distinct genetic pathways defined by 2 or more mutations including a) a signature (major) mutation a Q148H/K/R, N155H, or Y143R/H/C at the catalytic site of the Integrase and b) 1 or more additional minor mutations (Figure 1.12) (Hazuda et al., 2007).

FIs

Enfuvirtide is the first fusion inhibitor approved. It is a small syntetic peptide that mimics the HR2 region of gp41. Binding of enfuvirtide to the HR1 region of gp41 prevents interaction between HR1 and HR2 for formation of a harpin structure and subsequent fusion pore (Poveda et al., 2005). Resistance to Enfuvirtide is mediated by the appearance of mutations in the Enfuvirtide target region encompassing the residues 36-45 of the gp41 HR1 domain (Fig. 1.13) (Greenberg et al., 2004; Miller et al., 2004; Reeves et al., 2005).

CCR5 entry inhibitors

CCR5-antagonists target the interaction of the viral gp120 with the host cell surface CCR5 chemochine receptor, binding the human coreceptor. Virologic failure of these drugs frequently is associated with outgrowth of D/M or X4 virus from a preexisting minority population present at levels below the limit of assay detection. Mutations in HIV-1 gp120 that allow the virus to bind to the drug-bound form of CCR5 have been described in viruses from some patients whose virus remained R5 after virologic failure

of a CCR5 antagonist. Most of these mutations are found in the V3 loop, the major determinant of viral tropism (Nolan et al., 2009). There is as yet no consensus on specific signature mutations for CCR5 antagonist resistance. Some CCR5 antagonist-resistant viruses selected in vitro have shown mutations in gp41 without mutations in V3; the clinical significance of such mutations is not yet known (Huang et al., 2009).

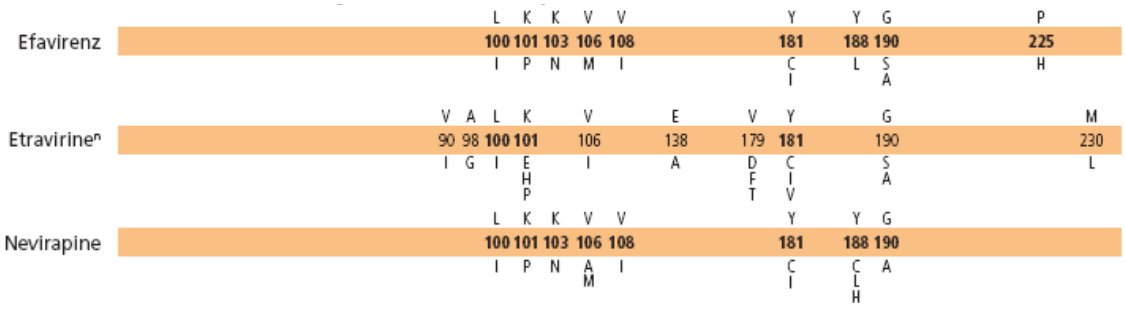


Figure 1.11. Mutations associated with NNRTI resistance (Johnson et al., 2009)

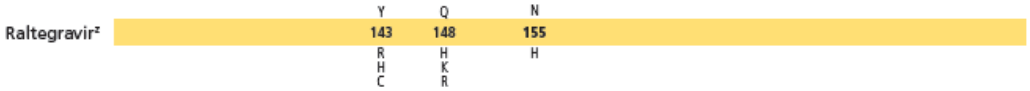


Figure 1.12. Mutations associated with Raltegravir resistance (Johnson et al., 2009)

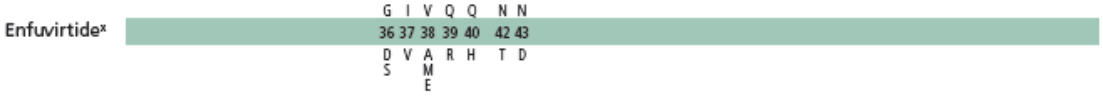


Figure 1.13. Mutations associated with Enfuvirtide resistance (Johnson et al., 2009)

1.2.4 Combination therapy

Currently, so called "highly active antiretroviral therapy (HAART) is recommended for the treatment of HIV-infected patients. HAART is defined as is defined as the reported use of three or more antiretroviral medications, one of which has to be a PI, an NNRTI, one of the NRTIs abacavir or tenofovir, an integrase inhibitor (e.g., raltegravir), or an entry inhibitor (e.g., Maraviroc or enfuvirtide) (DHHS/Kaiser Panel, 2008).

The introduction of the highly active antiretroviral therapy (HAART) has provided an extraordinary clinical benefit in HIV-infected patients in lowering morbidity and mortality (Palella et al., 1998; Valenti et al., 2001) (Fig. 1.19) nevertheless, toxicity profiles, limited tolerability and development of drug resistance reduce the durability of efficacy.

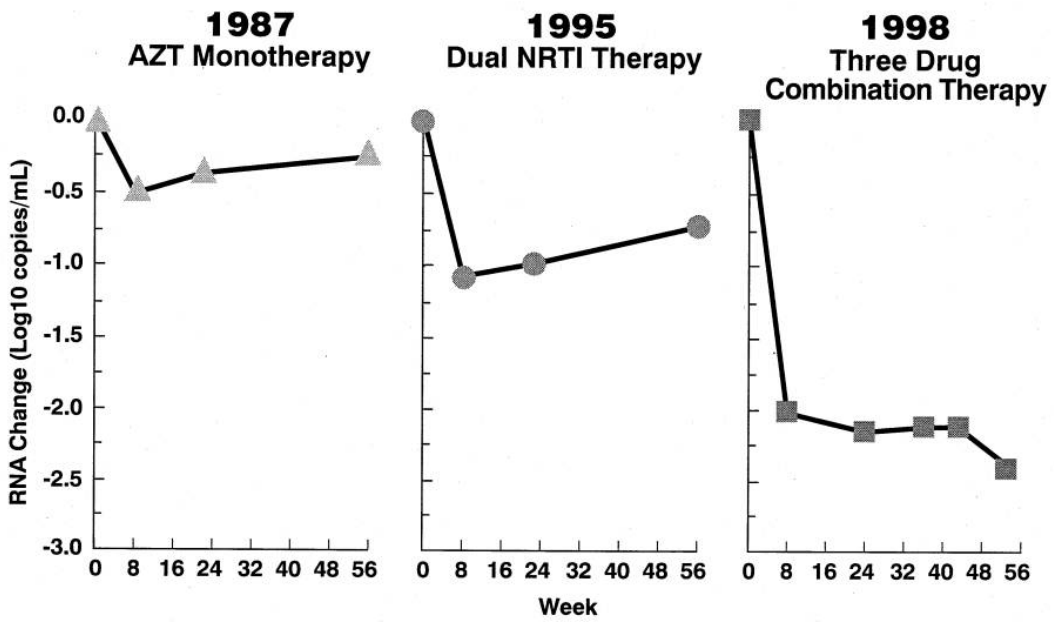


Figure 1.7. Effect of monotherapy, dual therapy, and HAART on viremia over time

1.2.5 Detection of drug resistance

There are 2 general types of resistance assays used in clinical practice: genotypic assays (i.e., HIV-1 gene sequencing to detect mutations that confer HIV-1 drug resistance) and phenotypic assays (i.e., cell culture–based viral replication assays in the absence or presence of drugs). Both tests have shown their benefits in the clinical practice of treatment-experienced HIV-infected patients, and were become of clinical value for antiretroviral-naïve individuals too (Durant et al., 1999; Baxter et al., 2000).

Genotypic resistance tests were to date considered as the standard of care for monitoring the response to HAART in HIV-1 infected patients. This assay identifies nucleotide changes in the viral genome compared to a viral reference strain. The profile of mutations is interpreted using algorithms that correlate known mutational profiles to phenotypic susceptibility data and/ or clinical outcome. Genotypic tests may, therefore, identify mutations or genetic profiles that are not necessarily resulting in phenotypic resistance but may furnish evidence of archived drug resistance. This test can be performed with commercial assay kits or in-house protocols.

Phenotypic drug-susceptibility assays determine the concentration of an antiretroviral agent that is required to inhibit HIV-1 replication by 50% (IC50) or 90% (IC90). The results are compared to the concentration of drug necessary to inhibit a reference strain of susceptible virus. The difference in IC50 or IC90 defines the susceptibility of the virus to the different antiretroviral agents.

Phenotypic resistance tests and genotypic sequence analyses are generally capable of identifying viral populations that make up at least 20% of the quasispecies. Drug resistant variants, partly overgrown by or reverted to wild-type may, therefore, be missed, suggesting that these tests might underestimate the presence of drug resistant

viruses. More sensitive resistance tests, such as allele-specific PCR, parallel-allele-specific sequencing, standard cloning, Ling Amp, or the Ultra deep pyrosequencing (UDPS), may be very useful for this indication. Originally designed for high throughput sequencing of mammal and bacterial genomes, the UDPS technique is particularly well-suited for an in depth analysis of a population of heterogeneous genomes like those of retroviruses. To date the parallel pyrosequencing technique can detect rare genetic variants constituting as little as 1% of the population. The procedure used for HIV-1 is reported in figure 1.14. Currently, the length of sequencing reads per clone is between 300 to 400 contiguous base pairs. This allows studying the genetic linkage of clinically relevant resistance mutations clusters, like mutations surrounding the NNRTI binding pocket, the integrase catalytic site, most mutations in the protease, and the complete V3 loop of gp120.

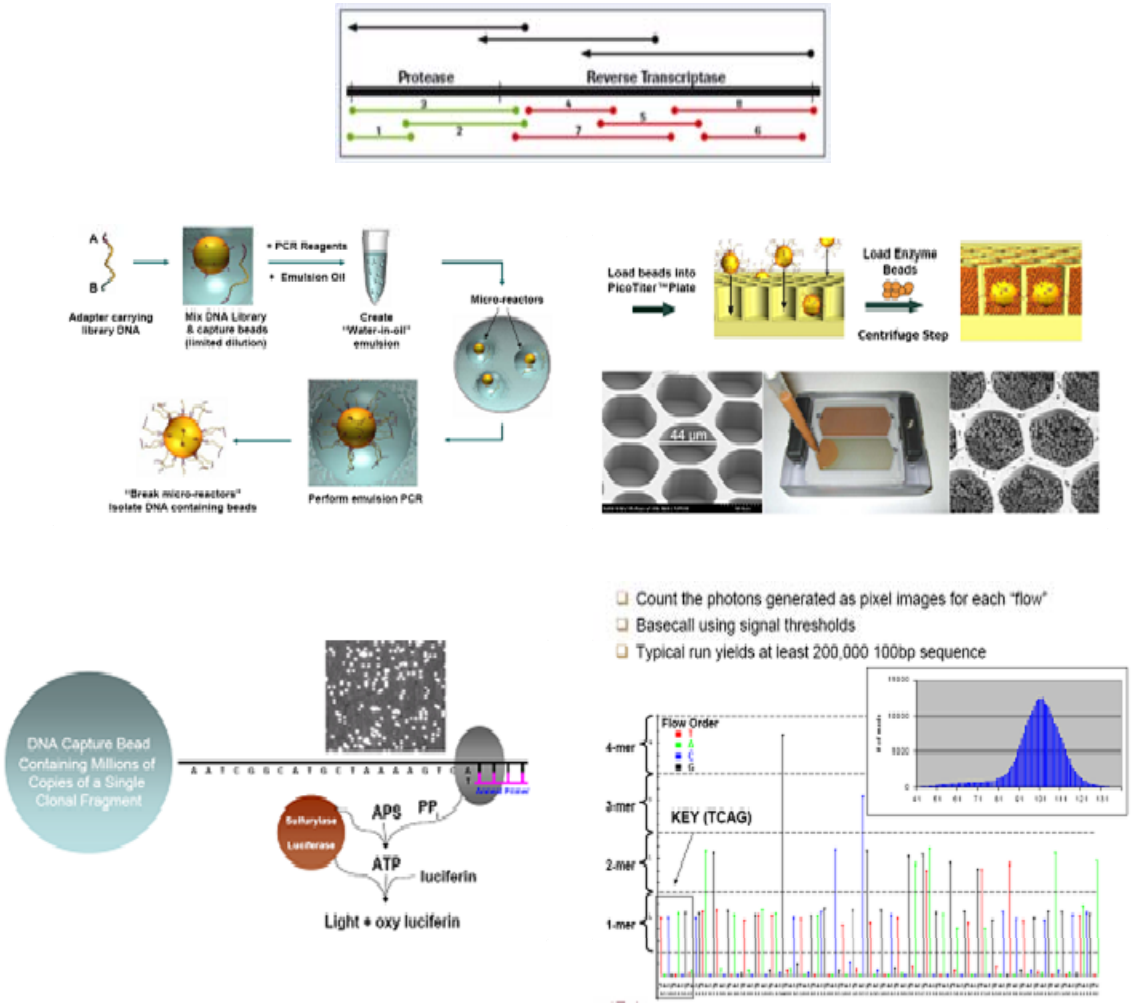


Figure 1.14. Schematic procedure for 454 in HIV. The target sequence is amplified with primers tagged with a sample-specific identification. An water/oil emulsion is prepared so each droplet contains 1 bead and 1 amplicon; each amplicon is then amplified by emulsion PCR within the droplet. The emulsion is disrupted and 28uM beads are loaded in 44 uM wells, so, theoretically, there is only one bead per well. Sequencing enzymes including luciferase are included in each well. Pyrosequencing proceeds by series of flows of known nucleotides. When a nucleotide hibridizes with its complementary pyrophsfate is released and this results in the release of chemioluminescent signal reordered by a CCD camera and integrated in a computer. Vertical bars in the flowchart represented the number of bases. Source: 454 sequencing website (www.454.com)

1.3 Transmission of HIV-1 drug resistance

1.3.1 Evolution of drug resistance in treatment naïve patients

In treated patients carrying drug resistant HIV variants, cessation of the complete regimen usually results in a rapid reappearance of the original drug-sensitive wild-type virus (Devereux et al., 1999). However, resistant variants may persist as minority populations in plasma and are maintained as provirus in resting cells within lymphoid tissue. As soon as therapy is reintroduced, the resistant variants can be rapidly reselected (Karlsson et al., 1999).

The frequent detection of drug resistance among HIV-infected treatment failing patients can in turn increase the risk of new infections driven by drug resistant viral strains.

In antiretroviral-naïve individuals, wild-type may dominate resistant variants with impaired fitness, if the infecting quasispecies contained both wildtype and resistant variants. If, however, through a founder effect, the diversity of infecting quasispecies is restricted to resistant variants, and no minority of wild-type variants is present, dominance by wild-type is not possible. In the absence of drug pressure, the transmitted drug resistant quasispecies may revert back to wild-type or mutate to other more fit variants. Viruses harbouring a single resistance-related point mutation, such as M184V in the reverse transcriptase (RT) gene, may revert more rapidly than variants that have evolved after multiple mutations (Brenner et al., 2002). Also, persistence of transmitted resistant variants for several months or years in the plasma of the newly infected subject has been described (Brenner et al., 2000; Little et al., 2003).

Furthermore, intermediate stages between resistant variants and wild-type or atypical variants are occasionally detected at codon 215 of viruses isolated from antiretroviral-naïve patients. These variants emerge from the resistance-related mutants T215Y and

T215F, which need two nucleotide changes for reversion to wild-type (De Ronde et al., 2001; Garcia Lerma et al., 2001).

Viruses containing these partial revertants or atypical mutations are selected because they display an increased fitness compared to the resistant variants. In vitro experiments have shown that variants with the atypical mutation 215D, or partial revertant 215S, can replicate as efficiently as wildtype, which may explain their persistence in vivo. Although the partial revertants or atypical mutants do not, by themselves, confer phenotypic resistance, they are only one step away from the resistant variants 215Y or 215F, compared to the two mutational steps that are needed from wildtype to 215Y/F. This may indicate an increased risk for developing resistance under subsequent treatment with zidovudine or stavudine (Violin et al., 2008).

In the absence of selective drug pressure in the new host, resistant variants may completely revert to wild-type, leaving no evidence of transmitted resistance in the plasma. However, one might expect that the initial resistant quasispecies will still be present as archived provirus in resting cells or as minority variants in plasma of the untreated subject.

1.3.2 Mechanism of transmission of drug resistant virus

Individuals may be exposed to varying amounts of virus depending on the route of HIV-1 transmission. Sexual and vertical transmission generally involves smaller amounts of virus and infected cells than transmission due to direct blood-to blood contact as occurs during intravenous drug use or blood transfusion (Zhu et al., 1993; Overbaugh et al., 1999; Wolinsky et al., 1993).

Regardless the route, transmission is accompanied by a loss of both genotypic and phenotypic viral heterogeneity (Delwart et al., 2002). As a result, the viral population

proliferating in the blood of recently-infected recipients is generally more homogeneous than the markedly heterogeneous quasispecies in chronically-infected donors (Zhu et al., 1993; Delwart et al., 2002; Wolfs et al., 1992; Zhu et al., 1992). The restriction in quasispecies implies the occurrence of a bottleneck process during transmission and establishment of infection. This process is determined by the number of transmitted virions, by the diversity of the transmitted viral quasispecies and by host factors, such as the mucosal barrier, the density of target cells and the immune system (Quiñones-Mateu et al., 2002).

Recent studies suggest that multiple strains are frequently transmitted, resulting in more genomic variation in the recipient than previously reported, though still not as heterogeneous as the donor population (Overbaugh et al., 1991; Learn et al., 2002). Subsequently, variants among the transmitted population that are most capable of widespread dissemination might be positively selected. The viruses that succeed in establishing infection do not have to be the variants that displayed the highest fitness under drug pressure in the donor quasispecies –as demonstrated by the ability of minor variants from the donor quasispecies to accomplish infection in the recipient (Zhu et al., 1993). This finding might be explained by stochastic processes that influence the diversity of the viral variants in the inoculum. Another reason for a shift in quasispecies might be that characteristics preferred during chronic infection differ from viral properties selected during transmission and adaptation to the new host.

The first case of transmission of drug resistant HIV was reported 10 years ago by Erice, et al, 1993. A patient who had never been exposed to therapy carried a viral variant resistant against zidovudine, not reflecting a natural variation in drug susceptibility. A few years after the introduction of combination therapy, Hecht, et al.,1998 described the

transmission of an HIV variant resistant to multiple RT and Protease inhibitors. Since then, transmission of virus resistant to antiretroviral drugs has been repeatedly documented through many infection routes: sexual contact, needle sharing among intravenous drug users, perinatal transmission and accidental exposure of health care workers (De Ronde et al., 1996; Siegrist et al., 1994; Conlon et al., 1994; Anonymous, 1993).

More recently, cohort studies have been published describing patterns of baseline drug resistance profiles in a significant percentage of therapy- naïve patients (Yerly et al., 1999; Boden et al., 1999; Brodine et al., 1999).

Multiple studies have reported impaired enzyme function and decreased viral fitness of HIV-1 isolates harboring drug resistant mutations, often resulting in a moderate or low viral load in the patient (Nijhuis et al., 2001). One might speculate that these strains are also less capable of accomplishing infection in a new host. On the other hand, if the transmitted quasispecies also contains drug-sensitive variants, resistant variants with impaired fitness may be at selective disadvantage during infection and/ or initial amplification in the new host.

Transmission of HIV-1 is strongly influenced by the level of viremia in the HIV-infected donor. Consequently, it seems logical to relate the rate of transmission of HIV to the overall level of viremia in the HIV-infected population. In this perspective, access to HAART has, by reduction of HIV-RNA concentrations, the potential to decrease transmission of HIV on a population level (Quinn et al., 2000; Hosseinipour et al., 2002). In pregnant HIV-infected women, the use of HAART has indeed shown a profound reduction of HIV incidence in their off-spring. Despite this success in preventing vertical transmission, HAART is currently not effective as a “population

prevention measure". In contrast, prolonged survival of infected patients, continuous injecting drug use and sexually related transmission, have resulted in an increased prevalence of HIV-infections in countries with access to HAART (HIV/AIDS Surveillance in Europe, 2006 and 2007).

The impact of HAART on the spread of HIV and its drug resistant variants may vary between different transmitters. For instance, the beneficial effect of therapy on HIV-RNA load will not reach individuals who are unaware of their infection status. Persons with primary infection among this population may be very infectious, because viremia in the plasma is extremely high during the acute phase of infection (Hubert et al., 2000). In contrast, the viremia in semen during acute infection appears not to be different from that during established infection, although this has only been studied to a limited extent (Dyer et al., 1997). Nevertheless, data from the Swiss cohort prescribe an important role of new diagnoses and primary infections on the spread of HIV-1. One third of acutely-infected patients in the cohort appeared to be infected by persons who had acquired HIV very recently themselves (Yerly et al., 2001). Although seroconverters are rarely assumed to be the source of drug resistant viruses, secondary transmission of resistant virus has been described in recent HIV infection and may become more frequent in the future.

Other possible transmitters are chronically-infected individuals that have been diagnosed with HIV, but not yet exposed to therapy. Toxicity and complexity of the current antiretroviral regimens have resulted in new guidelines favouring less aggressive strategies for initiating therapy in this population. This more conservative approach is leading to an increased number of individuals enduring unsuppressed viremia. Since a significant dose-response relation between transmission and the level of viral load has

been described, this new strategy may lead to an increased risk of transmission at the population level (Yerley et al., 2009; Huè et al., 2009).

Antiretroviral-experienced individuals make up a third group of possible transmitters (Goudsmit et al., 2001). Ideally, persons taking antiretroviral therapy should be less efficient in transmitting HIV, at least if their viral load is successfully suppressed (Quinn et al., 2000). Unfortunately, in many individuals on HAART, the viral load is not below the detection of the commercially available assays. Furthermore, the limited amount of therapeutic options supports continued use of HAART regimens that, even though unsuccessful from a virology point of view, are capable of sustaining a prolonged immunologic response. Since patients experiencing viral rebound frequently harbour drug resistant variants, they are the most likely source of transmission of resistant viruses. However, it has been suggested that the frequency of transmission of drug resistant viruses is less than expected, due to their impaired viral fitness.

1.3.4 Estimation rate of transmission of drug resistant virus

To date, there is a growing literature about the rate of transmission of HIV-1 drug resistant virus. In the United States and in Europe, where there is a wide access to highly active antiretroviral therapy (HAART), the prevalence of HIV-1 drug resistant strains ranges between 3.3% and 14.0% in recently infected patients and between 6.1% and 12.5% in chronically infected ones (Novak et al., 2005; Wensing et al., 2005; Yerly et al., 2007; UK, 2007; SPREAD, 2008). These different rates may reflect variations in access to therapy, therapeutic strategies, adherence or risk-related behaviour, but first of all for a lack of uniformity in the definition of transmitted resistance. Only with a standard list of mutations is it possible to compare the prevalence of transmitted

resistance from different times and regions and facilitate meta-analyses of surveillance data collected by different groups at different times. Compiling such a standard list, however, is not simple because of the rapidly changing field of ARV therapy and the large numbers of mutations associated with ARV drug resistance (Bennett et al., 2009).

Shafer at al., in 2007, provided a provisional list for identifying surveillance drug resistance mutations (SDRMs) on the basis of four criteria. The first criterion was that SDRMs should be recognized as causing or contributing to drug resistance – defined as being present on three or more of five expert lists of drug resistance mutations. The second criterion was that mutations should be non-polymorphic and should not occur at highly polymorphic positions. The third criterion was that the mutation list had to be applicable to the eight most common HIV-1 subtypes. The fourth criterion was that the list should be parsimonious, excluding mutations resulting exceedingly rarely from drug pressure.

The list of 2007 was uploaded in 2009, when new drug resistance mutations have been identified including mutations arising from the increased use of non-thymidine-analog containing regimens, the expanded use of two new protease inhibitors (PIs), and the recent approval of a new non-nucleoside RT inhibitor (NNRTI) (Bennet et al., 2009) (Table 1.2).

Table1.2 Surveillance Drug Resistance Mutation (SDRM) (Bennet et al., 2009)

Position	AA	PI	NRTI	NNRTI
41	L		●	
65	R		●	
67	N		●	
	G		●	
	E		●	
69	D		●	
	ins		●	
70	R		●	
	E		●	
74	V		●	
	I		●	
75	M		●	
	T		●	
	A		●	
	S		●	
77	L		●	
115	F		●	
116	Y		●	
151	M		●	
184	V		●	
	I		●	
210	W		●	
215	Y		●	
	F		●	
	I		●	
	S		●	
	C		●	
	D		●	
	V		●	
	E		●	
219	Q		●	
	E		●	
	N		●	
	R		●	
100	I			●
101	E			●
	P			●
103	N			●
	S			●
106	M			●
	A			●
179	F			●
181	C			●
	I			●
	V			●
188	L			●
	H			●
	C			●
190	A			●
	S			●
	E			●
225	H			●
230	L			●

Position	AA	PI	NRTI	NNRTI
23	I	●		
24	I	●		
30	N	●		
32	I	●		
46	I	●		
	L	●		
47	V	●		
	A	●		
48	V	●		
	M	●		
50	V	●		
	L	●		
53	L	●		
	Y	●		
54	V	●		
	L	●		
	M	●		
	A	●		
	T	●		
	S	●		
73	S	●		
	T	●		
	C	●		
	A	●		
76	V	●		
82	A	●		
	T	●		
	F	●		
	S	●		
	C	●		
	M	●		
	L	●		
83	D	●		
84	V	●		
	A	●		
	C	●		
85	V	●		
88	D	●		
	S	●		
90	M	●		

1.3.4 Clinical implications of transmitted drug resistance

Major concerns exist about the clinical implications of transmitted drug resistance. Although it might be expected that the response to antiretroviral therapy is diminished in the case of primary resistance, only limited data assessing clinical outcome are available at present. In a large North American cohort, therapeutic outcome for patients infected with HIV harboring major mutations was not different from patients infected with drug-susceptible viruses (Alexander et al., 2001).

However the majority of patients in this cohort were not prescribed drugs to which the virus they carried exhibited resistance. The small group of patients who did initiate therapy to which the virus exhibited possible resistance at baseline, displayed a relatively inferior virological outcome (Alexander et al., 2001).

Having transmitted drug resistance mutations seems not to be predictive of virological failure when patients received a fully active treatment also for a recent European study (Wittktop et al., 2010). HIV-1 infected patients enrolled in this project had a poor virological response only when the HAART regimen was based upon drugs to which the virus has lost susceptibility.

In three other studies from the United States and France, the time to achieve viral suppression was significantly prolonged in individuals carrying virus with major phenotypic or genotypic drug resistance at baseline compared to subjects with fully-susceptible virus at baseline (Little et al., 2002; Harzic et al., 2002; Grant et al., 2002; Peuchant et al., 2008).

Additionally, individuals from the ICONA cohort (the Italian Cohort Naïve for Antiretrovirals) harboring revertants or atypical mutants at position 215 of RT, had an increased risk of experiencing virological failure compared to those not carrying

revertants (Riva et a., 2002). These revertants have an increased ability to select the RT T215Y/F mutations that limit the efficacy of thymidine analogues. Of the 13 patients who carried the 215 mutants, 9 experienced virological failure.

Some groups report a lower baseline viral load (Harzic et al., 2002; Descamps et al., 2001) or a higher CD4 cell count (Grant et al., 2002) in antiretroviral-naïve individuals carrying primary drug resistant variants in comparison to individuals with drug-sensitive virus. These differences might reflect variations in duration of infection. Alternative interesting hypothesis suggested by the authors is a decreased viral replication capacity of the transmitted resistant viruses.

As described above, minority strains with transmitted drug resistance related mutations might not always be detected by conventional resistance testing, therefore a single mutation or a revertant might be an indicator that more extensive resistance has been transmitted. In a preliminary study in HIV-1 infected individuals experiencing failure of a first-line NNRTI-based regimen, a strong association between the presence of minority drug resistant variants and virological failure was observed (Paredes et al., 2010).

In this line, a recent case-control study found that 7% of individuals who experienced their first virologic failure had minority drug resistance mutations at baseline, and that minority resistance was rarely found in treatment successes (Johnson et al., 2008). These findings suggest that also the minority drug resistant HIV variants may have clinical consequences and that the presence of such variants in individuals who have not previously taken ART may reduce the efficacy of some ART regimens.

In summary, preliminary data show that transmission of resistance, even in minority species, can compromise therapy outcome. For these reasons, the new guidelines

recommend to perform the genotypic resistance testing in all drug-naïve patients, before beginning a first line antiretroviral regimen (Hirsch et al., 2008; Department of Health and Human Services, 2009).

1.4 Rationale of the work

The development of resistance to the currently available antiretroviral drugs against HIV-1 infection is one of the major limitations to the maintenance of a successful treatment. The frequent detection of resistance among HIV-infected treatment failing patients (Pillay et al., 2005; Wensing et al., 2005; Spread Programme, 2008) can in turn increase the risk of new infections driven by drug resistant viral strains (Wensing and Boucher, 2003). The prevalence of drug resistant HIV in newly diagnosed individuals naïve to antiretroviral therapy is likely to be influenced by the prevalence of drug resistance in the treated population, even if recent studies assumed that the transmission of drug resistant HIV-1 viruses reflects not only infection from drug-experienced patients, but also the circulation of resistant strains between drug-naïve individuals (Yerley et al., 2009; Huè et al., 2009).

Once transmitted, a drug resistant virus can either revert to wild-type or more frequently persist for months to years into the host (Barbour et al., 2004) as minority species, or stored in proviral DNA of infected cells. In drug naïve patients these minority drug resistant HIV-1 strains may become dominant at the time of HAART initiation, increasing the probability of virological failure to the first-line antiretroviral therapy (Little et al., 2002; Harzic et al., 2002; Grant et al., 2002; Peuchant et al., 2008; Roquebert et al., 2006).

In this light, the aim of this thesis was to characterize the distribution and the spread of drug resistant HIV-1 in Italy. The main objectives were to ascertain how many people naïve to the antiretroviral therapy are infected with drug resistant virus and which resistance profiles are circulating.

In addition, using a phylogenetic approach, this thesis attempted to investigate the

population dynamics of transmitted resistance and the potential contribution of untreated patients to the spread of antiretroviral resistance. The identification of potential epidemiological networks and sequence interrelationships between acute/early and chronic infections in both drug-naïve and drug-experienced individuals can provide new tools in prevention strategy as well as in management of treatment programs in a local, specific and well-characterized territory.

Finally, the last objective was to define new genetic markers in the pol gene that can predict the presence of a transmitted drug resistant minority species. Our attention has been focused on few selected mutations, T69S, L210M and K103R, found at 3 reverse transcriptase (RT) resistance positions and easily detected by standard population sequencing (GRT). Their prevalence in drug naïve patients is generally low (T69S, 2.4%; L210M, 0.9%; K103R, 1.7%, prevalence estimated in 2089 HIV-1 B subtype infected drug naïve patients) and no association with phenotypic resistance to RT inhibitors, in either clinical isolates or site-directed mutagenesis experiments was to date proved (Harrigan et al., 2005; Berkhout et al., 2006). Certainly, the analysis by UDPS of minority quasispecies, has allowed to better define the role of these mutations as potential markers of the existence of resistant minority variants, and to determine their impact on clinical virologic outcomes, thus enhancing the understanding and management of resistance in the future. This is even more relevant from the practical point of view, since the methods to detect minority quasispecies resistant to antivirals (such as UDPS, etc) are today used only for research purpose, and it is difficult to foresee their routine utilization in clinical practice. Having a surrogate able to provide similar information with lower cost and higher feasibility may thus provide a great clinical advantage.

2 Methods

In this chapter, dataset and methods used in each study described in this thesis were reported.

2.1 Characterization of the patterns of drug resistance mutations in newly diagnosed HIV-1 infected patients naïve to the antiretroviral drugs

2.1.1 Study population

The study included 263 HIV-1 infected individuals enrolled between January 2004 and March 2007 in the SENDIH (Studio Epidemiologico Nuove Diagnosi Infezione HIV-1) programme, a multicenter study aimed to collect behavioural, virological and molecular data on persons with newly diagnosed HIV infection. Characteristics and methods of the study have been previously described by Orchi et al., 2008. Individuals with a first HIV-1 positive test performed in 10 public Counselling and Testing centres (CTC) in Lazio Region, Italy, were invited to participate in the study. At the diagnosis, clinical and immunologic data, and blood sample have been collected from all participants to investigate the molecular characterization of the virus and to identify recently acquired infections.

Informed consent was obtained from participants and the ethics committee of the National Institute for Infectious Diseases L. Spallanzani, Rome approved the study. All of the information gathered during the study was analyzed in a completely anonymous way.

2.1.2 The IgG avidity assay

To estimate time since infection, we calculated the Avidity Index (AI) on the first available serum sample, using an automated anti-HIV enzyme immunoassay (EIA) (Suligoi et al., 2003 and 2008; Selleri et al., 2007). The method is based on the rationale that antibodies produced in the early phase of an infection show a low avidity for the antigen, and the antibody avidity increases progressively with the time after exposure to an immunogen. Thus, a low avidity is likely to indicate a recent infection. In particular, an AI <0.80 has been reported to fairly define an infection acquired in the 6 months prior the diagnosis (recent infection) while AI ≥0.80 is generally used in literature to define long-standing infection. Misclassifications of recent infections as long-standing infections (and vice versa) could not be excluded for patients with an AI index between 0.80 and 0.90 (Suligoi et al., 2003).

2.1.3 HIV sequencing

HIV genotype analysis was performed on plasma samples by means of a commercially available kit (ViroSeq HIV-1 genotyping system; Abbott Laboratories) (Ceccherini et al., 2004). The polymerase chain reaction was performed in all the 263 samples and was successful for 255 samples (yeld 97%), that were then sequenced (yeld of 97%). Briefly, RNA was extracted, retrotranscribed by murine leukemia virus reverse transcriptase (RT), and amplified with Amplitaq-Gold polymerase enzyme by using two different sequence-specific primers for 40 cycles. *Pol*-amplified products (containing the entire protease and the first 335 amino acids of the reverse transcriptase open reading frame, 1302 nt) were full-length sequenced in sense and antisense orientations by an automated sequencer (ABI 3100) by using seven different overlapping sequence-specific primers

(Ceccherini et al., 2004). Sequences having a mixture of wild-type and mutant residues at single positions were considered to have the mutant(s) at that position.

2.1.4 Phylogenetic analysis

All 255 HIV-1 sequences (1302 nt) were aligned and compared with reference sequences for the Major HIV-1 subtypes, available at: http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html using CLUSTAL X (Thompson et al., 1994). The sequences were then manually edited with the Bioedit program (Hall, 1999), and gaps were removed from the final alignment. All sequences were analyzed using the REGA HIV-1 subtyping tool (de Oliveira et al., 2005).

Separate trees were then generated using F84 Model of substitution with both NJ and Maximum Likelihood (ML) tree building methods (Swafford, 1999), for both non-B pure subtypes and putative recombinant forms.

Phylogenetic trees were performed with different evolutionary model according to the Hierarchical Likelihood Ratio Test (HLRT) implemented in the Model Test V3.0 software (Posada and Crandall, 1998). The statistical robustness within each phylogenetic tree was confirmed with a bootstrap analysis using 1000 replicates for the Neighbor-Joining (NJ) tree. All calculations were performed with PAUP*4.0 software (Swafford, 1999).

Simplot software version 3.2 (Ray, 2002) was used to generate similarity plots and bootscan plots, for genetic diversity and intersubtype recombination analysis.

2.1.5 Determination of drug resistance mutations

To estimate the prevalence of resistant strains, we used the list of drug resistance mutations associated with transmitted drug resistance, that is reported in the Table 1.2 of this thesis (Bennett et a., 2009).

We also determined the prevalence of the RT polymorphism V60I that has been associated with the persistence of tymidine analogues mutations 1 (TAMs1) in drug naïve patients (Lindström et al., 2006).

Other polymorphisms at positions already associated with drug resistance were also investigated.

2.1.6 Statistical analysis

(i) Quantitative measurements and mutation prevalence. For quantitative measurements, data sets with non-normal distributions were compared non-parametrically using Mann-Whitney U test. Categorical data were analyzed by using Fisher exact test. A p value less than 0.05 was used to determine statistical significance. Logistic regression analysis was used to examine the association between epidemiological, clinical and virological factors.

(ii) Mutation covariation. We calculated the binomial correlation coefficient (phi) for all the possible pairwise combinations between all mutations related with drug resistance. The covariation analysis was performed in the 213 HIV-1 B subtype infected patients.

All calculations were performed using a script implemented in the R software, version 2.7.1 (http://www.r-project.org).

Statistically significant pairwise correlations were those with a P value <0.05. For each pair, two positions each with a mixture of two or more mutations were excluded from

the covariation analysis, since it is impossible to discriminate whether these mutations fall in the same viral genome. We used the Benjamini-Hochberg method to identify pairwise combinations that were significant in the presence of multiple-hypothesis testing; a false discovery rate of 0.05 was used to determine statistical significance. In order to analyze the covariation structure of mutations in more detail, we performed mutational clusters, defined as clusters of three or more mutated positions in which each position was significantly correlated with each other, identified by a computational technique that evaluated all possible clusters that can be formed from the significant correlated pairwise combinations of mutated positions (Svicher et al., 2001).

2.2 Epidemiological network analysis in HIV-1 B infected patients diagnosed in Italy between 2000-2008

2.2.1 Study population

A total of 884 HIV-1 B subtype pol gene sequences (containing the full-length protease [PR] and the first 344 reverse transcriptase [RT]) codons from 306 drug-naïve (42 recently [PHI <6 months after seroconversion], and 264 chronically infected) and 578 drug-treated HIV-1 infected patients were analyzed. Patients were defined chronically or recently infected on the basis of quantity of antibody present using a Sensitive/Less-Sensitive ("Detuned") Assays (Janssen et al., 1998). All sequences were collected through routine drug resistance testing between 2000 and 2008 in a single center (Division of Infectious Disease, Bergamo) located in the Northern part of Italy. For drug-naïve patients, the sample closest to the date of HIV-1 diagnosis was selected for this analysis, while, for drug-experienced individuals, the last (in order of time) was used.

The sequences were coded for anonymity, but information on geographical region, self-reported route of transmission, and date of sampling was retained.

2.2.2 HIV-1 sequencing

Viral RNA was extracted from the patients' plasma, reverse transcripted, amplified and sequenced using the The Viroseq HIV-1 Genotyping System (Celera Diagnostics, CA, USA) according to the manufacturer's instructions.

Capillary electrophoresis was undertaken on the ABI-Prism-3130 Genetic Analyzer. Resistance mutation analysis was performed using the ViroSeq System Software.

Sequence data were submitted to Stanford Resistance Database tool (http:hivdb.stanford.edu) to obtain an estimate of the level of susceptibility to antiretrovirals and the identification of the HIV-1 subtype. In addition all sequences were analyzed using the REGA HIV-1 subtyping tool [http://dbpartners.stanford.edu/RegaSubtyping].

2.2.3 Phylogenetic analysis

To avoid the influence of convergent evolution at antiretroviral drug resistance mutations on the phylogenetic analysis, a codon-stripped dataset from which 44 codons associated with major resistance in PR (position 30, 32, 33, 46, 47, 48, 50, 54, 58, 74, 76, 82, 84, 88, 90) and RT (position 41, 62, 65, 67, 70, 74, 75, 77, 90, 98, 100, 101, 103, 106, 108, 115, 116, 138, 151, 184, 179, 181, 188, 190, 210, 215, 219, 225, 230) was analyzed.

The first 1302 nucleotides of pol gene from 884 HIV-1 B subtype infected patients were analyzed. All the sequences were aligned, removing gaps and cutting to identical sequence lengths using ClustalX software (Thompson et al., 1994).. The manual editing was performed using Bioedit software (Hall, 1999).

Phylogenetic approach was used to establish clustered transmission and sequence interrelationships between acute/early and chronic infections in both treated and not treated patients.

Multiple alignments spanning subtype specific HIV-1 pol sequences were analyzed by Bayesian phylogenetic methods.

Bayesian phylogenetic tree was reconstructed by Mr Bayes (Huelsenbeck and Ronquist, 2001) using a GTR + I + G model of nucleotide substitution and a proportion of invariant sites and gamma distribuited rates among sites. MCMC search was conducted

for $5*10^6$ generations, with tree sampled every 100 t h generation and a burn in of 50%. Statistical support for specific clades was obtained by calculating the posterior probability of each monophyletic clade and a posterior consensus tree was generated after a burn in of 50%. Transmission cluster were recognized on the basis of the same tree and a posterior probability was used as statistical support of each cluster. Cluster group size was determined using nodes with a posterior probability of 1.

Likelihood mapping of 10,000 random quartets of all HIV-1 B sequences was generated using TreePuzzle software (Schmidt et al., 2001). Each dot represents the likelihoods of the three possible unrooted trees for a set of four sequences (quartets) selected randomly from the data set: dots close to the corners or the sides represent, respectively, tree-like, or network-like phylogenetic signal in the data. The central area of the likelihood map, represents star-like signal. The percentage of dots in the central area is given at the basis of each map.

2.2.4 Genotypic resistance analysis

According to International Guidelines, a total of 88 mutations associated with transmitted drug resistance were analyzed, all reported in the Table 1.2 of this thesis (Bennett et a., 2009). Polymorphisms at positions already associated with drug resistance were also investigated.

For each tree, the phylogenetic clustering of at least one drug resistant sequence from a drug-naïve patients was considered evidence for the existence of a drug resistant viral lineage. Differences among clustered and non-clustered drug resistance mutations were assessed using Fisher's exact tests.

2.2.5 Time-scaled phylogenies

For each cluster group involving ≥6 sequences, the dated trees, evolutionary rates and population growth were co-estimated by using a Bayesian MCMC approach (Beast version 1.4.8. http://beast.bio.ed.ac.uk) (Drummond et al., 2007) implementing a GTR+Invariant+Gamma model. As coalescent priors, different parametric demographic models (a constant population size, and exponential and logistic growth) and a non-parametric Bayesian skyline plot (BSP) were compared under strict and relaxed clock conditions, and the best models were selected by means of a Bayes factor (BF, using marginal likelihoods) implemented in Beast (Suchard et al., 2001). In accordance with Kass and Raftery, 1995, the strength of the evidence against H_0 was evaluated as follows: 2lnBF <2 no evidence; 2-6 weak evidence; 6-10 strong evidence; >10 very strong evidence. A negative value indicates evidence in favour of H_0. Only values of ≥6 were considered significant.

Chains were conducted for $100x10^6$ generations, and sampled every 10000 steps for cluster A to D, whereas for cluster E the analysis was conducted for $50x10^6$ generations, and sampled every 5000 steps. Convergence was assessed on the basis of the effective sampling size (ESS) after a 50% burn-in using Tracer software version 1.4 (http://tree.bio.ed.ac.uk/software/tracer/). Only parameter estimates with ESS's of >200 were accepted. Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals.

The maximum clade credibility tree has been produced by the original version of TreeAnnotator (Drummond et al., 2007) as the tree in the posterior sample that has the maximum sum of posterior clade probabilities.

2.3 "Sentinel" mutations in standard population sequencing can predict the presence of RT major mutations detected only by 454-pyrosequencing

2.3.1 Study population

Among 2089 HIV-1 B subtype infected patients, naïve to the antiretroviral therapy, enrolled between January 2004 and December 2009 in the Spallanzani Hospital (Rome) through routine drug resistance testing, 30 individuals were retained for the study.

All 30 patients answered to the following criteria: no previous exposure to antiretroviral drugs (all patients were drug-naïve), a viral load >10.000 copies/ml at the time of testing, the complete absence of primary transmitted resistance at standard genotypic resistant test (GRT) (Bennett et al., 2009), and the detection by standard GRT of one atypical mutation at positions already associated with drug resistance (Johnson et al., 2009). In particular, the atypical mutations T69S, L210M, and K103R were present in 10/10/10 RNA samples, respectively.

In addition, 10 HIV-1 B subtype infected individuals, naïve to the antiretroviral therapy, with a viral load >10.000 copies/ml at the time of testing, and with wild-type virus at all the positions associated with drug resistance, were considered as controls.

All patients were coded for anonymity, but demographic characteristics, self-reported route of transmission, and date of sampling were retained. Virological and immunological parameters were also available for each patient.

2.3.2 HIV-1 sequencing

Standard population sequencing

HIV genotype analysis was performed on the 40 plasma samples by means of a commercially available kit (ViroSeq HIV-1 genotyping system; Abbott Laboratories) (Ceccherini et al., 2004). Briefly, after an ultracentrifugation of 2 hours to collect all viral particles, RNA was extracted, retrotranscribed by murine leukemia virus reverse transcriptase (RT), and amplified with Amplitaq-Gold polymerase enzyme by using two different sequence-specific primers for 40 cycles. *Pol*-amplified products (containing the entire protease and the first 335 amino acids of the reverse transcriptase open reading frame, 1302 nt) were full-length sequenced in sense and antisense orientations by an automated sequencer (ABI 3100) by using seven different overlapping sequence-specific primers (Ceccherini et al., 2004). Sequences having a mixture of wild-type and mutant residues at single positions were considered to have the mutant(s) at that position.

Ultra-deep pyrosequencing

After RNA extraction and retrotrascription, like to the standard sequencing, ultra deep pyrosequencing (UDPS) of reverse transcriptase was performed for 9 coupled plasma/cellular (PBMCs) samples and 31 additional plasma samples from the 40 drug-naïve patients enrolled.

Ultra-deep pyrosequencing was carried out with the 454 Life Sciences platform (GS-FLX, Roche Applied Science). PCR products were clonally amplified on capture beads in water-in-oil emulsion micro-reactors, and pyrosequencing was performed by using one of 16 lanes of a 70 × 75 mm PicoTiterPlate for each sample, following the standard approach for PCR amplicons sequencing. For each sample an SFF file was obtained,

from which nucleotide sequence data were extracted.

UDPS coverage, error rate estimation and correction algorithms

UDPS generated a median of 7,404 reads per sample with a median read length of 245 bases. This resulted in a median coverage of 1200 reads per base.

To measure the accuracy of the ultra-deep pyrosequencing, a pNL43 plasmid clone containing the region of interest was sequenced in parallel by ultra-deep pyrosequencing and by the Sanger method. The plasmid clone was obtained from a patient's sample by inserting a PCR amplicon spanning nucleotide positions 1,446 to 4,457 (reference strain Consensus B) into a pCR4-TOPO vector (Invitrogen Corp.). Sanger sequencing of the clone was performed on ABI Prism 310, using the BigDye Terminator cycle sequencing kit, following the manufacturer's instructions (Applied Biosystems Warrington, UK). Any differences between the two methods were considered to be a GS-FLX sequencing error.

With the aim of considering only the sequences leading to functional products, following correction algorithm were adopted (Rozera et al., 2009). Nucleotide sequences from each sample were divided into two separate files, one for the forward reads and one for the reverse ones. These sequences were then translated into amino acids with EMBOSS (Rice et al., 2000) using all possible frames; only those translated with the right open reading frame (ORF) were retained. Multiple alignments of the amino acid sequence files were then constructed using the software MUSCLE (Edgar, 2004) (default options) and trimmed at the 5' and 3' termini to include only a region potentially covered by both forward and reverse reads. To reduce the error rate, sequences containing ambiguous bases (Ns) were also discarded; this reduced the error rate due to the possible presence of reads coming from multitemplated beads (Huse et

al., 2007). Then, for each sample we compared the forward sequence datasets with the reverse ones and clustered them, reporting only identical matches between the two.

Sequences obtained from the patients have been compared to the sequences of reference HIV strains present in the laboratory to rule out possible contamination with these amplicons.

Finally, to carry out the haplotipes reconstruction we followed the approach described by Erikson et al., 2008.

2.3.3 Determination of drug resistance minority variants

To estimate the prevalence of drug resistance minority variants in our patients, we used the list of drug resistance mutations reported in bold in the Figures 1.10 and 1.11 of this thesis (Johnson et al., 2009). The percentage of HIV-1 sequences containing each mutation was calculated by dividing the number of mutant sequences by the total number of HIV-1 sequences and multiplying by 100. We also convert for each sample the percentage of sequences containing each mutation in copies number/ml by the following formula: % of sequence reads containing each mutation * Baseline Viral load.

According with the number of virus templates submitted for UDPS, the distribution of errors on the plasmid clone and the multiple testing on 330 amino acids performed, drug resistance mutations detected at level >2.0% of viral species in both forward and reverse direction were considered authentic minor variants. Drug resistance mutations detected at level >0.1% and <2.0% of viral species in both forward and reverse direction were considered authentic minority variants only if the relationship presented by Wang et al. between the number of required ultra-deep pyrosequencing reads per position (Required coverage) and the detection limit of the frequency of a minority sequence variant (Detection threshold) was guaranteed (Wang et al., 2008).

2.3.4 Statistical analysis

The association between atypical mutations revealed by standard GRT and drug resistance minor variants revealed by UPDS was assessed by Fisher exact test.

To define the role of minority drug resistance variants on the virological outcome, we compared the median time to reach the virological success (defined as at least 2 consecutive determination of plasma HIV-1 RNA <50 copies/ml) between subjects with no drug resistance mutations and subjects with minority drug resistance. The viral load mean change from baseline to 4, 8, 12, and 24 weeks after HAART initiation between these 2 groups of patients was also compared. For these analyses a Wilcoxon rank-sum test was used.

All *P* values and confidence intervals (CIs) presented are unadjusted for multiple comparisons.

3 Results

In this chapter, results obtained in each study described in this thesis were reported.

3.1 Characterization of the patterns of drug resistance mutations in newly diagnosed HIV-1 infected patients naïve to the antiretroviral drugs

3.1.1 Study population

Table 3.1 summarizes the main characteristics of the 255 out of 263 newly diagnosed patients, whose samples were successfully amplified and sequenced. Epidemiological information was available for 249 out of 255 individuals.

One hundred ninety three (193, 77.5%) individuals were males and more than 75% of patients were Italian (n=188). Among 61 foreigners, 25 patients originated from South America (41.0%), 17 from Sub-Saharan Africa (27.9%), 14 from Eastern Europe (23.0%) and 5 from North and Central America (8.2%).

Based on results of Avidity Index, 58 (22.7%) patients were classified as recent infected, while the remaining 197 (77.2%) were classified as long-standing infected patients. At the time of median diagnosis, CD4 cell count was higher in individuals with a recent infection compared to those with a long-standing infection (480 [interquartile range, IQR: 366-655] cell/mm3 vs 330 [IQR: 146-484] cell/mm3, P<0.001, data not shown) while median viremia was not significantly different in the two groups of patients (4.8 [IQR: 4.0-5.4] log10 HIV-1 RNA copies/ml vs 4.9 [IQR: 4.4-5.4] log10 HIV-1 RNA copies/ml, P=0.09). A major proportion of Italian patients was found in the group of recently HIV-1 infected patients than in the long-standing infected one (49 [87.5%] vs 139 [72.0%], P=0.02, data not shown).

Several factors that might affect the risk of becoming infected with drug resistant virus were explored (Table 3.1). Patients who had acquired HIV through homosexual intercourses were more likely to harbour a virus with at least one primary resistance mutation (OR 7.7; 95% CI: 1.7–35.0, P=0.008). In particular, homosexuals had a significant increased risk of harbouring HIV with resistance mutations to NRTI (OR 9.5, 95% CI: 1.2–76.6, P=0.03), while risk of resistance mutations to NNRTI and PI was not significantly associated with HIV risk factor (P=NS). This may reflect the higher and earlier access to treatment of this class than other groups of patients.

3.1.2 Circulation of HIV-1 subtypes in newly diagnosed patients in Italy

The figures 3.1 and 3.2 represent the 22 non-B pure subtypes and the 20 putative recombinant forms circulating in Italy.

Phylogenetic analysis showed that B subtype was the most predominant in the overall population (213 [83.5%]), followed by C subtype and CRF02_AG (10/255 [3.92%] each one). Regarding the country of origin, the majority of patients infected by B subtype was Italian (170 [79.8%]). Among the 42 individuals infected with a non-B subtype, 18 (42.9%) were Italian, 7 (16.7%) were from a European country, and 17 (40.5%) were from outside Europe. The prevalence of C and A subtypes was higher in non-Italian compared to Italian patients (7 [11.4%] vs 3 [1.6%], P=0.003, and 3 vs 0 [0.0%], P=0.01). Thus, nearly half of patients infected with a non-B subtypes were Italian.

Interestingly, we also observed the presence of CRF03_AB in one recently and in one long-standing infected patients, coming from Italy and Eastern Europe, respectively. This is the first study that reports the circulation of CRF03_AB in Italy (Figure 3.2).

Table 3.1 Distribution of HIV-1 drug resistance mutations by selected demographic, clinical and virological characteristics of 255 newly diagnosed patients

Characteristics	All patients n=255	Patients with at least one drug resistance mutation (NRTI, NNRTI, PI)[d] n=15	Patients with wild type virus n=240	*P-value*[e]	OR (95% CI)[f]
Italian [a] N (%)	188 (75.5)	14 (93.3)	174 (74.3)	0.13	4.8 (0.6-37.5)
Male [a] N (%)	193 (77.5)	14 (93.3)	179 (76.5)	0.13	4.8 (0.6-37.5)
Median age (years) (IQR) [a]	37 (31-43)	38 (34-46)	37 (30-43)	0.35	
Risk exposure N (%)					
Heterosexual	108 (47.6)	2 (13.3)	106 (50.0)	0.01	0.15 (0.03-0.70)
Homosexual	110 (48.6)	13 (86.7)	97 (45.8)	**0.008**	**7.7 (1.7-35.0)**
Intravenous drug use	9 (4.0)	0 (0.0)	9 (4.2)	1.00	0.0 (0.0-6.1)
Unknown, no.	28	0	28		
Viral Load, Median (IQR), (log copies/ml)	4.9 (4.3-5.4)	4.5 (4.3-5.2)	4.9 (4.3-5.4)	0.40	
CD4+ count, Median (IQR), cell/mm3	372 (171-517)	336 (132-450)	385 (194-527)	0.50	
Co-infection N (%)					
HBV	33 (13.3)	0 (0.0)	33 (13.7)	0.23	0.0 (0.0-1.6)
HCV	26 (10.4)	0 (0.0)	26 (10.8)	0.38	0.0 (0.0-2.2)
HIV-1 subtypes					
Subtype B	213 (83.5)	15 (100)	198 (82.5)	0.99	inf (2.2-inf)
Other Subtypes	42 (16.5)	0 (0.0)	42 (17.5)	0.99	0.0 (0.0-1.2)
Infectious status					
Long-standing Infection, > 6 months[b]	197 (77.2)	13 (86.7)	184 (76.7)	0.38	2.0 (0.4-9.0)
Recent Infection, ≤6 months[c]	58 (22.7)	2 (13.3)	56 (23.3)	0.38	0.50 (0.11-2.3)

[a] Among 255 patients, epidemiological information was available for 249 patients. The 6 patients without epidemiological information are included in the group of 240 patients carried wild type virus. Thus, the percentages were calculated on 249 patients in the second column and on 234 patients on the forth column.
[b] Long-standing infection was estimated by a ≥0.80 avidity index.
[c] Recent infection was estimated by a <0.80 avidity index.
[d] Mutations that have been associated with transmitted drug resistance (Shafer et al., 2007) are taking into account.
[e] For quantitative measurements, data sets with non-normal distributions were compared non-parametrically using Mann-Whitney U test. Categorical data were analyzed by using a chi-square test accomplished by a logistic regression analysis.
[f] CI, confidence interval, odds ratio (OR) are calculated with a logistic regression analysis.

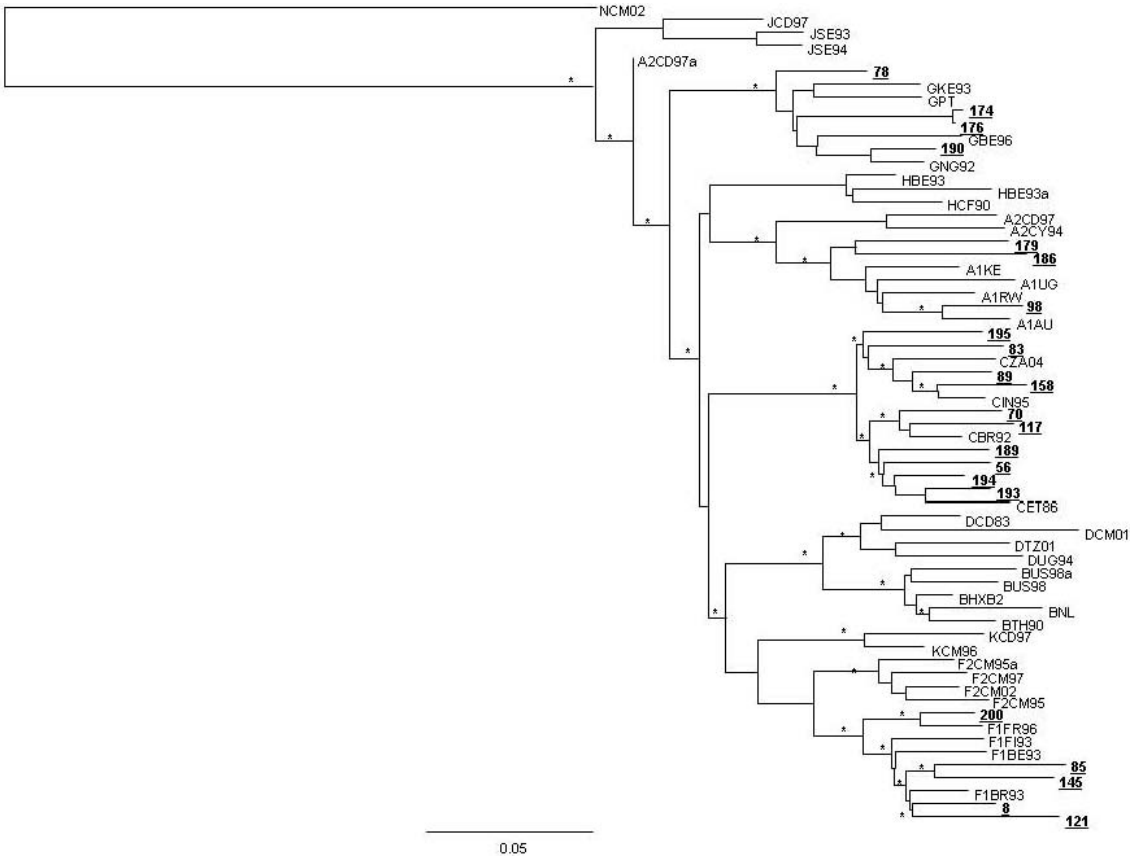


Figure 3.1: Phylogenetic relationships based on *pol* gene (1302 nt) of the HIV-1 *pol* gene between the HIV-1 non-B pure subtypes circulating in Italy (shown in bold and underlined) and representative strains of HIV-1 M group (subtypes A, C, D, F1, F2, G, H, J, K) from the Los Alamos HIV Sequence Database. The scale bar indicates 5% nucleotide sequence divergence.* indicates the *P value* < 0.001 (zero length branch test) and the bootstrap values more than 70%.

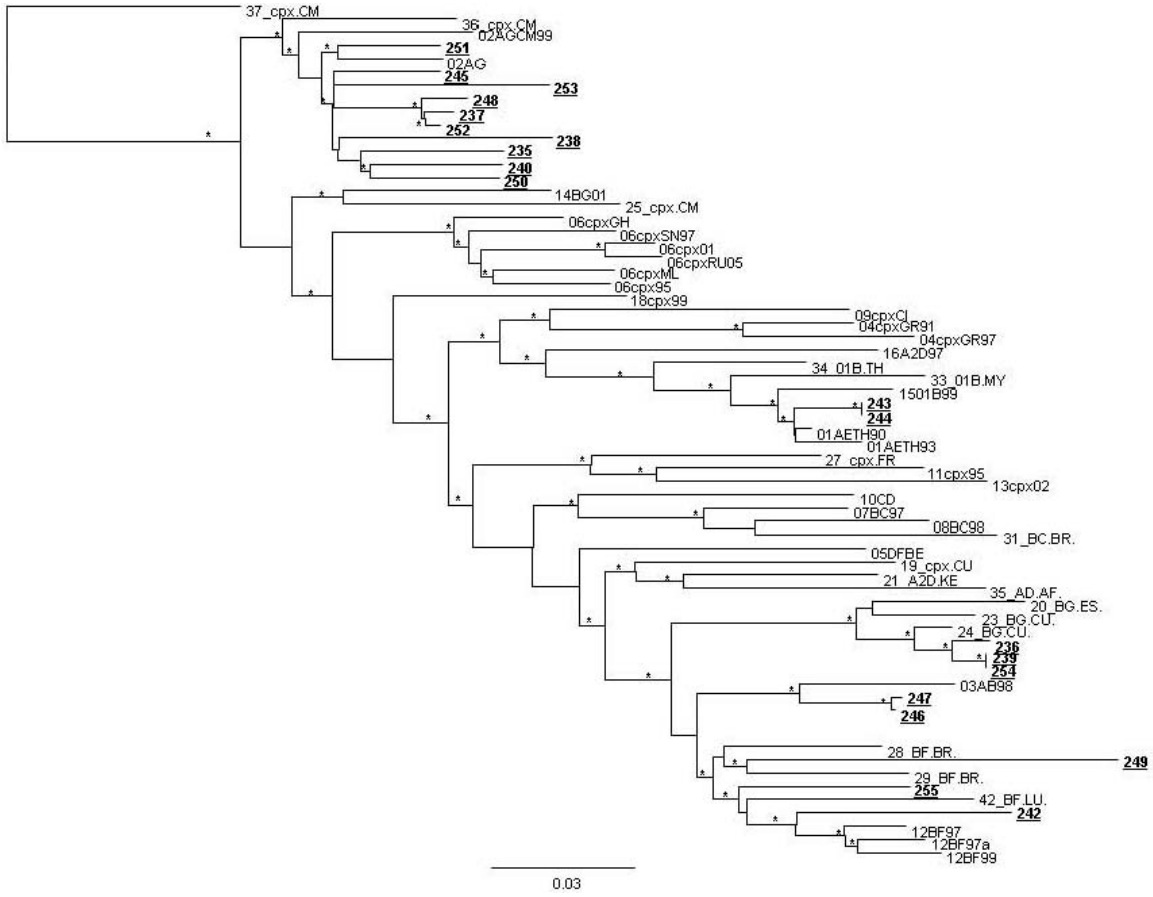


Figure 3.2: Phylogenetic relationships on *pol* gene (1302 nt) of the HIV-1 *pol* gene between the HIV-1 putative recombinant forms circulating in Italy (shown in bold and underlined) and the reference sequences of the CRFs of the HIV-1 M group from the Los Alamos HIV Sequence Database. Bootstrap values <90% are not shown. The scale bar indicates 3% nucleotide sequence divergence.* indicates the *P value* < 0.001 (zero length branch test) and the bootstrap values more than 70%.

3.1.3 Prevalence of major mutations associated with drug resistance

Among the 255 newly diagnosed, 15 (5.9%) carried HIV-1 strains with at least 1 major mutation associated with transmitted drug resistance (Table 3.2) (Bennett et al., 2009); all of them were infected with subtype B.

Of note, 9 (3.5%) of these patients carried HIV-1 strains with two or more mutations associated with transmitted drug resistance. Five out of 9 patients (55.5%) were resistant to 2 drug classes (all with HIV-1 B subtype). Among these 5 patients, 4 (80.0%) were resistant to both nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), and 1 (20.0%) to NRTIs and protease inhibitors (PIs). None carried resistance to all 3 drug classes.

Major mutations associated with NRTI resistance were observed in 10 (3.9%) patients (Table 3.2). The revertant mutations at codon 215 were the most frequently observed (8 [3.1%]), followed by L210W (6 [2.3%]) (Table 2). Among the 8 patients with the revertant forms of T215Y/F, 5 had T215D (resulting from a single nucleotide substitution of TAC/TAT [Y] to GAC/GAT [D]), 2 had T215S (resulting from a single nucleotide substitution of either TAT/C [Y] or TTT/C [F] to TCT/TCC [S]) and 1 had a mixture of T215D/S/Y. The only TAM2 observed was D67N, found in one patient together with the TAM-1 M41L, L210W and T215Y. Similarly the 3TC-selected mutation M184V was found in only one patient (together with L210W). Thus, the majority of NRTI resistance is sustained by TAM-1 (comprising the T215Y revertant forms), suggesting that such mutational pattern confers a fitness level greater than TAM-2.

Among 9 patients (3.5%) carrying NNRTI-resistance mutations, 7 had K103N (conferring high level of resistance to both nevirapine and efavirenz) (Table 3.2). This

mutation was found together with L100I and P225H in two and one patient, respectively; both mutations are known to occur almost exclusively with K103N in patients failing an antiretroviral regimen containing efavirenz (Bacheler et al., 2000).

3.1.4 Association among drug resistance mutations in newly diagnosed HIV-1 infected patients

i) Correlated pair of mutations

A covariation analysis was performed in order to determine significant patterns of pairwise correlation between drug resistance mutations in drug-naïve patients (Table 3). The most strongly correlated pairs of major mutations in drug naïve patients are as follows: L210W+M41L (phi=0.6) found in 3 patients, L210W+T215D (phi=0.9) and L210W+K103N (phi=0.4) found in 5 and 3 patients, respectively. The T215D was also strongly correlated with V60I (phi=0.31, P=0.02), a common polymorphism that is known to rescue the replicative capacity impaired by the TAM1 mutations in the absence of drug pressure (Lindström et al., 2006).

ii) Clusters of mutations

The topology of the dendrogram (Figure 3.3) shows the existence of one significant (bootstrap> 0.75) cluster of major mutations in newly diagnosed HIV-1 B subtype infected patients. In particular, the cluster involved four mutations associated with transmitted resistance (the TAM1 - M41L, L210W, the revertants T215D/S and the NNRTI-resistance mutation K103N) together with the polymorphism V60I (bootstrap=0.97).

Table 3.2 Prevalence of resistant HIV-1 strains

Mutation[a]	Prevalence			Infectious status		*P-value*[d]
	Total[b]		in combination[c]	Long-standing Infection > 6 months (N=197)	Recent Infection ≤ 6 months (N=58)	
	All patients N (%/255)	Subtype B N (%/213)	N (%)			
Major Drug resistance						
Any	15 (5.9)	15 (7.0)	9 (60.0)	13 (6.6)	2 (3.4)	0.09
NRTI						
Any	10 (3.9)	10 (4.7)	8 (80.0)	8 (4.1)	2 (3.4)	1
Any TAM	7 (2.6)	7 (3.3)	7 (100)	6 (3.0)	1 (1.7)	1
Any TAM1	7 (2.6)	7 (3.3)	7 (100)	6 (3.0)	1 (1.7)	1
Any TAM2	1 (0.4)	1 (0.5)	1 (100)	0 (0.0)	1 (1.7)	0.2
M41L	4 (1.6)	4 (1.9)	4 (100)	3 (1.5)	1 (1.7)	1
D67N	1 (0.4)	1 (0.5)	1 (100)	0 (0.0)	1 (1.7)	0.2
T69D	1 (0.4)	1 (0.5)	0 (0.0)	0 (0.0)	1 (1.7)	0.2
M184V	1 (0.4)	1 (0.5)	1 (100)	1 (0.5)	0 (0.0)	1
L210W	6 (2.3)	6 (2.8)	6 (100)	5 (2.5)	1 (1.7)	1
T215Y	2 (0.8)	2 (0.9)	2 (100)	1 (0.5)	1 (1.7)	0.4
T215D/S	8 (3.1)	8 (3.8)	6 (75.0)	8 (4.1)	0 (0.0)	0.2
NNRTI						
Any	9 (3.5)	9 (4.2)	5 (55.5)	8 (4.1)	1 (1.7)	0.7
L100I	2 (0.8)	2 (0.9)	2 (100)	2 (1.0)	0 (0.0)	1
K101E	2 (0.8)	2 (0.9)	0 (0.0)	2 (1.0)	0 (0.0)	1
K103N	7 (2.7)	7 (3.3)	5 (71.4)	6 (3.0)	1 (1.7)	1
P225H	1 (0.4)	1 (0.5)	1 (100)	1 (0.5)	0 (0.0)	1
PI						
Any	1 (0.4)	1 (0.5)	1 (100)	1 (0.5)	0 (0.0)	1
L90M	1 (0.4)	1 (0.5)	1 (100)	1 (0.5)	0 (0.0)	1
Polimorphism at major resistance related positions						
NNRTI						
Any	8 (3.1)	8 (3.8)	1 (12.5)	7 (3.6)	1 (1.7)	0.69
K101Q/R	6 (2.3)	6 (2.8)	1 (16.7)	5 (2.5)	1 (1.7)	1
K103Q/R	3 (1.2)	3 (1.4)	1 (33.3)	3 (1.5)	0 (0.0)	1

[a] Mutations that have been associated with transmitted drug resistance (Bennett et al., 2009) are reported.
[b] Number (%) of drug resistance mutations.
[c] Number (%) of drug resistance mutations that occurs in combination with other major mutations in a sequence. The percentages were calculated in patients containing each specific mutation.
[d] Data were analyzed by using Fisher exact test.
Abbreviations: PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; TAMs, thymidine analogue associated mutations.

Table 3.3 Significantly correlated pairs of mutations

Mutation 1	Frequency N (%)[a]	Mutation 2	Frequency N (%)[a]	Covariation Frequency N (%)	Phi[b]	*P-value*[c]
RT						
M41L	4 (1.9)	L210W	6 (2.8)	3 (1.4)	0.6	0.02
L210W	6 (2.8)	T215D	6 (2.8)	5 (2.3)	0.9	<0.001
L210W	6 (2.8)	K103N	7 (3.3)	3 (1.4)	0.4	0.04
T215D	6 (2.8)	V60I	34 (16.0)	5 (2.3)	0.3	0.02

[a] The frequency was determined in 213 HIV-1 B isolates from drug-naïve patients.
[b] Positive correlations and negative correlations with phi >0.15 and phi <-0.07 are shown, respectively.
[c] All *P* values for covariation were significant at a false discovery rate of 0.05 following correction for multiple-hypothesis testing (Benjamini-Hochberg method).

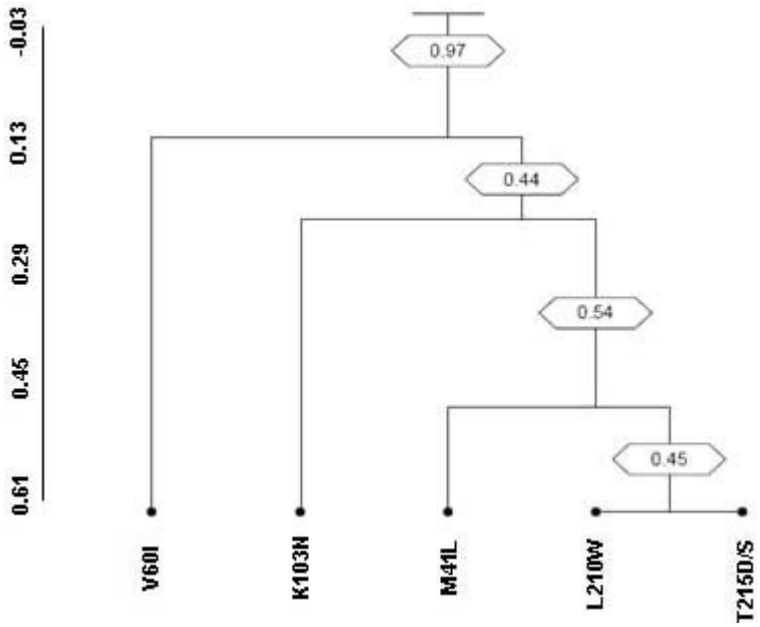


Figure 3.3: Dendrogram obtained from average linkage hierarchical agglomerative clustering, showing significant clusters of RTI resistance mutations. The length of branches reflects distances between mutations in the original distance matrix. Bootstrap values, indicating the significance of clusters, are reported in the boxes.

3.2 Epidemiological network analysis in HIV-1 B infected patients diagnosed in Italy between 2000-2008

3.2.1 Study population

This study includes 884 HIV-1 B subtypes infected patients followed in a single clinical center in North Italy between January 2000 and July 2009. Among them, 42 (4.8%) were classified having a recent infection (PHI <6 months after seroconversion), 264 (29.9%) as chronically infected patients and 578 (65.4%) as drug-experienced HIV-1 infected patients. The main exposure groups were all represented: men having sex with men (MSMs; n=156), heterosexual transmission (Heterosexual; n=330), injection drug users (IDUs; n=382), and other exposure group (n=16). All the other epidemiological and clinical data of our cohort are summarized in Table 3.4.

Table 3.4. Demographic, clinical and virological characteristics of the 884 patients

	Overall	**Drug-naïve**	**Drug-experienced**
Number of patients	884	306	578
***Male,* N (%)**	681 (77)	258 (84)	423 (73)
***Italian,* N (%)**	845 (95)	276 (90)	569 (98)
HIV exposure, N (%)			
MSM	156 (18)	93 (31)	63 (11)
Heterosexual	330 (38)	146 (50)	184 (32)
IDUs	382 (44)	56 (19)	326 (57)
***Unknown,* N**	16	11	5
Age (years), Median (IQR)	38 (34-43)	40 (33-46)	38 (34-42)
Viral Load, Median (IQR) Log Copies/ml	4.3 (3.7-4.9)	4.8 (4.5-5.5)	4 (3.5-4.5)
CD4 Cell/mm^3	344 (182-499)	317 (124-471)	353 (210-507)

3.2.2 Phylogenetic analysis

The phylogenetic tree involving all the 884 HIV-1 B sequences is shown in Figure 3.4. In this approach, we define clusters as clades identified with a posterior probability of 1. Tree topology revealed that 89 (29.1%) out of 306 drug-naïve patients grouped into 47 significant clusters. Thirty-six [76.6%] involved 2 patients, while the remaining 11 involved from 3 to 18 patients. In addition, 16 (34.0%) out of 47 significant clusters involved drug-naïve patients only, while the remaining 31 (66.0%) involved both naïve and HAART treated patients (Figure 3.5).

Among the 42 recent infections, 7 (16.7%) and 5 (11.9%) were significantly clustered with untreated and treated chronically infected patients, respectively.

The phylogenetic structure of HIV epidemic was also investigated by likelihood mapping analysis (Figure 3.6). The evaluation of 10,000 random quartets with the likelihood-mapping method showed a more than 90% of the randomly chosen quartets from the alignment were distributed in the corner of the likelihood map, which represent tree-like signal. All the clusters identified using the genetic distance approach meet this criterion on the Bayesian MCMC tree treated chronically infected patients, respectively.

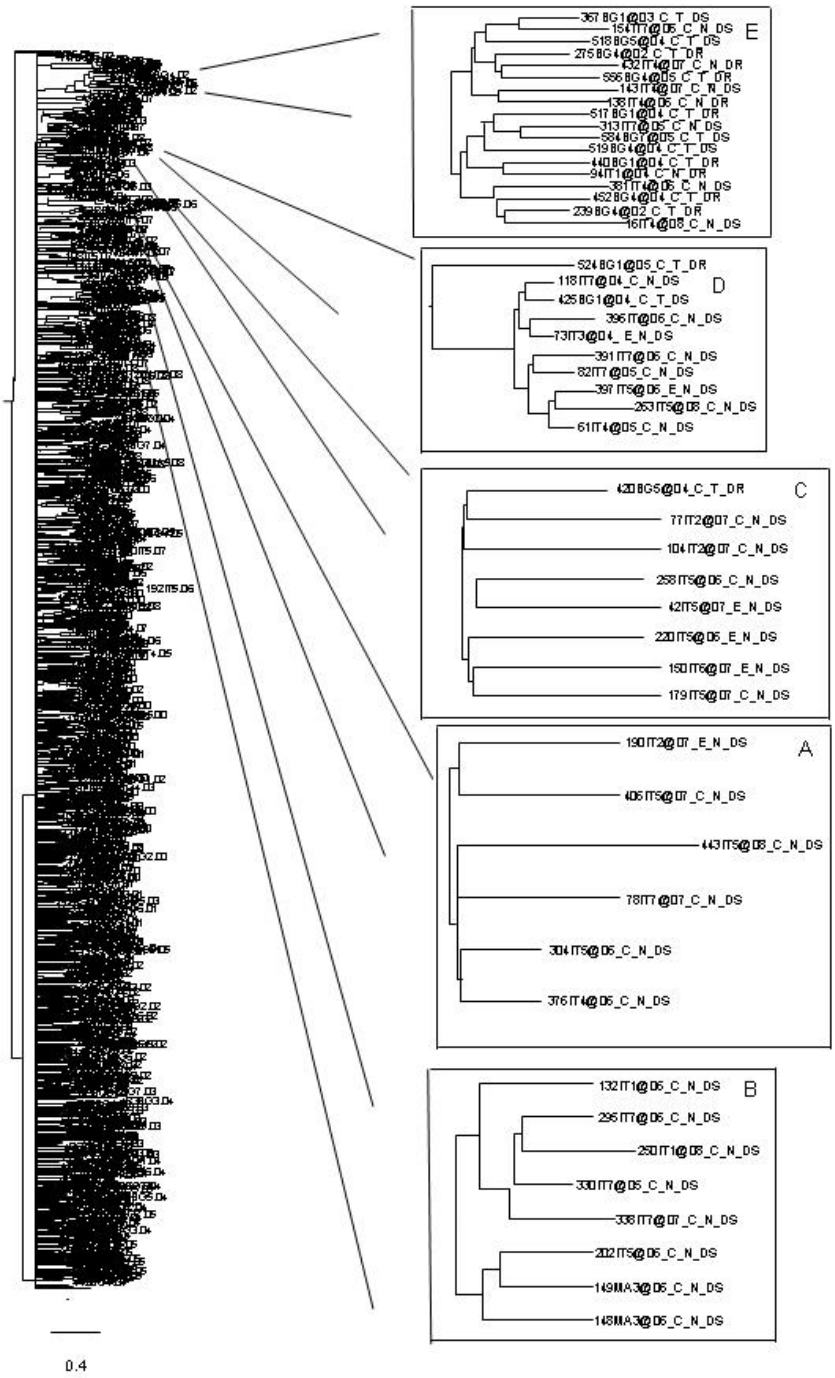


Figure 3.4 "Bayesian phylogenetic tree of the 884 HIV-1 B subtype pol gene sequences implementing relaxed molecular clock, after exclusion of 44 codon positions associated with drug resistance. Branch lengths are expressed as the number of nucleotide substitutions per site. The five dated clusters in the trees are highlighted. For each cluster the statistical support (p< 0.001 zero length branch test and the posterior probability [100%]) has been computed. Each patient in the five transmission clusters is identified by a code including information about early or chronical infection (E or C), HAART naïve or treated status (N or T), drug resistant or drug-susceptible virus (DR or DS).

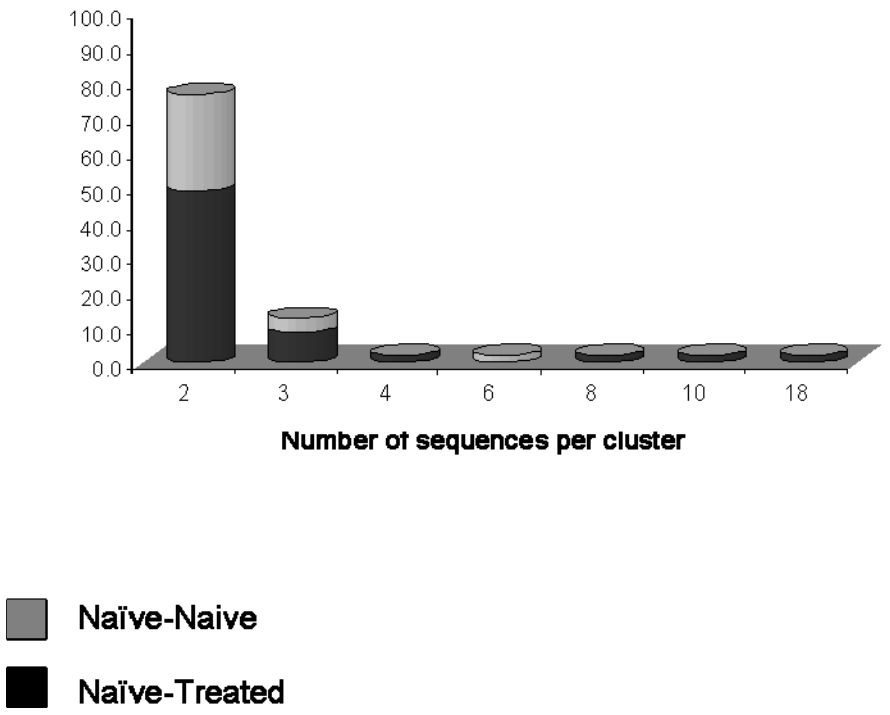


Figure 3.5 The distribution of drug-naïve and drug-experienced patients in the 47 clusters. Overall, 76.6% of the chains have 2 persons/cluster, whereas the remaining individuals are in clusters having >2 persons/cluster. Single clustering of drug-experienced patients and non-clustered drug-naïve patients is also depicted.

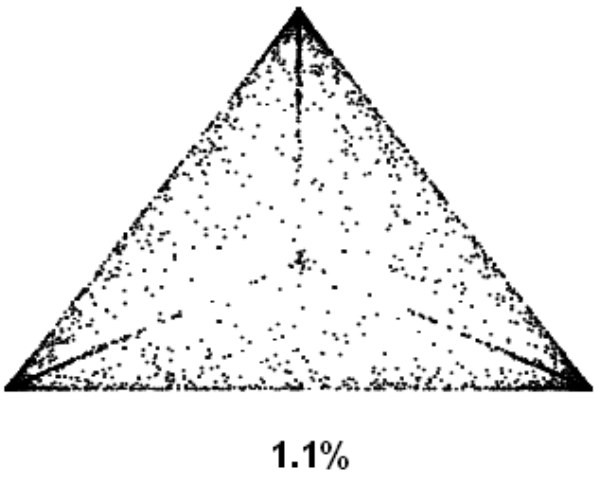


Figure 3.6 Likelihood mapping of HIV-1 B *pol* sequences. Each dot represents the likelihoods of the three possible unrooted trees for a set of four sequences (quartets) selected randomly from the data set (see Materials and Methods): dots close to the corners, the center, or the sides represent, respectively, tree-like, star-like, or network-like phylogenetic signal in the data. The percentage of dots in the center of the likelihood map is indicated.

3.2.3 Identification of antiretroviral drug resistant HIV-1 lineages in drug-naïve patients

Among the 306 drug-naïve patients HIV-1 B subtype infected patients, 36 (11.8%) carried a viral strains with at least 1 major mutation associated with transmitted drug resistance (Table 3.5) (Bennett et al., 2009). The overall prevalence of mutations conferring resistance to NRTIs, NNRTIs, and PIs was 5.9, 5.6, and 1.0%, respectively. In addition, only one patient among the 42 recently infected ones carried drug resistance virus (the NNRTI mutation G190A). This data suggests a decreasing trend of drug resistance in recent infections compared to chronic infections (P=0.04), also described in the first part of these results (see 3.1.3)

Of the 36 drug resistant strains, 23 (63.9%) remained unclustered, while 13 (36.1%) were involved in 9 significant clusters. Of this latter group, 8 (61.5%) strains clustered with HAART treated patients, while 5 (38.5%) with drug-naïve patients.

In particular, the 8 drug resistant strains clustering with HAART treated patients were involved in 6 significant clusters. These treatment-dependent transmitted drug resistance mutations consisted of phylogenetically linked mutations shared by drug-treated and drug-naïve patients. Therefore, these clusters of resistant viruses probably reflect transmission from treated to untreated individuals. These clusters involved in 2 cases the TAM 1 resistance mutations M41L and T215Y (this last one present as recombinant form T215S in the drug-naïve patient), the NNRTI resistance mutations G190A+V179F, K103N and Y181C in 3 cases, and finally in one case the NRTI mutations M41L, M184V and the NNRTI mutation G190E.

Differently, the 5 drug resistant strains clustering with drug-naïve patients were present in 3 significant clusters. These treatment-independent transmitted drug resistance

mutations were identified as resistance mutations circulating only within phylogenetically linked drug-naïve patients and probably represent drug resistant HIV-1 populations naturally circulating within untreated individuals. Drug resistance mutations involved in these clusters were the TAM2 D67N and K219Q, the revertant T215D, and the NNRTI resistance mutation K103N.

Of note, nearly half of NRTI and NNRTI resistance mutations (38.9% and 35.3%, respectively) were included in epidemiological networks, while PI resistance mutations were never found in clustered transmission.

In addition to clusters involving drug-naïve patients with transmitted drug resistance, we could also identify 11 clusters, involving as drug-naïve as drug-experienced individuals, where drug resistance mutations were observed only in drug-experienced patients. All these clusters involved viruses with complex patterns of drug resistance mutations, which have a negative impact on drug susceptibility and viral replicative capacity.

Table 3.5. Prevalence of drug resistance mutations in clustered and no-clustered drug-naïve resistant strains

	Naïve N=306	Recently Infected N=42	Chronically Infected N=264	Clustered[b]	Cluster Naïve-Treated[c]	Cluster Naïve-Naïve[c]
At least one Drug resistance mutation	36 (11.8)	1 (2.4)	35 (13.3)	13 (36.1)	8 (61.5)	5 (38.5)
At least one NRTI resistance	18 (5.89)	0 (0.0)	18 (6.8)	7 (38.9)	4 (57.2)	3 (42.8)
At least one TAM	17 (5.6)	0 (0.0)	17 (6.4)	4 (23.5)	2 (50.0)	2 (50.0)
M41L	8 (2.6)	0 (0.0)	8 (3.0)	2 (25.0)	2 (100.0)	0 (0.0)
D67N	5 (1.6)	0 (0.0)	5 (1.9)	2 (40.0)	0 (0.0)	2 (100.0)
T69D	3 (1.0)	0 (0.0)	3 (1.1)	1 (33.3)	0 (0.0)	1 (100.0)
K70R	4 (1.3)	0 (0.0)	4 (1.5)	0 (0.0)	-	-
L74V	1 (0.3)	0 (0.0)	1 (0.4)	0 (0.0)	-	-
M184V/I	6 (2.0)	0 (0.0)	6 (2.3)	1 (16.7)	1 (100.0)	0 (0.0)
L210W	3 (1.0)	0 (0.0)	3 (1.1)	0 (0.0)	-	-
T215rev	8 (2.6)	0 (0.0)	8 (3.0)	2 (25.0)	1 (50.0)	1 (50.0)
T215F	2 (0.7)	0 (0.0)	2 (0.8)	0 (0.0)	-	-
T215Y	3 (1.0)	0 (0.0)	3 (1.1)	0 (0.0)	-	-
K219Q/E	7 (2.3)	0 (0.0)	7 (2.7)	2 (28.6)	0 (0.0)	2 (100.0)
At least one NNRTI resistance	17 (5.6)	1 (2.4)	16 (6.1)	6 (35.3)	4 (66.7)	2 (33.3)
K101E	3 (1.0)	0 (0.0)	3 (1.1)	0 (0.0)	-	-
K103N/S	11 (3.6)	0 (0.0)	11 (4.2)	3 (27.3)	1 (33.3)	2 (66.7)
V106A	1 (0.3)	0 (0.0)	1 (0.4)	0 (0.0)	-	-
V179F	1 (0.3)	0 (0.0)	1 (0.4)	1 (100.0)	1 (100.0)	0 (0.0)
Y181C	2 (0.7)	0 (0.0)	2 (0.8)	1 (50.0)	1 (100.0)	0 (0.0)
G190A/E/S	7 (2.3)	1 (2.4)	6 (2.3)	2 (28.6)	2 (100.0)	0 (0.0)
At least one PI resistance	3 (1.0)	0 (0.0)	3 (1.1)	0 (0.0)	-	-
D30N	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	-	-
I54L	1 (0.3)	0 (0.0)	1 (0.4)	0 (0.0)	-	-
N88D	2 (0.7)	0 (0.0)	2 (0.8)	0 (0.0)	-	-
L90M	2 (0.7)	0 (0.0)	2 (0.8)	0 (0.0)	-	-

3.2.4 Description of the major lineages

The 5 major clusters (≥6 sequences), (posterior probability=1) were further analyzed to better identify a possible epidemiological networks. For each cluster, an exponential model of population growth was significantly favored over a constant and a logistic population size model(data not shown).

Among the 5 clusters analyzed (Figure 3.7 and Table 3.6), 3 (cluster A, B and E) revealed some degree of internal structure; indeed for each cluster two subclades (Figure 3.7) could be identified.

In particular, cluster A included samples from 2006 to 2008 from 6 drug-naïve patients, 5 with chronic and 1 with a recent infection; all patients harbored viruses with no drug resistance mutations but one of them carried an atypical mutation at a known drug resistance position L210S (Figure 3.7_A).

Cluster B included samples enrolled from 2005 to 2008 from 8 drug-naïve patients, all with chronic infection. Again, all patients harbored viruses with no drug resistance conferring mutations but with an atypical mutation at a known drug resistance position (PR V82I). In this cluster, the existence of two subclades seems to be closely related with risk factor and nation of origin; indeed, in the first subclade all patients were MSMs, with the exception of one patient who declared to be heterosexual, and all patients came from Italy, while in the second subclade all 3 patients were heterosexual and 2 of them came from Morocco (Figure 3.7_B).

Cluster E is the most complex lineage identified in our population; it is composed by two subclades and includes 6 drug resistant strains from HAART-treated patients, and 3 resistant strains from drug-naïve patients, observed between 2002 and 2008. The RT mutations M41L, M184V, and G190E were detected in both drug-experienced and

drug-naïve patients. In addition, the NNRTI resistance mutation K103N was found in the first subclade, and G190A/E in the second.

This lineage analysis also showed some evidence of reversions to wild type. TAMs D67N, K70R, L210W, T215Y/F and K219E, the NNRTI mutations K103N and G190A, and the PI resistance mutations D30N and L33F were lost before transmission to drug-naïve individuals; their absence in the earliest sequences of the cluster suggests a long interval between transmission events or a transmission route through individuals not present in this data set (Figure 3.7_E). Finally, clade C and D included viruses sampled from 8 and 10 subjects enrolled between 2004 and 2008. Both these clusters involved recently infected patients (3 in cluster C and 2 in cluster D). Drug resistance mutations were found only in HAART-treated patients, while only one drug-naïve patients carried the L210S atypical mutation.

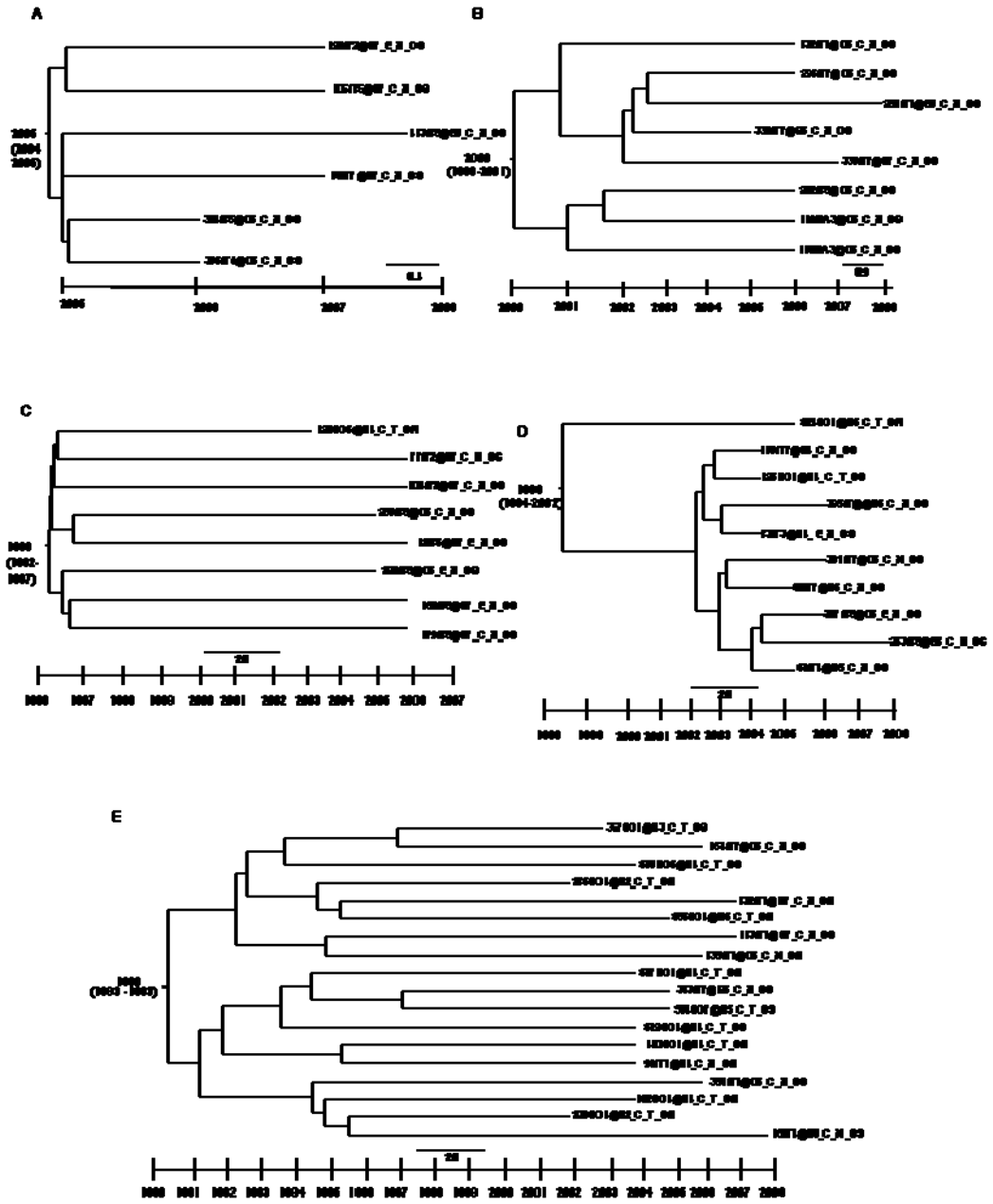


Figure 3.7 Relaxed clock time-scaled phylogenies for the five largest transmission clusters. The line at the bottom represents time (in years). Each patient in the five transmission clusters is identified by a code including information about early or chronical infection (E or C), HAART naïve or treated status (N or T), drug resistant or drug-susceptible virus (DR or DS).

Table 3.6. Details of the five transmission networks circulating in North Italy

Cluster	Sequence	Sampling Date	Risk Group	Shared drug resistance mutations			Atypical mutations	Treatment History	Nation of Origin	New Infection
				NRTI	NNRTI	PI				
A	190IT2.07	2007	Heterosex	-	-	-		Naïve	Italy	yes
A	406IT5.07	2007	Heterosex	-	-	-		Naïve	Italy	-
A	443IT5.08	2008	Heterosex	-	-	-		Naïve	Italy	-
A	78IT7.07	2007	Heterosex	-	-	-	$L210S_{rt}$	Naïve	Italy	-
A	304IT5.06	2006	MSM	-	-	-		Naïve	Italy	-
A	376IT4.06	2006	Heterosex	-	-	-		Naïve	Italy	-
B	132IT1.06	2006	MSM	-	-	-		Naïve	Italy	-
B	295IT7.06	2006	MSM	-	-	-	$V82I_{pr}$	Naïve	Italy	-
B	250IT1.08	2008	Heterosex	-	-	-		Naïve	Italy	-
B	330IT7.05	2005	MSM	-	-	-		Naïve	Italy	-
B	338IT7.07	2007	MSM	-	-	-	$V82I_{pr}$	Naïve	Italy	-
B	202IT5.06	2006	Heterosex	-	-	-	$V82I_{pr}$	Naïve	Italy	-
B	148MA3.06	2006	Heterosex	-	-	-	$V82I_{pr}$	Naïve	Marocco	-
B	149MA3.06	2006	Heterosex	-	-	-		Naïve	Marocco	-
C	420bg5.04	2004	Heterosex	M41L/D67N/T69D/ M184V/L210W/T 215Y	-	-		Treated	Italy	-
C	77IT2.07	2007	MSM	-	-	-		Naïve	Italy	-
C	104IT2.07	2007	Heterosex	-	-	-		Naïve	Italy	-
C	258IT5.06	2006	Heterosex	-	-	-		Naïve	Italy	-
C	42IT5.07	2007	Heterosex	-	-	-		Naïve	Italy	yes
C	150IT6.07	2007	Heterosex	-	-	-		Naïve	Italy	yes
C	220IT5.06	2006	Heterosex	-	-	-		Naïve	Italy	yes
C	179IT5.07	2007	MSM	-	-	-		Naïve	Italy	-
D	524BG1.05	2005	MSM	M184V	K103N/K101E	-		Treated	Italy	-
D	118IT7.04	2004	MSM	-	-	-		Naïve	Italy	-
D	425BG1.04	2004	MSM	-	-	-		Treated	Italy	-
D	396IT7.06	2006	MSM	-	-	-		Naïve	Italy	-
D	73IT3.04	2004	Heterosex					Naïve	Italy	yes
D	391IT7.06	2006	MSM	-	-	-	$L210S_{rt}$	Naïve	Italy	-
D	82IT7.05	2005	MSM	-	-	-		Naïve	Italy	-
D	397IT5.06	2006	Heterosex	-	-	-		Naïve	Italy	yes
D	263IT5.08	2008	Heterosex	-	-	-		Naïve	Italy	-
D	61IT4.05	2005	MSM	-	-	-		Naïve	Italy	-
E	367bg1.03	2003	MSM	-	-	-	$V179I_{rt}$	Treated	Italy	-
E	154it7.06	2006	Heterosex	-	-	-	$V179I_{rt}$	Naïve	Italy	-
E	275bg4.02	2002	IDU	M184V	K103N	-	-	Treated	Italy	-
E	518bg5.04	2004	IDU	-	-	-	-	Treated	Italy	-
E	432it4.07	2007	Heterosex	M184V	-	-	$V179I_{rt}$	Naïve	Italy	-
E	556bg4.05	2005	Heterosex	M41L/D67N/ L210W/T215Y	K103N	-	$K101Q_{rt}$;$V179I_{rt}$	Treated	Italy	-
E	143it4.07	2007	Heterosex	-	-	-	$V179I_{rt}$	Naïve	Italy	-
E	138it4.06	2006	Heterosex	M41M/L	-	-	$V179I_{rt}$	Naïve	Italy	-
E	517BG1.04	2004	MSM	D67N/K70R/ M184V/K219E	-	-	$V179I_{rt}$	Treated	Italy	-
E	313IT7.05	2005	Heterosex	-	-	-	$V179I_{rt}$	Naïve	Italy	-
E	584BG7.05	2005	Heterosex	-	-	-	$V179I_{rt}$	Treated	Italy	-
E	519BG4.04	2004	Heterosex	-	-	-	$V179I_{rt}$	Treated	Italy	-
E	440BG1.04	2004	MSM	-	G190E	-	$V179I_{rt}$	Treated	Italy	-
E	94IT1.04	2004	MSM	-	G190E	-	$V179I_{rt}$	Naïve	Italy	-
E	381IT4.06	2006	Heterosex	-	-	-	-	Naïve	Italy	-
E	452BG4.04	2004	Heterosex	M41L/D67N/K70R/ M184V/L210W/T215F/ K219E	G190A	-	$V179I_{rt}$	Treated	Italy	-
E	239BG4.02	2002	Heterosex	M184V		D30N/L33F	$V179I_{rt}$	Treated	Italy	-
E	16IT4.08	2008	MSM	-	-	-	$V106I_{rt}$; $V179I_{rt}$	Naïve	Italy	-

3.2.5 Timing of origin of the major lineages

The time of origin of the resistant lineages was determined using a Bayesian MCMC approach. Each viral sequence in the time-scaled phylogenies represents a different patient. For any two sequences, the branches connecting them through their most recent ancestor (MRCA) seems to include at least one possible epidemiological event. The distance between their recent ancestor and the previous node estimates the upper bound of time between epidemiological events. Figure 3.7_A-E also shows the variation in internode distances. This representation is scaled by calendar year, from which we can infer the periods over which these clustered transmissions occurred.

According to these estimates, the five clusters originated between 1990 (95% highest posterior density, 1983 to 1993 [cluster E]) and 2005 (95% highest probability density, 2004 to 2005 [cluster A]). For clusters B and D, (MRCA between 1986-01 and 1994-02 respectively) this spans much of the 2000s, from 2002 to 2006, while the transmissions that link cluster C and E occurred in the late 1990s, from 1995 to 2006 (MRCA between 1992-97 and 1983-93 respectively), the last cluster had the transmission period spanning in the late 2000s , from 2005 to 2007 (MRCA between 2004 to 2005).

3.3 "Sentinel" mutations in standard population sequencing can predict the presence of RT major mutations detected only by 454-pyrosequencing

3.3.1 Study population

Table 3.7 shows the epidemiological and viro-immunological characteristics of the 40 patients analyzed.

Regarding the epidemiological characteristics, homosexual and heterosexual transmissions were the two most common transmission route. In particular, heterosexual transmission was more frequently detected in patients with T69S mutation, while homosexual transmission was more frequently found in patients with L210M mutation (P=0.03).

While viral load was comparable among the four classes, CD4 cell count was lower in patients with the K103R at GRT (P=0.02).

Table 3.7. Demographic, clinical and virological characteristics of the 40 patients

	Overall	Samples with, at GRT test				P-value[a]
		Control virus	K103R	T69S	L210M	
Number of patients	40	10	10	10	10	
Date of HIV-1 diagnosis, Median (IQR)	2005 (2003-2006)	2002 (2000-2006)	2005 (2003-2005)	2005 (2002-2006)	2006 (2005-2008)	ns
Time since HIV-1 diagnosis (years), Median (IQR)	0.8 (0.1-3.7)	2.9 (0.1-6.5)	0.3 (0.2-4.1)	0.9 (0.2-3.2)	0.4 (0.1-2.0)	ns
Male, N (%)	34 (85.0)	9 (90.0)	10 (100.0)	6 (60.0)	9 (90.0)	ns
HIV exposure, N (%)						
MSM	9 (22.5)	3 (30.0)	0 (0.0)	1 (10.0)	5 (50.0)	**0.03**
Heterosexual	8 (19.5)	1 (10.0)	2 (20.0)	5 (50.0)	0 (0.0)	**0.03**
IDU	7 (17.1)	3 (30.0)	3 (30.0)	1 (10.0)	0 (0.0)	ns
Other or Unknown	16 (39.0)	3 (30.0)	5 (50.0)	3 (30.0)	5 (45.5)	ns
Age (years), Median (IQR)	39 (33-44)	42 (40-44)	44 (41-49)	38 (36-39)	33 (27-41)	ns
Patients starting a first line regimen, N (%)	32 (80.0)	9 (90.0)	7 (70.0)	10 (100.0)	6 (60.0)	
Viremia (log copies/ml), Median (IQR)	5.0 (4.5-5.5)	5.0 (4.8-5.6)	5.0 (4.6-5.3)	5.3 (4.5-5.7)	4.8 (4.3-5.2)	ns
CD4 cell count (cells/ul), Median (IQR)	239 (66-345)	142 (46-272)	70 (25-189)	170 (64-327)	559 (270-897)	**0.02**
DNA sample availability	9	2	4	1	2	

[a] Statistically significant differences among the 4 groups of patients were assessed by Chi Squared test for trend for categorical data and by Kruskal Wallis test for continuous variables.

3.3.2 Coverage profile and UDPS results

The mean number of reads obtained for each position of the reverse transcriptase (RT) in RNA and in DNA samples was showed in figure 3.8.

Focusing our analysis on the position known to be associated with drug resistance, we found that UDPS detected RT drug resistance minority variants in the RNA samples of 18 patients (45.0%) (Table 3.8). Thirteen individuals carried only NRTI minority variants, 3 only NNRTI minority variants, 2 both NRTI and NNRTI minority variants.

The tymidine analogues mutations (TAMs) were the most frequently observed (5 patients [12.5%]) with a sequence reads value encompassing the 0.40% for T215Y and K70R, to the 1.75% for M41L. These mutations were followed by the NRTI mutation F77L, involved in the Q115M complex and observed in 4 patients (10%) with a sequence reads of 0.27%, 0.55%, 0.76%, and 0.92%, respectively.

Additional mutations, including the T215 revertants A/N/I/D, and the NNRTI minor V106I, G190E and V179D, were found in 4 and 6 persons respectively, with a sequence reads >0.30% and <1.40% for the revertants, and >0.40% and <1.20% for the NNRTI minor .

RT drug resistance minority variants were also found in 5 DNA samples (55.6%). Of these 5 samples, 4 showed drug resistance mutations also in the respective RNA samples (44.4%). Interestingly, the NRTI resistance minor variant M184I was found in 4 DNA samples, and never in RNA samples, (44.4% vs 0.0%, P=0.003).

Clinical characteristics and UDPS results of the 18 antiretroviral-naïve patients with drug resistance minority variants were described in table 3.9.

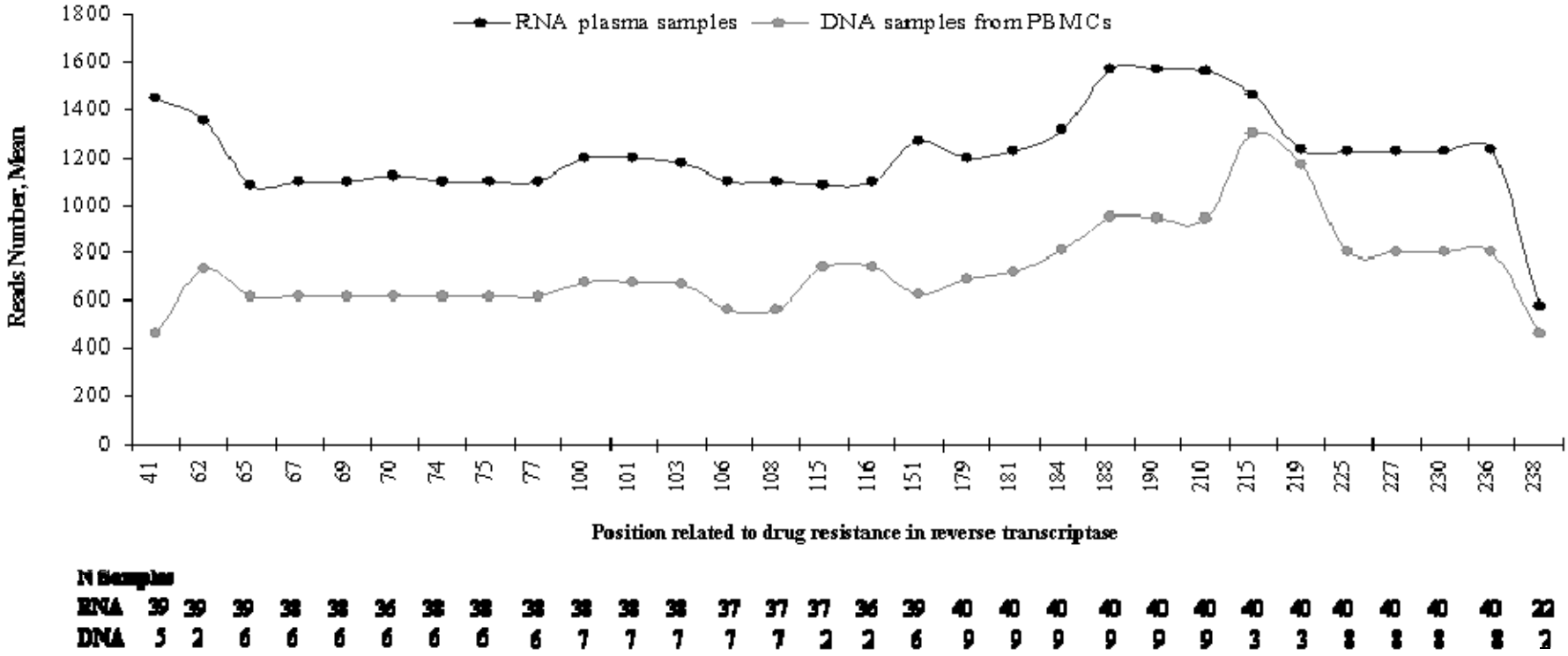


Figure 3.8 Coverage profile for each drug resistance position in Reverse Transcriptase. The mean number of reads for Reverse Transcriptase drug resistance related positions analyzed in the study are shown in the figure. We analyzed only positions per sample where at least 300 reads were generated for each position (reads-number/position range analyzed: 303-3371).

Table 3.8 Prevalence of drug resistance minor variants in 40 RNA-plasma samples and in 9 DNA-samples from PBMC.

	RNA plasma samples				DNA samples from PBMCs	
	HIV-1 RNA log copies/ml Mean (Min-Max)	**Prevalence[a] N (%/40)**	**% drug resistant Reads[b] Mean (Min-Max)**	**Drug resistance loads Log Copies/ml[c] Mean (Min-Max)**	**Prevalence[a] N (%/9)**	**% Reads Mean (Min-Max)**
At least one RT drug resistance mutation	5.16 (4.02-5.70)	18 (45.0)	1.06 (0.27-4.20)	3.16 (1.79-3.89)	5 (55.6)	8.37 (0.47-15.14)
NRTI plus NNRTI resistance	5.53 (5.24-5.70)	2 (5.0)	1.33 (1.11-1.55)	3.69 (3.29-3.89)	3 (33.3)	8.44 (3.41-15.15)
NRTI resistance alone	5.02 (4.03-5.70)	13 (27.5)	0.82 (0.27-1.81)	2.90 (1.79-3.54)	2 (22.2)	0.47-13.25
NNRTI resistance alone	5.28 (4.48-5.62)	3 (7.5)	1.91 (0.55-4.20)	3.31 (2.84-3.61)	0 (0.0)	-

a Prevalence of drug resistance minor variants in RNA and DNA samples.
b Percentages of reads minority variants.
c Estimated number of minority variants log copies/ml.

Table 3.9 Clinical characteristics, UDPS results, ARV therapy and virologic response for 40 antiretroviral-naïve persons analyzed by standard direct PCR Sanger sequencing.

Patient number	Year of diagnosis	CD4 count (cells/ul)	HIV-1 RNA (log copies/ml)[a]	Atypical mutation at DR position detected at GRT	Minority Drug resistance (% of reads with mutation)[b]	HAART[c]	Virological response and follow-up therapy [c,d]
3012	16/02/2005	90	5.7	None	None	3TC ABC TDF LPV/b	Virologic suppression at 233 days
4845	17/02/2006	281	5.62	None	**V106A** (0.99)	FTC TDF EFV	Virologic suppression at 147 days
7070	01/09/2007	19	4.95	None	**F77L (0.92)** T215I (1.1)	FTC TDF EFV	Virologic suppression at 71 days
2773	NA	174	4.8	None	None	3TC AZT LPV/b	No virological response; interruption of 7 months; follow-up ARV of FTC TDF ATV/b, leading to virologic suppression at 80 days.
7253	20/11/2006	487	5.02	None	None	NA	Virologic suppression at 139 days
7644	NA	1269	4.06	None	None	-	-
5171	01/01/2000	21	4.47	None	**M184V (0.54)**	3TC AZT LPV/b	Virologic suppression at 29 days
5202	01/08/2000	31	5.7	None	None	FTC TDF LPV/b	Partial suppression; follow-up ARV of FTC DDI ATV/b, leading to virologic suppression at 137 days.
5732	NA	243	5.06	None	None	FTC TDF LPV/b	Virologic suppression at 111 days
7169	NA	111	5.44	None	None	NA	Virologic suppression at 109 days
2990	01/01/2005	25	5.47	K103R	None	NA	Virologic suppression at 295 days
3726	01/01/2000	337	4.87	K103R	**F77L(0.76)**	FTC TDF	Virologic suppression at 74 days
202	01/07/2005	69	5.7	K103R	T215D=1.35	NA	No virological response after 18 weeks
4402	NA	8	4.48	K103R	T215A(0.32) **M184V(0.67)**	NA	Virologic suppression
6335	NA	189	5.2	K103R	None	NA	Virologic suppression at 195 days
2729	01/09/2004	25	4.12	K103R	None	NA	Virologic suppression at 122 days
2799	01/01/2003	147	5.32	K103R	None	-	-
3267	15/04/2005	70	4.91	K103R	None	3TC AZT LPV/b	Virologic suppression at 97 days
7817	01/07/2008	NA	5.09	K103R	**V108I(0.56)**	-	-
8648	17/04/2005	344	4.15	K103R	None	-	-

Patient number	Year of diagnosis	CD4 count (cells/ul)	HIV-1 RNA (log copies/ml)[a]	Atypical mutation at DR position detected at GRT	Minority Drug resistance (% of reads with mutation)[b]	HAART[c]	Virological response and follow-up therapy [c,d]
2580	15/08/2003	235	4.03	L210M	**M184V(1.14) K219Q(0.8)** V179D(1.06)	3TC D4T NVP	Virologic suppression at 138 days
4027	01/10/2005	1134	5.7	L210M	**M41L(0.76) L74V(2.38) V108I(1.51)**	FTC TDF LPV/b	Virologic suppression at 84 days
5581	15/12/2004	1128	4.36	L210M	**F77L(0.27)**	-	-
7755	27/06/2008	270	5.24	L210M	**M41L(1.75) M184V(0.49) T215FY(0.40) K103N(2.6) G190A(0.35)**	NA	Virologic suppression at 147 days
8889	27/07/2009	820	4.08	L210M	**F77L(0.55)** G190E(0.95)	-	-
8893	NA	NA	4.21	L210M	**A62V(0.82) F77L(0.57)** G190E(1.1)	FTC TDF EFV	Virologic suppression at 73 days
7567	09/01/2006	271	4.55	L210M	None	-	-
8932	01/01/2009	NA	5.47	L210M	**V75I(0.89)** G190E(0.47)	NA	No virological response after 18 weeks
4780	01/07/2004	783	5.23	L210M	None	FTC TDF LPV/b	Virologic suppression at 123 days
4095	23/12/2005	336	4.79	L210M	None	-	-
1749	01/03/1999	727	4.3	T69S	None	3TC TDF LPV7b	Virologic suppression at 60 days; at 5mo changed to 3TC TDF EFV, confirming virological suppression
3268	01/11/2003	63	4.63	T69S	**D67N(1.81)**	FTC TDF EFV	Virologic suppression at 94 days
3742	01/01/2006	65	5.7	T69S	**V75I(0.70)** V106I (0.85)	NA	Virologic suppression at 110days
4004	01/01/2002	264	5.34	T69S	**L210W(0.53)** V106I (4.6)	3TC TDF LPV/b	Virologic suppression at 83 days
5603	01/05/2006	457	4.48	T69S	**K103N (4.2)**	FTC TDF LPV/r	Virologic suppression at 135 days; at 10mo changed to FTC TDF EFV, confirming virological suppression
6534	01/05/2007	30	5.7	T69S	T215N (0.38)	FTC TDF EFV	Virologic suppression at 108 days
6565	12/06/2006	345	4.28	T69S	**A62V(1.70) K70R(0.40)**	3TC AZT NVP	Virologic suppression at 89 days
7587	01/01/2002	271	5.23	T69S	None	3TC ABC LPV/b	Virologic suppression at 128 days
2667	15/10/2004	20	5.7	T69S	None	3TC TDF AZT IDV/b	No virological response; follow-up ARV of 3TC AZT LPV/b, leading to virologic suppression at 130 days.
8502	01/03/2009	76	5.93	T69S	None	FTC TDF EFV	Frequent interruptions; no virological responce

a Plasma HIV-1 RNA level.
b The percentages for the minority variants are shown in parentheses. Drug resistance mutations are shown in bold, while revertants and minor resistance mutations are shown in grey.
c 3TC, lamivudine; ABC, abacavir; APV, amprenavir; ATV, atazanavir; d4T, stavudine; ddI, didanosine; EFV, efavirenz; FTC, emtricitabine; IDV, indinavir; LPV,
lopinavir; TDF, tenofovir; ZDV, zidovudine; /r, ritonavir provided for pharmacologic boosting.
d Virologic suppression was defined as at least 2 consecutive determination of plasma HIV-1 RNA <50 copies/ml.

3.3.3 Role of atypical RT mutations in predicting minority resistance variants

Prevalence of drug resistance minority variants

UDPS revealed that patients with T69S and L210M at standard GRT were more frequently infected by minority RT drug resistance variants than controls patients (5 [50%] and 7[70%] vs 3[30.0%], even if P not significant). This is not true for patients with K103R at standard GRT, for which UDPS revealed 3 cases of minority drug resistance species (30.0%). Restricting our analysis to NRTI drug resistance, we found that patients with L210M virus were more frequently infected by minority NRTI resistance variants than K103R and control virus (7 [70.0%] vs 4 [20.0%], P=0.015).

Interestingly, minority drug resistance variants reached the 1% of sequence reads only in patients with T69S and L210M. In particular, we found that 3 patients with T69S and 4 patients with L210M carried minority drug resistance variants with a sequence reads >1%, versus none control and K103R sample (3[30.0%] and 4[40.0%] vs 0[0.0%], P=0.03 and P=0.008). Analyzing each specific mutations, the NRTI resistance mutations reached the 1% of sequence reads with the tymidine analogues mutations (TAMs) M41L and D67N, the cytidine analogue mutation M184V, the abacavir resistance mutation L74V, and the A62V. The NNRTI resistance mutations reached the 1% of sequence reads with the K103N (2 patients) and with V108I (one patient) (Figure 3.9A). The higher prevalence of minority drug resistance variants especially in patients with L210M was confirmed after the conversion of sequence reads in copies number. As shown in Figure 3.9_B, in 3 patients carrying L210M virus, 6 drug resistance mutations reached a load > 2,000 copies numbers/ml.. These mutations were the TAM M41L, the abacavir resistance mutation L74V, the V75I, involved in the 151 complex , and the NNRTI resistance mutations V106A, V108I and K103N.

Combination of drug resistance mutations

To sequence the entire RT of HIV-1, the UDPS methodology availed itself of 5 amplicons. So, for drug resistance mutations at positions near each other, it was possible to establish how many reads with more than one drug resistance mutation could be detected in each sample.

Seven out of the 40 patients analyzed showed more than one drug resistance mutation detected by UDPS. At standard GRT, these patients carried L210M (6 patients) or T69S (1 patients). Only for 3 patients, the samples 7755, 2580 and 4027, all carrying L210M at GRT, drug resistance mutations were sufficiently near each other to be in the same haplotype. In particular, the 7755 sample showed a 0.43% of sequence reads resistant to both NRTI and NNRTI (5 reads showed the combination of RT mutations M184V, T215F, G190A, and Y181C). In the sample 2580 all the minority variants expressing the TA mutation M41L carried also the L74V (2.49%). In the same way, in the sample 4027, all the minority species with K219Q carried also the NRTI resistance mutation M184V (1.58% of sequence reads).

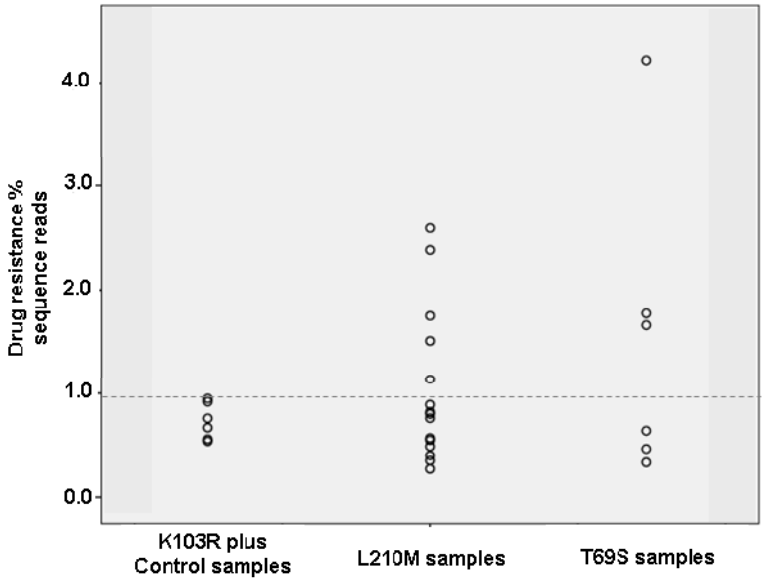
These data suggest that, the mutation L210M detected at standard GRT could be a "sentinel" not only of drug resistance minority species, but, also, of complexes and multi-drug resistance minority variants.

Association of L210M and T69S with specific drug resistance mutations

Analysing specific drug resistance mutations, we found that TAMs were exclusively found as minority species in patients with L210M and T69S atypical mutations. In particular, 6 patients, 3 with T69S and 3 with L210M, carried the TAMs D67N (1.81%), K70R (0.40%), L210W (0.53%), M41L (0.76% and 1.14%), T215FY (0.40%) and K219Q (0.80%). This positive association between the T69S and L210M detected at

standard GRT and minor variants resistant to TAs were supported by a P=0.03, when compared with controls and K103R viruses.

A



B

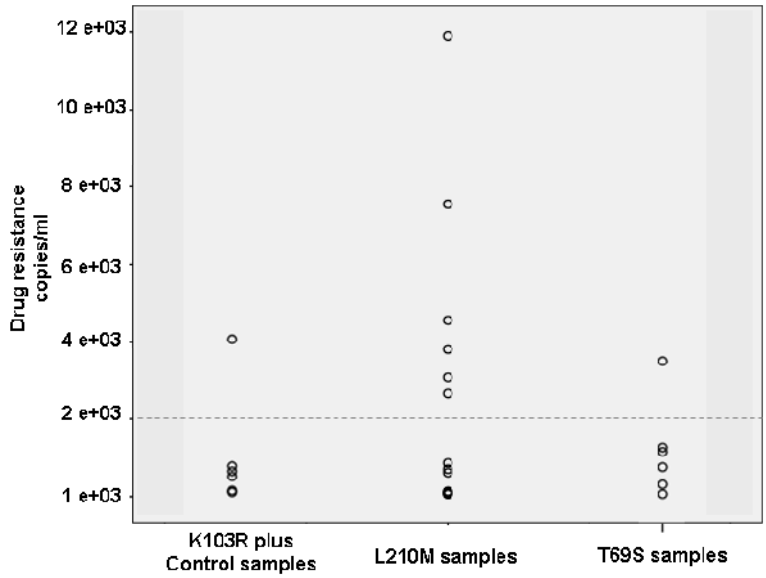


Figure 3.9 Percentage of sequence reads with RTI resistance mutations (A) and RTI resistant loads in the 40 drug naïve patients analyzed. Each dot represents one drug resistance mutation.

3.3.4 Response to HAART

Among the 40 patients analyzed, 32 (80.0%) started an antiretroviral regimen after genotypic test (after 2009). All patients started their antiretroviral regimen with comparable viral load (5.0 [4.4-5.5] log copies/ml for patients with drug resistance minority variants and 5.3 [4.1-5.7] log copies/ml for patients without drug resistance minority variants, P=NS) and CD4 cell count (307 [214-548] cell/ul for patients with drug resistance minority variants and 271 [160-415] cell/ul for patients without drug resistance minority variants, P=NS). Twenty-nine patients (90.6%) reached the virological success (defined as two consecutive determinations of HIV-1 RNA >50 copies/ml) in a median time of 16.7 weeks (IQR: 13-21) after HAART initiation. The only 3 patients that did not achieve virological success within the 18 weeks of HAART treatment (viral load copies/ml: mean 263 [min-max:252-2045]) carried HIV-1 virus with an atypical RT mutation; in particular one patient carried L210M, one T69S and one K103R, respectively.

To define the role of minority variants on response to HAART, we also decided to examine whether low-abundance drug resistance mutants could impact initial decreases (to 4, 8 and 12 weeks) of HIV-1 RNA level on treatment initiation. Among subjects with available viral load data, the mean change in viral load at week 4 and 8 from HAART initiation was not significantly different between subjects with drug resistance minority species and subjects with no resistance mutations (P=0.34 and 0.87); a significantly smaller mean change in viral load was found at week 12 in patients with drug resistance minority variants (n=11, mean change=4.7 log10 copies/ml), compared with subjects with no drug resistance mutations (n=9, mean change=5.3 log10 copies/ml) (P=0.02).

4 Discussion

At population level, several components contribute to the global burden of HIV-1 drug resistance: the rate of failure of antiretroviral treatment in the treated population, the resistance patterns associated with therapy failure, risk behaviour and transmissibility of drug resistant viruses. This thesis is thought to better define this last phenomenon, and to give a systematic analysis of the magnitude of transmission of drug resistant HIV variants.

4.1 Characterization of the patterns of drug resistance mutations in newly diagnosed HIV-1 infected patients naïve to the antiretroviral drugs

The first part of this thesis shows that the prevalence of HIV-1 drug resistant strains in newly diagnosed patients is 5.9%, in the range described by other European and American reports (Novak et al., 2005; Wensing et al., 2005; Yerly et al., 2007; UK, 2007; Payne et al., 2008; Spread, 2008), and that the majority (60.0%) of these HIV-1 strains carries more than one major drug resistance mutation.

A trend toward a decreased rate of resistance was found in recent infections comparing with chronic infections (3.4% *versus* 6.6%; P=0.09), that may reflect the increasing use of potent drugs and highly active antiretroviral regimens in recent years. At the same time, this supports the analysis of Blower that has estimated a progressive decrease in the proportion of acquired resistance from 1996 to 2005 (Blower et al., 2001). These data suggest that the phenomenon of extensive and continuously increasing transmission of resistant strains followed by their disappearance from blood (while remaining in reservoir) is not as widespread as previously foreseen.

In addition, we found that the prevalence of mutations associated with transmitted drug resistance was higher in MSM than in heterosexual patients, in line with other European and American reports (Spread, 2008; Weinstock et al., 2004); this factor could be related with the higher and earlier access to treatment of this class than other groups of patients.

Among major drug resistance mutations, the most common mutations observed were the NNRTI resistance mutation K103N and the NRTI T215 revertants (3.1% each one). Interestingly, the first one, K103N, was known to confer high level of resistance to nevirapine and efavirenz (without altering the viral replicative capacity), but not to the recently approved etravirine (Vingerhoets et al., 2007). The presence of the T215 revertants suggests a previous infection with a HIV-1 strains containing T215Y/F (De Ronde et al., 2001; Garcia-Lerma et al., 2004), and has been associated with an increased risk of virologic failure in patients receiving a first line regimen with thymidine analogue (Violin et al., 2008).

We also found that about 60% of our patients harbours HIV-1 strains with more than one major drug resistance mutation. In addition, by performing a cluster analysis, we observed, in our cohort of newly diagnosed HIV-1 B subtype infected patients, the existence of a complex mutational cluster involving the revertant forms T215D/S and the TAM1 M41L, L210W (known to confer cross-resistance to all NRTIs), the K103N (known to confer cross-resistance to EFV and NVP) and the polymorphism V60I. This mutation has been shown to rescue the replicative capacity impaired by the major drug resistance mutations in the absence of drug pressure, and to contribute to the persistence over time of major drug resistance mutations in drug naïve patients (Lindström et al., 2006). Consistent with this finding, patients infected by HIV-1 strain with V60I and

TAM1 had an higher viremia than those infected by HIV-1 strain with TAM1 only (5.30 *versus* 3.9 log copies/ml, respectively, P=0.09); however, the limited sample size allow us to describe only a trend, that needs to be confirmed in a larger dataset.

When we performed the clustering analysis in another cohort of 152 naïve patients from Central Italy, diagnosed between 1997-2000, we found that major mutations occurred alone in 10 (71.4%) of 14 patients with HIV-1 drug resistant virus, more than 2 major mutations were observed only in 1 patient, and no clusters have been identified in both PR ad RT (Alteri et al., 2008). This finding is in agreement with the Spread programme that showed that 71% of naïve patients with drug resistant virus, diagnosed for HIV-1 infection between 2002 and 2003, harboured strains with only a single major mutation. Thus, two important results emerge from our study: i) newly diagnosed patients carry HIV-1 strains with more drug resistance mutations than that observed in previously diagnosed patients, ii) such mutations are organized in well defined clusters, that can seriously compromise the success of a not sufficiently potent first line regimen. These findings can have important clinical implications. In particular, our results strongly support the use of genotypic test in newly diagnosed patients. This test can help clinicians to set-up and individualize initial therapy especially in patients with extensive drug resistance. It is conceivable that in these patients the drop and long-term maintenance of viral load below 50 copies/ml can be warranted only by using a combination of potent drugs, even belonging to new class (as integrase inhibitors).

One point that is missing in most of the epidemiological studies addressing transmitted drug resistance is the global frequency of use of antiretroviral drugs (mainly NNRTI and PI) in treated patients. This may allow to compare more efficiently the different results obtained in the different countries. In addition, in the attempt to better clarify our

results, we collected a cohort of 2,344 patients failing HAART regimen between 2001-2007 in Central Italy (Santoro et al., 2008). In this cohort, we observed that the percentage of patients failing an NNRTI containing regimen and the percentage of patients with NNRTI resistance mutations remained stable from 2001 to 2004, then showed a progressive decrease from 2004 to 2007 (from 40% to 26.2% and from 50% to 36.0%, respectively). This decreasing trend may be in line with the decreased percentage of drug naïve patients with NNRTI-resistance mutations (3.6% in 2004 to 0.0% in 2007). Regarding PIs, we observed that the increasing use of PIs boosted with Ritonavir (RTV) (from 18.4% in 2001 to 32.1% in 2004 and to 56.4% in 2007) is associated with a decrease in the percentage of patients with PI resistance mutations (from 58.0% in 2001 to 41% in 2004 and to 28.0% in 2007). This decreasing trend coupled with detrimental effect on viral fitness of PI resistance mutations may explain the complete absence of transmitted PI resistance mutations observed in our cohort of recently infected patients (0.0%).

Regarding the subtype distribution in our study, even if B subtype remains the prevailing one, we observed an increase of non-B subtype and of the putative recombinant forms compared to patients diagnosed in Central Italy before 2000 (16.5% vs 5.6%, P=0.001 and 7.8% vs 0.6%, P=0.001, respectively) (personal communication); in particular, we now report for the first time the circulation of the CRF03_AB in Italy. Of note, almost half of patients carrying non-B subtypes infected are Italian and Caucasian, confirming a diffusion and circulation of non-B subtypes within Italian population significantly increased when compared to the recent past.

4.2 Epidemiological network analysis in HIV-1 B infected patients diagnosed in Italy between 2000-2008

This part of this thesis reports for the first time that many (34%) of HIV-1 subtype B transmission clusters indentified in Italy were only composed by drug-naïve individuals and that the 14% of transmitted drug resistance was linked to transmission clusters composed only of newly diagnosed individuals. The phylogenetic analysis was performed on a large cohort of drug-naïve recently/chronically infected individuals and drug-experienced patients represent almost all infected individuals in a restricted geographical area. The phylogenetic structure of the observed epidemics, characterized by high population density and a strict epidemiological link among individuals, indicated a strong signal for a cluster distribution as occurred for the spread of HIV-1 subtype B in Western Europe and North America (Walker et al., 2005).

By analyzing the prevalence of transmitted drug resistance, we found that among the 42 recently infected patients only a single patient carried drug resistant strains. This result is consistent with the other studies led in Italy and in other European countries (Novak et al., 2005; UK, 2007; Payne et al., 2008), and may suggest a reduction in transmitted drug resistance. However, we should acknowledge that other European studies reported a stable frequency of transmitted drug resistance in patients at the time of primary HIV-1 infection (Chaix et al., 2009), while the Swiss cohort reported an increasing trend of transmitted drug resistance, in particular for NNRTI resistance (Yerly et al., 2009). These differences can be explained by the different use of antiretroviral drugs in HIV-1 treated patients in the different settings analyzed. In the attempt to better explain our results, we have performed a further analysis in our cohort of HIV-1 treated patients.

This analysis has shown a progressive decrease in the use of NNRTI starting from 2004, that could be in line with the lower percentage of NNRTI-resistance mutations in recent infections compared to chronic infections (2.4% vs 6.1%).

A different scenario was observed in chronically infected patients, where 13.3% carried at least one mutation associated with transmitted drug resistance. In particular, NRTI and NNRTI resistance mutations were found at a frequency of 5.9% and 5.6%, respectively, while mutations associated with PI resistance only in 1.0% of patients. Again, these results were consistent with other American and European reports (Novak et al., 2005; UK, 2007; Payne et al., 2008), even if other European studies have reported a lower prevalence of NNRTI resistance mutations and a higher prevalence of NRTI and PI resistance mutations in their cohort of drug-naïve patients (Wensing et al., 2005; Spread, 2008; Yerly et al., 2007). These discrepancies among the different studies highlight the need of local analysis, since only such type of studies can provide a proper answer to the prevalence of resistance in drug-naïve patients in that particular settings. Extrapolation of data from other areas, even if contiguous, might bring to erroneous conclusions with potentially relevant clinical consequences.

Approximately 36% of these drug resistance strains were involved in significant clusters: 61% of them clustered with HAART treated patients, while 38% with only drug-naïve patients. This result, consistent with that shown by Hue (2009) in United Kingdom and Yerly (2009) in the Swiss Cohort, suggests that, beyond treatment-experienced patients, also drug-naïve patients can contribute to the transmission of drug resistance viruses. In particular, these lineages are indicative of treatment-independent reservoirs of resistance and represent a potential long-term risk to the continued success of antiretroviral therapy.

The treatment-indipendent transmitted drug resistance mutations involved in 3 epidemiological events were the TAMs2 D67N and K219Q, the revertant T215D, and the NNRTI resistance mutations K103N. Their presence may be related with the limited effect of these mutations upon viral replicative capacity, and therefore with the increased chances to establish infection in the new host; in particular, the mutations D67N and K103N were known to confer high level of drug resistance without altering the viral replicative capacity (Domaoal et al., 2006), while the mutations K219Q and T215D, frequently found in HIV-1 lineages (Huè et al., 2009), may not significantly change drug-susceptibility on their own and impair viral fitness (Cong et al., 2007 ; De Ronde et a., 2001). Not surprisingly, PI resistance mutations, observed only in 1% of our population, were never found in these treatment-indipendent transmission networks. The lower prevalence of PI resistance mutations in transmission networks is consistent with data from other cohorts (Brenner et al., 2007 & 2008) and could be related with the negative impact on viral replicative fitness of the PI mutations (Pillay et al., 2000).

Differently, the 61.5% of drug-naïve patients with transmitted drug resistance clustered in 6 epidemiological networks with also HAART treated patients. In these clusters, as well as in the 11 clusters involving drug resistant strains from HAART treated individuals and drug-sensitive strains from drug-naïve individuals, we could frequently observe reversion events of drug resistance mutations. This could be explain by the substantial loss of HIV-1 genetic diversity during transmission (Keele et al., 2008) and by the fitness cost of the multidrug resistance variants, thus contributing to create a bottleneck for forward transmission of such drug resistant variants (Turner et al., 2004).

An evidence of reversion events could be provided by 2 of the major analyzed clusters (cluster D and cluster E; Figure 3.7 D & E). It is noteworthy that these clusters involved

HAART treated patients with multiple resistance mutations, that could be easily lost before transmission to drug-naïve individuals; their absence in the earliest sequences of the cluster could be also suggested by a long transmission interval or transmission through individuals not present in the data set. Differently, the persistence of other drug resistance mutations as the G190E could be explained by their skill to not alter the viral replication capacity (Domaoal et al., 2006). The persistence of such drug resistant viruses in drug-naïve patients could also result from short transmission intervals, such that resistance mutations are transmitted to the next individual before reversion occurs.

The five major clusters was also characterized by a dated phylogenies reconstruction to provide new insights into the temporal structure of resistance transmission among treated and untreated HIV patients. According to the time-scaled phylogenies, these clusters originated between 1990 (Cluster E) and 2005 (Cluster A). The 3 drug resistance lineages originated between 1990 and 2000, which corresponds to the early years of ART usage and to the time when the transmission of drug resistance mutations was at its highest level in Europe and in United States (Grant et al., 2002). Interestingly, clusters involving only drug-susceptible strains originated more recently, between 2000-2005 (A e B), thus supporting the reduction of transmitted drug resistance in drug-naïve patients recently highlighted in Europe (Spread, 2008; UK, 2006; Yerly et al., 2009).

In conclusion, our findings highlight the role of newly diagnosed individuals, not yet exposed to antiretroviral drugs, in the transmission of drug resistant HIV-1 strains, despite the reduction of drug resistance in new infections. Overall, these findings provide new insights for the planning and management of treatment programs in developing countries.

4.3 "Sentinel" mutations in standard population sequencing can predict the presence of RT major mutations detected only by 454-pyrosequencing

In our cohort of 40 HIV-1 infected patients naïve to the antiretroviral drugs and with no resistance detected by bulk sequencing, minority drug resistant variants could be detected in 45% of patients by using the ultra deep pyrosequencing technology. This prevalence is relatively higher that that found in previous data obtained in drug naïve patients (Johnson et al., 2008; Simen et al., 2009; Metzner et al., 2009; Peuchant et al., 2008). However, it is important to note that most results on minority variants were obtained by investigating only few mutations by the allele specific PCR technology (as the K103N and M184V in the reverse transcriptase and L90M in the protease), thus the estimation of the frequency of minority-resistant variants in these studies would certainly have been higher if more mutations had been searched for.

Overall, this finding suggests that ultra-deep sequencing can detect a significantly proportion of minority-resistant variants despite negative results of standard genotyping, more than doubling the prevalence of resistant variants, as previously demonstrated by other studies (Wang et al., 2007; Varghese et al., 2009; Simen et al., 2009).

The drug resistance mutations more often detected by UDPS in this study were the TAMs (12.5% of patients, with a sequence reads >0.40%) for the NRTI resistance mutations and the K103N (5% of patients, with a sequence reads >2.0%) for the NNRTI resistance mutations. Interestingly, also the NRTI T215 revertants were frequently observed in our drug naïve population (25% of patients with a sequence reads >0.30%).

It is interesting to note that also by standard GRT the most common drug resistance mutations observed in drug naïve individuals were the NNRTI resistance mutation K103N, the NRTI T215 revertants (3.1% each one), and TAMs (2.6%) (see table 3.2). The null or the modest fitness cost conferred by these mutations to the virus supports persistence of these mutants in the untreated population and highlights the potential for a frequent transmission. In particular, the NNRTI mutation K103N, known to confer high level of resistance to nevirapine and efavirenz, and the T215 revertants do not alter the replicative capacity of the virus (Bacheler et al., 2001; Domoal et al., 2006; Garcia-Lerma et al., 2001), while the TAMs T215Y/F directly confer an advantage to the viral fitness if compared with wild-type (Huz et al. 2006).

The patterns of drug resistant minority quasispecies found in our patients deserve another important consideration. The prevalence of minority quasispecies of drug resistant viruses at baseline appears to be dependent on specific mutations detected at standard GRT. In particular, patients with HIV-1 virus carrying the atypical mutations L210M and T69S at baseline, were more frequently infected by minority RT drug resistance variants. In particular, patients with L210M were more frequently infected by minority NRTI resistance than patients without these mutations (60.0% vs 20.0%, P=0.0015). This association is reinforced when we compared the prevalence of tymidine analogues mutations between patients with L210M and T69S and patients without these mutations (P=0.03).

In particular, the L210M could be “sentinel” not only of undetected drug resistance but also of complexes and multi-drug resistance minority variants. Indeed, all the 3 patients harbouring minority HIV-1 strains with more than one major drug resistance mutation, carried L210M at standard GRT. In all the 3 cases, these multi drug resistance strains

involved the tymidine analogues mutations (in particular, M41L, T215F, K219Q), known to confer cross-resistance to all NRTIs (Whitcomb et al. 2003), in combination with the NNRTI G190A and Y181C (known to confer cross-resistance to EFV and NVP and in less extent to ETV), the NRTI L74V (conferring resistance to ABC and DDI) (Johonson et al. 2009), and with the M184V. This last mutation is known to be associated with 3TC and FTC resistance, but when present as minority species does not necessarily lead to virological failure, probably due to its ability to impair viral fitness (Van Rompay et al., 2002).

Interestingly, T69S and L210M mutations detected at standard GRT seem to be also predictive of an high detection threshold of drug resistance minority variants, frequently up to the 1% of sequence reads and the 2,000 copies/ml. This is important in the context of the virological response in patients starting an antiretroviral regimen.

At this regard, we have also investigate the virological response to HAART in our patients according to the presence or absence of resistant variants in the minority population. The detection of a baseline resistance in the minority population was shown to be associated with a less pronounced decrease in plasma HIV-1 RNA from baseline to 12 weeks on therapy. This finding is in accordance with that reported by previous studies, suggesting that transmitted resistance, even if in minority species, could lead to suboptimal response to first-line therapy (Johnson et al., 2009; Simen et al., 2009; Paredes et al., 2010). However, the detection of minority resistant variants by UDPS was not associated with a clear virological failure to HAART in our study: 90.6% of patients starting an antiretroviral regimen reached the virological suppression in a median time of 16.7 weeks (IQR: 13-21), while the 3 patients that not reach virological success have the last follow up at 18 weeks after HAART initiation. This finding could

be explained by the absence of a long-term follow-up for our patients, all starting an HAART regimen after 2009.

In any case, the clinical implications of minority drug resistance are still unclear. Some observations suggest that minority drug resistance variants can emerge as major virus populations after initiation of salvage therapy in pre-treated patients (Kapoor et al., 2004; Lecossier et al., 2005; Roquebert et al., 2006). In particular, detection of baseline minority NNRTI resistant variants could worse the virological response in patients starting a first-line efavirenz-based antiretroviral therapy (Metzner et al., 2009; Paredes et al., 2010). Conversely, other studies showed no association between minority K103N variants and virological failure on early therapy (Metzner et al., 2007; Peuchant et al., 2008). Surely, the different threshold for the minority variants detection used in these studies may explain their discrepant results. To avoid this problem, establish a threshold quantity of minority drug resistance variants at baseline predictive to virological failure is warranted. At this regard, Goodman and his group has been the first to define that the presence of K103N above the 2,000 copies/ml strongly correlate with virological failure (Goodman et al., 2009). It is interesting to note that in our cohort of patients, the NNRTI mutation K103N was found in one L210M expressing patient at a sequence reads of 2.6%, corresponding to more than 4,564 copies/ml, suggesting an higher risk of virologic failure for this patient during the first NNRTI-containing regimen.

5 Conclusion

At population level, several components contribute to the global burden of HIV-1 drug resistance: the rate of failure of antiretroviral treatment in the treated population, the resistance patterns associated with therapy failure, risk behaviour and transmissibility of drug resistant viruses. This thesis is thought to better define this last phenomenon, and to give a systematic analysis of the magnitude of transmission of drug resistant HIV variants.

First of all, this thesis has shown that the prevalence of viruses with transmitted drug resistance mutations in newly diagnosed individuals in Italy is approximately 6%, in the range described by other European and American reports, and that the 3.5% of patients carried HIV-1 resistant strains with more than one major drug resistance mutation.

The second aim of this thesis has been to identify the source of transmitted drug resistance variants in Italian population. Different groups of infected individuals contribute to the transmission of HIV: individuals with primary infection, chronically infected individuals naïve to the antiretroviral drugs, and finally HAART treated subjects with detectable viremia. This thesis has shown that, beyond drug treated patients, also drug naïve patients can contribute to the transmission of HIV-1 drug resistant strains. Indeed, the 14% of patients carrying transmitted drug resistance were detected in transmission clusters composed only of newly diagnosed individuals.

Last, but not least, this thesis suggested that specific mutations revealed at standard GRT, as the RT mutations L210M and T69S, can act as “sentinel”s of hidden drug resistance mutations (even in complex patterns), detectable only by ultra deep pyrosequencing. It is well known that, due to their low replication capacity in absence of drug pressure, drug resistance variants generally fade away after transmission, and

may became present only as minority quasispecies in the population. The genotypic test used in clinical practise can not detect viral species under the threshold of 20% and the use of more sensitive assay is today limited only for research purpose. So, a detailed map of association between “sentinel” mutations, detected by genotypic test, and minor drug resistance variants, only detectable with high resolution methods, may help to select patients at high risk of carrying resistance in reservoirs, hence providing a surrogate marker of hidden drug resistance mutations easily detectable by routine testing.

In conclusion, this thesis was able to characterize the HIV-1 transmitted drug resistance and the transmission dynamics of HIV-1 drug resistant strains in Italy. These data were essential to improve the surveillance and the preventive programmes on HIV infections. This thesis has also characterized “sentinel” mutations able to predict minority drug resistance variants, undetectable by standard genotypic tests. Taking into account these genetic markers right from diagnosis can help clinicians to improve the management of HIV-1 infected patients.

6 References

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Summary

To date 25 drugs are available for the HIV-1 treatment. The combined use of these drugs, known as Highly Active Antiretroviral Therapy (HAART) has successfully suppressed replication of HIV-1 and has dramatically improved the prognosis of HIV-1 infected patients. However, if viral rebound occurs during therapy, viruses with mutations conferring drug resistance can be selected and potentially transmitted not only as predominant species but also as minority variants.

Because an individual can be newly infected with resistant HIV-1, first line treatment is also jeopardised. To date, there is a growing literature about the rate of transmission of HIV-1 drug resistant virus. In the United States and in Europe, where there is a wide access to HAART, the prevalence of HIV-1 drug resistant strains ranges between 3.3% and 14.0% in recently infected patients and between 6.1% and 12.5% in chronically infected ones. Fortunately, several assays, as phenotypic resistance test and genotypic sequence analyses, are to date used to assess antiretroviral drug resistance even when present as minority species (ultra deep pyrosequencing). These assays are of particular interest for the clinical point of view, since they may facilitate the management of HIV-1 infected patients.

In this light, the aim of this thesis was to characterize the distribution and the spread of drug resistant HIV-1 in Italy, with particular attention to the population dynamics of transmitted resistance and the role of untreated patients to the spread of drug resistance. An additonal objective of this thesis was to define new genetic markers in the pol gene of HIV that can predict the presence of transmitted drug resistant minority species. This approach could be useful in clinical practice where the use of more sensitive assay, as ultra deep pyrosequencing, are too expensive to be apply. In addition, the identification

of these “sentinel” mutations could improve the genotypic interpretation and could help the selection of patients potentially reservoir of drug resistance.

In the first part of this thesis, we examined the prevalence of transmitted drug resistance in Italy and focussed our attention on a cohort of newly diagnosed infected patients enrolled in the SENDIH programme. Among these patients, the 5.9% showed signs of transmitted resistance; in particular, 3.9% of patients was infected with HIV nucleoside reverse transcriptase inhibitor (NRTI)-resistant viruses, 3.5% with HIV non-NRTI-resistant viruses and 0.4% with HIV protease inhibitor (PI)-resistant viruses. Most importantly, the 3.5% of patients carried HIV-1 resistant strains with more than one major drug resistance mutation. Comparing chronic infections with recent infections, we also found a decreased rate of resistance in recent infections (P=0.09), reflecting the increasing use of potent drugs and highly active antiretroviral regimens in recent years. Homosexual individuals were also more likely to harbour a virus with at least one primary resistance mutation (OR 7.7; 95% CI: 1.7–35.0, P=0.008).

Overall, the prevalence of drug resistant HIV-1 strains among newly diagnosed individuals in Italy is consistent with the data from other European countries. Nevertheless, we found complex patterns of drug resistance mutations highlighting an additional potential risk for public health and strongly supporting the extension of wide genotyping to newly diagnosed HIV-1 infected patients.

The second aim of this thesis has been to identify the source of transmitted drug resistance variants in Italian population. It is known that different groups of infected individuals contribute to the transmission of HIV: individuals with primary infection, chronically infected individuals naïve to the antiretroviral drugs, and finally HAART treated subjects with detectable viremia. Using a phylogenetic approach, the second part

of this thesis attempted to investigate the population dynamics of transmitted resistance and the potential contribution of untreated patients to the spread of antiretroviral resistance. A total of 884 HIV-1 B subtype pol gene sequences from 306 drug-naïve (42 recently and 264 chronically infected) and 578 drug-treated HIV-1 infected patients were collected through routine drug resistance testing between 2000 and 2008 in Northern Italy.

By exploring the source of transmitted drug resistance, we could demonstrate that beyond drug treated patients, also drug naïve patients can contribute to the transmission of HIV-1 drug resistant strains. Indeed, the 14% of transmitted drug resistance was linked to transmission clusters composed only of newly diagnosed individuals. This result is consistent with another recent study led in the UK (Huè et al., 2009).

The third part of this thesis showed how detection of specific mutations revealed at standard GRT and never associated with drug resistance can be applied to the surveillance of primary drug resistance, bypassing the use of more sensitive assay too expensive for the clinical practice. Among 2089 HIV-1 B subtype infected patients, naïve to the antiretroviral therapy, enrolled between 2004 and 2009 through routine drug resistance testing, 30 individuals were retained for the study. All 30 patients did not show primary transmitted resistance at GRT, but showed one atypical mutation at position already associated with drug resistance. In particular, the atypical mutations T69S, L210M, and K103R were present in 10/10/10 samples, respectively. Additional 10 patients with wild-type virus at all the positions associated with drug resistance, were considered as controls.

First of all, minority drug resistant variants could be detected in 45% of our drug-naïve patients by using the ultra deep pyrosequencing technology. This finding confirms that

ultra-deep sequencing can detect a significantly proportion of minority-resistant variants despite negative results of standard genotyping, more than doubling the prevalence of resistant variants.

The patterns of drug resistant minority quasispecies found in our patients deserve another important consideration. Specific mutations revealed at standard GRT, as the RT mutations L210M and T69S, can act as “sentinel” of hidden drug resistance mutations (even in complex patterns), detectable only by ultra deep pyrosequencing.

So, a detailed map of association between “sentinel” mutations, detected by GRT, and minor drug resistance variants, only detectable with high resolution methods, may help to select patients at high risk of carrying resistance in reservoirs, hence providing a surrogate marker of hidden drug resistance mutations easily detectable by routine testing.

In conclusion, this thesis was able to characterize the HIV-1 transmitted drug resistance and the transmission dynamics of HIV-1 drug resistant strains in Italy. These data were essential to improve the surveillance and the preventive programmes on HIV infections. This thesis has also characterized “sentinel” mutations able to predict minority drug resistance variants, undetectable by standard genotypic tests. Taking into account these genetic markers right from diagnosis can help clinicians to improve the management of HIV-1 infected patients.

List of Publications

Svicher V, D'Arrigo R, Alteri C, Andreoni M, Angarano G, Antinori A, Antonelli G, Bagnarelli P, Baldanti F, Bertoli A, Borderi M, Boeri E, Bon I, Bruzzone B, Callegaro AP, Cammarota R, Canducci F, Ceccherini-Silberstein F, Clementi M, D'Arminio Monforte A, De Luca A, Di Biagio D, Di Gianbenedetto S, Di Perri G, Di Pietro M, Fabeni L, Fadda G, Galli M, Gennari W, Ghisetti V, Giacometti A, Gori C, Leoncini F, Maggiolo F, Maserati R, Mazzotta F, Micheli V, Meini G ,Monno L, Mussini C, Nozza S, Paolucci S, Parisi SG, Pecorari M, Pizzi D, Quirino T, Re MC, Rizzardini G, Santangelo R, Soria A,Stazi F, Sterrantino G, Turriziani O, Viscoli C, Vullo V, Lazzarin A, Perno CF on behalf of OSCAR group. Performances of genotypic tropism testing in clinical practice using the enhanced sensitivity version of Trofile as reference assay: results from the OSCAR Study Group. New Microbiologica. Submitted.

Callegaro A, Svicher V, Alteri C, Lo Presti A, Valenti D, Goglio A, Salemi M, Ciccozzi M, Perno CF, Maggiolo F Epidemiological network analysis in HIV-1 B treatment-naive infected patients diagnosed in Italy between 2000-2008. Infection genetics and evolutions. Submitted

Zehender G, Lai A, Ebranati E, Galli M, Perno CF, Santoro MM, Alteri C, Giuliani M, Lo Presti A, Ciccozzi M Population Dynamics of HIV-1 Subtype B in a Cohort of Men-having-Sex-with-Men in Rome, Italy. Journal of Acquired Immunodeficiency Syndrome. Accepted

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