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Section of General Microbiology and Virology
& Microbial Biotechnologies

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ANALYSIS OF HIV-1 GENOTYPIC MUTATIONS IN PATIENTS WITH MULTIPLE VIROLOGICAL FAILURE

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INTRODUCTION

The target of antiretroviral therapy in patients with HIV/AIDS is to reach a complete and durable suppression of plasma viral replication in order to restore or maintain the immune function and to minimize the development of viral drug resistance.

The prospects for treatment-naive people have never been better, with the availability of more than 20 different antiretroviral agents in four different classes, that has made long-term control of HIV replication a readily achievable outcome for the majority of HIV-infected patients initiating therapy in latest two years [1]. Unfortunately, the patients who were infected with HIV earlier have not been able to take advantage of the major improvements in therapy achieved with the HAART era.

As consequence, a large proportion of HIV-infected patients currently in care are infected with resistant viruses which require expert attention to ensure the best possible options for treatment [2].

While specific recommendations are available to treat patients starting antiretroviral therapy for the first time, the situation for treatment-experienced patients is more difficult. Each patient has arrived at a particular point in the treatment sequence that represents the accumulated effects of prior treatment choices, levels of adherence, drug interaction events, and specific viral characteristics [3].

The success of the therapy might be due to individual patient's situation, some of them able to recover to a full suppression of HIV replication, while in some others an immunological stabilization is the only long-term strategy

Continuous viral replication during therapy leads to accumulate drug-resistance mutations, resulting in increased viral load and a greater risk of disease progression. Patients with drug-resistant HIV-1 infection have three therapeutic options: a change to a salvage regimen with the aim of fully suppressing viral replication; a decision of interruption of

therapy; or maintenance of a partially effective regimen [4]. The first strategy is the best choice for patients failing their first or perhaps their second regimen. However, the best approach remains unclear for patients who have failed multiple treatment regimens and who have limited options for complete viral suppression.

The understanding of the pathogenic mechanism underlaying the drug-resistant HIV-1, the clinical consequences of virological failure, the potential benefits and limitations of diagnostic assays, and the efficacy of new drugs, is necessary in order of an effective management of such patients [5].

An important phase for the development of new drugs is the dynamic research of their molecular targets.

The antiretroviral drugs currently used, or still in phase of study, in the therapy of the AIDS can be classified on the bases of their targets in:

1. *Reverse transcriptase inhibition*:
   a) nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs);
   b) Non-nucleoside reverse transcriptase inhibitors (NNRTIs);
2. *Protease inhibition* (PIs);
3. *Cellular fusion inhibition*;
4. *Modulator of cellular receptor CD4 expression*;
5. *Antagonists of the chemokine receptors*
6. *integrase inhibition*
7. *transcription inhibition*
8. *ribonuclease H inhibition*

Table 1 shows the date of the antiretroviral drugs entry into clinical use.

Table 1. Entry of antiretroviral agents into clinical use

1987	Zidovudine	1998	Efavirenz
1991	Didanosine		Abacavir
1992	Zalcitabine	1999	Amprenavir
1994	Stavudine	2000	Lopinavir
1995	Lamivudine	2001	Tenofovir
	Saquinavir	2003	Emtricitabine
1996	Ritonavir		Enfuvirtide
	Indinavir		Atazanavir
	Nevirapine		Fos-amprenavir
1997	Nelfinavir	2005	Tripranavir
	Delavirdine	2006	Darunavir

Current anti-HIV drug regimens, termed Highly Active Antiretroviral Therapy (HAART), consist of a combination of at least three antiretroviral drugs, with two or more nucleotide reverse transcriptase inhibitors (NRTIs) being a staple of most regimens [6]. The most common combination given to those beginning treatment consists of two NRTIs combined with either an NNRTI or a "boosted" protease inhibitor. Ritonavir (in small doses) is the drug most commonly used to boost a protease inhibitor. Table 2 lists the drugs combination used in the treatment of HIV infection [7].

Table 2. Drugs combination and date of their approval

Brand Name	Generic Names	Approval Date
	Multi-class Combination Products	
Atripla	efavirenz, emtricitabine and tenofovir	12-July-06
	Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	
Combivir	lamivudine and zidovudine	27-Sep-97
Emtriva	emtricitabine, FTC	02-Jul-03
Epivir	lamivudine, 3TC	17-Nov-95
Epzicom	abacavir and lamivudine	02-Aug-04
Hivid	zalcitabine, dideoxycytidine, ddC	19-Jun-92
Retrovir	zidovudine, azidothymidine, AZT, ZDV	19-Mar-87
Trizivir	abacavir, zidovudine, and lamivudine	14-Nov-00
Truvada	tenofovir disoproxil fumarate and	02-Aug-04
Videx EC	enteric coated didanosine, ddI EC	31-Oct-00
Videx	didanosine, dideoxyinosine, ddI	9-Oct-91
Viread	tenofovir disoproxil fumarate, TDF	26-Oct-01
Zerit	stavudine, d4T	24-Jun-94
Ziagen	abacavir sulfate, ABC	17-Dec-98
	Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)	
Rescriptor	delavirdine, DLV	4-Apr-97
Sustiva	efavirenz, EFV	17-Sep-98
Viramune	nevirapine, NVP	21-Jun-96
	Protease Inhibitors (PIs)	
Agenerase	amprenavir, APV	15-Apr-99
Aptivus	tipranavir, TPV	22-Jun-05
Crixivan	indinavir, IDV,	13-Mar-96
Fortovase	saquinavir (no longer marketed)	7-Nov-97
Invirase	saquinavir mesylate, SQV	6-Dec-95
Kaletra	lopinavir and ritonavir, LPV/RTV	15-Sep-00
Lexiva	Fosamprenavir Calcium, FOS-APV	20-Oct-03
Norvir	ritonavir, RTV	1-Mar-96
Prezista	darunavir	23-Jun-06
Reyataz	atazanavir sulfate, ATV	20-Jun-03
Viracept	nelfinavir mesylate, NFV	14-Mar-97
	Fusion Inhibitors	
Fuzeon	enfuvirtide, T-20	13-Mar-03
	Entry Inhibitors - CCR5 co-receptor antagonist	
Selzentry	maraviroc	06-Aug-07

DRUGS THAT INHIBIT REVERSE TRANSCRIPTASE

The enzyme reverse transcriptase (RT) is used by retroviruses to transcribe their single-stranded RNA genome into single-stranded DNA and to subsequently construct a complementary strand of DNA, providing a DNA double helix capable of integration into host cell chromosomes. Functional HIV1-RT is a heterodimer containing subunits of 66 kDa (p66) and 51 kDa (p51). The subunit p66 contains two domains, the N-terminal polymerase domain (440 residues) and the C-terminal RNase H domain (120 residues). p51 is processed by proteolytic cleavage of p66 and corresponds to the polymerase domain of the p66 subunit. Portions of both p51 and the polymerase domain of p66 can be described as a "right hand" that contains three subdomains: fingers, palm, and thumb (Fig.1). The connection subdomain connects the hand of the polymerase domain and the RNase H domain in p66, which provides the ribonuclease activity of HIV-RT. Although p51 contains a connection subdomain, it lacks an RNase domain.

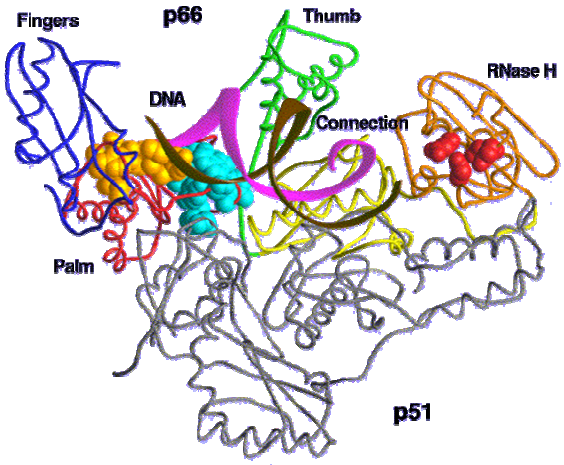
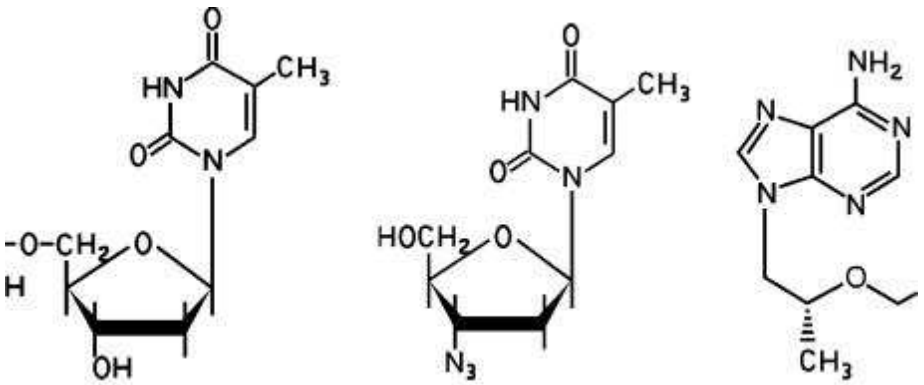


Figure 1: Structure of HIV-1 RT complexed with dsDNA showing the relative location of the dNTP-binding pocket (in gold), the NNRTI binding site (in cyan), and the RNase H active site (in red).

Nucleoside analogue reverse transcriptase inhibitors

Due to its essential role in synthesizing the double-stranded proviral DNA from single-stranded HIV-1 RNA genome, the HIV-1 RT is a major target of current antiviral therapies directed against HIV-1. Nucleoside analogue reverse transcriptase inhibitors (NRTIs) represent an important class of compounds to treat infection with the human immunodeficiency virus type 1 (HIV-1). Six different NRTIs are today in clinical use. These compounds are intracellularly phosphorylated and compete with natural 2'-deoxyribonucleoside triphosphate (dNTP) pools for incorporation into the growing DNA chain.



The nucleoside analogues such as zidovudine (azidothymidine, AZT) are comprised of a base (thymidine in the case of AZT) attached to a ribose sugar in which the normal 3’ hydroxyl has been replaced by an azido group.

The presence of the 3’ OH is required for elongation of the growing DNA chain. Replacement of the OH at the 3’ position prevents bonds from being formed with this nucleoside. Incorporation of AZT into the

growing DNA chain in place of the normal nucleoside leads to a "chain termination" that stops polymerization of the growing DNA molecule.

The nucleotide analogues (eg, tenofovir) inhibitory mechanism of action is identical to that of the nucleoside analogues, with the main difference being structural in that tenofovir is an acyclic deoxyadenosine. Both nucleoside and nucleotide RT inhibitors must enter the cell and become phosphorylated in order to act as synthetic substrates for RT. Both classes of agents can prevent infection of susceptible cells but will have no effect on cells that already harbour HIV.

Non-nucleoside analogues reverse transcriptase inhibitors

In addition to NRTIs, which are both competitive inhibitors and chain-terminators, the nonnucleoside reverse transcriptase inhibitors (NNRTIs) consist of structurally dissimilar hydrophobic compounds that directly bind reverse transcriptase at a hydrophobic pocket near the catalytic site (the binding site is formed by amino acids from codons 100-110, 180-190 and 220-240 [8]), and alter the enzyme ability to change conformation. This increased enzyme rigidity prevents its normal polymerization. The side effects of the NNRTIs are generally less than those of the nucleoside analogues; however, the main disadvantage of these agents is the rapid development of resistance. As a result, the NNRTIs are never used for monotherapy of HIV infection.

PROTEASE INHIBITOR DRUGS

Newly assembled HIV particles are not fully functional or infectious until they have undergone a final "maturation." This maturation involves cleavage of viral protein precursors by HIV protease enzymes. These enzymes are encoded by HIV and offer a unique and attractive target for preventing HIV maturation.

HIV protease enzymes are symmetrical dimers with a central core that binds the peptides which require to be modified by the enzyme.

Three domains of the PR are frequently found in literature: the active site cavity, the dimerization domain, and the flaps (Fig. 3).

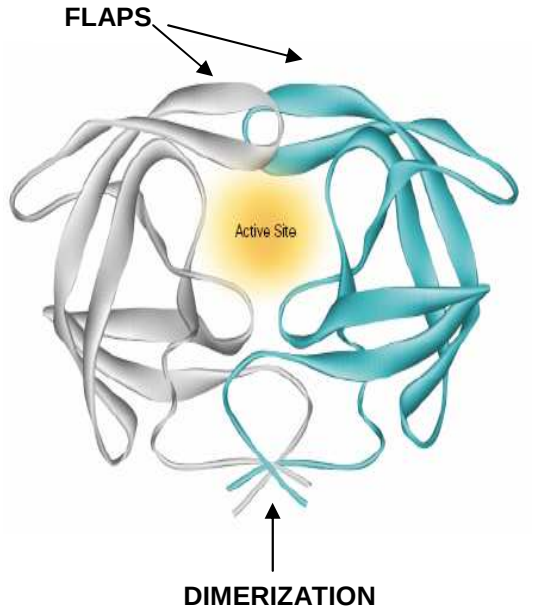


Fig 3. Structure of HIV-1 protease

Protease inhibitors are designed to fit and bind at the catalytic site of the enzyme with high affinity and thereby block its activity.
Inhibition of HIV protease enzymes still allows viral particles to be formed and released from host cells; however, the particles released are immature and not infectious.

FUSION INHIBITORS.

Fusion of HIV with the host cell membrane is an essential step in the viral infectious activity.
Entry of HIV-1 into target cells is a multistep process involving attachment (mediated by gp120 binding to CD4 lymphocytes), chemokine coreceptor binding, and association of 2 trimeric helical coils (HR-1 and HR-2) located in the ectodomain of gp41 into a 6-helix bundle that brings the virus and cell membranes into close approximation, allowing membrane fusion [9]. A number of drugs currently in development block HIV-1 infection by interfering with one

or more of these steps. The recently approved fusion inhibitor enfuvirtide (known as T-20) blocks the association of HR-1 with HR-2 by binding to the trimeric HR-1 complex, thereby inhibiting fusion and blocking virus entry [10].

In August 2007 a new type of entry inhibitor known as maraviroc was licensed in the US. In Europe, it is available through an expanded access programme for people with few remaining treatment options, but still awaits full licensing. This new drug is known as a CCR5 inhibitor as it blocks the CCR5 co-receptor on human immune cells, preventing the HIV attachment to the cells surface.

While HAART regimens have decreased both the mortality and morbidity of HIV-infected patients, several factors contribute to therapy failure. The highly error-prone nature of HIV-1 RT combined with a robust rate of viral replication provides the virus with an ideal context for the emergence of resistant variants. In addition, the significant toxicity associated with the current anti-HIV drugs (showed in table 3) often leads to noncompliance, which may result in a treatment failure [11]. For these reasons, there is a high interest in the development of more potent anti-HIV inhibitors that are less likely able to lead to drug-resistant variants and with a lower toxicity.

Table 3. Adverse effects of antiretroviral drugs.

Drug class	Example	Adverse effects
Nucleoside,	Zidovudine	**Lactic acidosis**
Nucleotide analogues	Didanosine	**Nausea**
	Lamivudine	
	Abacavir	
	Stavudine	
	Emtricitabine	
	Tenofovir	
Non-Nucleosides	Nevirapine	**Rash**
	Efavirenz	**Hepatotoxicity**
Protease inhibitors	Ritonavir	**Hyperglycemia**
	Saquinavir	**Nausea**
	Amprenavir	**Diarrhea**
	Indinavir	
	Nelfinavir	
	Atazanavir	
	Tipranavir	
	Fosamprenavir	
Fusion Inhibitors	Enfuvirtide (T-20)	**Reaction at injection site**

FAILURE DEFINITION

On the basis of clinical trials and cohort studies, approximately 20–30% will experience treatment failure on their first regimen. Treatment failure can be defined in many ways [12]. These include:

- *virological failure* (inability to achieve virological suppression or the occurrence of virological rebound),
- *immunological failure* (progressive CD4 cell count decline), or
- *clinical failure* (HIV disease progression).

One or more of these may be present in each patient, and anyone of them may suggest the necessity to change the treatment.

It is critical to identify factors associated with treatment failure in order to adequately address them in the next regimen. Adherence problems are often present and, if they are, specific reasons for suboptimal adherence (dosing frequency, pill burden, drug–drug interactions, adverse event experiences, coexistent substance abuse, and so on) should be evaluated if possible to choose the next regimen.

At present, there are few recommended therapeutic options for the management of patients with highly resistant viral strains and severe therapeutic failure. The use of an antiretroviral regimen containing five to six active drugs, so called mega-HAART, has been reported to be at least partly effective in these patients [13].

DRUG RESISTANCE.

Drug resistance to human immunodeficiency virus (HIV) is a major factor in the failure of antiretroviral therapy [14].

Several factors related to the life cycle and replication of HIV are key contributors toward the rapid and widespread emergence of resistance.

First, the HIV reverse transcriptase (RT) enzyme is notoriously "low fidelity" (ie, the enzyme is somehow nonselective during the copying process) and is prone to errors when copying viral RNA into DNA. By some estimates, HIV RT makes one error in each HIV genome per

round of replication [15]. This translates into approximately 1 mutation for every 2000 nucleosides. Most of these errors are base substitutions, other mutations that can also occur are insertions or duplications. Second, HIV has an exceptionally high rate of replication; several billion new viral particles may be produced each day in the untreated patient.

Since the half-life of cells infected with HIV is generally 1-2 days, HIV must infect new cells at a very high rate to maintain the infection at a stable level. This high rate of replication coupled with the high rate of error for RT means that numerous HIV "variants" are rapidly formed and propagated. Patients who are infected with HIV can have multiple variants of the virus present in their system. These variants can have greatly different sensitivities to antiretroviral agents, a factor that can significantly complicate the selection of drugs and the course of therapy. Additional factors that may contribute to the development of HIV drug resistance, include poor patient compliance, subtherapeutic blood levels of antiretroviral agents, and inappropriate choice of antiretroviral agents. Patients should be told to take their HIV medications as prescribed and not to miss any doses. Pharmacokinetic factors that can affect blood levels of antiretroviral agents include poor oral absorption and alteration of drug metabolizing enzymes by other agents, as well as various drug-drug interactions.

While some HIV variants may exhibit intrinsic or "primary" resistance to antiretroviral agents, most drug resistance develops as a result of exposure to these agents. Antiretroviral resistance can still occur even during successful therapy of HIV infection [16]. Any mutations that confer a selective advantage to a particular viral variant will allow that particular viral variant to predominate. To some extend, the use of antiviral agents exerts a "selective pressure" leading to the development of more resistant viruses. The use of multiple drugs in combination may represent a strategy able to reduce the ability of resistant viral variant to survive treatment.

The major implications of drug resistance include a change in viral fitness, possible changes in viral tropism, an interaction in the susceptibility to other drugs.

In particular, cross-resistance (defined as resistance to drugs to which a virus has never been exposed) within a given class of antiretroviral agents has been found to affect all classes of drug currently available [17]. Compared with single-drug resistance, this cross-resistance may result in class-wide resistance (CWR), which could substantially reduce the clinical utility of antiretroviral drugs of the same class and reduce future treatment options [18].

Resistances may be classified as follow:

- *Genotypic resistance*: the presence of genotypic changes that reduce the sensitivity to one or more drugs;
- *Phenotypic resistance*: the capability of a virus to grow in the presence of a drug concentration in witch a wild type virus replication is blocked.

According to the time of acquisition of drug resistance, we can differentiate a primary resistance, which is drug-resistance mutations detected in antiretroviral naive-patients, and a secondary resistance, which is drug-resistance mutations detected in antiretroviral experienced-patients.

Drug pressure drives selective forces for genetic changes in the viral genome. Mutations arising under antiretroviral therapy allow virus to escape from the inhibitory effect of the drug on virus replication. Two major classes of drug resistance mutations have been identified as:

Primary mutations:

Selected early in the process of resistance to one drug. However, they may be selected or favoured after the appearance of some secondary mutations. These mutations have an high degree of specificity for one drug witch significantly compromises the susceptibility of the virus for that drug;

Secondary mutations:

Tend to accumulate in viral genomes already containing one or more primary mutations, they may have little or no effect on the level of resistance, but they may increase viral replication by increasing viral fitness.

Genetic barrier is a terminology referred to the number of mutations required of reducing or loosing the drug antiretroviral activity. Genetic barrier may be classified as

- low: loss of antiviral activity by the appearance of a single mutation. It is a relatively easy hurdle for the virus to overcome.
- High: loss of antiviral activity after the appearance of multiple mutations

However, it is not necessary a high level of resistance for virological or immunological treatment failure.

RESISTANCE TO ANTIRETROVIRAL DRUGS

1. NRTI.

Resistance to NRTIs occurs through 2 mechanisms: the first is mutation of the residues that results in reduced incorporation of the NRTI into the growing DNA chain. It is now well accepted that primer unblocking (i.e. nucleotide excision) is the mechanism of zidovudine resistance. Primer unblocking is the mechanism by which pyrophosphate or ATP can remove the terminal nucleotide from a growing DNA chain [19]. This reaction (in essence the reversal of DNA synthesis) can result in removal of zidovudine, thereby relieving the block to reverse transcription.

The second mechanism of NRTI resistance is associated with enhanced removal of drug from its site of attachment at the end of the DNA chain. These RT mutations allow ATP or pyrophosphate (both of which are in high concentration within the cell) to bind at the active site

adjacent to the bound nucleoside analogues. The high energy ATP or pyrophosphate can then attack the bond that binds the drug to DNA, thereby liberating the drug and terminating its effect.

Multi-NRTI resistance mutations, also known as nucleoside analogue-associated mutations (NAMs), are associated with resistance to numerous NRTIs. TAMs (thymidine analog mutations) are a subset of NAMs that are selected by the thymidine analogues zidovudine and stavudine and are associated with cross-resistance to all NRTIs currently approved by the US FDA.

The M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E are known as TAMs. These resistance mutations *increase* the rate of nucleotide excision by reverse transcriptase. In contrast, others reverse transcriptase mutations (e.g. M184V, Y181C) *slow* the rate of excision.

Others important resistance mutations implicated in the cross-resistance are the complex Q151M (A62V, V75I, F77L, F116Y, Q151M) (20,21) and the 69 insertion complex, that consists of a substitution at codon 69 (typically T69S) and an insertion of 2 or more amino acids (S-S, SA, S-G, or others). The 69 insertion complex is associated with resistance to all NRTIs currently approved by the US FDA when present with 1 or more thymidine analogue-associated mutations (TAMs) at codons 41, 210, or 215. Other amino acid changes at codon 69 without the insertion may also be associated with broad NRTI resistance.

Several studies analysed V118I substitution, that seems decrease the susceptibility to multiple nucleoside analogues by a reduction in rates of their incorporation [22]. Several clinical data revealed that mutations E44D and V118I, when present in a background of classical AZT-mutations (M41L, D67N, L210W, and T215Y), confer dual resistance to AZT and 3TC [23]. V118I lies in close proximity to residues Y115 and F116, which interact with the incoming nucleotide. Changes at these positions were shown to have profound effects on the ability of RT to discriminate between correct and incorrect nucleotides. The prevalence of the E44D/A and/or V118I mutation increases with the

number of antiretroviral treatments, and this suggests that the mutation might be involved in a more broad-spectrum nucleoside resistance.

2.NNRTI

Resistance to this class of agents occurs mainly through mutation of hydrophobic RT residues within the binding pocket for the NNRTIs. Since all of the NNRTIs bind to essentially the same region of RT, mutations in this area will affect binding of all of the agents in this class. This may in part explain the high rates of HIV cross-resistance within this class of agents [24].

The most common mutations in viruses isolated from patients treated with NNRTI are Y181C and K103N. Mutations of residue 190 (mostly G190A/S) represent approximately 15% of NNRTI-resistant variants and confer variable levels of drug resistance and fitness [25]. Variants carrying the G190E mutation are linked to reduced susceptibility to NNRTI, but show impaired replication with significantly reduced polymerase, RNase H and protease activities.

Numerous NRTI mutations, such as the TAMs M41L, L210W, and T215Y mutations, may lead to viral hypersusceptibility to NNRTIs in NRTI-treated individuals. The presence of these mutations may improve subsequent virologic response to NNRTI-containing regimens in NNRTI treatment-naive individuals [26],[27].

Several studies demonstrate that different combinations of V118I, H208Y, and T215Y produce NNRTI hypersusceptibility, while single mutations V118I, H208Y, and T215Y don’t show this hypersusceptibility [28] [29].

The exact mechanism responsible for the action of these polymorphisms is still unknown. Several authors propose a mechanism of stabilization in the three-dimensional structure of the enzyme that could be explained by the structural vicinity of the mutations at

positions 208, 211, and 214 to the ZDV-associated mutations L210W and T215Y/F..

Figure 2 shows the DNA polymerization active site of HIV-1 RT with commonly observed drug-resistance mutation sites for NRTI and for NNRTI.

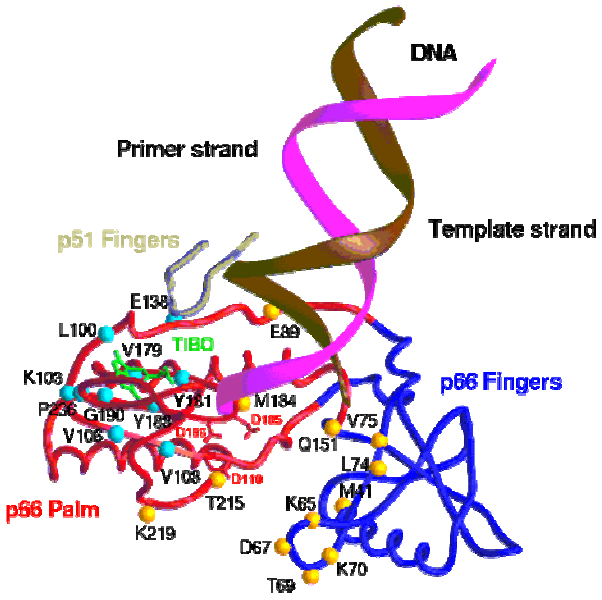


Figure 2. A view of the DNA polymerization active site of HIV-1 RT with commonly observed drug-resistance mutation sites for nucleoside inhibitors (in gold) and for non-nucleoside inhibitors (in cyan).

3. PI

Resistance to protease inhibitors occurs primarily as a result of amino acid mutations that arise within or proximal to the catalytic binding site to the drug. Replacement of key amino acids within the protease enzyme can significantly alter the affinity of the enzyme for binding protease inhibitors. In addition, the geometry of the catalytic site is altered and enlarged by these mutations. Since the protease inhibitors bind the catalytic sites with significantly higher affinity than the natural substrates, mutations in this region will have a greater effect on drug binding than on the endogenous peptides.

Resistance mutations in the protease gene are classified as "major" or "minor" (table 4)

Table 4. HIV protease inhibitor resistance mutations

Protease inhibitor resistance mutations according to the IAS-US A panel for ARV resistance					
Protease Inhibitor drug	Cross-resistance mutations		Unique mutations		Number of resistance mutations
	Major	Minor	Major	Minor	
Saquinavir/r	L90M, G48V	L10IRV,24I,4VL,62V,71VT,73S, 77I,82AFTS,84V			2 or more
Indinavir/r	46IL,82AF T,84V	L10IRV,20MR,24I,32I,36I,54V, 71VT,73SA,77I,90M			3 or more
Nelfinavir	L90M	L10FIRV,L24I,M36I,M46IL, A71VT,G73S,V77I,V82AFTS, I84V,N88DS	D30N		2 or more
Fosamprenavir/r	I50V	L10FIRV,V32I,M46IL,I47V, I54LVM,G73S,V82AFST,L90M			3 or more
Lopinavir/r	V32I,I47V A,V82AFT S	L10FIRV,K20MR,L24I,L33F, M46IL,I50V,F53L,I54VLAMTS, A71VT,G73S,I84V,I90M		L63P	6 or more
Atazanavir/r	I84V,N88 S	L10IFVC,K20RMITV,L24I,V32I, L33IFV,M36ILV,M46IL,G48V, F53LY,I54,LVMTA,I62V,A71VITL, G73CSTA,V82ATFI,L90M	I50L	G16E,E34Q, D60E, I64LMV,I93LM	3 or more
Tipranavir/r	L33F,V82L T,I84V	L10V,K20MR,E35G,M36I,K43T, M46L,I47V,I54AMV,L90M		I13V,Q58E, H69K,T74P, N83D	7 or more
Darunavir/r	I50V,I54M L, I84V	V11I,V32I,L33F,I47V,G73S	L76V	V11I,L89V	3 or more

Major mutations in the protease gene are defined either as those selected first in the presence of the drug, or those shown at the biochemical or virological level leading to an alteration in drug binding or an inhibition of viral activity or viral replication.

Major mutations have an effect on drug susceptibility phenotype. In general, these mutations tend to be the primary contact residues for drug binding. Minor mutations generally emerge later than major mutations, and by themselves do not have a significant effect on phenotype. In some cases, they may improve replicative fitness of the virus containing major mutations [30].

Certain PI resistance mutations cause limited or no cross-resistance to other agents. Nelfinavir can select for D30N; atazanavir for I50L; and fosamprenavir for I50V. Importantly, 150V can reduce susceptibility to lopinavir if other PI resistance mutations are present. When these individual mutations are selected, the response to ritonavir-boosted PIs is usually preserved.

UPAMS are the universal protease-associated mutations (L33I/V/F; V82A/F/LT; I84V and L90M) that confer broadly cross resistance among all available protease inhibitors.

4. FUSION INHIBITORS

Although just introduced to clinical practice, varying susceptibility of different HIV strains to enfuvirtide has already been documented [31].

Mutations in HR-1 that reduce enfuvirtide susceptibility are selected by in vitro passage of HIV-1 in the presence of the drug and have been identified in isolates obtained from patients receiving enfuvirtide in clinical trials [32]. In particular, amino acid substitutions at gp41 codons 36–45, which are part of the binding site for enfuvirtide, are found in virus samples recovered from patients experiencing protocol-defined treatment failure of enfuvirtide and are associated with an average 20-fold increase from the baseline IC50 of enfuvirtide [33]. The 500-fold range of enfuvirtide susceptibility among pretreatment isolates with wild-type sequences in HR-1 suggests that sequence variation in other regions of the HIV-1 envelope modulate susceptibility to this drug.

Genotypic diversity among HIV-1 subtypes may lead to different pathways to drug resistance.

In order to provide effective pharmaceutical care to their HIV patients, it is essential that the practitioners understand the mechanisms of HIV drug resistance as well as the various factors that can contribute to its emergence.

Resistance testing is critical to the construction of an active regimen in treatment-experienced patients [34]

The test must be done while the patient is on therapy in order to identify reliable resistance mutations. It is also important to be aware of prior resistance mutations that may have developed but whose presence may not be evident on the most recent assay. In most instances drug resistance that has been acquired from prior treatment failure cannot be reliably detected from a resistance test if the patient has been off therapy for a few months or if the current regimen does not continue to force the virus to sustain the mutations in question [35].

Resistance results must be analyzed aware of the current and previous treatment regimens of the patient, in order to properly modified the patient therapy.

The interpretation of HIV genotypic assays in clinical settings is very difficult because of the large numbers of drug resistance mutations and because these mutations interact one with another one and emerge in complex patterns.

The type of resistance test (genotype or phenotype) that is most helpful in making good treatment decisions likely differs for specific patient situations. Nonetheless, the most difficult it is likely to be to interpret the resistance genotype. The interactions among various mutations may be unpredictable as they relate to each drug, making the interpretation of genotypic results a big challenge. In this setting it is almost always simpler to interpret phenotypic tests. The genotype may complement the phenotype, and many experienced clinicians prefer to have both genotype and phenotype resistance test results available to guide drug selection in these often very difficult patients. The main goal of resistance test interpretation is to identify at least two active drugs that can be prescribed to suppress viral replication [36].

The greatest availability of genotype tests and its lower cost have allowed its wider introduction compared to the phenotype assays.
The question that remains now is how best to help a physician with interpreting genotypic information. It seems that 3 different approaches are helpful:

1. the clinician consults with the expert on a case-by-case basis. However this is not always feasible because many clinicians may not have access to an expert advisory.
2. "Rule-based" system. These rules are described in a table or generated from a computer as part of a genotype report, such the TrueGene™ HIV report that is supplied with the commercially available Visible Genetics genotype test. These rules are set by a panel of experts based on current knowledge, and require continual updating.
3. "database-driven predicted phenotype". In this form of interpretation, the patient's genotype is matched with other similar genotypes found in a genotypic/phenotypic relational database.

There are four sources of data that form the basis for drug resistance knowledge:

1. Genotypic-phenotypic correlations on laboratory isolates (often confirmed by site-directed mutagenesis studies);
2. Genotypic-phenotypic correlations on clinical HIV-1 isolates;
3. Correlations between HIV-1 genotype and the treatment history of patients from whom sequenced virus isolates are obtained;
4. Correlations between HIV-1 genotype and the virologic response to a new treatment regimen.

RESISTANCE ASSAYS

The development of genotypic and phenotypic tests has help to guide the therapeutic management in HIV-infected patients. There have

been a number of both prospective and retrospective studies that have demonstrated the clinical benefit of resistance testing. Currently, it exists several techniques that allow us to analyze the HIV genotype and phenotype.

Available testing includes HIV-1 genotype, phenotype and virtual phenotype determined from plasma samples using automated assays.

Genotypic testing/genotyping: test conducted to determine the nucleotide sequence of the virus genome. The results are provided as a list of changes in any amino acid or mutation that is different from the wild type reference strain. Such mutations are expressed by the position they have in a certain gene (codon), preceded by the letter corresponding to the amino acid seen in the wild type virus, and followed by the mutated amino acid. Example: M184V would correspond to the substitution of the amino acid Methyonine by Valine in codon 184 of the retrotranscriptase gene.

Phenotypic testing/phenotyping: test conducted to determine the susceptibility of a virus to drugs in a virus culture assay. The results may be expressed as:

IC_{50}, IC_{90}, IC_{95}: Concentration (in μg/ml or μM) of the drug needed to inhibit the viruses growth in vitro by 50%, 90% or 95%, respectively.

$$\text{Fold changes} = \frac{IC_{50}\text{ from the patient isolate}}{IC_{50}\text{ from a sensitive laboratory strain}}$$

Virtual Phenotype

It is the predicted HIV drug resistance from the genotype. When a genotype for patient sample is generated, the genetic code for the RT-PR regions is added into a software system. This system identifies all the mutations that can affect resistance to each drug and then interrogates the database for genotypes from previous samples that match these patterns of mutations. When all the matches are identified, the software retrieves the phenotypes for these samples and, for each

drug, averages the data for the matches. This produce a Virtual Phenotype, with a fold change in IC_{50} for each drug that is typically based on data from hundred of real phenotypes with the same pattern of mutations [37].

COMPARISON BETWEEN THE RESISTANCE TESTS

Genotypic assays analyze the HIV genome in order to detect specific mutations responsible for drug resistance. Interpretation of genotypic assay results is done by matching the results from the individual virus against lists of frequently updated HIV mutations that are known to confer drug resistance. These genotypic assays are relatively inexpensive and may now be performed rapidly on site with commercially available assay kits. However, genotypic testing can only identify documented HIV mutations and may not detect new mutations that arise in a particular HIV variant. In addition, since different mutations confer different degrees of drug resistance, it is often difficult to predict the actual degree of clinical drug resistance in a virus with multiple mutations.

In contrast, phenotypic resistance assays examine the actual drug susceptibility of a particular HIV variant. HIV genes for reverse transcriptase and protease enzymes are amplified and inserted into a recombinant virus which is then exposed to various anti-HIV drugs. Phenotypic testing provides information on the sensitivity of a particular HIV variant in comparison to a control isolate with full drug sensitivity. One practical difficulty associated with phenotypic testing is translating observed decreases in viral drug sensitivity in the assay into actual decreases in clinical sensitivity. What degree of phenotypic resistance needs to be present for each drug in order to see actual decreases in clinical effectiveness for that drug? It is only through large-scale clinical trials that an actual correlation might be made between changes in phenotypic sensitivity and actual drug resistance. So far these

clinical/phenotypic correlations have only been done for a few anti-HIV drugs [38].

The nature of phenotypic testing makes assays more technically difficult and expensive, thus such testing is only carried out at dedicated commercial facilities.

The virtual phenotype is faster and less expensive than a phenotypic test.

The ability to detect HIV-1 non-B subtypes is still not well known on both genotypic and phenotypic assays. However, some studies have reported amplification and successful resistance analysis for all group M (A-H) subtypes [39,40].

RELEVANCE OF RESISTANCE TESTING IN CLINICAL CARE

In addition to resistance testing also other factors should be considered in order to choose the best pharmacological therapy for a rescue intervention in patients with virological failure. Between these, we mention:

- Drug treatment history
- Plasma viral load
- CD4+ lymphocytes
- Medication tolerance
- Adherence
- Concomitant medications
- Blood and cellular drug levels

Many practicing physicians and clinical investigators might not be aware about the fact that the most conventional knowledge about drug resistance derives from studies performed solely during the pre-clinical and early clinical development of a new drug. Information arising after a drug has been approved by the FDA and has been incorporated as a clinical tool often not included on many of the "gene charts" that many

physicians and reference labs rely on for interpreting genetic sequence data. The final resistance report is often limited to the amino acid positions that are known to be involved in drug resistance [41]. Thousands of patients may have been treated inappropriately because sequence interpretations based on such oversimplified charts which in consequence might can be inaccurate. Additionally, complacent reliance on simple yet inaccurate data has masked the need for the additional research necessary to interpret genotypic tests in a clinically meaningful manner [42].

In this study we described the genotypic state of 12 HIV-1 positives patients with an history of multiple failure. Our data are based on two consecutive genotypic resistance tests and the therapy choices made for these patients. The aim was to evaluate the usefulness of analyzing other nucleotide polymorphism (not considered in the resistance report because not associated with drug resistance, or because mutations in resistance sites, but of a yet unclear significance).

The analysis were performed in two steps:

1) Complete analysis of the two consecutives tests;
2) New interpretation of the same tests following guide lines G12 updated to 2006.

In the first step we evaluate if the development of new resistance mutations in the second test was already observable in the research report of the previous test between the mutations in sites already known to be associated to a resistance .

In a second time we made a new interpretation of the first sequences and we observed the differences in the mutations, witch lead to a difference of the two reports.

We have then analysed the therapeutic choices made for these patients on the basis of the resistance test in order to determine the weight of these additional informations in the best choice of the best therapy.

METHODS

Samples preparation

EDTA plasma samples were collected and stored in a freezer at -80°C until use.

HIV-1 RNA quantitation

The viral load determination, parameter evaluated to monitor HIV-positives patients, is necessary to decide the resistance test performability. The test can be performed with viral loads>1000 copies/ml, even if a more low viral load (250-500 copies/ml) is sufficient for certain patients.

HIV-1 RNA copy number was assessed using until 2006 the HIV-1 Amplicor™ Monitor (Roche) and since 2006 the Versant® HIV-1 RNA 3.0 assay (b-DNA) (Bayer).

The bDNA (branched DNA) technology, in contrast to PCR, amplifies the non-isotopic signal of a direct hybridization to the target sequence and the signal amplification is linear. The assay is a sandwich nucleic acid hybridization procedure for the direct quantification of HIV-1 RNA in human plasma.

Briefly, the enclotted (EDTA) whole blood samples are centrifuged at 2000 rpm for 10 minutes and plasma is stored at -20°C until use. HIV-1 is first concentrated from plasma by centrifugation (14000 g for 70'). After HIV-1 genomic RNA is released from the virions, the RNA is captured to a microwell by a set of specific, synthetic oligonucleotide capture probes. A set of target probes hybridize both the viral RNA and the pre-amplifier probes. The capture probes, comprised of 17 individual capture extenders, and the target probes, comprised of 81 individual target extenders, bind to different regions of the pol gene of the viral RNA. The amplifier probe hybridizes to the pre-amplifier forming a branched (bDNA) complex.

Multiple copies of an alkaline phosphatase (AP) labelled probe are then hybridized to this immobilized complex. Detection is achieved by

incubating the complex with a chemiluminescent substrate. Light emission is directly related to the amount of HIV-1 RNA present in each sample, and results are recorded as relative light units (RLUs) by the analyzer. A standard curve is defined by light emission from standards containing known concentrations of HIV-18E5/LAV virus. Concentrations of HIV-1 RNA in specimens are determined from this standard curve. The lower detection limit of RNA quantification for this assay is 50 copies/ml.

HIV-1 resistance evaluation from RNA.

For HIV-1 sequencing, plasma RNA was extracted using the *High Pure Viral Nucleic Acid Kit (Roche Applied Science)*.

Virus lysis is accomplished by incubation of the sample (plasma) in a special lysis/binding buffer in the presence of proteinase K. Subsequently, nucleic acids bind specifically to the surface of glass fibers in the presence of a chaotropic salt. The binding reaction occurs within seconds due to the disruption of the organized structure of water molecules and the interaction of nucleic acids with the glass fibers surface. Since the binding process is specific for nucleic acids, the bound nucleic acids are purified from salts, proteins and other impurities by a washing step and are eluted in low salt buffer or water.

Reverse transcriptase polymerase chain reaction (RT-PCR) and direct DNA sequencing of protease and reverse transcriptase genes were performed using the *TruGene® HIV-1 Genotyping Kit* and the *OpenGene™ DNA Sequencing System (Visible Genetics-Bayer Diagnostics, Toronto, Canada)* according to the manufacturer's instructions.

This test allows to detecting HIV genomic mutations (in the protease and part of the reverse transcriptase regions of HIV), that confer resistance to specific types of antiretroviral drugs. These two regions code for the main targets of antiretroviral treatment. Development of viral resistance to these drugs is associated with

mutations within these coding regions. The mutations are identified by sequencing an RT-PCR product corresponding to these regions, and comparing the sequence to a wild type virus reference standard.

The TRUGENE HIV-1 Genotyping Assay consists of several processes:

1. Reverse transcription of target RNA to generate cDNA using RT-PCR amplification of target cDNA using HIV-1 specific primers;
2. CLIP sequencing of the PCR amplicons using HIV-1 specific primers;
3. Separation of the CLIP sequencing reactions by electophoresis on a polyacrylamide gel, and detection by laser-induced fluorescence;
4. Analysis of the forward and reverse CLIP sequences using the OpenGene DNA System Software. The end result is a Trugene HIV-1 Resistance Report for each sample.

The HIV-1 *pol* genes for the protease and reverse transcriptase regions are 297 and 1680 nucleotides in length (99 and 560 amino acids) respectively and are located at positions 1835-4678 (including the RNase H region) in the *pol* region of the HIV-1_{LAV-1} genome (GenBank number K02013). Due to the highly polymorphic nature of HIV-1, a mixture of several primers targeted at the most common viral variants are used in this kit.

The resistance report include relevant mutation, associated to resistance toward a particular drug, on the basis of four possible "evidence rules":

I – rule based on two or more independent studies of virological response and other in vitro data.

II – rule based on in vitro data (phenotype's data and/or in vitro demonstration of mutation) and preliminary data of virological response;

III – rule based on in vitro data.

IV – rule based on Consensus Panel extrapolation. Do not exist in vitro or in vivo data.

Apart from the resistance report, the software elaborate an other research report, with all polymorphism not involved in the drug resistance.

Results.

Description of the study population

Of the 450 patient HIV-infected attending our Immunology Centre about a 10% are in an advanced stage of infection and in an advanced line of treatment. The analysed sequences derive from 12 HIV-1 positives patients for witch a genotypic resistance test was performed for reason of virological failure. We considered two consecutive resistance tests for each patient, performed between 2001 and 2004 and between 2003 and 2007 respectively, and analysed by several "libraries" (guide-line rules), from "g4" to "g11".

All patients was in a relatively advanced stage of infection. According to the Center for Disease Control and Prevention classification, 6 (50%) patients were in stage B3, 3 in stage C3, 2 in stage B2 and 1 in stage C1.

All patients begun therapy between 1992 and 1996 and received several previous treatment (more of 6 therapy cycles with all three class NRTI, NNRTI, PI), with multiple treatment failure.

Median viral load was of 16700 HIV-1 RNA copies/ml (range 3570 to 380000 HIV-1 RNA copies/ml) at the time of first test and 6090 HIV-1 RNA copies/ml (range 1800 to 81367 HIV-1 RNA copies/ml) at the time of second test. The time mean elapsed between the two tests was 21.17 ± 2.39 months.

Viral load, therapy regimen at the time of the two genotyping analysis (that we labelled time 1 and time 2), the following therapy switch, the response of the patients (intending as positive response a viral load <50 copies/ml within six months) and the months elapsed between the two resistance tests are showed in table 7. During this period not necessarily was maintained the same therapeutic regimen, but for certain patients were made empiric modification on the basis of previous therapeutic history and previous resistance test.

Tab 7. Therapy and switch therapy following the resistance test interpretation

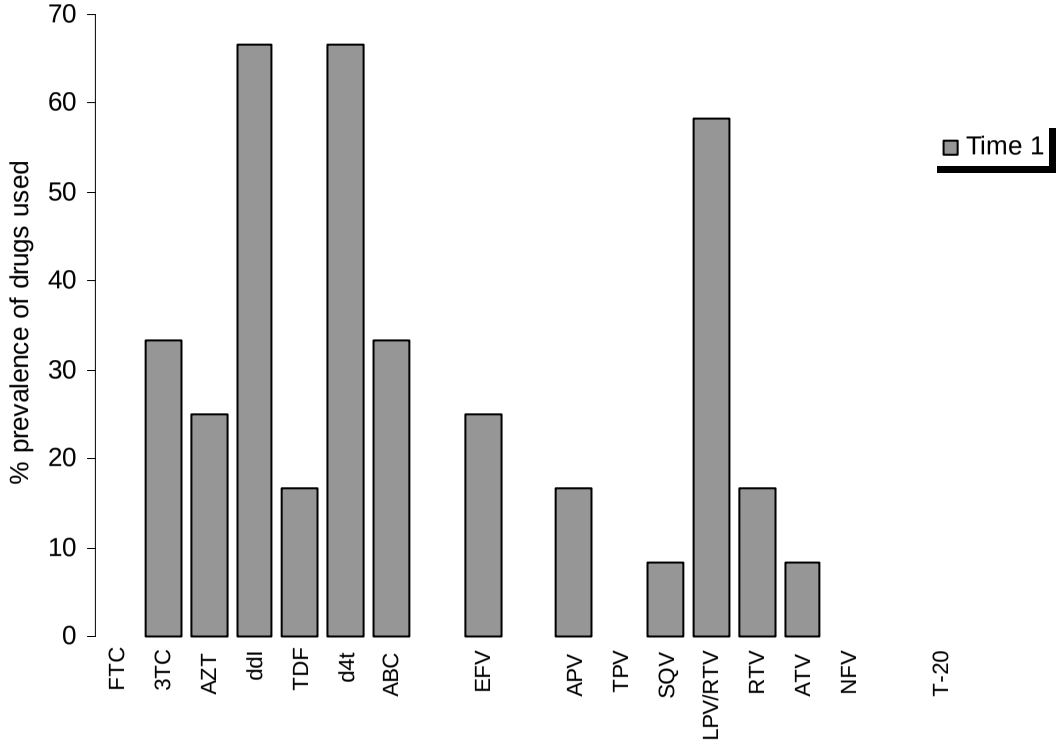
Time 1					M. diff	Time 2			
P	VL (cps/ml)	Therapy	Switch	Resp		VL (cps/ml)	Therapy	Switch	Resp
1	26100	d4T,EFV, LPV/r	3TC,ddI,LPV/r	N	17,59	4420	FTC, 3TC, ddI, LPV/r	3TC,ddI,TDF, LPV/r	Y
2	6950	ddI, d4T, ABC,LPV/r	ddI,TDF,EFV	N	9,27	3170	3TC, d4T, LPV/r	NO SWITCH	N
3	39100	AZT, ddI, LPV/r	3TC,EFV,LPV/r	Y	23,11	1800	3TC, TDF, LPV/r	NO SWITCH	N
4	6070	ddI, d4T, EFV	ddI,d4T,SQV, RTV	N	35,90	5110	FTC, d4T, LPV/r	NO SWITCH	Y
5	8460	ddI, ABC, LPV/r	NO SWITCH	N	27,55	29400	TDF,ABC, LPV/r	NO SWITCH	N
6	16300	d4T,ABC, LPV/R	3TC,d4T,EFV	Y	15,32	5790	TDF, d4T, LPV/r	3TC,TDF, LPV/r	Y
7	7620	3TC, ddI, TDF, d4T, LPV/R	NO SWITCH	N	18,77	6390	3TC, AZT, LPV/r	3TC,AZT, LPV/r	N
8	380000	ABC, AZT, 3TC, ddI, SQV	3TC,d4T,NFV	N	12,13	67500	TDF, FTC, ddI, TPV, RTV	ddI,TDF, LPV/r	N
9	29400	3TC,TDF, d4T,APV, RTV	NO SWITCH	N	25,41	45500	TDF,EFV, RTV, ATV	NO SWITCH	N
10	>100000	ddI, d4T, APV,RTV	3TC,TDF,EFV	N	25,74	81367	FTC, TPV, RTV	AZT,TDF,FTC	N
11	3570	3TC, AZT, ATV	AZT,TDF,EFV	Y	12,13	5232	AZT, TDF, EFV	TDF,FTC, LPV/r	Y
12	17100	ddI, d4T, LPV/r	SUSPENSION 6 MONTHS	N	31,07	13900	3TC, TDF, d4T, EFV, APV, RTV	3TC,TDF, LPV/r	N

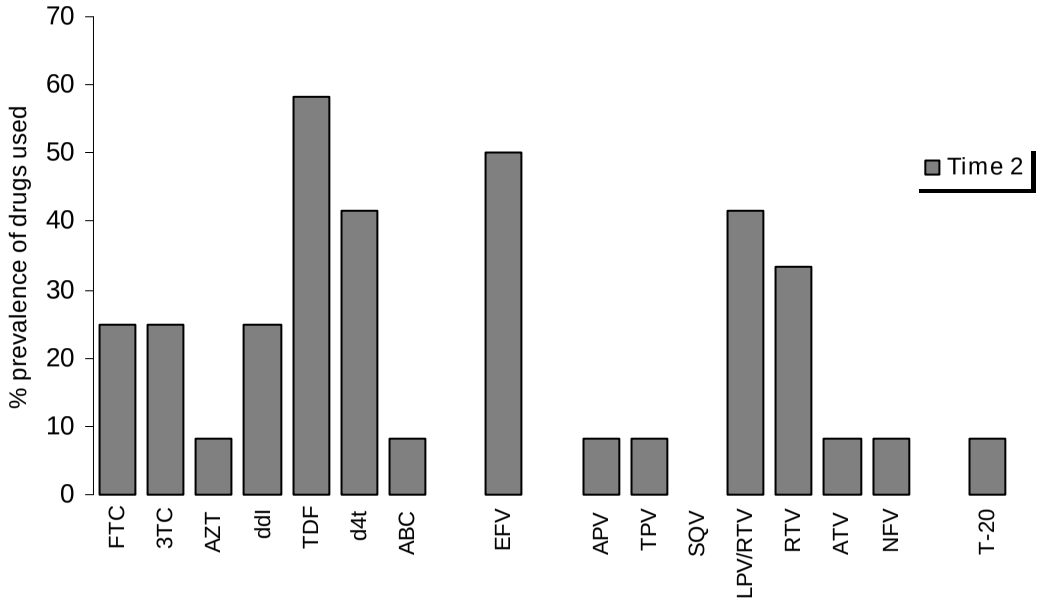
There wasn't a therapy switch for 3 patients (25%) at time 1, and for 5 patients (41.7%) at time 2, two of which being the same.

There was a positive response following the resistance test for 3 patient at time 1, and for 4 patients at time 2.

The more used drugs at time 1 were didanosine and stavudine between NRTIs (68%), and lopinavir/ritonavir between PIs (58%), while at time 2 were tenofovir (58%) and stavudine (42%) between NRTIs, and lopinavir/ritonavir (42%) between PIs (figure 1).

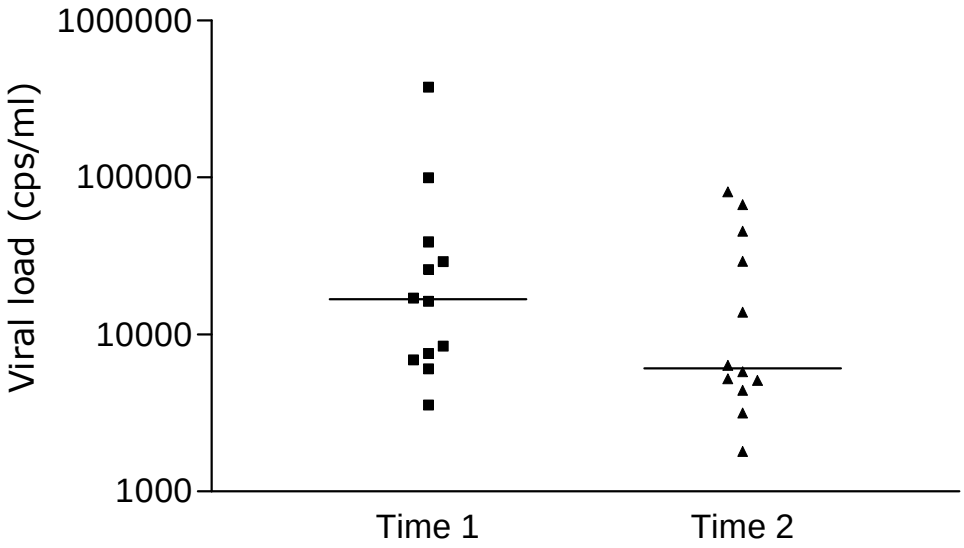
Figure 1. Prevalence of drug used





The mean viral load of the overall at time 2 is resulted lower than at time 1, but this difference was not statistically significant (figure 2)

Figure 2. Viral load



No significant differences were found in viral load between responders and non responders patients.

In tables 8 (A/B), 9 (A/B) e 10 (A/B) we listed all the mutations witch appear in the resistance report and in the research report too.

In particular, in table 8 (A/B) we listed the mutations present in the resistance report.

Table 8.A. Resistance mutations. Time 1.

P	NRTI mutations	NNRTI mutations	PI mutations
1	M41L, Q161Q/L, T215Y	K103N, Y188L	L10I, K20R, M36I, M46L, G48V, I54V, A71V, V82S, L90M
2	E44D, A62V, K65R, L74V, V75I, M184V, L210W, T215N/T/S/Y	V118I	L10I/L, K20K/I/M, M36I/M, M46I/M, F53L/F, I54I/V, L63P, A71A/V, G73S/G, V82A/V, L90M/L
3	M41L, D67N, L210W, T215Y	/	M46I, I54L, L63P, I84V, L90M
4	M41L, L210W	K101E, Y181C, G190A	L10F, K20M, L24I, M36I, M46I, I54V, L63P, A71V, V82A
5	M41L, D67N, L210W, T215Y	V118I	L10F, K20R, L24I, M36I, M46I, L63P, A71V, G73S, V82A, I84V
6	M41L, D67N, L210W, T215Y	/	M36I/M, M46I, I47I/V, I54V, L63P, A71V, G73S, I84V, L90M
7	M41L, D67N, M184V, L210W, T215Y	V118I	L10I, L33F, M46I, F53L, L63P, A71V, G73S, V82A, L90M
8	/	/	L63P
9	M41L, M184V, L210W, T215Y	V118I	K20R, M36I, I54V, L63P, A71V, I84V, L90M
10	F116Y, Q151M, T215D	/	L10I, M36I, M46I, I54V, L63P, A71T, L90M
11	D67N, K70R, M184V, T215F, K219Q	/	M36I, M46L, I50L, L63P, A71V, G73S, L90M
12	M41L, E44D, D67N, M184V, L210W, T215Y	V118I	L10I, M46I, I54I/L, L63P, I84V

Table 8.B. Resistance mutations. Time 2.

P	NRTI mutations	NNRTI mutations	PI mutations
1	M41L, M184V, T215Y	K103N, Y188L	L10I, K20R, M36I, M46L, G48V, I54V, A71V, V82S, L90M
2	M41L, E44D, A62V, L74V, L210W, T215Y	K101K/E,V118I,Y181C,G190S	L10I, K20I, L33I, M36I, M46I, I54V, L63P, A71V, G73S, V82A, L90M
3	M41L, D67N, M184V, L210W, T215Y	K103N	L10F, M36I, M46I, I54L, L63P, A71V, G73T/S, I84V, L90M
4	M41L, L210W, T215Y	K101E, V118I, Y181C, G190A	L10I, K20M, L24I, M36V, M46I, F53L/F, I54V, L63P, A71V, V82A
5	M41L, D67N, L210W, T215Y	V118I	L10F, I13V, K20R, L24I, L33I, E34Q, M36I, M46I, L63P, A71V, G73T, V82A, N83D, I84V
6	M41L, D67N/D, M184V, L210W, T215Y/C	K103N, V108I	L10F, M36I, M46I, L63P, A71V, G73S, I84V, L90M
7	M41L, D67N, M184V, L210W, T215Y	V118I	L10I, L33F, M46I, F53L, I54V, L63P, A71V, G73S, V82A, L90M
8	M184V, T215Y	/	L63P, L90M/L
9	M41L, L210W, T215Y	L100I, K103N, V118I	K20R, M36I, M46L, I54V, L63P, A71I, I84V, L90M
10	Y115F, F116Y, Q151M, M184V, T215D	K103S, Y188H, H208Y	L10I, I13V, K20I, M36I, M46I, I54V, L63P, A71T, G73T, I84V, L90M
11	D67N, K70R, T215F, K219Q	K103N, Y181C, P225H/P	M36I, M46L, I50L, L63P, A71V, G73S, L90M
12	M41L, E44D, D67N, V75M, M184V, L210W, T215Y, K219Q	V118I, G190S	L10I, M46I, L63P, I84V

At time 1 all patients but 1 (pz. 8) show three or more mutations at the transcriptase gene and five or more mutations at the protease gene. The presence of thymidine-associated mutations (TAMs) were more common.

Five patients of 12 (patients 3, 6, 8, 10, 11, about 42%) don't show mutations toward NNRTIs. Between the 7 patients that show these NNRTIs mutations, the prevalent mutation (in 5 patients) was the V118I.

.

At time 2 appear one or more mutations toward the NNRTIs as well in those patients with no mutations at time 1, but in no one of those appears the V118I mutation.

It's likely to be a lack of therapy adherence at time 1 for patient 8, highlighted by any mutation in RT in spite of the therapeutic regimen. For what this patient concern we can consider the resistance test an helpful tool in the determination of the reason of the failure, and in particular to detect it was due to the virus undisturbed replication rather than on a virus resistance.

In table 9 (A/B) we listed silent mutations or other mutations in resistance sites, and in table 10 (A/B) we listed the mutations in codons not known to be associated to a resistance in that time. No significant differences were found between responders and non responders patients considering the number of these polymorphisms.

Only in two patients of 12 (patients 2 and 4) some of mutations in sites of resistance, and only for the transcriptase gene, evolved in resistance mutation included in the report at time 2, and these were Y181Y, G190G and M41I/M for patient 2 and T215T for patient 4.

With new interpretation we observed in the report some adjunctive mutations (table 11 A/B).

Table 9.A. Resistance sites . Time 1

	Silent mutations		Others mutations	
P	**RT**	**PR**	**RT**	**PR**
1	L100L	/	V179I	L63A
2	K103K,V106V,Q151Q,Y181Y, G190G,F227F	/	M41I/M,D67D/G	L24L/F
3	Y115Y	/	/	L10F,K20V
4	K70K,L100L,Q151Q,T215T, P225P,F227F	/	V179F,M230W	/
5	K66K,P225P	/	E44A,K219N	L33I
6	K101K	/	K219K/X	L10F,K20I
7	A62A,K101K,K103K,V179V, G190G,P225P	/	/	/
8	A62A,K70K,F116F	N88N	/	M36M/L
9	G190G	/	/	L10F
10	D67D,L100L,Q161Q,P225P	/	K70S,A98S	K20I,G73T, V82C
11	L100L	V32V	A98A/S, V179I	E35D
12	L100L,Q161Q	/	/	K20K/T

Table 9.B. Resistance sites . Time 2

	Silent mutations		Others mutations	
P	**RT**	**PR**	**RT**	**PR**
1	L100L,P225P	L24L	V179I,F227N/I/Y/F	L63A
2	F77F,Q151Q	/	D67S	K20I,L24F, L33I
3	/	/	K219K/R	K20V
4	K70K,L100L,Q151Q,H208H, P225P,F227F	H69H,L89L	/	E35D
5	T69T,P225P	/	E44A,K219N	E35N
6	K101K,F227F	L33L	/	K20I
7	A62A,K103K V179V G190G P225P	/	/	/
8	F116F	L33L,N88N	K103R	/
9	G190G		/	/
10	D67D,L100L,Q161Q,P225P	V11I,T12S,I15V, Q18QP/E/A,S37D ,I62V,I72T,I93L	K70S, A98S	L33V,E34A, V82C
11	L100	V32	A98A/S	E35D
12	L100L,Q161Q	/	/	K20T

Table 10.A. Other mutations not evaluated in resistance report . Time 1.

P	RT Polymorphisms	PR Polymorphisms
1	T39A,K43K/Q,I135M,I142T,D177N,V189I,I195L,G196R, L214F	L23I,S37N,I62V,I64V,I93L
2	K43K/Q/X,K46K/R,S48P/L/S/F,V60I/V,S68S/G,Q91Q/X,E122K/E,D123E,R125R/S,Y127Y/F,A129A/V ,P133P/L,I135T/I,136N/Y,N137N/I,E138E/D,I142I/F,R143R/S/X/C,I178I/M,G196E/G,T200T/A, H208H/Y,R211K/R,L214F,V245E	I15I/V,L33I/L,E35E/D,S37N,K55K/R,Q58Q/E, I62I/V,V77I/V
3	K43Q,V111I,E122K,D123E,D177E,I178M,V189I,E203D,Q207E, H208Y,R211K,L214F	W6X/S/W,I15V,E35D,S37D,Q61R,I72L,G73S, L76V,I85V
4	C38C/F,K43Q,K49R,E53K/E,V60I,D121Y,S134S/G,I135T,N136K/N,E138A/V,T139T/P,Q145Q/X, P150T/P,W153X/W/C,F160S, S163S/R,M164M/L,E169E/D,K173K/T,I178M,D185D/G,T200L, R206R/S,Q207G,R211K,L214F,T216T/S,D218E/D,Q222Q/X,E233E/X,H235Q/H,D237?,T240T/A	Q7Q/H,I13I/L,I15V/L,Q18Q/E,D29D/V,S37N, G40E/G,R41K,V56R/S/I/G/V,D60E,Q61E,I62V, P79P/L,I85V,T91N/T,I93L
5	T39T/S,K43E,K46K/X,I50M/I/L/F,P52P/L,E53D,E122K/T,D123E,I132K/I,E138E/G,T139Q/P/E/A, R143T/R,L149L/V,D177E,E194A, G196E,R211K,L214F,V245E,L246P/L,P247?	L5?,W6X/W,I13V,A22V,E34Q,E35N,S37D,D60E, I62R/S/I/G/V,I72L,N83D,F99?
6	T39?,R83K,K102Q,E122K,D123E,I135L,E138G,P140Q/P,R143K/R,Q145K/Q,Y146X/Y,P150P/S, K173E,Q174Q/X,I178L,R206R/S/I,H208Y,R211K,L214F,K220K/I,Q222Q/H,K223K/I/X/L,H235H/L, K238K/I,I244K/I,V245E	L33M/L,S37N,I62V,I72R,I85V,I93L
7	T39A,K43E,F61X/Y/L/F,R83K,L120L/F,E122K,A129A/V/S/L,I135K/I,Y144X/Y,P157P/R,T200A,L214F, V245E	V11I/F,I13I/L,I15V,L23I,T26T/I,E34Q,S37D, K43T,G51A,K55R,Q61Q/X,F99Y/F
8	C38G/V/C/F,E40E/D/V,K43K/N,R83K,V90I/V,F124N/I/Y/F,F130Y/F,S134R/S,Y144K/N/X/Y,L214F, E233E/D,L234P/L/S/F,H235N/H, W239X/W,V245E	I151V,S37N,I72V
9	T39A,D123E,I142V,K173Q,R211K,L214F,V245K	L33F,E35D,S37N,R41K,I62V,H69K,T74P,I93M,F 99?
10	T39?,K49R,V60I,S68G,L92L/F,E122K,D123E,I135L,N137S,T165K/T,E169D,P170H,Q174Q,T200A, H208Y,R211K,L214F	V11I,T12S/L,I13V,K14K/M,I15V,E35D,S37D, I62V,I72T,I93L
11	E40Q/E,K49K/R,V60I,R83K,E122K,I135T/I,N136K/N/T,E138E/A,S156S/L,S163S/R,M164M/I, T165T/A,Q174Q/R,D177D/G,Q182Q/P,D186D/Y,V189G/V,D192E/D,L193L/F,E194E/G,Q197Q/P, H198H/D,R199R/X/W,T200T/A,I202G/V,E204E/G,L205M/I/L, Q207Q/P,L209P/L,L214F	S37N,K45K/R,I62V,I64I/V
12	C38?,K43K/Q,V60I,D123N,K166R,D177E,I178M/I,T200T,R211N/D,L214F,W239M,T240A,Q242R, V245E	W6X/W,I13V,S37N,I62V,I64V,L76V

Table 10.B. Other mutations not evaluated in resistance report . Time 2.

P	Polymorphisms RT	Polymorphisms PR
1	T39A,S105T,I135M/V,I142T,D177N,V189I,I195I/L,G196E,R206K/R,L214Y/F,D218E/D,E233K/E, D237D/A,W239X/W	K14K/Y,L23I,S37N,K45K/R,I62V,I64V,I72V,T 91S, I93L
2	K43Q,D123E,I35T,V148G/V,V189I/V,G196E,R206R/M,Q207Q/L,H208Y,R211K,L214F,K220K/I, E224E/D,G231G/C,H235H/L,D237D/Y,V241V/L,V245E	I15V,E35D,S37N,K55R,Q58E,I62V
3	E40E/D,K43Q,V111I,E122K,D123E,E138Q/E,G141G/W,I142N/I/H/L,D177E,I178I/M,V189I, E203N/D,R206R/I,Q207E, H208Y,R211K,L214F,V245G/V,	I15L/V,Q18Q/H,E34Q,E35D,S37D,R57K/R,Q6 1R, I62V,I72L,L76V,I85V
4	K43K/Q/E,K49R,V60I,D121Y,T131T/A,I135T,N137N/Y,E138A,S156S/L,K166R,I178M,T200L,Q207G, R211K, L214F,Q222Q/H,P226P/L/S,L228R,K238N,V245E	I15V,S37N,R41K,D60E,Q61E,I62V,I85V,I93L, T96T/I
5	E42E/G,K43E,E53D,D121H,T139A,I142I/F,R143R/I/G/V,P157P/S,D177E,E194A,G196E,R206R/S/I, R211K,L214F,V241E/V,V245E	K14K/X,A22V,S37D,K55R,D60E,I62V,I72L,T9 1S, Q92Q/E,F99?
6	K49K/N,R83K,K102Q,E122K,D123E,A129T/A,I135I/L,E138G,K173E,I178L,H208Y,R211K,L214F, L228?, W229R/W,Y232N/Y,E233K/E,L234N/I/H/L,H235N/H,D237N/D,V241E/V,I244K/I,V245K/E	S37N,I62V,I72R,I85V,I93L
7	T39A,K43E,S48S/L,P52P/L,R83K/R,E122K,F124I/F,A129E/A,F130Y/F,P133H/P,S134R/S,V148M/V, T200A, R211K/R,L214F,Q242Q/L,P243P/L,V245K/E	W6G/W,L23I,E34Q,S37D,K43T,G51A,K55R
8	T39T/S,E40K/E,S48S/L,R83K,N136K/N/T,E138D/E,P140Q/P,T200A,R206R/I,L214F,D218D/Y, H221H/L, Q222Q/H,K223K/I/X/L, P226P/S,K238K/I/M,I244K/I,V245E	K14K/M,I15R/I,G16A,T26T/S,S37N,I72V,I93 N/I,G94G/W/C,C95C/W/F/L,L97L/F,N98K/N
9	T39A,D123E,I142V,P157P/R,K173Q,R211K,L214F,V245K	T12T/I,I15I/V,E35D,S37N,R41K,I62V,H69K,T 74P, I93M
10	T39A,K49R,V60I,S68G,V111I,E122K,D123E,I135L,N137S,I142I/F,R143R/I,W153G/X/W,G155E/G, E169D, P170H,I178L,T200A,I202I/V,R211K,L214F,L228R,D237E,V245E/V	V11I,T12S,I15V,Q18QP/E/A,S37D,I62V,I72T, I93L
11	C38F,T39?,E40E/D/G/V,K49R,N57K/N,V60I,R83K,E122K,I132T/I,P133H/P,S134N/S,T139T/R, K154K/E, G155R/G,Q174Q/R,T200T/A,I202V,L214F,L228H/L	S37N,K45R,I62V,I64V,N98N/I
12	K43K/R,V60I,K82K/R,R83K/R,D123N,I135T,Q145Q/H/X/Y,K166R,D177E,I178L/F,R211N/D,L214F, D218E, I244K/I,V245T	I13V,S37N,I62V,I64V,L76V

Table 11.A. New interpretation of resistance mutations. Time 1.

P	NRTI mutations	NNRTI mutations	PI mutations
1	M41L, Q161Q/L,T215Y	K103N, Y188L	L10I, K20R, M36I, M46L, G48V, I54V, I62V, A71V, V82S, L90M, I93L
2	E44D, A62V, K65R, L74V, V75I, M184V, L210W, T215S/Y	V118I, H208H/Y	L10I/L, I15I/V,K20K/I/M, L33I/L, M36I/M, M46I/M, F53L/F, I54I/V, Q58Q/E, I62I/V,L63P, A71A/V, G73S/G, V82A/V, L90M/L
3	M41L, E44K/N/E/D, D67N, L210W, T215Y	H208Y	L10F, I15V, K20V, M46I, I54L, L63P, G73S, L76V, I84V, I85V, L90M
4	M41L, L210W	K101E,Y181C, G190A	L10F, K20M, L24I, M36I, M46I, I54V, D60E, I62V, L63P, A71V, V82A, I85V, I93L
5	M41L, D67N, L210W, T215Y	V118I	L10F, I13V, K20R, L24I, L33I, E34Q, M36I, M46I, D60E, I62R/S/I/G/V, L63P, A71V, G73S, V82A, N83D, I84V
6	M41L, D67N, L210W, T215Y	H208Y	L10F, K20I, M36I/M, M46I, I47I/V, I54V, I62V, L63P, A71V, G73S, I84V, I85V, L90M, I93L
7	M41L, D67N, M184V, L210W, T215Y	V118I	L10I, V11I/F, I15V, L33F, E34Q, K43T, M46I, F53L, L63P, A71V, G73S, V82A, L90M
8	/	/	M36M/L, L63P
9	M41L, M184V, L210W, T215Y	V118I	L10F, K20R, D30N, L33F, M36I, I54V, I62V, L63P, H69K, A71V, T74P, I84V, L90M, I93M/L
10	F116Y, Q151M, T215D	H208Y	L10I, V11I, I13V, I15V, K20I, M36I, M46I, I54V, I62V, L63P, A71T, G73T, L90M, I93L
11	D67N, K70R, M184V, T215F, K219Q	/	K20K/R, M36I, M46L, I50L, I62V, L63P, A71V, G73S, L90M
12	M41L, E44D, D67N, M184V, L210W, T215Y	V118I	L10I, I13V, K20K/T, M46I, I54I/L, I62V, L63P, L76V, I84V

Table 11.B. New interpretation of resistance mutations. Time 2.

P	NRTI mutations	NNRTI mutations	PI mutations
1	M41L, K65K/R, D67N/D, Y115Y/F, M184V, T215Y	K103N, Y188L	L10I, K20R, M36I, M46L, G48V, I54V, I62V, A71V, V82S, L90M, I93L
2	M41L, E44D, A62V, L74V, L210W, T215Y	K101K/E,V118I,Y181C,G190S,H208Y	L10I, I15V, K20I, L33I, M36I, M46I, F53L/F, I54V, Q58E, I62V, L63P, A71V, G73S, V82A, L90M
3	M41L, D67N, M184V, L210W, T215Y, K219K/R	K103N, H208Y	L10F, I15L/V, K20V, E34Q, M36I, M46I, I54L, I62V, L63P, A71V, G73T/S, L76V,I84V, I85V, L90M
4	M41L, L210W, T215Y	K101E, V118I, Y181C, G190A	L10I, K20M, L24I, M36V, M46I, F53L/F, I54V, D60E, I62V, L63P, A71V, V82A, I85V, I93L
5	M41L, D67N, L210W, T215Y	V118I	L10F, I13V, K20R, L24I, L33I, E34Q, M36I, M46I, D60E, I62V, L63P, A71V, G73T, V82A, N83D, I84V
6	M41L, E44E/D, D67N/D, M184V, L210W, T215Y/C	K103N, V108I, H208Y	L10F, K20I, M36I, M46I, I62V, L63P, A71V, G73S, I84V, I85V, L90M, I93L
7	M41L, D67N, M184V, L210W, T215Y	V118I	L10I, L33F, E34Q, K43T, M46I, F53L, I54V, L63P, A71V, G73S, V82A, L90M
8	M184V, T215Y	/	L63P, L90M/L
9	M41L, L210W, T215Y	L100I, K103N, V118I	I15I/V, K20R, M36I, M46L, I54V, I62V, L63P, H69K, A71I, T74P, I84V, L90M
10	Y115F, F116Y, Q151M, M184V, T215D	K103S, Y188H, H208Y	L10I, V11I, I13V,I15V, K20I, L33V, M36I, M46I, I54V, I62V, L63P, A71T, G73T, I84V, L90M, I93L
11	D67N, K70R, T215F, K219Q	K103N, Y181C, P225H/P	M36I, M46L, I50L, I62V, L63P, A71V, G73S, L90M
12	M41L, E44D, D67N, V75M, M184V, L210W, T215Y, K219Q	V118I, G190S	L10I, I13V, K20K/T, M46I, I62V, L63P,L76V, I84V

With the new interpretation it has been possible to determine that in all patients several resistance mutations (in red in the table) toward PI appear in both new reports, that mutations seen in the first interpretation as polymorphisms in sites of non-resistance, or neutral mutations in resistance sites, while as far as transcriptase mutations is concerned, only in some patients we observed other resistance mutations, the more frequent being the H208Y.

This mutations discordance caused a few differences in the drug-resistance interpretation.
The amino acid substitutions that confer resistance to the principal drugs in use, considered by latest library (g12) of OpenGene DNA System Software, updated at 2006, are listed in table 6. Some mutations are associated to resistance toward both NRTI and NNRTI.

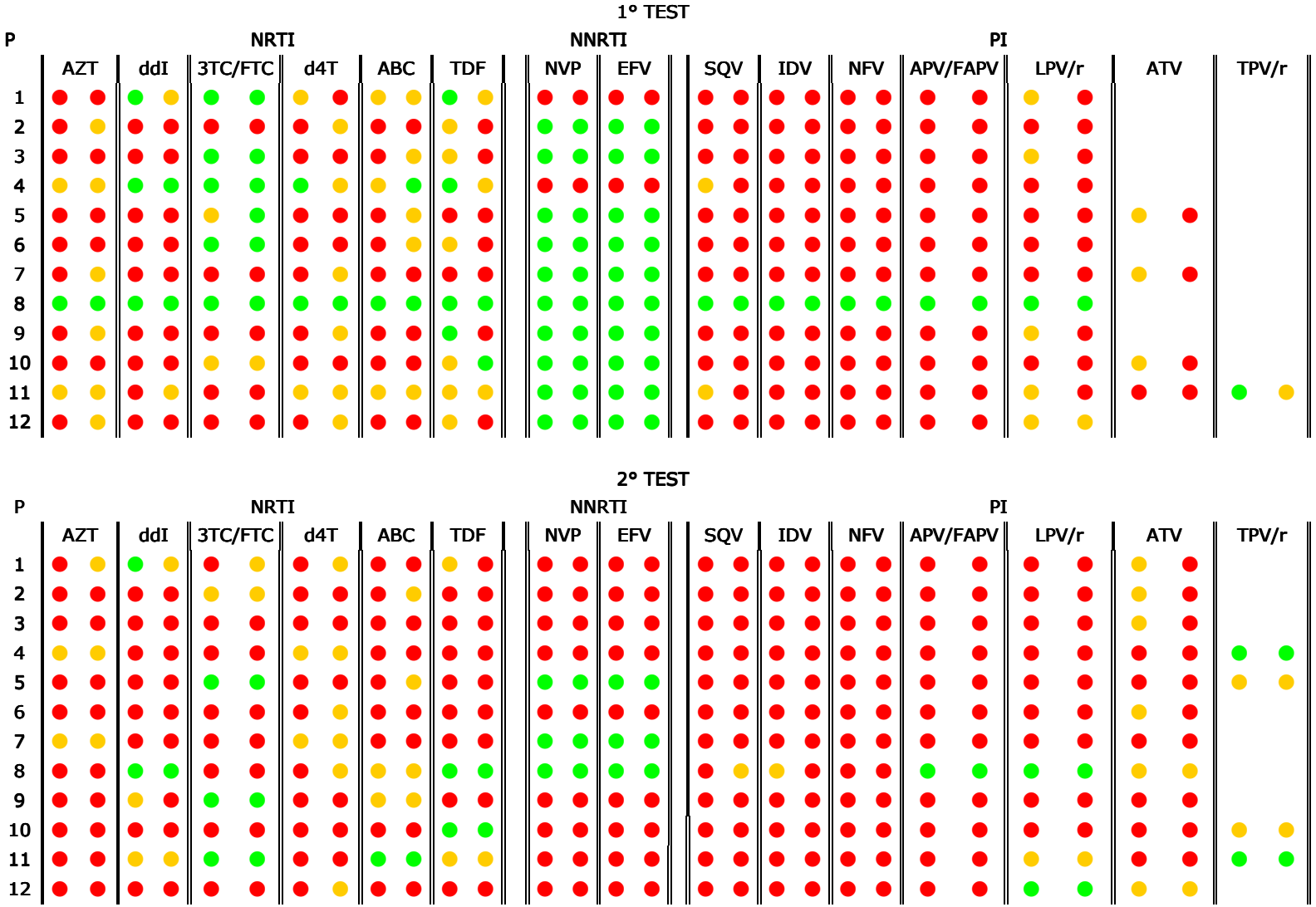
Table 6. Resistance mutations considered by library G12

NUCLEOSIDE RT INHIBITORS	NON NUCLEOSIDE RT INHIBITORS	PROTEASE INHIBITORS
Abacavir (ABC) Didanosine (ddl) Lamivudine (3TC)/ Emtricitabine (FTC) Stavudine (d4T) Tenofovir (TDF) Zidovudine (AZT)	Efavirenz (EFV) Nevirapine (NVP)	Amprenavir (APV)/Fosamprenavir (FPV) Atazanavir (ATV) Indinavir (IDV) Lopinavir + Ritonavir (LPV/r) Atazanavir + Ritonavir (ATV/r) Nelfinavir (NFV) Saquinavir + Ritonavir (SQV/r) Tipranavir + Ritonavir (TPV/r) Darunavir + Ritonavir (DRV/r)
associated mutations	**associated mutations**	**associated mutations**
M41L E44D A62V K65R D67N T69D/N T69XX/XXX/XXXX* K70R/E L74V V75A/I/M/S/T F77L W88G Y115F F116Y V118I Q151M Q161L Y181C M184I/V L210W T215C/D/F/S/V/Y K219E/Q/R	A98G L100I K101E/P/Q K103H/N/S/T V106A/M V108I V118I V179D/E Y181C/I Y188C/H/L G190A/E/S H208Y P225H F227L M230L	L10F/I/M/R/V V11I I13V I15A/V G16E K20I/M/R/T/V L24I/V D30N V32I L33F/I/V E34Q E35G M36I/L/V K43T M46I/L/V I47A/V G48M/V I50L/V F53L I54A/L/M/S/T/V Q58E D60E I62V L63P/T H69K A71I/L/T/V G73A/C/S/T T74P/S L76V V77I V82A/F/L/M/S/T N83D I84A/C/V I85V N88D/S L89I/M/V L90M I93L

Drug-susceptibility of first report and latest interpretation are shown in table 12.

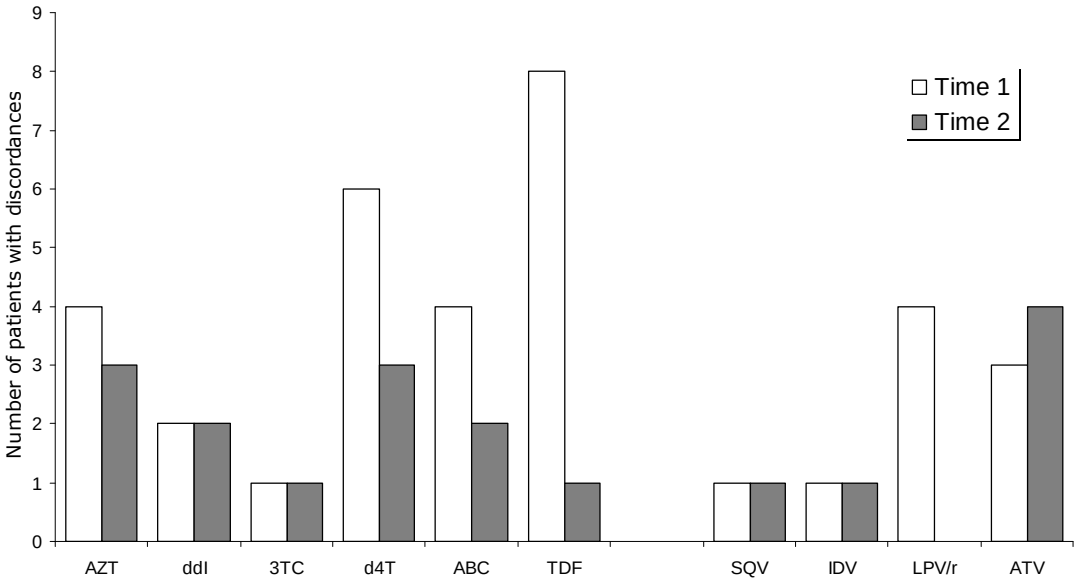
The major discordances between the two interpretations have been observed in the first test, as expected based on previous poorer information about the HIV resistance mechanisms. The most diverse drugs were within NRTIs, stavudine (d4T) and tenofovir (TDF) (Figure 3). The total number of non-responder patients except one at time 1 were in therapy with d4T or TDF. No discordances with new interpretation were noted for NNRTIs.

Table 12. Double interpretation of resistance tests for each patient



Red= Resistance, Yellow= Possible resistance, Green= No evidence of resistance

Figure 3. Drugs with more discordances of interpretation



At the state of art, the laboratory informations collected were compared with the clinical history of each patient and their therapeutic regimen, in order to understand if a different interpretation of the test performed could have lead to a different therapeutic choice.

The interpretation of genotipic test act to detect drug resistance wouldn't have been as effective as expected in order to choose a better therapy, mainly due to the personal individual differences present in a so dishomogeneous choort of patients, and in particolar because of the poor availability of effective drugs at that time.

This is confirmed by the fact that most of these patients are right now positively responding to drugs of the new generation.

Discussion

HIV drug resistance is a complicated and dynamic topic.
Considerable new information on the epidemiology, pathogenesis, and clinical significance of HIV-1 drug resistance and resistance testing was published during the last years.

In fact, genotyping analysis during the course of antiretroviral treatment might provide significant informations that should be carefully considered as guidelines for rational therapeutic strategies.

Despite considerable progress, much remains to be learned about antiretroviral drug resistance.
Treatment of advanced HIV infection is usually characterized by an extensive resistance to all available classes of treatment.

The availability of new drugs and the understanding of treatment principles that have engendered in these patients a greater success than in years past mean that the prospects for such patients have never been brighter. It is likely that additional new drugs and greater understanding of proper management strategies will continue to characterize future management approaches.
Nevertheless, a more profound understanding of genotypic data could allow significant improvements in the management of HIV infection with the current arsenal of antiretroviral drug.

The genotypic analysis is generally limited to the amino acid positions that are known to be involved in drug resistance, while an analysis of the complete amino acid sequences may yield to a wider amount of informations, e.g. on new putative resistance-associated amino acid positions.

In this study we tried to evaluate if a more complete resistance report will improve the strategy of the therapeutic choice for these complex patients.

Some of the informations not included in the report might be more useful than what expected if better analyzed, and more clearly pondered at the moment of the therapeutic choice, even if they seems have no evidence of resistance.

This kind of approach requires a deep and update knowledge of the last discovery about the drug resistance, and a wider experience of the physicians.

The limit of our study is the small number of patients analyzed, and the fact that this is a dishomogeneous group for what concern the characteristics analyzed, if not the presence of multiple therapeutic failure.

Much more is to know about drug resistance than the canonical mutations identified. Anyway, we can conclude that our study added additional knowledge on the genotypic test role to provide a useful resistance report.

We are aware of the difficult for the physicians to keep themselves update on all the new informations coming out daily in this field, but we recognize the necessity of it to a better management of so difficult patients.

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