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Correlation between resistance profile and immunosuppression in heavily treated HIV-1 infected Romanian patients

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Abstract

***Background and Objective**: As available data on HIV-1 strains from Romania indicate the prevalence of a particular subtype- F, not found in other European countries, we aimed at investigating the impact on drug susceptibility of mutations associated with drug resistance and their correlation with the virological and immune response to therapy.*

***Methods**: 38 long term survivors, HIV-1 parenterally infected in childhood, with repeated virological failures, were genotyped for drug resistance and subtype determination. A phylogenetic tree of aligned reversetranscriptase sequences was built.*

***Results**: 94.7% of all the patients strains were subtype F1, clustering together with other Romanian and Angolan F1 strains. Despite the long and complex treatments, 15.8% of patients had wild type virus, 68.4% were fully susceptible to protease inhibitors, 47.3% to non-nucleoside reverstranscriptase inhibitors, 28.9% to nucleoside reverstranscriptase inhibitors. Only 13.2% were resistant to all antiretroviral drug classes. A significantly higher total number of mutations were encountered in severely immunosuppressed patients, who presented also major mutations in the protease gene (V82A, I54V, G48V) and the major M184V mutation associated with type 2 thymidine analogs mutations in reverstranscriptase gene.*

***Conclusion:** A good immune status seems to be associated with a low range of mutations, indicating the impact of immune restoration or preservation on the therapeutic success rate. The slower post-HAART progression of mutational pattern of HIV- 1 subtype F1 in long term survivors may also influence the viral replicative fitness, a fact that can explain its steady prevalence in Romania.*

Key words: HIV, subtype F, antiretroviral resistance, immune status.

Introduction

During recent years significant progress has been made in the treatment of HIV-1 infection. Highly Active Antiretroviral Therapy (HAART), a therapy that combines at least three antiretroviral drugs, usually directed to the *reverse transcriptase* and *protease* of the virus, has led to impressive decrease in mortality and morbidity, especially in developed countries. In addition, the development of novel classes of drugs (including inhibitors that target virus fusion process; viral entry by attachement to CCR5 cellular coreceptors and another key viral enzyme - integrase) has radically changed the clinical outcome of HIV infection. However, problems associated with the complex treatment strategies, such as: long-term side effects, suboptimal drug potency and the necessity of nearly perfect adherence, are important factors leading to treatment failure.

Drug failure has been defined broadly as inadequate viral suppression (virologic failure, defined as a confirmed detectable HIV RNA), a decrease or unsatisfactory increase in CD4+ cell count, or clinical progression, including clinical signs and symptoms related to immunologic dysfunction. A persistently low CD4 count while on therapy is associated with a small, but appreciable, risk of AIDS- and non-AIDS-related morbidity and mortality. As no accepted specific definition for immunologic failure exists, resistance testing should be used to guide selection of therapy regimens. [1]. Emergence of drug resistance remains one of the major threats to the sustained inhibition of viral replication. HIV high replication rate together with the low fidelity of the viral reverse transcriptase work together generating enormous amounts of viral variants, including ones resistant to antiretroviral drugs that can be selected during a specific therapeutic regimen.

Since 1992, the HIV-1 strains isolated from patients all over the world have been classified into four distinct genetic groups: M (major) group, O (outlier) group, N (non-M, non-O) group and the recently described P, [2]. Within the M group nine subtypes have been identified so far (A, B, C, D, F, G, H, J, and K) and inter subtype circulating recombinant forms (CRFs) and unique recombinant forms (URFs) have been described. The variation in the genetic make-up of HIV-1 clades can be as high as 35%, which is highly relevant to antiretroviral development and efficiency, [3]. While the majority of data concerning drug resistance come from subtype B of HIV-1, 90% of HIV-1-infected people worldwide harbor non-subtype B variants [4]; subtype C of HIV-1 being currently prevalent in more than 50% of HIV infections worldwide, especially among heterosexually infected patients, while recombinant forms account for 18% of the global infections.

The Romanian HIV/AIDS epidemic started and evolved as a pediatric one, with the majority of the reported cases represented by children infected parenterally during mid 1990s. Romania is the first country in Central and Eastern Europe that provided universal access to treatment and care for HIV infected patients. In 2010, there were 10,245 people living with HIV/AIDS in Romania, out of which 8,734 are being actively monitored, and 7,306 are currently receiving antiretroviral therapy (ARV) according to international standards (report available online at www.cnlas, [5]; most of them are long term survivors, who acquired the infection during their childhood. Analysis of the data on HIV-1 strains circulating in Romania, both in pediatric and more recently in adult cases also, revealed a stable profile, with a high prevalence of subtype F1- a particular HIV1 clade -that has not been reported in other European countries, [6], [7], [8]. Subtype F1 sequences from Romania are most closely related to those described in Angola, where this subtype was reported in 8-16% of the HIV infected patients and only distantly related to the subtype F1 lineage circulating in South America, where F1 and BF1 CRF are found in more than 10% of the cases.[9]. A cohort of about 700 patients, parenterally infected during childhood, diagnosed with HIV infection before 1995, receive care and treatment through a unique public private partnership between Baylor International Pediatric AIDS Initiative with the local infectious diseases hospital from Constanta. A large number of them have been receiving ARV therapy for a relatively extended period of time (up to 10 years). A part of these patients were followed up for the virological status on a regular basis, at the Stefan S. Nicolau Institute of Virology in Bucharest. The purpose of this study was to analyze amino acids substitutions at codons recognized to confer drug resistance in these heavily treated patients, and to compare the post-HAART progression of mutational patterns of Romanian HIV-1 subtype F1 with similar strains isolated from other countries. We focused on the genomic diversity at a number of sites known to be associated with resistance to each three major classes of antiretroviral drugs: i.e., nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and *protease* inhibitors (PIs).

Materials and methods

Patients.

38 plasma samples collected from HIV-1 infected Romanian patients, long term survivors, parenterally infected in early childhood, who experienced multiple virological failures on HAART (defined as plasma HIV-1 RNA level >400copies RNA/ml after 12 weeks of treatment) were investigated. In all instances, samples were obtained with informed consent. All patients were heavily treated and received several therapeutic regimens over time, such as: 2NRTIs (for at least 24 months), 3NRTIs (6 months), 2NRTIs+1NNRTI (17months), 1NRTI+1NNRTI+1PI (22months), 2NRTIs+2PIs (48months). All patients received regimens containing NRTIs (2 or 3) and PIs (1 or 2), during the last 12 months and eleven patients received NNRTI along with NRTI and PI in their regimen. Administered NRTIs included: didanosine (DDI), stavudine (D4T), abacavir (ABC), zidovudine (AZT) and lamivudine (3TC) (combined as combivir (CBV) or separately), tenofovir (TDF). Administered PIs included: lopinavir/r (Kaletra), tipranavir (TPV), fosamprenavir (FPV), ritonavir (RTV), amprenavir (APV), indinavir (IDV). Efavirenz (EFV) and nevirapine (NVP) were the two NNRTIs administered in this group of patients.

Immunological and virological tests.

CD4+ cell counts were determined by flow cytometry using the TRITEST three-color reagent CD4/CD8/CD3 with TRU-COUNT tubes (Becton & Dickinson, USA). HIV viral load was determined with a commercial nucleic acid amplification test, COBAS AMPLICOR HIV-1 MONITOR TEST version 1.5 manufactured by Roche Diagnostic Corporation, USA. Following isolation of viral RNA, reverse transcription was performed to yield single-stranded complementary DNA and a 155 nucleotide target sequence within the highly conserved region of HIV-1 *gag* gene was amplified by PCR. The lowest detection limit of the test is 400 copies of HIV-1 RNA/mL with the linear range between 400 (log_{10}=2.6) and 750,000 (log_{10}=5.87) copies of HIV-1 RNA /mL.

Drug resistance genotyping was performed using the TRUGENE HIV-1 Genotyping Kit (SIEMENS Healthcare Diagnostics, USA), according to the manufacturer's package insert instructions. This is a sequence-based assay targeted at the protease region (codons 1 to 99) and *RT* region (codon 40 to 247) of the HIV-l genome. Briefly, extraction of plasma RNA, using the QIAamp Viral RNA Mini Kit (Qiagen, USA), was followed by RT- PCR with amplification of a 1,3-bp fragment of the pol gene, (entire protease and the first 250 codons of reverse transcriptase as a single amplicon). RT-PCR product from each specimen was used in each of 16 sequencing reactions, based on the CLIP principle and employing four pairs of primers labeled with Cy™ 5 and Cy™ 5.5 dyes, sequencing bi-directionally the protease reading frame (two pairs) and the beginning and middle of the reverse transcriptase reading frame (one pair each). Separation of the CLIP sequencing reactions by electrophoresis on a polyacrylamid gel, and detection by laser induced fluorescence was followed by analysis using the OPENGENE DNA system software, with a HIV-1 Resistance Report issued for each sample, that provided a list of nucleotide mutations detected in the protease and the reverstranscriptase and their potential impact on the antiretroviral susceptibility.

For subtype assignment, the sequences of the polymerase gene of HIV-1 obtained previously were submitted to the public Los Alamos National Laboratory HIV Sequence Database (http://hiv-web.lanl.gov/) and REGA database (http://www.bioafrica.net/rega-genotype/html/subtypinghiv.html, as described previously [10].

For **phylogenetic analysis** [11] the patient reverse transcriptase sequences were submitted to www.phylogeny.fr, trimmed to correspond to nucleotides 112-742 in the HBX2 reference reverse transcriptase (these boundaries were chosen based on the position of the sequencing primers on the target *RT* genes) and a reverstranscriptase tree was created. The parameters of the workflow included: multiple sequence alignment with ClustalW (v.2.0.3), removal of gaps using the built-in curer, a maximum likelihood method of constructing the tree implemented in the PhyML program and visualization with TreeDyn (v.198.3). Branches with less than 50% support were collapsed. Reference sequences included: a consensus subtype B reference virus HBX2 and several F strains: two F1 subtypes from Brazil (accession numbers: AY173958, FJ77101q0), two F1 from Romania (AB485658, HM191561), one F1 from Angola (FJ900266), one F2 from Belgium (EU248361), one F2 from Cameroon (AF377956), one F from Congo (FM164921), one BF from Brazil (AY771591), one BF1 from Brazil (AF005495). In addition, for RT, one F1 sequence from Romania (EU032009), and one F1 from Angola (EU068447) were chosen.

Accession numbers.

Genotyped reverse transcriptase sequences were deposited in GenBank under accession numbers JF268256, JF268257, JF280766-JF280791.

Statistical analysis.

Statistical analyses were performed using Stata IC version 11.1 (StataCorp, College Station, TX). Differences in the distribution of age and gender within three groups by CD4 count were tested using the Pearson χ^2. Mean group differences in number of mutations to different classes of drugs were compared using the Wilcoxon rank-sum test. $p<0.05$ was considered statistically significant.

Results

Subtype and phylogenetic analyses.

According to the REGA database, the majority of the infecting strains (36/38) were HIV-1 subtype F1. The remaining two sequences were classified as not assigned (NA) using the same method. The subtype was assigned based on sequences over 800 bp, clustering with a pure subtype, with bootstrap 70%, without recombination in the bootscan. All 38 patient sequences had 100% bootstrap in the F1 subtype. All Romanian strains clustered close together. The phylogenetic analyses based on the *RT* sequence (Figure1) show distinct clustering of all patient strains, together with other available Romanian F1 sequences, and more closely related to the F1 Angolan sequences than to the Brazilian ones.

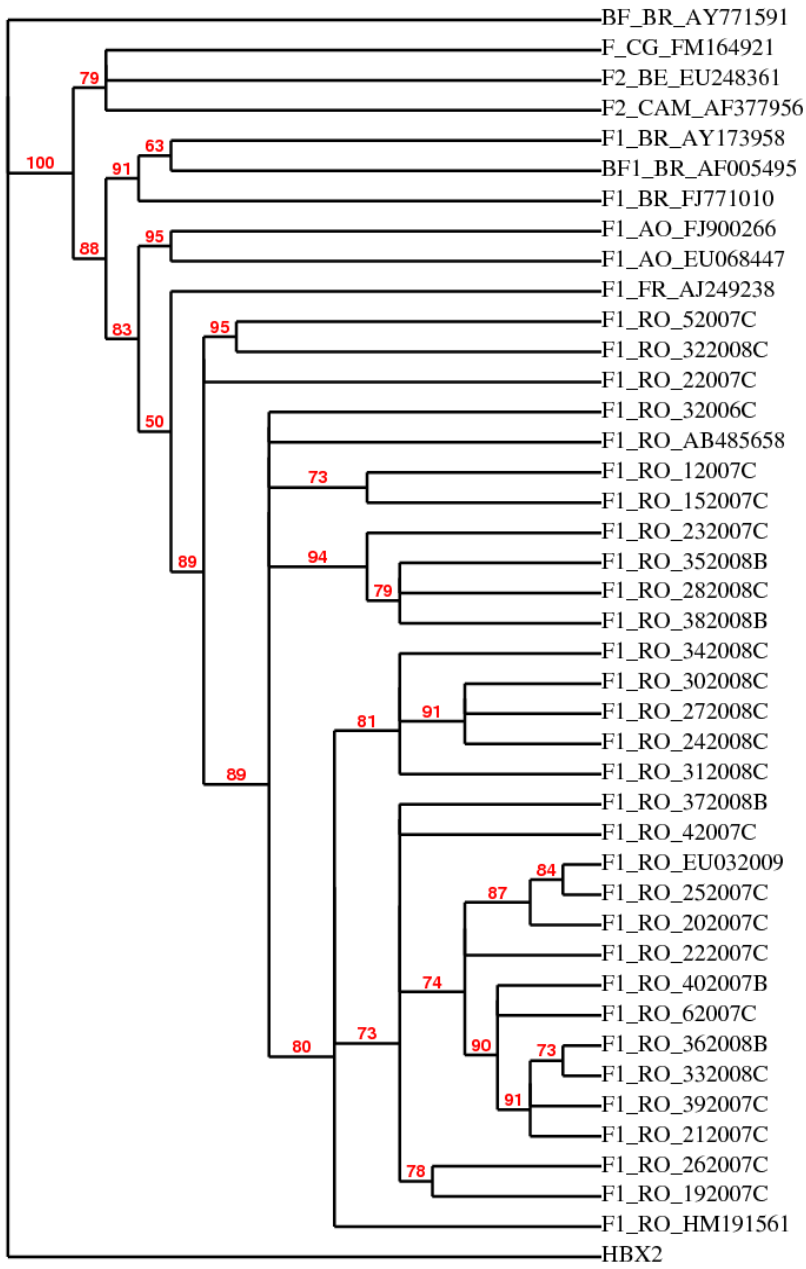

Figure 1. Phylogenetic tree based on the *reverse transcriptase* sequence (630 nt) Red font values represent percent branch support values. (See Material and Methods for phylogenetic analysis)

Virological and immunological characteristics of the patients. The average CD4 cell count was 296 cells/μL (range: 3-1533) and the average HIV-1 viral load (VL) was 4.66 ± 0.68 $\log_{10}$ RNA copies/ml (Figure 2). Twenty one out of the total of 38 patients (55.3%) had severe immunosuppression (CD4 count < 200 cells/ul); 11 patients (28.9%) had moderate immunosuppression (CD4 count 200-500 cells/ul), while 6 (15.8%) patients showed no sign of immunosuppression (CD4 count > 500 cells/ul).

There was a significant trend of increasing viral load from the group with the highest CD4 count to the group with the lowest CD4 count (p=0.01).

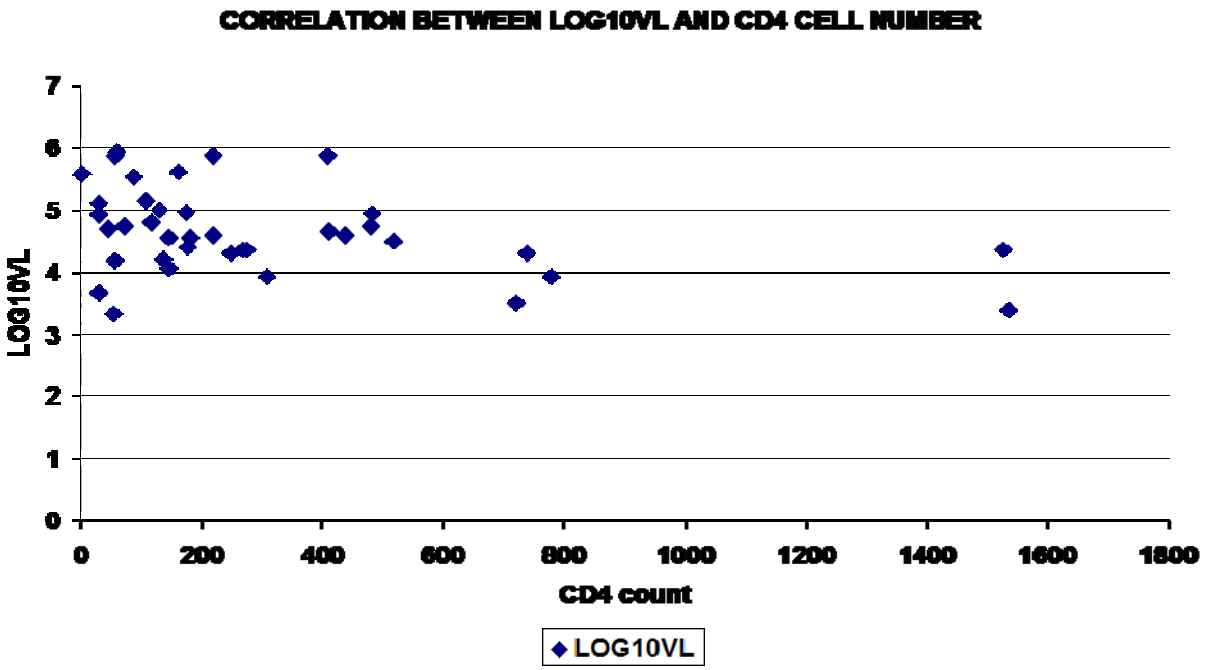

Figure 2. Correlation between $\log_{10}$ VL and CD4 cell number shows a significant trend of increasing viral load from the group with the highest CD4 count to the group with the lowest CD4 count. Each point represents a patient sample.

Evaluation of the susceptibility to antiretroviral drugs. Wild type virus (with no resistance mutations at all) was detected in 6 out of 38 patients (15.8%). Of all the study patients, 68.4% were susceptible to all PIs; 47.36%, to all NNRTIs and 28.9% to all NRTIs. Only 5/38 (13.1%) patients presented resistance to all classes of antiretroviral drugs (Figure 3).

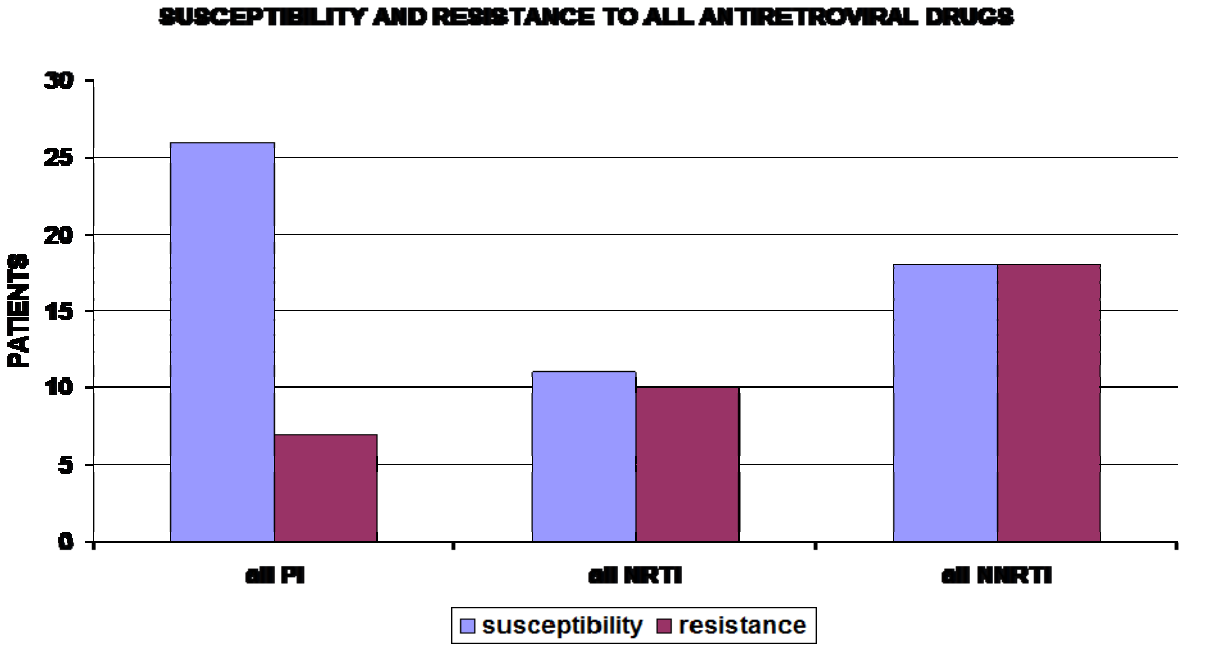

Figure 3. Predicted susceptibility and resistance to antiretroviral drugs, based on HIV-1 Resistance Report obtained after sequencing with TRUGENE® HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics, USA), see Materials and Methods section.

The highest total number of mutations was encountered in severely immunosuppressed patients - 36.52 ± 19.45 versus 29.55 ± 15.2 in those with moderate suppression and 22.83 ± 8.29 in those without immunosuppression (p=0.08) (Table 1).

Table 1. Analysis of viral load (VL) and number of resistance mutations to PI, NRTI, and NNRTI by CD4 groups. Results for viral load and number of mutations are given by average± SD/range; (*) stands for statistical significant

Average± SD (Range)	CD4 <200 cells/ul 21 patients	CD4 200-500 cells/ul, 11 patients	CD4 >500 cells/ul 6 patients	p value	χ^2 test for trend, p value
VL at genotyping $\log_{10}$ copies RNA/ml)	4.80 ± 0.69 (3.32-5.92)	4.75 ± 0.62 (3.93-5.88)	4 ± 0.47 (3.39 - 4.51)	0.03*	0.01*
No. of total PI mutations	13.9 ± 5.14 (4-25)	9.73 ± 5.42 (1-19)	6.16 ± 4.02 (0-11)	0.005*	0.001*
No. of major PI mutations	1.57 ± 2.38 (0-8)	0.81 ± 1.6 (0-5)	1 ± 1.55 (0-3)	0.71	0.49
No. of minor PI mutations	0.81 ± 0.81 (0-3)	0.64 ± 0.67 (0-2)	0.5 ± 0.55 (0-1)	0.75	0.41
No. of other PI mutations	11.52 ± 4.66 (0-20)	8.27 ± 5.04 (0-15)	4.66 ± 4.03 (0-11)	0.01*	0.003*
No. of NNRTI mutations	1.38 ± 1.32 (0-4)	0.64 ± 0.81 (0-2)	0.5 ± 0.55 (0-1)	0.2	0.07
No. of NRTI mutations	2.86 ± 2.76 (0-8)	2.36 ± 2.73 (0-8)	3.33 ± 2.07 (2-6)	0.39	0.5
Total no. of other RT mutations	18.38 ± 15 (0-58)	16.82 ± 11.1 (0-38)	12.83 ± 10.1 (0-22)	0.81	0.59
Total no. of mutations	36.52 ± 19.45 (9-81)	29.55 ± 15.2 (5-50)	22.83 ± 8.29 (13-32)	0.22	0.08

The mutational pattern for different antiretroviral drug classes is summarized in Table2.

Table 2. Summary of patents resistance to different antiretroviral (ARV) drugs classes.

Resistance to at least one ARV from	% of patients	Major mutations
NRTI	71.1	M184V, TAM 2 (D67N, K70R, T215F)
NNRTI	52.7	K103N, Y181C
PI	31.6	V82A, I54V, G48V

NRTI resistance mutations. 26.3% of the studied patients, irrespective of the immunological group, were resistant to all NRTIs. The major NRTI resistance mutation M184V was the most commonly detected (in 52.3% of the severe immunocompromissed patients versus 36.4% and 33.4% of patients with moderate or absent immunosuppression). This substitution confers high level resistance to lamivudine and emtricitabine, but increase susceptibility to zidovudine, stavudine and tenofovir. In fact none of the patients displayed high level resistance to tenofovir, despite the fact that 44.8% of them received this drug during antiretroviral treatment. Thymidine analog mutations, TAMs, selected by zidovudine (AZT) and stavudine (D4T) were usually accompanying this mutation; TAM-2 pathway (mutations D67N, K70R, T215F) was most commonly encountered in immunossupressed patients (28.6%), as well as other substitutions that by themselves, or while accompanying other mutations, confer NRTI resistance: K219E/K/Q (38.1%) and T69N (19%). 19.5% of the patients presented L74V/I mutation that occur in patients with virologic failure while receiving non-TAM regimens and causes intermediate resistance to DDI and ABC, and a slight increase in susceptibility to AZT and TDF. None of the patients presented K219N/R, the mutation that usually occurs in heavily NRTI treated patients, nor multi-nucleoside resistance, via the Q151 or the 68-69 pathway.

NNRTI resistance mutations. 52.4% of the severely immunosupressed patients were highly resistant to nevirapine and 42.85% to efavirenz (EFV) and delavirdine (DLV), a drug never used in our patients. 81.8% of the patients who had received one NNRTI in therapy combination during the last 6 months displayed a range of 1-4 NNRTI mutations. The major NNRTI resistance mutation, K103N, which causes high-level resistance to nevirapine and variable resistance to efavirenz, occurred in 28.9% of all studied patients. Interestingly, six out of the 11 patients with the K103N mutation had not been treated with NNRTIs. Five out of the 38 of patients presented already a mutation at codon 181 that compromise susceptibility to etravirine- a "next-generation" NNRTIs compound that was never used in these patients.

PI resistance mutations. Only 18.4% (7/38) of all patients were resistant to all PIs. High level of resistance to nelfinavir, (NFV) and saquinavir (SQV) was recorded in all immunological groups, but the highest total number of PIs mutations was recorded in severely immunosuppressed patients (p=0.005) (Table 1). Five out of the 21 patients with severe immunosuppression harbored major PI mutations V82A, I54V, and G48V associated with phenotypic resistance to ritonavir.

A series of minor and accessories PI mutations were present in the *protease* gene of all patients, with a significant trend of increasing number of mutations in those with major immunosuppression (p=0.003). The most commonly encountered polymorphisms that may induce early development of drug resistance, were L63T, present in 80.5% of the cases and M36I in 77.8% of the cases, followed by L89M (50%), K20R (72.3%) and L10V (63.9%) of the patients. Accessory PI mutations occurring at these codons usually up-regulate *protease* processivity to compensate for the decreased fitness associated with the major PI resistance mutations that occur at codon 189-193.

Additional PI-selected accessory mutations were found only in the severe immunosuppressed group and include the highly polymorphic mutations I13V (23.8%), D60E (14.3%) and K55R, an uncommon non-polymorphic mutation.

Discussion

In Romania, the steady prevalence of a single HIV-1 subtype, different from the ones circulating in the neighboring countries or in the rest of the European Union was interpreted as a proof of the origin of the epidemic in a single or limited source of infection. [12]. Our

study confirms these data, with the majority of patients, long term survivors, parenterally infected as children, more than 15 years ago, still harboring HIV-1 F1 subtype. The phylogenetic analysis showed that HIV-1 F1 sequences from all patients form a monophyletic sub-cluster with other Romanian strains, and are more closely related to the Angolan than to the Brazilian ones, in accordance with the findings of other authors [9], indicating a distinct spread of an original African F1 strain to Romania, Angola, and South America. These data substantiate that so far, HIV-1 subtype F1 remains dominant in Romania, without any reports of intersubtype recombinants being signaled in our country, despite the increased trend in travel and migration seen in Eastern Europe and the sporadic introduction of multiple distinct viral strain. The molecular characteristics that could confer a selective replicative advantage for this strain are worth being investigated.

Despite the long course of treatment and the administration of various therapy regimens, 15.8% of all patients did not present any mutation conferring drug resistance. The majority of patients, 68.4%, were still fully susceptible to all *protease* inhibitors and almost half, 47.36%, to all non-nucleoside reverse transcriptase inhibitors. Due to the small sample size and the absence of longitudinal follow-up, as well as to the fact that the use of population based sequencing prevented us from detecting minority strains containing mutations, we cannot exclude an underestimation of the degree of resistance. Self reported compliancy with therapy requirements was good in our patients, but the intricate relationhip between adherence and development of resistance must be always carefully considered.

The highest degree of resistance was present for nucleoside analogue reverse transcriptase inhibitors (71.1% patients), facilitated by the presence of TAMs, a fact that can easily be explained by the long time exposure to non-suppressive regimen based only on thimidine analogues. However, only1/4 of all patients showed cross-resistance to all nucleoside or nucleotide analogues, and NRTIs are supposed to remain useful in combinational antiretroviral therapies, as reports from a large European cohort (EuroSIDA) documented a slow rate of accumulation of TAMs with time and a ceiling effect for developing additional TAMs in treated patients, even in those who continued to receiving virologically failing regimens [13].

In our patients, the same TAM 2 resistance pathway conferring resistance to NRTIs encountered in subtype C-infected patients from Botswana (67N/70R/215Y) occurred in a higher percentage (63.8%) than TAM-1, as reported previously. [4]

In our study group, all patients presented high levels of viral load, but major resistance mutations to PI, NRTI, NNRTI occurred mainly in patients with impaired immunity. Severe immunosuppression may be a risk factor for mutations development, the most prevalent major mutations being in the *RT* region, inducing high level of resistance to all NNRTI and NRTI in a significant number of patients. It has been recently reported that subjects with more than one virological failure have slower rates of immune recovery than those without virological failure both in terms of CD4 annual increase and in the period of time needed to reach a CD4(+) cell count of >300 cell/ul [14].

We report for the first time the emergence of major PI resistance mutations in heavily treated HIV 1 infected patients, despite the relatively recent introduction of PI in our patient's therapy regimen and the natural higher genetic *PR* barrier to mutations [15]. High level resistance was recorded for nelfinavir (in 7/38, 18.4 % of the patients), indinavir and saquinavir (in 4/38, of the 10.5% patients). 92.1% of studied patients had at least 3 PI accessory mutations, which were not always associated with decreased predicted susceptibility to protease inhibitors. However, the presence of multiple secondary protease mutations was already reported in the HIV-1 F subtype strains isolated in Romania from antiretroviral drugs treatment-naıve patients, indicating transmitted drug resistance that may favor later development of PI major

mutations and can affect the efficacy of these potent antiretroviral drugs. Notably, in our patients a good immune status was associated with a low range of PI mutations, indicating that further use of PI inhibitors might be dependent on immune restoration or preservation.
Although 80% of mutations related to antiretroviral resistance in subtype B also seem to confer resistance in other subtypes, there are controversial reports regarding the impact of genetic diversity on the emergence of drug resistance, especially with regard to NNRTIs drug resistance. Group O viruses are resistant to this class of antiretroviral drugs, resistance develops faster in subtype C, due to selection of V106M [4] and resistance to nevirapine after its administration for prevention of mother-to-child transmission in Uganda was more frequently encountered in subtype D than in subtype A [16]. EFV and NVP were the only NNRTIs used to treat the patients in this study, but high level resistance to all NNRTIs was present in 47.3% patients (18/38). Notably in our study, a high percentage of patients displayed NNRTI associated mutations without evidence of exposure to this drug class. Regarding *protease* inhibitors susceptibility, in Brazilian subtype F1 isolates [17], presence of the L89M polymorphism in the *protease* gene was related to the maintenance of viral fitness, conferring a higher genetic barrier to the accumulation of the L90M resistance mutation, the latter being considered a predictor of failure to Kaletra-based regimens. A similar pattern was displayed by isolates from our study, 50% of these harboring the L89M polymorphism, while only 5.3% the L90M mutation. I93L, found in two F1 patients from this study, one susceptible to all PIs and another one with low degree resistance to some of the class compounds is a secondary resistance mutation in subtype B that causes hyper susceptibility to PIs in subtype C isolates. [18] The M89I mutation, found to emerge in F, G, and C subtypes after nelfinavir failure, and also associated with increased susceptibility to other PIs, except for lopinavir [4], was not found in our group.

Conclusion

In our patients a good immune status was associated with a low range of PI mutations, indicating that further use of PI inhibitors might be dependent on immune restoration or preservation. In spite of the multiple treatment regimen changes and several virological failures, susceptibility to antiviral drugs (mainly to PI and NNRTI) was preserved in these studied patients. This slower post-HAART progression of mutational patterns in HIV1 F1 subtype in long term survivors may greatly influence the viral replicative fitness and can account for the stable prevalence of this subtype in Romania.

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