# ACQUIRED DRUG RESISTANCE TO NRTI CLASS IN TREATMENT-EXPERIENCED HIV INFECTED PATIENTS FROM THE CONSTANTA COUNTY: THERAPEUTIC IMPLICATIONS

**Roxana-Carmen Cernat[1], MD,**
**Assoc. Prof. Irina Magdalena Dumitru[1,2], MD, PhD, D. Otelea[3], MD, PhD,**
**Prof. Sorin Rugina[1,2], MD, PhD**

*[1]Clinical Hospital of Infectious Diseases, Constanta*
*[2]"Ovidius" University of Constanta*
*[3]"Prof. Dr. Matei Bals" National Institute of Infectious Diseases, Bucharest*

## ABSTRACT

**Objective.** To determine the prevalence of acquired drug resistance (ADR) and of resistance patterns in treatment-experienced HIV infected patients from Constanta in order to establish the best therapeutic options in NRTI class.

**Material and methods.** A retrospective study which included 144 treatment-experienced HIV patients with confirmed viral failure. The strains isolated from these patients were analysed in the Molecular Genetic Laboratory of „Matei Bals" National Institute of Infectious Diseases, Bucharest and the resulting sequences were saved in FASTA format. The HIV-1 subtyping was based on „REGA HIV01&2 Automated subtyping tool version 2.0" algorithm. „Stanford HIVdb Program version 8.4" was used in order to determine the therapeutic options. For statistical calculations, the R-Project software was used. Graphic representations were performed using GNUPLOT program.

**Results.** The prevalence of the acquired drug resistance was 92.36%. The most frequent mutation occurred at the level of the codon 184. The TAM-2 path was more frequently selected compared to TAM-1. Association between TAM1 and TAM 2 were also found, mutation K65R being rarely met.

**Conclusions.** The prevalence of the acquired drug resistance in our study was high, The most valuable therapeutic option in the INRT class remains tenofovir, due to the mutational profile, which was selected on account of the extensive use of thymidine analogues.

**Keywords:** HIV, NRTI, Acquired Drug Resistance (ADR)

## INTRODUCTION

HIV resistance to antiretrovirals is defined by the ability of the virus to mutate and to multiply in the presence of an inhibitor. Therefore, the concentration of the drug must be increased in order to achieve viral suppression, but even so, increased concentrations only partially succeed in supressing viral replication. The first occurrence of this phenomenon dates back to 1989, with AZT as monotherapy, this drug being the first NRTI used. Subsequently, other molecules of this class were synthetized and used in combinations and thus they became the foundation on which the therapeutic regimens of HIV-infected patients were built.

The NRTI mechanism that causes inhibition of viral replication is well known: the molecules act as alternative substrate, complementing physiological nucleosides (deoxyadenosine, deoxythymidine, deoxyguanosine and deoxycytidin), from which they differ in a minimal change and, as such, the inclusion of NRTIs leads to the impossibility of synthesizing and elongating the viral DNA.

NRTIs are divided into two subgroups:

1. thymidine analogues: zidovudine (AZT) and stavudine (d4T) acting in activated CD4 cells,

Corresponding author:
Roxana-Carmen Cernat, Clinical Hospital of Infectious Diseases, 100 Ferdinand Boulevard, Constanta
E-mail: roxana.cernat@seanet.ro

2. non-thymidine analogues: which act in both activated and dormant CD4 cells, include lamivudine (3TC) and emtricitabine (FTC)-analogues of cytidine, as well as didanosine (ddI) and zalcitabine (ddC)

Nucleotide reverse transcriptase inhibitors have the same mechanism of action as the ones mentioned above, but require only double phosphorylation, being already monophosphorylated. They are active in many cell types, including those that are dormant (lymphocytes, monocytes, macrophages) and have good intracellular penetration. The representative of this subclass is tenofovir TDF. Recently a prodrug, tenofovir TAF (tenofovir alafenamide fumarate) proved to have properties superior to TDF (concentration in PBMCs 5-7 times higher, 90% lower in plasma) (1).

Resistance to INRT class is based on two distinct mechanisms:

**a.** Increased phosphorylation via ATP or pyrophosphate that results in excision of NRTIs already incorporated into DNA. It is determined by mutations in the codons M41L, D67N, K70R, L210W, T215Y, K219Q (9), mutations called Thymidine Analogue Mutations (TAMs). Their accumulation is gradually acquired in a therapeutic regimen containing thymidine analogues, their presence leads to cross-resistance between the class compounds. There are two distinct pathways of TAMs: TAM-1 is described by the occurrence of the mutations M41L, L210W, T215Y and TAM-2 by the occurrence of D67N, K70R and K219Q / E (2).

**b.** Steric inhibition caused by mutations that render the reverse transcriptase (RT) capable of recognizing structural differences between NRTI and physiological deoxynucleotides. In the presence of M184V, Q151M, L74V, or K65R, incorporation occurs in favour of the deoxynucleotides, avoiding NRTIs (3).

There are numerous studies indicating that a different selection of these pathways leads to different therapeutic responses. An example would be the selection of the TAM-1 pathway which, compared to the TAM-2 pathway, has a greater impact in terms of resistance to AZT, thus influencing more significantly and decreasing the susceptibility to ABC, ddI and TDF (4).

The HIV epidemic in Constanta is characterized by a significant number of cases originating from the paediatric cohort, most likely infected by parenteral mode between 1986 and 1990 (5), given the pre-existence of the virus in the adult population (6). From the epidemiological sources of the Ministry of Health, there is evidence that this local epidemic overtakes the national epidemic by one year (7). Having access to AZT since 1995, and subsequently to bi-therapy with ddC, 3TC, ddI, these patients have extensively used NRT-based regimens in mono-, bi- and tri-therapy. Another characteristic of the Constanta group was the access to lopinavir/ritonavir (LPV/r) after 2001 (8). In an early access trial, the most common therapeutic association was a combination of 2 NRTIs (AZT + 3TC or ddI + d4T) and LPV/r. If the resistance profile induced in this LPV/r population was already published, the profile induced by NRTIs has only been assessed in one national study (9) involving patients across the Romanian paediatric cohort.

## MATERIAL AND METHOD

### Population sample

A group of 144 patients diagnosed and confirmed with HIV infection, who are in the care of the Constanta Regional Centre, were included. The patients were under antiretroviral therapy for at least 6 months and were with confirmed virological failure (HIV-RNA > 1000 copies/mL, required level for RT genotyping). The following information was collected: demographic data, infection pattern, nadir CD4 values and zenith HIV-RNA, CD4 and HIV-RNA values at genotyping, year of diagnosis and the year of ART initiation, type of ART used, time of exposure to NRTIs, the number of therapeutic regimens, the time (in months) of virological and immunological failure, the cumulative mutations resulting from the genotyping tests, the number of genotyping assays performed.

## METHODS

Genotyping tests were carried out in the Laboratory of Molecular Genetics of the „Matei Bals“ National Institute of Infectious Diseases from Bucharest. For HIV-RNA sequencing, the Viroseq™ HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA) kit was used, for RT-PCR the GeneAmp System 9700 (Applied Biosystems) system

was used. The products obtained through amplification were bi-directionally sequenced with the ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). For the initial analysis of the sequence, the Sequencing Analysis Software Version 3.7 (Applied Biosystems) was used, and subsequently the sequences were assembled with ViroSeq 2.5 / 2.7 / 2.8HIV-1 Genotyping System Software (Celera Diagnostics, Alameda, CA).

The resulting sequence, representing the entire PR gene and two thirds of the RT gene, was saved in the FASTA format. HIV-1 subtyping was performed on the basis of the REGA HIV-1 & 2 Automated subtyping tool version 2.0 algorithm. The mutations in the reverse transcriptase structure were those listed in the IAS-USA Expert Group's Consensus article published in January 2017 (update to the 2015 version) (10), respectively: M41L, A62V, K65REN, D67N, T69ins, K70R, L74V, V75I, F77L, Y115F, F116Y, Q151M, M184VI, L210W, T215Y F, K219QE. FASTA files were processed with the Stanford HIVdb Program version 8.4 algorithm (2017-06-16).

The CD4, CD8 and CD4 / CD8 lymphocyte assays were performed in the Constanta Hospital for Infectious Diseases Hospital using FACSCount produced by Becton Dickinson. HIV-RNA determinations were performed using the Cobas TaqMan48 Roche system, with a detection limit of 48 copies /mL, and Cepheid GeneXpert from 2016, with a detection limit of 40 copies / mL.

The population group was analysed and quantified using descriptive statistics. For statistical calculations, the R-Project program was used. Graphical representations were made using the GNUPLOT program.

## RESULTS

The study group was somewhat balanced, with a slightly predominance of males (51.39%), mostly of urban provenance (62.5%). The median age was 29 years (range 5 to 68 years). The most important mode of HIV acquisition was parenteral, 65.27% of subjects belonging to the paediatric cohort; 11.11% were infected vertically (most of them before year 1990) and the remaining 23.61% by sexual contact

Although very young, the patients in this group had a long history of illness, the median duration of HIV infection being 12 years, 87.5% being already in C clinical category according to CDC Atlanta 1993 classification.

The median CD4 cell count at the last genotyping was 277 cells/mm$^3$ with a median nadir CD4 cell count of 78 cells/mm$^3$. HIV-RNA at the last genotyping (median value) was 4.31 $\log_{10}$ copies/mL, whereas during the course of the disease the patients in the batch showed higher viral loads, with a zenith HIV-RNA median value of 5.02 $\log_{10}$ copies/ml.

The median duration of ART administration was 10 years, with a median number of therapeutic regimens of 5 (1-12). A small proportion had a history of AZT monotherapy (4 patients, 2.78%), another 64 (44.44%) experienced dual therapy, all of them being exposed later to tri-therapy or even quadruple therapy. Although they experienced months of viral failure, most subjects, 70.83%, had a single resistance test during the course of the disease progression; for the other patients in the group, between 2 and 5 genotyping analyses were performed.

The most common viral subtype was 92.36% F1, with rare cases of B (2.78%), C (2.78%), A1 (0.7%) and two recombining circulating forms CRF02_AG (0.7%) and CRF14_BG (0.7%).

### Mutations associated with resistance to NRTIs

The prevalence of amino acid substitutions in the reverse transcriptase gene in the study group was 92.36%. The RT gene mutations for the studied group are described in Fig. 1.

The most frequent mutation selected in this class was at the level of codon 184, due to the exposure to 3TC or FTC. A total of 131 subjects (90.97%) selected M184V mutation, and 2 (1.39%) the M184I mutation. As this mutation is the first to occur in a failing regimen, and 3TC is one of the most commonly used drugs in both fixed combinations (ABC / 3TC, AZT / 3TC, AZT / ABC / 3TC), but also as an individual part of a therapy, it is understandable why it was found with such a high frequency. The median exposure to 3TC was 75.5 months; exposure to 3TC/FTC and resistance mutation being represented in Fig. 2.

To note the fact that the 2 patients who were not in therapeutic failure while on regimens including 3TC or FTC, had previously been exposed to 3TC, mainly to maintain susceptibility to TDF under

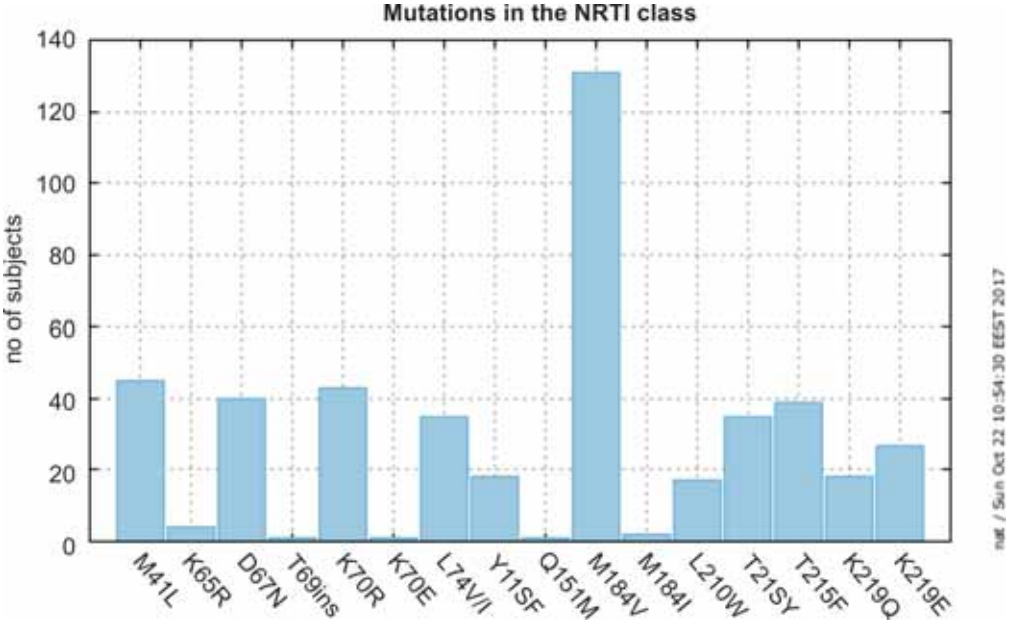


***FIGURE 1.*** *Mutations in the NRTI class*

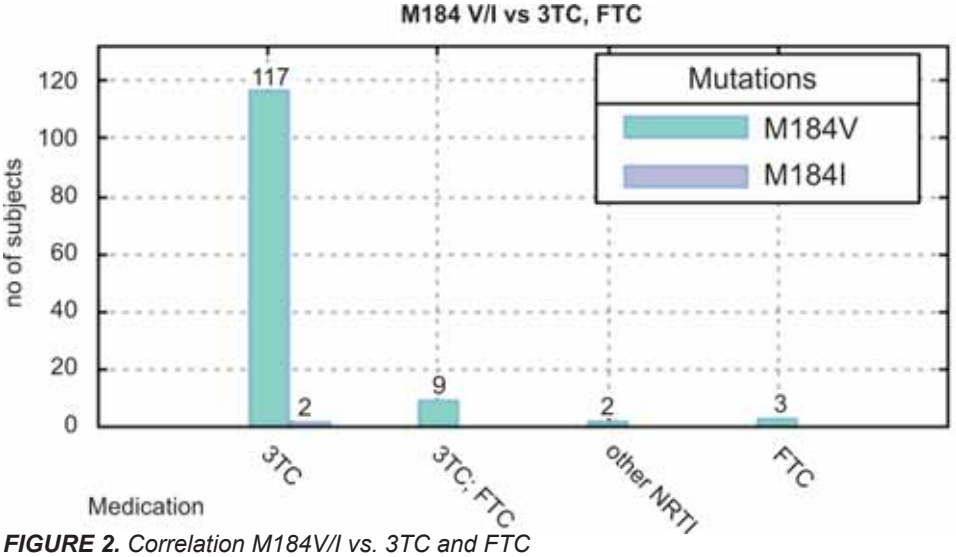


***FIGURE 2.*** *Correlation M184V/I vs. 3TC and FTC*

high triple-class resistance demonstrated by previous genotypes.

The use of thymidine analogues represented by AZT and d4T resulted in the occurrence of TAMs mutations. From the group of patients who developed these mutations in the TAM pathway as an acquired resistance mechanism, 89.5% (129/144) were exposed during their therapeutic history to AZT or d4T.

The mutations selected by these drugs are represented in Fig. 1. The most common was the TAM-2 path, respectively D67N, K70R, T215F, K219Q / E, the prevalence rates for each of these mutations being: 27.77% at D67N, 29.86% to K70R, 27.08% to T215F and 31.25% to K219Q/ E. The highlighted TAM-1 mutations were: M41L (31.25%), L210W (11.80%) and T215Y (24.30%). Particularly interesting is the association of the two-pathway mutations (Fig. 3). The most common associations were: T215Y at D67N, K70R, K219Q/ E and T215F at M41L.

A small percentage of the patients were exposed only to d4T, AZT being more commonly used as the only thymidine analogue (Fig. 4). While AZT was frequently associated with 3TC, d4T had been used in combination with ddI, before this association was prohibited for cumulative toxicity. Most patients had sequential therapy with AZT and d4T during the therapeutic history. The median exposure to the two thymidine analogues were 49 and 11 months, respectively.

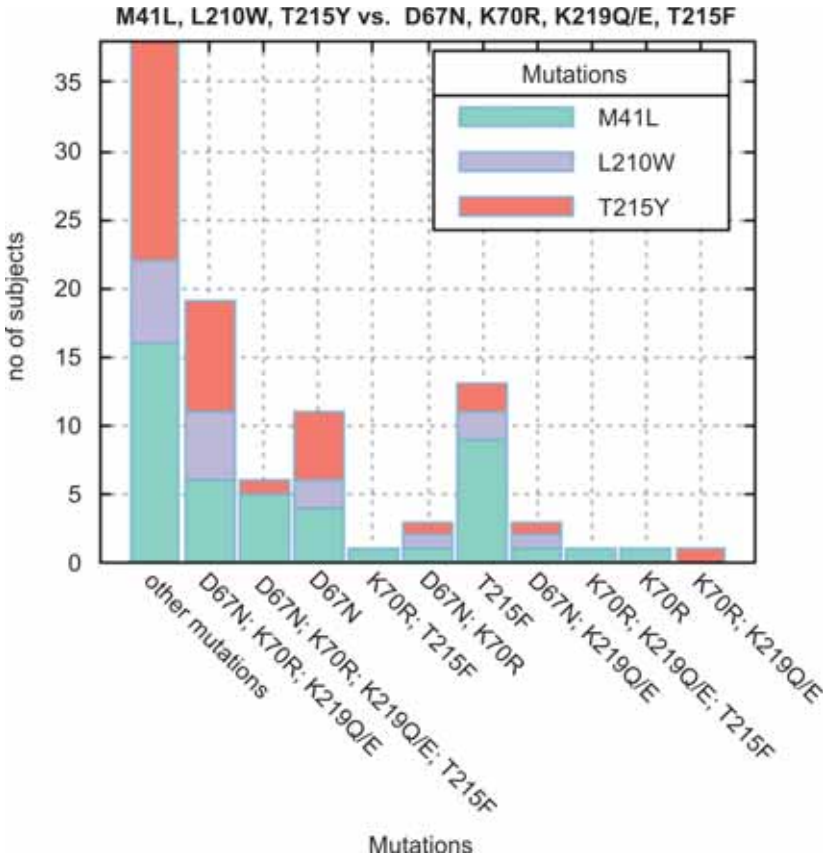


***FIGURE 3.*** *Mutation association on TAM1 and TAM2 pathways*

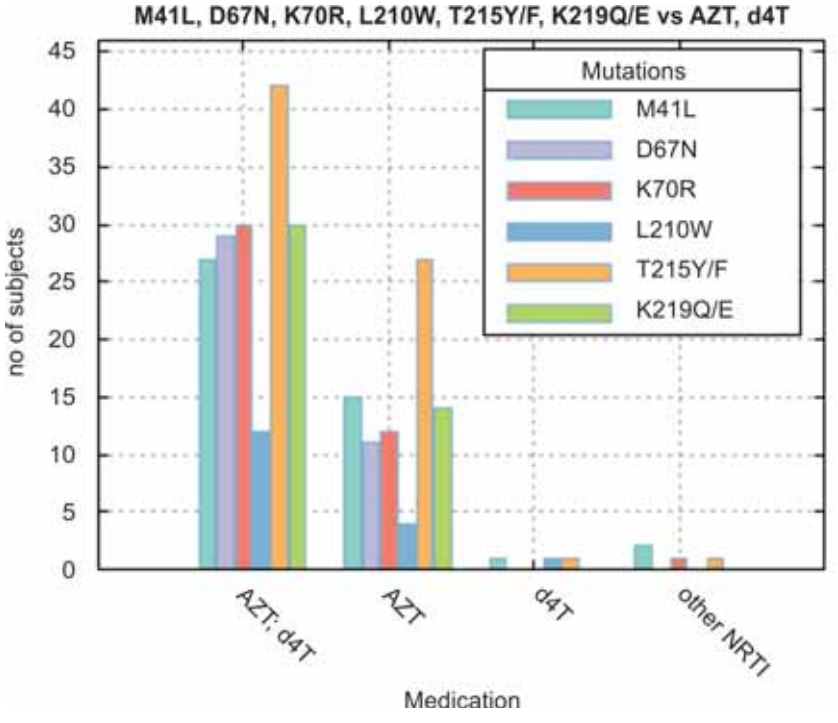


***FIGURE 4. TAM mutations associated with AZT, d4T***

The K65R mutation was present in 4 patients (2.77%) and was associated with the exposure to TDF or ABC (both in 2 cases). Due to the late access on the Romanian market, TDF was rarely used, only 19 patients experiencing virological failure under TDF- containing ART. Out of 144, 80 patients were exposed to ABC.

The uncommon selection of K65R mutation under these conditions was due to the presence of TAMs induced by pre-exposure to thymidine analogues, 15 of the 19 TDF failure patients presenting between 1 to 5 TAMs mutations. Moreover, 51 out of 80 of those who used regimens containing ABC had between 1 to 6 TAMs mutations. Furthermore, 2 out of 4 subjects, who developed K65R, had TAMs mutations.

The L74V/I mutation was present in 32 subjects (22.22%), as a consequence of long exposure to ABC or ddI. It is worth mentioning that 25 of them associated between 1 to 5 TAMs; a single patient associated K65R with L74V.

Therapeutic history of patients who selected the mutations K65R and L74V is represented in Fig. 5.

A total of 80 subjects had an ABC-containing regimen, 73 others had regimens which included ddI. A number of 15 used ddI and ABC successively, 2 other patients used ddI, ABC and afterwards TDF, and one used ABC followed by TDF. At the time of genotyping, 60 patients were treated with ABC and only 18 were in treatment regimens including ddI.

The presence of K65R determines a decrease in susceptibility to TDF, ABC and ddI, while L74V reduces susceptibility to ABC and ddI (at the same time increasing the susceptibility to AZT, TDF and reducing viral fitness, which is not the case with L74I).

Two patients showed MDR mutations: insertion at position 69, resulting in the loss of therapeutic response to the whole class of NRTIs and the Q151M complex with the only viable option the incomplete residual activity of TDF. Both strains occurred in the context of prolonged virological failure under ABC/3TC associated with EFV, respectively SQV/r (both with low genetic resistance threshold), which were not changed in time.

## Therapeutic options in the NRTI class

The stratification of therapeutic options in the NRTI class is shown in Fig. 6, which takes into account only the drugs currently in use.

Due to the extensive use of combinations consisting of 3TC and thymidine analogues or ABC, the result of the Stanford cumulative interpretation is not surprising at all. The highest rate of resistance was observed for 3TC or FTC because these drugs are the weakest link in a combination of ART. FTC, although very rarely used (for 13 patients out of 144), has the same resistance profile as 3TC. Maintaining 3TC in the therapy of the patients from this group would be useful only in maintaining low viral fitness and re-sensitization to thymidine analogues and TDF, as 131 of the 144 strains are resistant.

A second option that will be difficult to use due to a high degree of resistance is didanosine. Almost a half of the study population developed high resistance (74/144) to ddI, with only 12 strains being sensitive. ddI could only be useful in exceptional cases when its residual activity would be more important than its toxicity. The reason for this high degree of resistance is the presence of a large number of TAMs, rather than the selection of M74V/I or K65R. It is proven that the presence of 3 TAMs mutations determines resistance to ddI and this

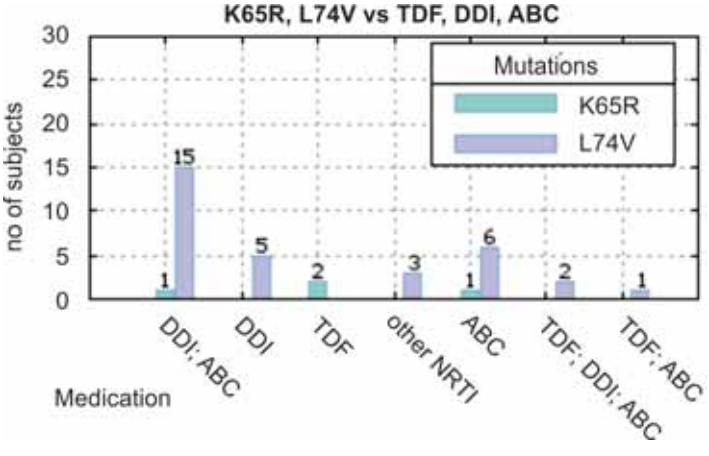


***FIGURE 5.*** *Correlation between K65R and L74V-therapeutic history TDF, ddI and ABC*

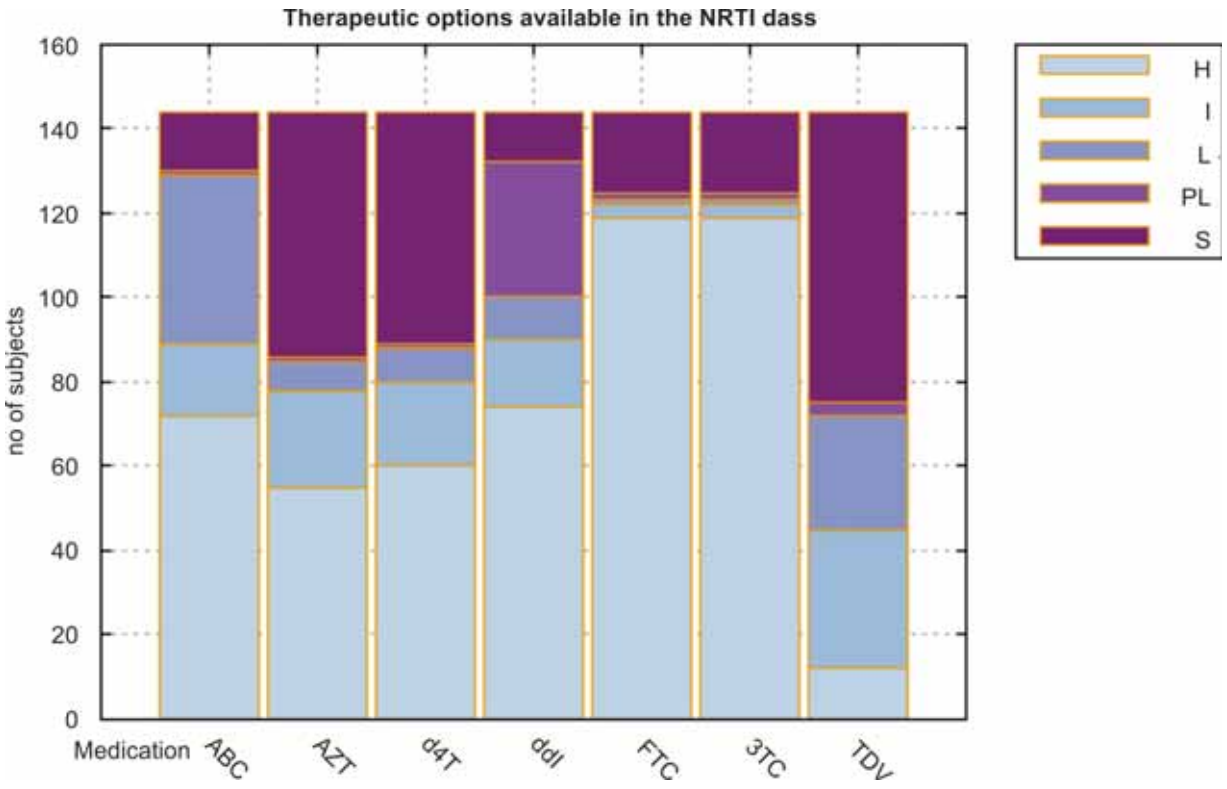


***FIGURE 6.*** *Therapeutic options available in the NRTI class of drugs*

type of profile is commonly found in our group. Surprisingly, in term of resistance, ABC dominates the competition with thymidine analogues. In other words, only 14 of the 144 strains maintained ABC sensitivity, while in the case of AZT and d4T, 58, and 55 respectively, of the strains maintained their maximum susceptibility.

The most potent NRTI remains TDF: 69 of the 144 strains were susceptible and only 12 developed high resistance. Even if it develops intermediate (33 cases) or low-level resistance, this drug may be a viable option because it has the best intracellular penetration and its therapeutic effect is less influenced by the existence of a number of TAMs smaller than 3.

## DISCUSSION

In the context of a study population with a long therapeutic history, overlapping with the introduction and use of ART in Romania (since 1995 AZT, then ddC, 3TC, d4T, ddI, the last introduced being TDF and FTC in 2008), the high prevalence of acquired resistance to the NRTI class is not surprising. Most of the subjects included in the study group belonged to the cohort infected by parenteral mode in the late 80s, with the F1 subtype. These patients were exposed to mono and dual therapy (AZT-based regimens later associated with ddC, ddI or 3TC) and had triple ART with regimens with re-used NRTIs. In terms of acquired resistance, this has generated a particular pattern. The weakest link in the therapeutic regimen was 3TC, its intrinsic antiviral activity being currently lost for these patients. The current role of 3TC could be to re-sensitize other NRTIs (TDF or AZT) and to reduce viral fitness. Although rarely used, FTC has the same resistance profile and the same effect. Therefore, in terms of cost-efficiency, using 3TC is more justified given the two above-mentioned reasons.

The most interesting particularity in the study group was the accumulation of TAMs, as a result of the use of AZT and/or d4T. The TAM2 pathway was more common, compared to TAM1, although we noticed numerous associations between mutations in the two pathways, even in strains that did not show a large number of TAMs. The constant exposure to thymidine analogues over the years could be the explanation for these associations and for the high number of highlighted mutations. A number of 36 out of 144 subjects had between 4

and 6 mutations, 7 of the 36 associating L74V and M184V, with significant reduction in susceptibility of these strains, the only remaining option with intermediate sensitivity being TDF. As none of them were exposed to AZT monotherapy, 23 of them experienced dual therapy, being patients from the paediatric cohort who had started treatment prior to year 2000. The predominant selection of the TAM2 pathway is in discordance with the results reported for B subtype (4), but it is consistent with published data in patients from the Romanian cohort (9).

The extremely rare selection of the K65R mutation is another characteristic of the highly drug experienced subjects from Constanta. Although access to TDF was limited, exposure to ABC could have favoured the selection of this mutation if there had not been a significant number of TAMs selected through previous use of thymidine analogues. TAMs mutations played a protective role in the acquisition of the K65R, which was not the case for L74V, where the therapeutic effect of ddI was lost. Interestingly, the association of TAMs and K65R is not concordant with other published data (12), which support the two-way antagonistic effect of these mutations.

The consequence of selecting these mutations determines the therapeutic response to TDF, the best preserved NRTI in terms of efficiency in this class.

## CONCLUSIONS

The extensive and successive use of NRTI during the therapeutic history of the highly drug experienced patients in Constanta generated a profile dominated by the accumulation of TAMs, being also associated with the emergence of mutations at codon 184, especially M184V. The mutational profile of TAMs is different from that described in other studies involving the B subtype strains, due to the TAM2 pathway dominance, the relatively frequent association between the two pathways, and, sometimes, by the association with the K65R mutation. The extremely rare selection of the K65R mutation is not necessarily secondary to the late TDF access (since there was an important exposure to ABC), but more probably to the pre-existence of TAMs. As a consequence, in the presence of these mutations, TDF remains the most useful therapeutic option in this group of patients. When TDF has partial activity, the use of 3TC in order to induce TDF re-sensitization is warranted even if 3TC has lost its intrinsic antiviral activity.

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