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# ANTIRETROVIRAL THERAPY IN ROMANIA: HAART?

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**Abstract.** This study **aimed** to investigate resistance mutation in patients under discontinued antiretroviral (ARV) therapy. **Material and methods.** Selection of reverse-transcriptase (RT) and protease mutations was investigated in 48 pediatric patients treated for at least 52 weeks by tritherapy including non-nucleoside inhibitors but whose treatment was discontinued for at least three times (< 1 week) during the follow-up, mainly due to lack of financial resources. Viral load (VL) was measured by Monitor Roche (detection limit: 200 copies/mL). **Results.** All the patients were infected by HIV-1 subtype F1. VL was undetectable in 5 patients. RT and protease were amplified in 44 and 42 patients respectively. Wild-type protease was observed in 29 patients, while wild RT was observed in 3 cases. 4 patients with detectable VL had non-nucleoside inhibitors of reverse-transcriptase (NNRTI) mutations (K103N=30; Y181C=6; Y188L=2; V106M=1). Four of them presented substitutions associated with high-level resistance to NRTI (M184V=2; T215Y=2), whereas 13 patients presented substitutions associated with resistance to PI (M46I; V82A). All the patients presenting substitutions associated with the resistance to PI also presented mutations associated with resistance toward NNRTI. **Conclusions.** Our results show a very high frequency of selection of NNRTI mutations in patients receiving a discontinued treatment containing this class of drugs. NNRTIs should be avoided in those situations where for economical reasons there are risks of discontinuations of treatment.
**Key-words: HIV-1, reverse-transcriptase, protease, ARV resistance mutations**

**Rezumat. Scopul** acestui studiu este de a investiga instalarea mutațiilor de rezistentă la pacienți sub triterapie antiretrovirala (ARV) administrată discontinuu. **Material şi metodă.** Am investigat selecționarea mutațiilor în structura proteazei şi revers-transcriptazei (RT) la 48 de copii tratați pentru cel puțin 52 de săptămâni cu diferite regimuri incluzând inhibitori non-nucleozidici de RT (NNRTI) şi prezentând cel puțin trei întreruperi terapeutice (< 1 săptămână) în timpul monitorizării, în principal datorate lipsei fondurilor. Viremia plasmatică (VL) a fost măsurată cu ajutorul testului Monitor Roche (limita de detecție: 200 cópii/mL). **Rezultate.** Toți pacienții erau infectați cu HIV-1 subtip F1. La 5 pacienți VL era nedetectabilă. RT şi proteaza au fost amplificate la 44 si respectiv 42 pacienți. Proteaze şi RT de tip sălbatic au fost observate la 29 şi respectiv 3 pacienți. 39 din cei 43 pacienți cu VL detectabilă prezentau mutații de rezistență la NNRTI (K103N=30; Y181C=6; Y188L=2; V106M=1). 4 pacienți aveau mutații asociate cu niveluri ridicate de rezistență la inhibitorii nucleozidici de RT (NRTI) (M184V=2; T215Y=2), iar substituții asociate cu rezistența la inhibitori de proteaze (PI) (M46I; V82A) au fost observate la 13 pacienți. Toți pacienții care prezentau rezistențe la PI asociau mutații de rezistență la NNRTI. **Concluzii.** Rezultatele noastre relevă o mare frecvență a selecției mutațiilor de rezistență la

NNRTI la pacienții care primesc tratamente discontinue cu această clasă de antiretrovirale. Administrarea NNRTI ar trebui evitată în situațiile în care există riscul întreruperilor terapeutice.
**Cuvinte cheie: HIV-1, revers-transcriptază, protează, ARV**

INTRODUCTION

Associations of the new antiretroviral drugs, in particular protease inhibitors (PIs), had a dramatic impact on the natural history of HIV-1 infection, generating unprecedented changes in AIDS mortality and quality of life of AIDS patients in Western countries (1, 2). Numerous studies reported the potency of these combinations resulting in durable immune suppression in a high proportion of patients (3-10). Moreover, these studies pointed on the prognostic value for the disease progression and therapeutic efficacy of plasma viral loads (VLs) (11-14). Major pitfalls of currently available antiretroviral drugs concern the limited action on virus in sanctuaries, which result in their inability of eradicating the virus from infected patients (15, 16). However, the long-term results of antiretroviral therapy generated a moderate optimism regarding our ability to transform HIV infection in a chronic life-lasting infection, in which virus replication can be at least partially controlled for long periods.

The major obstacle toward this aim is represented by the ability of HIV to rapidly develop resistance to antiretroviral (ARV) drugs (17-23). Therefore, the clinical guidelines include as major objective of ARV therapy the maximal suppression of viral replication (24). Although viral resistance is the hallmark of treatment failures, this is not the only factor involved in this failure: other factors are the clinical and biological status of HIV-1-infected patients, the compliance and the rate of adverse reactions. These aspects being considered, the clinician has a major responsibility in generating therapeutic success by choosing optimal drug associations, which will induce maximal suppression of viral replication in the shortest time with minimal adverse effects.

Highly active antiretroviral therapy (HAART) was initiated in Romania in 1998, when Romanian government and non-governmental organizations joined to provide the funds to cover ARV treatments for most of the HIV-1-infected patients. National guidelines for ARV treatment were released together with data concerning viral resistance to ARV drugs (25, 26). Development of Regional Laboratories created the infrastructure for monitoring treatment efficacy by measuring plasma viral loads (VLs) and basic immune cell populations in treated patients.

However, this national effort interfered with some incidental conditions, some of these being objective, and consisting in treatment discontinuities due to insufficient budgetary resources. Another aspect was related to underdevelopment of the technology selected for VL monitoring, LCx Abbott technology being FDA-approved only two years after marketed in Romania. Finally, one of the major causes of treatment failures was represented by alternation of ARV associations in a given patient, which is contrary to any current guidelines.

This study investigates the dynamics of ARV resistance in treated patients after 1 year of treatment in real-world circumstances (with interruptions and alternation) and potential consequences for long-term strategies of HAART in Romania.

## MATERIAL AND METHODS

**The study group** was formed by 48 HIV-1-infected children, aged 10-12 years (11±0.9 years), nursed in "Gulliver House", Iaşi and diagnosed as HIV-1-infected between 1990 and 1995. All these children were born from HIV-1 seronegative mothers, being nosocomially-infected (27).

These patients received ARV treatments, in successive associations (AZT monotherapy→ bitherapy→tritherapy) starting from 1996. Starting with 1999, all these patients were receiving HAART, the ARV associations including drugs belonging to the three classes. HAART history is presented in figure 1.

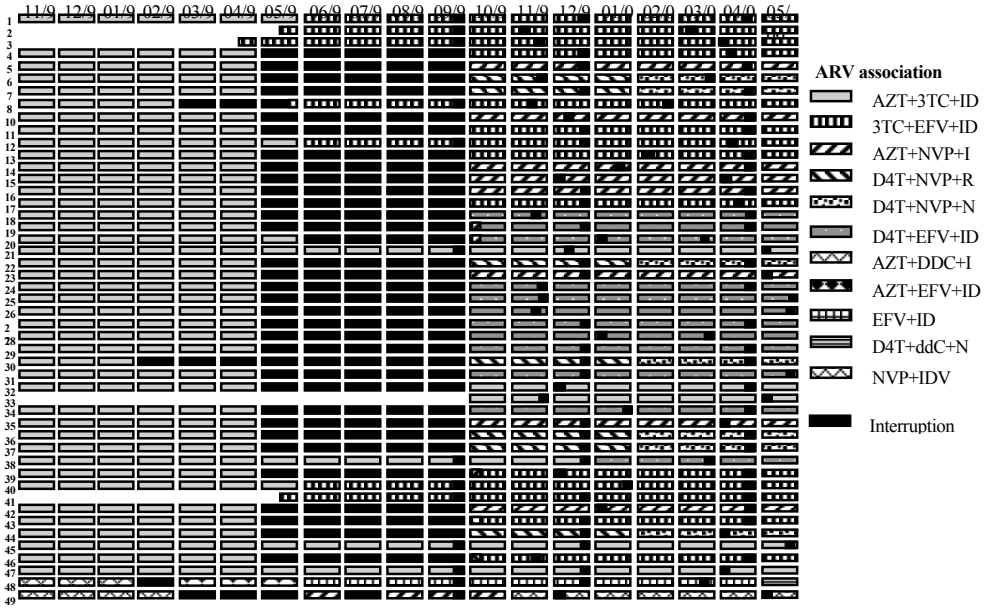


**Fig. 1 The history of antiretroviral associations and the therapeutic interruptions during the follow-up: November 1999 and May 2000.**

For 6 patients, we disposed of reverse-transcriptase and protease sequences sampled in 1994-1996, which allowed us to compare the wild genotype with mutant ones and to establish whether, observed resistance mutations occurred through ARV therapy (28).

EDTA-blood was sampled from all included patients. Blood was centrifuged at 1,700 RPM for 10 minutes. Plasma

was then separated and clarified through a new centrifugation (3000 g for 10 min). Clarified plasma samples were stored in liquid nitrogen (-192°C) and thawed when transported. Viral RNA was extracted within 5 hours using the lysis buffer from Amplicor HCV Specimen Preparation Kit (Roche Diagnostic Systems Inc. Branchburg, USA).

**Plasma Viral Load** was quantified using Monitor Roche 1.5 (Roche Diagnostic Systems Inc. Branchburg, USA) assay, which has a detection limit of 200 copies/mL. Results were confirmed by bDNA (Chiron, Emeriville, CA, USA), which has a detection limit of 500 copies/ml. This method is highly sensitive in quantifying non-subtype B samples.

**Phylogenetic analyses of RT fragments.** The *pol* nucleotide sequence alignment was obtained from the Los Alamos National Laboratory HIV Sequence Database (34). Newly derived Romanian sequences were aligned with the latter alignment using the CLUSTAL W profile alignment option. Phylogenetic trees were inferred from the nucleotide alignments by the neighbor-joining method using the reliability of HKY85 model of nucleotide substitution implemented using PAUP*. Branching order was assessed by performing 1000 bootstrap replicates, again using neighbor-joining and the HKY85 model. Phylogenetic trees were also inferred by maximum-likelihood using PAUP* with the HKY85 model incorporating an estimate of the transition/ transversion ratio and an alpha shape parameter (using four discrete categories) for a gamma distribution of rate heterogeneity among sites, both estimated on the neighbor-joining tree. The neighbor-joining tree topology was used as the starting tree in a heuristic search using TBR branch swapping.

**Genotypic testing** was done based on plasma RNA. Test interpretation was based on the protocol of *Agence Nationale des Recherches sur le SIDA* (ANRS) (18). The RT gene was amplified in a nested polymerase chain reaction (PCR) with outer primers RT-18/RTout and inner primers RT-19/RT-20 as previously described (28). Each nested PCR product was subjected to direct sequencing with sense primer $A_{20}$ and antisense primer $NE1_{20}$ (28).The protease gene was amplified in a nested PCR using outer primers 5'PROT-1/3'PROT-1 and inner primers 5'PROT-2/3'PROT-2 as previously described (29). Sequencing reactions for the protease gene were done using the same primers.

Sequencing was run with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (FS: Perkin Elmer) on an automated sequencer (Applied Biosystems 373A).

## RESULTS

Only 5 out of the 48 patients in the study group had undetectable VLs (<200 cp/mL) in the Monitor assay. VLs ranged between 200 and 500 copies/mL in other 6 patients. Remaining patients had VLs ranging from 558 and 11.8 x $10^5$ copies/mL (table 1).

**Table 1. Genotypic resistance testing and ARV susceptibility in the study group. RT and protease mutations are shown, as well as their effect on VLs**

| Patient | Viral load (Eq/mL) | Resistance mutations to PI | RT resistance mutations |
|---|---|---|---|
| 1 | 213,000 | **M46I, I54V, V82A** | M41L, T69D, L74V, L100I, **K103N**, **M184V**, L210W, **T215C** |
| 2 | 298,000 | Wild type | **K103K/N** |
| 3 | 240 | *Negative PCR* | **K103N** |
| 4 | 8,670 | Wild type | M41L, T69T/A, **K103K/N**, **T215S/C** |
| 5 | 450,000 | **M46I, I54V, V82A** | D67N, T69D, K70R, **K103N**, **Y181Y/C**, **T215F/C**, K219Q |
| 6 | 558 | **V82V/I** | Wild type |
| 7 | 226,000 | **I54V**, **V82A** | D67N, K70R, **K103N**, **Y181C**, **T215I**, K219Q |
| 8 | 8,510 | Wild type | K70K/R, **Y188Y/C**, G190G/A |
| 10 | 356 | *Negative PCR* | *Negative PCR* |
| 11 | 27,400 | Wild type | **K103N** |
| 12 | <200 | *Negative PCR* | *Negative PCR* |
| 13 | 359,000 | Wild type | **K103N**,**T215D** |
| 14 | 24,800 | **V82A** | D67N, **K101L/E**, **K103N**, **Y181Y/C**, K219Q |
| 15 | 10,100 | Wild type | T69T/I, **K103N**, **Y181C** |
| 16 | 75,200 | **M46M/L**, **V82A** | D67N, T69D, K70R, **K103N**, **Y181C** |
| 17 | 12,500 | Wild type | **K103N** |
| 18 | 1,180,000 | Wild type | K70R, **K103N** |
| 19 | 3,270 | Wild type | **K103N** |
| 20 | 28,900 | Wild type | **K103N** |
| 21 | 2,090 | Wild type | K70R, **K103N** |
| 22 | 20,000 | **I54V**, **V82A** | **Y181C** |
| 23 | 349,000 | **V82A** | D67N, T69D, K70R, **K103N**, **Y181C**, K219Q |
| 24 | 696,000 | **V82A** | **K103N**, P225P/H |
| 25 | 1,800,000 | Wild | **K101K/Q**, **K103N**, G190G/A |
| 26 | 419,000 | Wild type | D67D/N, T69A/D, K70K/R, **K103R**, **Y188L**, K219K/Q |
| 27 | 491,000 | Wild type | T69T/A, **K103K/N**, K219Q |
| 28 | 46,600 | Wild type | **K103N** |
| 29 | 1,390,000 | Wild type | T69S, **K103N** |
| 30 | 357 | Wild type | 69T/I |
| 31 | 625,000 | Wild type | D67G, **Y188L**, **T215I**, K219E |
| 32 | 3,470 | Wild type | T69S |
| 33 | 4,030 | Wild type | T69S |
| 34 | 40,800 | Wild type | **K103N** |
| 35 | 19,200 | Wild type | 67D/A, **K103N** |
| 36 | 20,000 | **M46L**, **I54V**, **V82A** | K70R, **K103N** |
| 37 | <200 | *Negative PCR* | Wild type |
| 38 | 17,000 | *Negative PCR* | *Negative PCR* |
| 39 | 3,950 | Wild type | **K103N** |
| 40 | 91,600 | **M46I**, **I54V**, **V82A** | D67N, T69D, K70R, **K103N**, K219Q |
| 41 | 4,300 | Wild type | **K103N** |
| 42 | <200 | Wild type | *Negative PCR* |
| 43 | 5,780 | Wild type | **K103N** |
| 44 | <200 | Wild type | Wild type |
| 45 | 408 | **I54V**, **V82A** | K70R, **M184V** |
| 46 | 45,900 | Wild type | **K103N** |
| 47 | 699 | **V82I** | K70R, K219K/Q |
| 48 | 2,950 | Wild type | **V106M** |
| 49 | 22,700 | *Negative PCR* | **K103N**, **Y181C**, **T215Y**, K219E |

RT and protease fragments could be amplified in 44/48 and 42/48 patients, respectively. The lower efficacy of protease amplification was probably due to a higher polymorphism of this gene fragments. RT fragments (750 bp) were used for tree construction. Phylogenetic analysis showed that all the children were infected with subtype F1 strains, which characterize the nosocomial AIDS epidemic in Romania (fig. 2) (27). Therefore, genotypic test interpretation considered that protease mutation L10V/I, K20R, M36I and L63T occur as genetic polymorphisms in subtype F1 strains, not being interpreted as responsible for drug resistance.

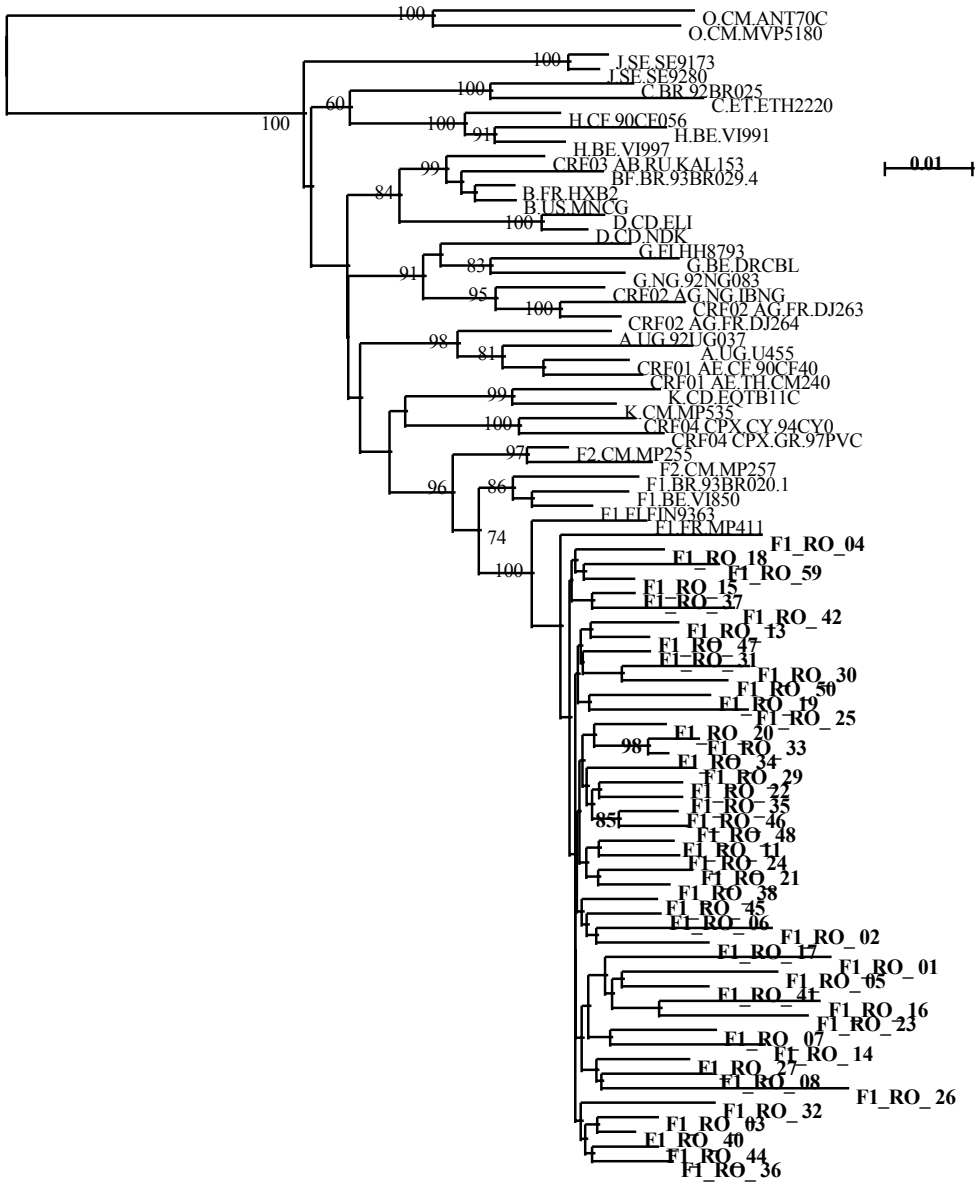


**Fig. 2 Phylogenetic tree of HIV-1 strains from patients. RT fragments (750 bp) were compared to reference RT strains from the Los Alamos Database. The tree was constructed by neighbour- joining, with 100 tree replications. Node values represent the bootstrap values (values > 75 are considered significant). The first letter in strain identification is the infecting subtype.**

Protease was amplified in 42 patients (table 1). Twenty-nine (69%) of the amplified strains were wild-type. Remaining sequences contained at least one mutation associated with resistance to PIs. V82A mutation, which is associated with major resistance to ritonavir (RTV) and indinavir (IDV) was present in 11/13 patients, remaining two patients presenting V82I and V82V/I polymorphisms. M46I mutation, which is associated with major resistance to ritonavir IDV was present in 3/13 protease sequences, whereas polymorphisms M46L and M46M/L were present in one patient each. Finally, mutation I54V, which is associated with moderate resistance to IDV and RTV, but responsible to cross-resistance to other PIs (such as saquinavir-SQV and nelfinavir-NFV) was present in 7/13 patients (table 1). To conclude, 27% of included patients harbored PI mutations which associate therapeutic failures, probably selected as a result of treatment interruptions. RT was amplified in 44/48 (92%) cases. Only 3/44 patients showed a wild-type RT (table 1). Mutations associated with resistance to ARV were observed in 41/48 (86%) of the studied RTs, being associated with therapeutic failures (detectable VLs).

The most important cause of therapeutic failure was NNRTI resistance. Thus, 28/41 of patients harbored K103N mutation, which is associated with high levels of resistance towards all the compounds in this therapeutic class. Three other patients harbored mixed viral populations K103K/N. The majority of nevirapine (NVP)-treated patients harbored specific mutations for this drug: Y181C (n=6), Y181Y/C (n=2), V106M (n=1), Y188L (n=2) (table 1).

Interestingly, one patient (#48) which received different ARV associations during the follow-up, harbored NVP-resistant strains (V106M) as only source of therapeutic failure, which were observed within several weeks after patient started NVP treatment (fig. 1). Two patients receiving NVP (#23 and #31) showed only specific NVP mutations (V181C), these strains being still susceptible to other NNRTI drugs.

In spite of long term NRTI monotherapy, NRTI mutations were only observed in 17/44 patients. Generally, NRTI mutations concerned multidrug resistance codons (D67N, K219Q, K70R), which associate moderate phenotypic resistance. Mutations of the codon 215, the most important causes of AZT failures, were observed in 6 patients, but coding amino acids were not described in association with reduced phenotypic susceptibility (T215C, T215D, T215F/C, T215SC, T215I); only one patient harbored T215Y mutation. Interestingly, K70R and K219Q mutations, which are generally the first to appear as markers of AZT resistance and prefigure the occurrence of major mutations, were observed in 10 and 7 patients, respectively. M184V mutation, which is associated with 3TC and abacavir (ABC) resistance, was only observed in 2 patients (table 1).

To conclude, RT analyses showed that most of the included patients harbored mutations associated with NNRTI

resistance. Selection of strains harboring these mutations generated therapeutic failures, as shown by high levels of VL.
Given these results, HIV-1 sequences obtained in 2000 were compared to available HIV-1 sequences obtained during our previous studies, in 1994-1996, prior to the use in ARV therapy of PIs or NNRTIs (28). Six of these sequences had been obtained from patients included in the present study group. This comparison revealed that none of the sequences from 1994-1996 were harboring drug resistance mutations. Therefore, we concluded that genotypic resistance patterns of subtype F1 are similar to those described for subtype B strains (30).

DISCUSSION

Our study points on two important aspects that have to be considered in ARV therapy in Romania. First, our results stress the obligation of confirming the diagnosis of HIV infection prior to administering ARV therapy. The use of NNRTIs in Romania call for type and subtype characterization, because HIV-2 is naturally resistant to NNRTIs (31), similarly to HIV-1 group O strains or to some of the group M strains (28).
On the other hand, our study showed that relevant mutations associated to resistance to ARVs in subtype F1 strains are similar to those described for subtype B, which allow the use of the same clinical criteria to interpret genotypic resistance tests (which were established using subtype B strains) (30). However, one should note that when interpreting genotypic resistance tests for subtype F1 strains we have to consider that some of the genetic polymorphisms of this subtype were described as minor resistance mutations for subtype B strains (30). These mutations (L10V/I, K20R, M36I and L63T) will not be considered when interpreting resistance tests because these are present in most of the subtype F1 strains (70-100%) prior to any treatment. These observations are supported by recent studies showing that these polymorphisms are present at a high frequency (>75%) in non-subtype B strains (32), with no impact on treatment efficacy.
Once the diagnosis is confirmed and treatment started, monitoring the therapeutic effect of ARVs by VL quantification has to be doubled by a continuous administration of drugs. Therapeutic interruptions are prohibited by the majority of available studies, especially when ARV association includes INNRT, for which viral resistance may occur very rapidly (in <1 week) if the VL is not strictly controlled (33). Although recent studies suggested that structured treatment interruptions (STIs) and the alternation of different ARV associations may represent logical approaches in HIV therapy, especially in those patients which are not controlling viral replication, these approaches should be carried out under strict control, following well defined guidelines and only after all clinical trials confirm the utility of such an approach. As a preliminary result, our study indicate that even if STIs will be admitted as alternative to current treatments, such approaches will only be

possible whether ARV association do not include NNRTI. As it can be observed form the analysis of our results, even a short term administration of a NNRTI may be the source of therapeutic failure (as in the case of patient #48).

Finally, our study revealed that use of NNRTI in ARV associations is associated with a high rate of therapeutic failure. This aspect may be considered as a particularity of HAART in Romania. Numerous clinical trials pointed on the high efficacy of NNRTI in HAART, these molecules having a high capacity of VL suppression. Clinical trials also showed that the control of VL is sustained only if the treatment is systematically administered, with no interruptions of treatment changes. The high rate of failure towards this group of drugs pointed out a major problem of HAART in Romania. This is not a peculiarity of Northeast Romania, being probably systematically observed. Irrespective of the causes generating these results, their signification is obvious: the majority of patients receiving ARVs in Romania may be therapeutic failures, making useless the government efforts to provide the infrastructure for HAART in Romania.

Our results show that until ARV treatment is not systematically and detailed monitored, the administration of NNRTI should be reconsidered in Romania. These drugs may constitute excellent salvation therapies. Also, our results call for an ample evaluation of viral resistance in Romanian patients, to evaluate the efficacy of ARV treatment and to establish a coherent strategy to prevent the emergence of highly resistant HIV strains.

## CONCLUSIONS

Selection of reverse-transcriptase (RT) and protease mutations was investigated in 48 pediatric patients treated for at least 52 weeks by tritherapy including non-nucleoside inhibitors and whose treatment was discontinued for at least three times (< 1 week) during the follow-up.

Only 5 out of the 48 patients in the study group had undetectable VLs (<200 cp/mL). VLs ranged between 200 and 500 copies/mL in other 6 patients. Remaining patients had VLs ranging from 558 and 11.8 x $10^5$ copies/mL. RT and protease fragments could be amplified in 44/48 and 42/48 patients, respectively.

Mutations associated with resistance to ARV were observed in 41/48 (86%) of the studied RTs, being associated with therapeutic failures (detectable VLs).

RT analyses showed that most of the included patients harbored mutations associated with NNRTI resistance.

The most important cause of therapeutic failure was NNRTI resistance.

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