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RESISTANCE PROFILES IN MULTIEXPERIENCED AND NA ÏVE HIV PATIENTS – CLINICAL AND THERAPEUTIC IMPLICATIONS

PH.D. THESIS ABSTRACT

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The reason of choosing the thesis theme is the public health risk of HIV infection in its most difficult form to combat: infection-resistant strains, irrespective of whether this resistance was transmitted or acquired.

The HIV pandemic is one of the greatest challenges humanity has faced over the last 40 years, the dynamics of this type of infection being directly related to humans as a vector in the transmission and dissemination of the disease. In Romania, the epidemiological situation is a special one. Firstly it is an F1 subtype enclave surrounded by other different viral subtypes (A and B). Also, the epidemic described in the pediatric population at the beginning of the '90s is a particularity of Romania in the global evolution of HIV infection. In Constanta, the level of the epidemic overtakes the rest of the country by one year at least [1]. Children infected during the period 1986-1990 are a local cohort, with some common features: first-year infection (either horizontally or vertically by mother-to-child transmission between 1987 and 1990 [1]), F1 subtype in almost all cases [2], co-infection with hepatitis concomitant with HIV infection [3], almost equal sex distribution [4]. These patients were registered, treated according to national guidelines and benefited from active surveillance of the infection as well as that of associated conditions. [5]. They have become long-term survivors who have experienced many therapeutic regimens and describe the phenomenon of "treatment fatigue" [4].

Over the years, epidemic dynamics have changed, the heterosexual transmission path becoming the most important. As some of these patients are working outside the country, we assume that the subtype population would have had to acquire new variants with different profiles of primary resistance. Also, in recent years, frequent cases of acute infection are detected in the MSM community, where an epidemic overlapping the global epidemic profile described in the early 1980s is outlined.

At the end of 2016, there were 1037 patients in our clinic, of which almost half came from the long-term survivors of the pediatric cohort (463 patients). They are therapeutically multiexperienced, some of them having significant therapy adherence problems. Reaching the adult age, they have become sexually active, therefore we expect a generation of secondary cases. In the case of those multiexperienced patients with few therapeutic options, where viral undetectability cannot be obtained, there is a risk of secondary cases with primary resistance. In patients with low adherence to treatment, transmission should be more frequent (due to important viral levels), but secondary cases will likely have wild strains or, if genotyping was performed late after the infecting moment, strains that have archived their acquired resistance mutations. Most patients from

Constanta are in antiretroviral therapy (891); 603 out of 891 experienced more than one treatment regimen, with the largest proportion being that of patients with more than 3 schemes of treatment (287 out of 891).

Two special situations were found in Constanta. Firstly, the pediatric cohort had access to LPV/r in an extended access study [6], as this form of treatment was not available in Romania. By the end of 2010, 199 of these (48.1%) continued treatment regimens that included LPV/r with an undetectable rate of 63.8% [7]. As exposure to LPV/r was made after other treatment regimens, there may have been cases in "functional monotherapy". This triggers the interest of knowing the developed resistance profile and consequently the therapeutic options they had left after LPV/r. Secondly, some of the Constanta patients developed tuberculosis as an AIDS-related manifestation (Constanta is an area with high endemicity for TB), requiring tuberculostatic therapy. Many patients had EFV-based therapy while optimized background therapy did not contain active NRTIs, so it would be useful to find out, in terms of antiretroviral resistance and subsequent availability to other ARVs, what kind of prognosis these patients have.

Data on HIV-1 resistance in Constanta is scarce. In this context, the research could bring new data on the HIV epidemic in Constanta in terms of resistance to antiretrovirals and circulating viral types.

The thesis aims to determine the primary resistance in newly diagnosed cases in Constanta (and its clinical and therapeutic implications) and the resistance profiles in multiexperienced patients (and the clinical and therapeutic implications). In the alternative, the establishment of circulating subtypes in Constanta and their dynamics over time is another desideratum.

The study was conducted on a group of subjects diagnosed and confirmed with HIV infection, under monitoring in Constanta Regional Center. Those included in this study are either newly diagnosed ARV-naïve patients, with detectable viral load, with genotyping analysis done as soon as posible after the infection was confirmed, or patients with antiretroviral therapy for at least 6 months considered to be in viral failure, having a viral load of > 1000 copies/ml, thus eligible for genotyping.

The genotypes were determined in the Laboratory of Molecular Genetics of the National Institute of Infectious Diseases "Prof. Dr. Matei Balş" in Bucharest, at the Institute of Virology "Ştefan S. Nicolau" in Bucharest and in the Quest Diagnostics, Monogram Bioscience and Virco laboratories. The complete sequences which have been obtained, representing the entire PR gene and two-thirds of the RT gene, were generated in

Fasta file format. HIV-1 subtyping was performed using HIV-1&2 REGA algorithm. In order to determine therapeutic options after the proven failure of the regimen, the Stanford HIVdb Program version 8.4 algorithm was used. Mutations in the structure of revertranscriptase, protease and integrase were considered, according to the positions listed in the consensus article of the International AIDS Society (IAS) Expert Group – USA, published in January 2017 (update to 2015) [8]. In the case of primary resistance, the WHO consensus on resistance mutations developed for epidemiological surveillance was used, the last update being made in 2009 [9]. In order to determine the prediction of tropism, a 10% cut-off was used as recommended by the „European Consensus Group on clinical management of HIV-1 tropism testing "[10].

The data was organized and mathematically structured in MySQL databases. The BASH and "make" software applications were used for project management, and the M.A.W.K. was used to recognize patterns in the database. For statistical calculations, the R-Project program was used. "Single sample", "two sample" and "multi sample" analyses were used. The average, median, standard deviation, the confidence interval, and p-value were calculated for each batch and/or subgroup. Depending on the type of variables analyzed, T.student test, χ^2-test and Fisher test were used.

The first part of the study focused on the determination of transmitted resistance, as TDR may be one of the predictive factors for the success or the failure of the first therapeutic regimen. A therapy that causes viral failure will have consequences for the next regimens. The studied group comprised of 153 newly diagnosed HIV-infected patients who underwent genotyping tests during 2007-2017. All patients were naïve to antiretroviral therapy when genotypic resistance testing was performed. Genotyping was performed for all at the pol gene for revertranscriptase and protease and only for 26 patients at the level of integrase. The tropism test was performed for 22 patients by genotyping at the V2-V3 loop of the env gene.

Most patients included in the batch were male (64.05%), and a significant number (30.07% out of 153) admit sexual transmission through MSM. Only a few parenterally infected patients from the cohort were included (8.5%). The median age of the entire batch was 36.5 years [3 and 76 years]. The age range grouping most of the patients is between 18 and 40 years (72.55%), the most sexually active age. Depending on the path of acquisition of the disease, there is a statistically significant difference between the median age in sexually infected subjects versus those in the cohort (32 years versus 21 years, $p = 0.0002$). Most of the naïve patients from Constanta come from the urban environment 111

(72.55%). The transmission source was known in 35.92% of patients; 8.50% meet the characteristics of the pediatric cohort, and most of the group 56.21% do not know the source of transmission, but the transmission route is sexual.

The median CD4 lymphocyte count of the whole group was 333 cells/mm3, sexually infected patients showed a significantly higher CD4 average value than the parenteral average CD4 count (343 versus 142 cells/mm3, p = 0.01). HIV-RNA plasma viral load at the time of diagnosis ranged from 0.301-7.0 log10 copies/ml, with an average value of 4.75966 log10 copies/ml. There are no statistically significant differences between median values in the sexually infected and parentally infected group (4.834 versus 4.929, p=0.59). There is a statistical correlation between median RNA-HIV and severe immunodepression (p=1.99e-10); the more deficient the immune status, the higher the viral values are.

The time frame from the date of diagnosis to the date of genotyping is less than one year in over half of the patients (54.90%) and between 1-2 years in another 33.99% of the subjects, so there is a high probability that the processed data will reflect a correct percentage of TDR.

The prevalence of primary resistance in the study group was 6.53% (10 of the 153 patients presenting at least one WHO consensus mutation developed for epidemiological surveillance, updated in 2009). All 10 patients showed primary resistance to a single class of antiretrovirals, most frequently the mutations being present in the reverse transcriptase sequence. The prevalence of the TDR to NRTIs was 1.96%, to NNRTIs of 2.61% and to PIs 1.96%. No primary resistance to INSTI was found in patients whose genotyping was performed at the integrase gene level. In determining tropism prediction, 5 strains of the 26 were tropic CXCR4, 4/5 were non-F: C and CRF14_BG strains.

There was no case of TDR in the MSM community, among which a recent epidemic of HIV-1 diverse subtypes is apparent (the most commonly involved is the local subtype F1, but subtypes B, C, A and G were also present).

Mutations associated with resistance to NRTIs were present in 3 isolate strains: M41L, M41L+L74V, A62V. Mutations associated with resistance to NNRTI (K103N and V108I) were detected in 4 strains, without associating other resistance mutations to NRTI and PI classes. Three other patients had primary resistance mutations only to protease inhibitors: Q58E, T74P and V812M. Most of the strains isolated and genotyped with TDR are subtype F (6), while in the case of non-F subtypes, A, B and the recombinants CRF02_AG and CRF14_BG were isolated.

The majority of isolated strains in the studied group presented accessory mutations in the PR gene, often these being polymorphisms. The mutations highlighted with a high rate of polymorphism were: M36I, L89M, K20R, L10I/V, I62V, I93L, L63P, G16E, T74S. Genotyping at the RT gene level has identified some accessory mutations, the most common being in V179 and E138.

The calculation of genotypic resistance scores according to the Stanford algorithm for the 10 patients indicates that, although the strains did not have a total sensitivity to the three antiretrovirals, the used combination scored at least 2. The therapeutic regimens containing IP/r determined the therapeutic success in these cases. The therapeutic combination with EFV, with a genotypic resistance score of 2.5, failed viral control. For the other patients, the therapeutic scheme orientation based on the genotypic resistance score was beneficial and avoided viral failure.

The initiation of ARV therapy in 135 patients determined an undetectable percentage of only 57.75% at week 24 and it increased to 69.52% until the end of the study. This was considerably lower compared to naïve patients in other countries. For 16/17 of the genotypes performed the wild virus was demonstrated, the strains showing the same mutations as the initial strains, which suggests that there is a significant percentage of non-adherents to therapy, even under properly established and well supervised treatment.

We started from the hypothesis that the majority of HIV-positive people has access to antiretroviral therapy according to the requirements of the national guidelines and that most of the patients are multiexperienced. As a result, the number of potential sources of acquired resistance should have been large enough to generate secondary cases with TDR (primary resistance). This, however, is not confirmed by the TDR prevalence value determined in the study, especially regarding the three classes of classical medication used. The prevalence rate (6.53%) is higher than the national TDR value (4.6%) [4], but it is lower than the European one (8.4%) [11] and the regional values: Bucharest 14.75% [12] 7.3% [13].

The explanation for the low prevalence of primary resistance in isolates taken between 2007-2017 results from the analysis of the viral suppression rate for patients receiving ARV treatment: it varies from 53% in 2013 to 66% in 2017 but does not reach the level recommended by UNAIDS of 90%. Other causes are the number of patients who give up therapy, the loss of tested subjects who are not enrolled and treated in the medical system, the insufficient testing.

The study of multiexperienced patients group commenced bearing in mind that sequential exposure to suboptimal schemes, allowed viral replication and generated mutant strains with acquired resistance (ADR).

A total of 235 patients were included in the study group. They had at least one therapeutic failure under antiretroviral therapy, which required a genotyping analysis that guided the next therapeutic scheme. The lot consisted largely of the cases from the Constanta pediatric cohort (67.23%), with a slight female predominance (53.19%), the rest of the cases acknowledging the following ways of transmission: sexual (24.68%), vertical (7.66%), occupational exposure (0.43%). A larger number comes from the urban environment (61.70%), just over half have an educational level of up to 8 classes (53.19%). The patients in the study group are young, the median age being 29 years [5 and 71 years] but they have a significant number of years of exposure to therapy. The number of years from the time of diagnosis to the last genotyping, expressed as the median duration of HIV infection, is 12 years, ranging from 6 months to 26 years.

TB is the most common coinfection (54.47%) in the studied group.

The median CD4 value of the batch at the last genotyping performed was 250 cell/mm^3 (with a range of 1-1533 cell/mm^3), while the nadir CD4 median value was 95 cell/mm^3 (the limits being 1-1118 cell/mm^3). The group includes patients with advanced category C infection (87.2%). The average of HIV viral loads at the last genotyping was 4.383815 log10 copies/ml and the mean average HIV-RNA zenith during disease progression was 5.041393 log10 copies/ml. An important percentage (43.40%) was exposed to dual teraphy. The median number of therapeutic regimens up to the last genotyping analysis was 5, with an interval ranging from 1 to 12 regimens. Approximately one-quarter of the patients in the group, who had multiple treatment failures, had between 2 and 5 genotypes performed, the remaining patients had only one genotype determination done in the course of disease progression.

The results of the genotypes performed led to the conclusion that only 144 of the patients (61.28%) can be considered in real therapeutic failure with at least one mutation in the structure of revertranscriptase, protease, or integrase in the positions listed by the IAS-USA expert group, published in January 2017 (update to the 2015 version). For the rest of the batch (91 patients, 38.72%) the genotyping test indicated wild HIV strains.

The prevalence of acquired resistance in the studied group is 56.59% (for NRTIs), 40.85% (for NNRTI) and 38.72% (for PI), but excluding patients whose genotyping only

revealed wild virus (noncompliance to therapy) prevalence rates rise: 92.36% (NRTI), 66.67% (NNRTI) and 63.19% (IP).

The most common mutation selected in the INRT class was M184V, found in 131 subjects, two others selecting M184I. The use of thymidine analogues represented by AZT and d4T resulted in the occurrence of TAMs mutations. The most common in this case was the TAM2 pathway, respectively D67N, K70R, T215F, K219Q/E. These rates were: 27.77% for D67N, 29.86% for K70R, 27.08% for T215F and 31.25% for K219Q/E. Mutations were not always selected on a single TAM pathway; there were also combinations of mutations. The K65R mutation was present in a small number of cases (4), and in 3 patients (out of 4) it was associated with TAMs. In contrast, a mutation at the level of L74V/I codon was determined in 22.22% of patients from the subgroup of patients with acquired resistance, the mutation being consequent to consistent exposure to ABC or ddI. Two patients showed MDR-specific mutations: insertion at position 69 and Q151M complex.

The Stanford interpretation indicated TDF as the first therapeutic option in the NRT class to treat patients in the acquired resistance group, with only 12 strains showing high resistance, 69 retaining their full sensitivity, and the rest developing low and intermediate resistance.

For the NNRTI class, the most common mutation was K103N (56.16%) followed by Y181C/I/V (13.19%), Y188L/C/H (10.41%) and G190A/S/E mutations (9.01%). As a result, the most valuable therapeutic option for patients with acquired resistance remains ETR, 60.41% strains maintaining their overall susceptibility to this drug.

Among primary mutations associated with PI resistance, 3 are seen in patients with ADR in this class: V82A (52.82%), I54V (59.31%) and M46L/I (41.66%). These three mutations are correlated, in particular with the administration of LPV/r. The rest of them are found in combinations, but much less than the first three (in order of frequency: G48A, L76V, I50V, V32I, I84V, L90M etc.).

Comparing the two sub-groups (those with ADR versus those with wild strains) it is found that in the group of those with resistant strains there is a much greater number of accessory mutations than in the group of those with wild strains.

The Stanford interpretation indicates that DRV/r remains a viable therapeutic option for multiexperenced patients in viral failure under regimens containing other IPs. Only 7 patients in our group developed high resistance to DRV/r, in all cases the number of RAM-DRV being 4 or 5, and one of them being I50V. A number of 93 out of 144,

respectively 64.58%, of the strains are sensitive, but given DRV significant residual activity, it can also be used in low, potentially low or intermediately resistant strains.

Of the 11 subjects in a failing treatment regimen containing RAL, only two showed resistance mutations, both co-infected with TB. The selected mutations were E138K + Q148R in one patient, respectively T79A + Y143R in the second, both of which determined high resistance to RAL.

A total of 82 strains from the 235 patients enrolled in the study were analyzed for viral tropism using a genotyping test at the V2-V3 region of the env gene. The results indicated that a number of 23 patients, all of them in the cART, had CXCR4 tropic strains. As a result, MRV cannot be used in these patients.

In both subgroups (with ADR and wild-type strains), a modest viral suppression rate was found: 59.09% in the group of those with acquired resistance and 45.05% in the group of wild-type viruses.

The mortality rate of the group of patients with wild-type HIV strains is 37.46%, worryingly high compared to the one registered in the resistant strain group – 13%. Although at the start of the study these patients were in better conditions with fewer months of immune failure (median immunologic failure was 48 months) and viral failure (median period of viral failure was 36 months) and they were less exposed to therapy (median duration of ART administration is 8 years and the median number of treatment regimens is 4), they have evolved poorly as a consequence of noncompliance to the prescribed treatment. Mortality in this group was mainly determined by TB. For the group of patients with resistant strains, the predominant causes of mortality were non-AIDS. It is concluded that non-adherence to treatment is more often the cause of mortality than multi-drug resistance.

Having known the distinct characteristics of the Constanta group, which were confirmed by the group analysis, the premises would have been set for a much higher acquired resistance rate. The large number of noncompliant patients makes this supposition not to be confirmed. The ADR prevalence rate in Constanta differs from those in many European countries. In a European study involving 15 states, ADR was 80.7% (NRTI 73.5%, NNRTI 48.5% and IP 35.8%) [14]. In Germany, in a 10-year study (2001-2010), this rate reached 64%, with predominantly NNRTI class (55%) followed by NRTI (51%) and PI (30%). [15]. In Italy, in a cohort of multi-experienced patients, between 2007 and 2009, the prevalence of acquired resistance to at least one class decreased from 70% to 66% between these years; the class with highest ADR is NRTI (65% in 2007 and 56% in

2009), followed by PI (39% in 2007 but down to 27% in 2009) and NNRTI (36% in 2007, to 31%) [16]. In Romania too, these data are discordant. In a group of patients with adherence problems (6/38 patients) from the "Victor Babes" Hospital in Bucharest, the detected resistance to NRTIs was of 71.1% (through M184V and TAMs mutations), to NNRTIs was of 52.7% (through K103N and Y181C), while resistance to PIs was of 31.6% (the mutations being V82A, I54V, G48V) [17].

In Iasi Regional Center, in a group of 40 patients, 92.5% had resistance to NNRTI (the most frequent mutations being at codons 67, 70, 219 followed by 41 and 215), 85% had resistence to NNRTI (K103N present in 70 % of the subjects) and 32.5% showed resistance to PI (the most common mutations being V82A, I54V and M46L) [18].

The results of the study highlight the dual nature of the analyzed group, given the percentage of nonadherent patients, which explains the "polluted" prevalence rates of ADR (when the calculation includes the nonadherent sub-group). The group with ADR, has a extremely high prevalence rate, underlining a worrying situation that requires to closely monitor those patients with limited therapeutic options. Patients with wild-type strains, that have median viral levels greater than those in ineffective viral suppression under ART (4.514547 vs. 4.310460, $p = 0.03$), are potentially more effective sources of infection than those in treatment. Genotyping was an instrument of "measuring adherence" in these patients, but the determination of non-adherence by this method was not followed by the expected results in terms of viral suppression.

Summing up the studied groups (the group of 144 naïve patients and the group of 235 multiexperienced patients), the dynamics of viral subtypes were analyzed from the earliest cases up to 2017. As expected, the dominant subtype is F1 within the total of the 388 HIV strains. If sporadic subtype B strains occur before 1999, these strains, C and B, appear systematically in a small number after that year. In 2004, the first subtype A strains were isolated and it is the moment when the circulating recombinant forms are introduced as CRF02_AG. The year 2007 is the year with the highest biodiversity: besides F1, there appear subtypes C, A, B, G, as well as the circulating recombinant forms CRF02_02, CRF06_cpx and CRF01_AE. Since 2008 the viral population remains polymorphic, but with the predominance of the F1 subtype, which predicts a more active dynamics in the future years, with the emergence of minority subtypes at the expense of the majority strain.

In conclusion, the special conditions that shaped the outbreak of the HIV epidemic in Constanta led to two main patient profiles. The noncompliant patient profile is the one that influences the most the dynamics of the epidemic.

Regarding TDR, if medication is prescribed under current guidelines, undetectability can be obtained, but compliance to treatment is essential.

The absence of TDR in the MSM community, where the most dynamic infection rate is currently found, can create the premises for implementing PrEP in order to limit the epidemic.

The level and particularities of ADR in the subgroup of patients who have demonstrated adherence to therapy limit the therapeutic options for several drugs: TDF, ETR, DRV/r plus those of the new INSTI class and, sometimes, coreceptor antagonists.

For non-adherent patients, a new management strategy needs to be rethought, as the current one is not cost-effective either for them individually (the mortality rate is extremely high) or for society (they are potential sources that enhance the epidemic's expansion while important resources are invested at the same time).

The HIV epidemic in Constanta started as a monoclonal expansion of the F1 subtype, but in time we are witnessing a diversification of the subtypes. At this moment there are circulating, alongside F1, whose prevalence is maintained, the B, C, A, G subtypes, as well as various recombinants forms such as CRF01_AE, CRF 02_AG, CRF06_cpx, CRF14_BG.

Key-words: HIV infection, Constanta, transmitted resistance, acquired resistance, ARV therapy.

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