

Temporal characterization of drug resistance associated mutations in HIV-1 protease and reverse transcriptase in patients failing antiretroviral therapy

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SUMMARY

The aim of this study was to evaluate the prevalence of genotypic resistance for each drug-class, and of single resistance-mutations in 1,075 HIV-1 infected multi-treated patients undergoing their first genotypic resistance-test (GRT) after virological failure, over the years 1999-2003. First GRT was requested in 2003 for patients at earlier stages of failure, with less advanced disease, higher CD4-cell-count, lower HIV-RNA, and lower drug-experience with respect to 1999. Prevalence of resistance to all three drug-classes decreased from 33.3% in 1999 to 14.8% in 2003 ($p < 0.001$). Patients with protease-inhibitor (PI) resistant viruses decreased from 68.1% in 1999 to 34.1% in 2003 ($p < 0.001$); patients with nucleoside reverse transcriptase-inhibitor (NRTI) resistant viruses remained unchanged (85.4% in 1999; 86.4% in 2003); patients with non-NRTI (NNRTI) resistant viruses increased from 36.1% in 1999 to 52.3% in 2003 ($p = 0.005$) (corresponding to an increased NNRTI-use and decreased PI-use). From 1999 to 2003, resistance-mutations to drugs with high genetic-barrier significantly decreased (L90M/V82A/M46I/I54V/G73S/I84V/G48V for PIs; M41L/D67N/L210W/V118I for NRTIs, $p < 0.05$), while mutations to drugs with low genetic-barrier increased (D30N in protease, M184V/K103N/V108I in reverse transcriptase, $p < 0.05$). Taken together, earlier recruitment to first GRT in patients with less severe disease, and with lower prevalence of drug-resistant viruses may further improve therapeutic strategies aimed at longer and greater control of HIV-related disease progression.

KEY WORDS: HIV-1, Antiviral therapy, resistance, genotypic test

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INTRODUCTION

Antiviral therapies for the treatment of human immunodeficiency virus type 1 (HIV-1) infection have been standardized in recent years, and rec-

ommendations for their correct management are currently available (http://aidsinfo.nih.gov/guidelines/adult/AA_102904; Yeni *et al.*, 2004). Nevertheless, when viral replication is not completely suppressed, drug resistant HIV-1 strains can be selected through the accumulation of mutations in the reverse transcriptase (RT) or protease (PR) genes, thus leading to therapeutic failure (Hirsch *et al.*, 2000; Vandamme *et al.*, 2004). The emergence of resistance represents a frequent and major limitation of antiviral therapy (Clavel and Hance, 2004). However, the assessment of

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resistance in clinical settings helps in the management failure, as demonstrated by literature evidence supporting resistance testing (mostly genotypic, GRT) as a valuable tool to guide the choice of antiretroviral therapy (Baxter *et al.*, 2000; Cingolani *et al.*, 2002; Clevenbergh *et al.*, 2000; De Gruttola *et al.*, 2000; Tural *et al.*, 2002).

The emergence of drug resistance is continuously increasing in drug-naïve patients (due to transmission of resistant variants) (Little *et al.*, 2002; Simon *et al.*, 2002; UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance, 2001; Wensing *et al.*, 2005) and is very high in patients failing HAART (Hertogs *et al.*, 2000; Kantor *et al.*, 2004; Miller *et al.*, 2001; Shafer *et al.*, 1998). In the United States, three different studies (two for genotype and one for phenotype) (Kagan *et al.*, 2004; Rhee *et al.*, 2004; Richman *et al.*, 2004) analyzed the dynamics of resistance changes among infected patients. On 6,153 isolates derived from patients treated between 1997 and 2003, 78.7% had virus strains resistant to at least one antiretroviral drug (ARV) (Rhee *et al.*, 2004). Confirmatory to this point, two other studies show that about 80% of samples from treated patients tested at the end of 1998 harbored virus strains resistant to at least one ARV (Kagan *et al.*, 2004; Richman *et al.*, 2004), while their prevalence declined to 65% in September 2002 (Kagan *et al.*, 2004). Genotypic HIV resistance to antiretroviral drugs was also examined in a French population between 1997 and 2002: on the overall population, the proportion of patients carrying virus strains resistant to at least one PI, at least one NRTI, and at least one NNRTI was 47%, 78.3%, and 38.9% respectively; only 17.8% carried no resistance-mutations (Tamalet *et al.*, 2003). Finally, the prevalence of ARV resistance in England in 1999, 2000, and 2001 was 69%, 88%, and 55% respectively (Scott *et al.*, 2004).

The confidence of clinicians on GRT has increased and improved over time. Nevertheless, the best time to use GRT remains a matter of debate, depending from country to country, and even in the same hospital, from physician to physician. It should also be mentioned that the availability of new drugs characterized by different mutation profiles (including boosted PIs) may have further changed the prevalence and characteristics of resistance-mutations.

The identification of HIV-mutations present at first GRT may contribute to a better understanding of the evolving dynamics of resistance to antiviral drugs, and to help the physician in defining their pattern(s) of development. Based on these considerations, we then characterized the prevalence of genotypic resistance over the years 1999-2003 in our set of HIV-1 infected patients undergoing their first GRT under treatment failure. We analyzed the prevalence of resistance for each drug class and of individual resistance-mutations in association with immunologic, virologic, and therapeutic parameters.

MATERIALS AND METHODS

Patients

The study included 1,075 HIV-1 infected drug-treated adult patients followed in clinical centers in Rome and surroundings, undergoing their first GRT for routine clinical purposes, after virological failure between 1999 and 2003. HIV-1 infected children, drug-naïve patients, patients under therapy interruption (for any reason), and those carrying non-subtype B strains were excluded from this analysis. For each sample, genotypic mutations and clinical history of the patients, including demographic, immunologic, virologic, and therapeutic parameters (past and at GRT) were recorded in an anonymous database. The last treatment before GRT was recorded in the database for all samples, while the data of other parameters considered in the analysis were available in the database for around 80% of samples. Overall, at the time of first GRT, 99.2% of patients were in current treatment with at least one nucleoside-nucleotide RT inhibitor (NRTI) [abacavir (ABC), didanosine (DDI), lamivudine (3TC), stavudine (D4T), tenofovir (TFV), zalcitabine (DDC), zidovudine (AZT)], 36.2% were in non-nucleoside RT inhibitor (NNRTI) [efavirenz (EFV), nevirapine (NVP)], 60.4% were in PR inhibitor (PI) [amprenavir (APV), indinavir (IDV), nelfinavir (NFV), lopinavir (LPV), ritonavir (RTV), saquinavir (SQV)]. From year 2000, RTV, due to its capability to enhance the bio-availability of other PIs, was generally administered as booster in combination with others (LPV, SQV, IDV, and APV). Therefore, the RTV trend must be evaluated in

association with other PIs over that period. In particular in the patients treated with LPV, this PI was always administered boosted with RTV. From 2002 AZT + 3TC were administered in the combination drug Combivir.

Genotypic resistance test

Direct sequencing of RT and PR genes was performed on plasma samples in our centre by means of a commercially available kit (the ViroSeq™ HIV-1 Genotyping System, versions 1 and 2, Applied Biosystems) and an automatic sequencer (ABI 377 and ABI 3100, Applied Biosystems, Foster City, California, USA) (Perno *et al.*, 2001; Ceccherini-Silberstein *et al.*, 2004). Briefly, RNA was extracted from plasma samples, retrotranscribed, and amplified with specific primers. The PCR products covered the pol region coding for all amino acids of the PR and the first 320/335 amino acids of RT, which is the area where most (relevant) mutations conferring resistance to antiretroviral fall. Sequence data were analyzed by dedicated HIV genotyping system software that automatically assembles the 7 sequence segments into a consensus sequence, which is then compared to a consensus B reference strain. Sequences having a mixture of wild-type and mutant residues at single positions were considered to have a mutation at that position. When the mixture was between two different mutations, both mutations were considered and reported.

The majority of nucleotide sequences of patients treated with at least a PI within the HAART regimens (Ceccherini-Silberstein *et al.*, 2004; Svicher *et al.*, 2005) have already been submitted to GenBank (accession no.: AY855351-AY855439, AY855441-AY855458, AY855460-AY855502, AY855504-AY855836, and AY995408-AY995555); the others are in the process of being submitted.

Mutations

The mutations considered in the analysis were those reported to be associated with resistance to antiretroviral drugs available for clinical use (Johnson *et al.*, 2004; Stanford HIV sequence database 2004: <http://hivdb.stanford.edu>). In particular, for PI, L10F/I/R/V, K20M/R, L24I, D30N, V32I, L33F, M36I, M46I/L, I47A/V, G48V, I50L/V, F53L, I54A/L/M/S/T/V, L63P, A71T/V, G73A/S, V77I, V82A/F/S/T, I84V, N88D/S, L89M,

L90M, I93L mutations were considered. For NRTI, M41L, E44D, A62V, K65R, D67N, T69D, T69 insertion, K70R, L74V, V75I/M/T, F77L, Y115F, F116Y, V118I, Q151M, M184I/V, L210W, T215F/Y, K219E/Q were considered. Finally, for NNRTI, A98G, L100I, K101E/P, K103N/S, V106A/M, V108I, V179D, Y181C/I/V, Y188C/H/L, G190A/E/Q/S, P225H, F227L, M230L, P236L, K238N/T, Y318F were considered.

The median number (and inter-quartile range: IQR) for each year of these PR (both major and minor) and RT resistance associated mutations to antiretroviral drugs was calculated at first GRT. Resistance to each drug-class was defined according with two available studies (Rhee *et al.*, 2004; Tamalet *et al.*, 2003), whose definition of resistance is very close as follows:

- 1) for PI by the presence of at least one of the mutations at the above mentioned 14 non-polymorphic PR sites 24, 30, 32, 46, 47, 48, 50, 53, 54, 73, 82, 84, 88, 90;
- 2) for NRTI by the presence of at least one of the mutations at the above mentioned 18 positions;
- 3) for NNRTI by the presence of at least one of the mutations at the above mentioned 16 positions.

Statistical analysis

On the samples with available data, the median and IQR of CD4 cell counts, plasma HIV-RNA levels at the time of genotype test, and number of known RT and PR mutations were calculated over the years 1999-2003; statistical analysis was performed by the Mann-Whitney non-parametric t-test. Statistical differences over the years of CDC-stage, of proportion of patients carrying resistant virus strains, of mutation frequencies, and of drug administrations were calculated by chi-square test (based on a 2-by-2- contingency table). P values <0.05 were considered statistically significant for both tests. The results of statistical analysis were reported mainly considering the differences between 1999 versus 2003.

RESULTS

Study population

A total of 1,075 HIV-1 infected multi-treated adult patients undergoing their first GRT after viro-

logical failure between 1999 and 2003 were analyzed: 144 patients underwent GRT in 1999, 232 in 2000, 286 in 2001, 237 in 2002, and 176 in 2003. Patient characteristics are shown in Table 1 stratified by the years 1999-2003.

From 1999 to 2003 a significant decrease of CDC-stage C in patients undergoing their first GRT (from 52% to 35%, $p=0.01$) was observed. Of interest, the median CD4 cell count at first GRT increased from 268×10^6 cells/l in 1999 to $322 \times$

10^6 cells/l in 2003 ($p=0.038$). Similarly, median plasma HIV RNA levels at first GRT over the same period decreased from 4.87 to 3.73 \log_{10} copies/ml ($p<0.001$). Interestingly, in 3.6% of samples tested for GRT the plasma viremia was <1000 copies in 1999, compared to 19.4 % in 2003 ($p<0.001$). Patients had generally had substantial previous treatments, as confirmed by a high number of drugs used pre-GRT. The number of patients undergoing GRT for the first time and also at

TABLE 1 - Characteristics of 1,075 HIV-1 infected multitreated patients at the time of the first GRT after virological failure over the period 1999-2003

Characteristic	1999	2000	2001	2002	2003	<i>p</i> value ^a (1999-2003)
Number of patients	144	232	286	237	176	
Men, (%)	75.7	69.0	71.3	69.8	78.0	0.748
Age (years), median (min-max)	40 (26-69)	38 (24-62)	39 (25-71)	41 (27-75)	40 (25-71)	0.050
CDC stage, (%)						
A	11.8	12.4	15.8	14.5	20.0	0.117
B	36.3	40.4	46.3	46.4	45.0	0.205
C	52.0	47.2	37.8	39.1	35.0	0.010
CD4 (10^6 cells/l), median (IQR)	268 (116-443)	299 (152-451)	337 (184-534)	370 (238-512)	322 (173-484)	0.038
HIV RNA level (\log_{10} copies/ml), median (IQR)	4.87 (4.28-5.36)	4.47 (3.98-4.98)	4.04 (3.63-4.67)	3.70 (3.22-4.30)	3.73 (3.24-4.45)	<0.001
Patients at 1st failure, (%)	16.7	11.2	15.7	16.9	18.2	0.736
Patients treated with (%):						
<5 drugs	35.4	31.9	39.7	33.9	37.0	0.862
5-8 drugs	48.6	57.3	51.2	52.8	54.9	0.315
>8 drugs	16.0	10.8	9.1	13.3	8.1	0.046
Patients PI treated at GRT, (%)	87.5	78.4	51.7	46.6	47.2	<0.001
Patients NRTI treated at GRT, (%)	97.2	99.1	99.7	99.6	100	0.086
Patients NNRTI treated at GRT, (%)	28.5	31.0	39.5	40.3	38.6	0.073
Overall PI experienced, (%)	97.2	92.3	78.3	79.3	77.3	<0.001
Overall NRTI experienced, (%)	100	100	100	100	100	1
Overall NNRTI experienced, (%)	36.1	41.8	50.0	57.8	59.1	<0.001

The values are calculated on the samples with the available data (range: 80% of all samples). *In bold are indicated statistically significant differences in demographic, immunologic, virologic, and therapeutic parameters between 1999 versus 2003.

their first virological failure remained stable over the years (17-18%, Table 1), while the number of patients at first GRT that had been treated with >8 antiviral drugs decreased between 1999 and 2003 (from 16% to 8%, $p=0.046$). The proportion of patients treated with all three drug classes at the time of GRT decreased over the years (from 16.7% in 1999 to 2.3% in 2003, $p<0.001$), while the proportion of patients receiving two drug classes remained stable around 80%, and the proportion of patients receiving only one drug class (100% NRTI in 1999; and 99% NRTI and 1% PI in 2003) increased from 3.5% to 17% ($p<0.001$). At the time of genotypic analysis, the proportion of patients receiving treatment with PI progressively decreased between 1999 and 2003 (from 87.5% to 47.2%, $p<0.001$), with a median time of PI exposure in the last regimen of 296 days in 1999 and 595 days in 2003 ($p<0.001$). The proportion of patients receiving treatment with NNRTI increased (from 28.5% to 38.6%), with a median time of NNRTI exposure in the last regimen of 219 days in 1999 and 907 days in 2003 ($p<0.001$). The overall PI-experience before first GRT decreased (from 97.2% to 77.3%, $p<0.001$), while the overall NNRTI-experience increased (from 36.1% to 59.1%, $p<0.001$).

Genotypic resistance

To study the prevalence of mutations associated with resistance at first GRT, we analyzed the overall presence of mutations for each drug class. The median number of PI mutations dropped from 6 (IQR: 2-8) in 1999 to 3 (IQR: 1-5) in 2003 ($p<0.001$). The median number of NRTI resistance-mutations remained substantially steady over the years (3, IQR: 1-5 in 1999; 2, IQR: 1-4 in 2003); the median number of NNRTI resistance-mutations by contrast increased from 0 (IQR: 0-1) in 1999 to 1 mutation (IQR: 0-2) in 2003.

On the overall population for year, the proportion of isolates carrying resistance-mutations to at least one drug class (PI, NRTI, or NNRTI), two drug classes (i.e. to at least one RT inhibitor and one PI), and to all three drug classes (i.e. at least one NRTI, one NNRTI and one PI) is reported in Fig. 1. The proportion of patients carrying a virus resistant to at least one NRTI remained constant over the years (85.4% in 1999; 86.4% in 2003), even if the prevalence of NRTI resistance calculated without M184V mutation (related to use of 3TC) declined over the years from 75.7% in 1999 to 61.9% in 2003 ($p=0.012$). The proportion of patients with a virus resistant to at least

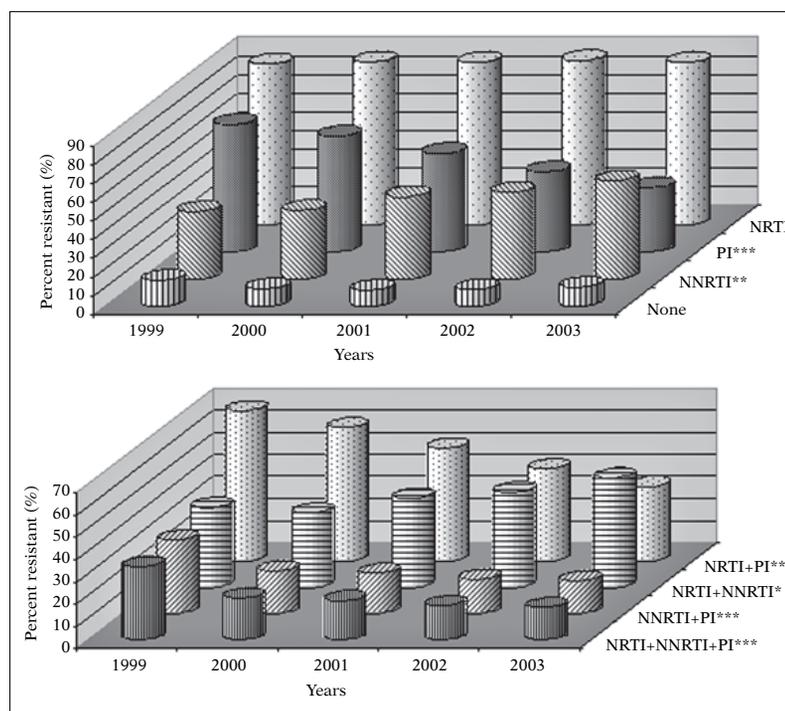


FIGURE 1 - Percent of resistance to any drug-class (A) and of multi-class resistance (B) over the years 1999-2003. Statistically significant differences between 1999 versus 2003 were assessed by a chi-square test: *indicates $p<0.05$; ** $p<0.01$; *** $p<0.001$.

one NNRTI increased (from 36.1% in 1999 to 52.3% in 2003, $p=0.005$) (Fig. 1a). In patients treated with NRTI or NNRTI at GRT, the resistance to both classes was very high (around 85% for NRTI, >90% for NNRTI) and was constant over the years. By contrast, the proportion of patients with PI resistant viruses on the overall population progressively decreased (from 68.1% in 1999 to 34.1% in 2003, $p<0.001$), consistent with the decreased use of PIs over the years. In addition, in patients treated with at least one PI at GRT, the resistance to this class was lower in 2003 than in 1999 (59% versus 70.6%, $p=0.007$) consistent with the increased use of boosted PIs (from 12.6% in 1999 to 21.7% in 2003, $p=0.085$). Patients harboring (at first GRT) a virus resistant to all three drug classes sharply decreased

from 33.3% in 1999 to 14.8% in 2003 ($p<0.001$) (Fig. 1b), while patients with no resistance associated mutations remained rather constant (13.9% in 1999 to 10.2% in 2003, $p>0.05$) (Fig. 1a).

Temporal modification of PIs and resistance associated mutations

From 1999 to 2003, in line with international trends, the prescription at first GRT of un-boosted PIs such as IDV and SQV decreased, respectively, from 34.7% to 9.1% ($p<0.001$) and from 21.5% to 1.7% ($p<0.001$), counterbalanced by an increased use of LPV (from 0% to 8.5%, $p=0.001$) (Fig. 2a). The use of other PIs was not statistically different over the years.

With the exception of D30N (that increased from

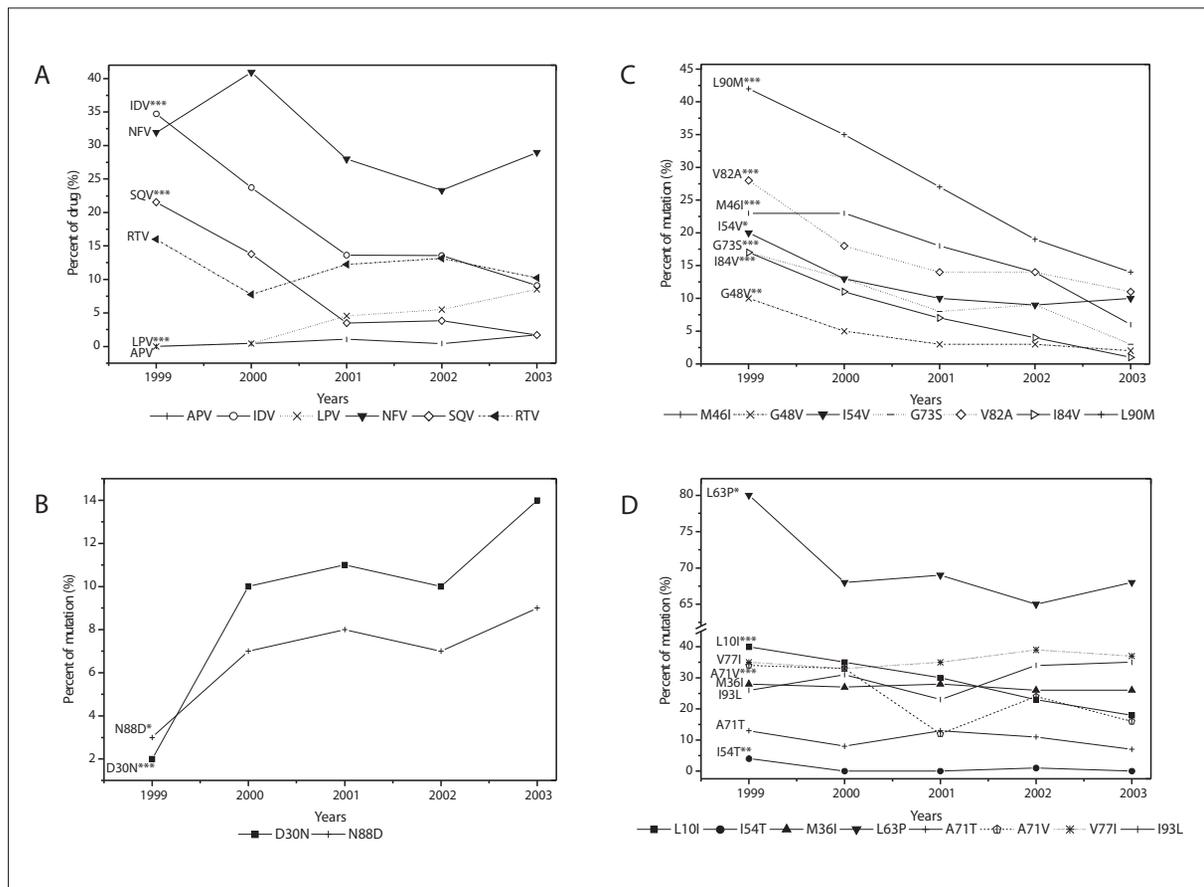


FIGURE 2 - PI use and PI-associated mutations over the years 1999-2003. (A) Percent of PI use. (B) Frequency of mutations D30N and N88D. (C) Frequency of major PR mutations. (D) Frequency of minor PR mutations. The PR mutations characterized by a frequency less than 10% over the time period analyzed, and whose difference between 1999 and 2003 was not statistically significant are not reported in C and D. Statistically significant differences between 1999 versus 2003 were assessed by a chi-square test: *indicates $p<0.05$; ** $p<0.01$; *** $p<0.001$.

2.1% to 14.2%, $p=0.001$) (Fig. 2b), all major PI resistance-mutations (Fig. 2c) dramatically decreased at first GRT from 1999 to 2003. In particular, the I84V mutation almost disappeared (from 16.7% to 1.1%, $p<0.001$), while L90M and V82A had a remarkable drop from 42.4% to 13.6% ($p<0.001$) and from 28.5% to 10.8% ($p<0.001$), respectively. This general decrease of major mutations was not shared by minor mutations (Fig. 2d) whose prevalence from 1999 to 2003 remained substantially stable, with exception of L10I (40.3%-18.2%, $p<0.001$) and A71V (34.0%-16.5%, $p=0.001$), whose decrease suggests (in contrast with other minor mutations) a direct and strong correlation with major PR mutations in the development of resistance. Among minor mutations, I54T, present at low prevalence in 1999 (4.2%), disappeared in 2003 ($p=0.007$) (probably associated with a decrease of the use of drugs, such as IDV or SQV, able to select such mutation). Only N88D increased from 2.8% to 9.1% ($p=0.036$) over the years 1999-2003 (Fig. 2c), confirming its association with D30N (increasing) and the use of NFV as first PI (also increasing from 2.1% in 1999 to 6.3% in 2003).

Other relevant mutations (L10F, L10V, K20M, K20R, L24I, V32I, L33F, M46L, V82T, L89M) were characterized by a low average prevalence (3-10%), and with a not statistically significant difference between 1999 and 2003. All other PI resistance-mutations under analysis remained stable over the years with an average prevalence <3% (data not shown).

Temporal modification of NRTIs and resistance associated mutations

The use of NRTIs at the time of first GRT changed over the years 1999-2003. A decrease of D4T (from 63.9% to 48.3%, $p=0.008$), DDI (from 32.6% to 20.4%, $p=0.019$), and DDC (from 6.3% to 1.1%, $p=0.028$) was observed, corresponding to an increased use of 3TC (from 53.5% to 80.7%, $p<0.001$), AZT (from 25.0% to 44.3%, $p=0.001$) and ABC (from 1.4% to 6.8%, $p=0.037$) (Fig. 3a). In particular, AZT administration in combination with 3TC (Combivir) increased from 16% in 1999 to 38.6% in 2003 ($p<0.001$). The use of TFV was still low in 2003 (2.8%). Among NRTI-associated mutations, a remarkable decrease of NAMs M41L (from 50.7% to 31.3%, $p=0.001$), D67N (from 40.3% to 26.1%, $p=0.011$), L210W (from

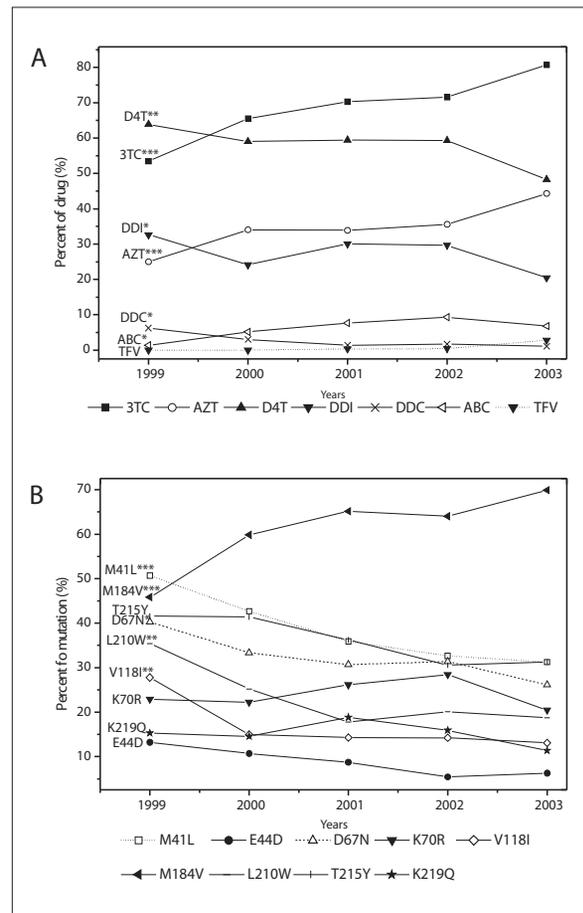


FIGURE 3 - NRTI use and NRTI-associated mutations over the years 1999-2003. (A) Percent of NRTI use. (B) Frequency of NAMs and M184V. The NRTI-associated mutations characterized by a frequency less than 10% over the time period analyzed, and whose difference between 1999 and 2003 was not statistically significant are not reported in B. Statistically significant differences were assessed by a chi-square test: *indicates $p<0.05$; ** $p<0.01$; *** $p\leq 0.001$.

35.4% to 18.8%, $p=0.002$), and V118I (from 27.8% to 13.1%, $p=0.002$) was observed from 1999 to 2003. The prevalence of all other NRTI-associated mutations remained stable over the years (T215Y $\geq 30\%$, K70R and K219Q around 20%, and E44D around 9%) (Fig. 3b). The prevalence of mutation M184V at first GRT increased during the years (from 45.8% to 69.9% in 2003, $p<0.001$) in a fashion similar to 3TC use (Fig. 3). Other relevant RT mutations had a non-statistical difference between 1999 and 2003. In particular, A62V, L74V, T215F, K219E mutations had a prevalence between 4% and 10%, while K65R,

V75I, V75M, V75T, F77L, Y115F, F116Y, Q151M, M184I mutations were below 4%.

Temporal modification of NNRTIs and resistance associated mutations

At first GRT the use of EFV increased from 1999 to 2003 (from 6.3% to 17.6%, $p=0.004$), while NVP remained stable (Fig. 4a). In line with the overall increased use of NNRTIs (from 28.5% to 38.6%), K103N mutation increased from 17.4% in 1999 to 27.8% in 2003 ($p=0.038$); mutations P225H and K238T, completely absent in 1999, reached a prevalence of 2.8% ($p=0.041$) and 4% ($p=0.016$) respectively in 2003, possibly associ-

ated with the specific increase in EFV use. The V108I mutation (that by itself does not necessarily affect drug activity) fluctuated until 2002 and sharply decreased to 10.8% ($p=0.024$) in 2003 (Fig. 4b). Mutation Y181C decreased over the years, but without statistical significance (from 18.1% in 1999 to 13.1% in 2003, $p=0.282$). The frequency of all other NNRTI mutations remained within 6% without statistically significant differences over the years (data not shown).

DISCUSSION

The understanding of the prevalence and patterns of HIV drug resistance represents a major task in antiviral therapy, particularly in view of long-term strategies aimed to maintain the virus under control for long periods of time. In this work, we examined the genotypic, virologic and immunologic data from an Italian set of 1,075 HIV-1 infected multi-treated adult patients, undergoing the first GRT after virological failure between 1999 and 2003. To maintain uniform criteria, we analyzed only genotypic data from subtype B virus therefore our results cannot be directly extrapolated to other viral subtypes. Further analyses will be required in the future, also because the number of patients carrying different subtype viruses increased in the recent years. The same approach will be required for HIV-infected children, excluded from this analysis because of their different immune-virologic characteristics and drug availability.

In our large set of patients, over 90% of individuals carried viruses with at least one key mutation conferring resistance to at least one anti-retroviral drug, thus indicating a high prevalence and circulation of resistant viruses among drug-treated patients. However, the prevalence of NRTI/NNRTI/PI "triple" resistance declined over recent years from 33.3% in 1999 to 14.8% in 2003, and this decline was also confirmed in 2004 (9.9%) and 2005 (9.6%). This decrease can be explained by a sharply decreased prescription of all three drug classes at the time of GRT, and decreased prescription of un-boosted PIs (in particular IDV and SQV) counterbalanced by an increased use of boosted PIs (less prone to select for resistance both to PIs and NRTIs at failure,

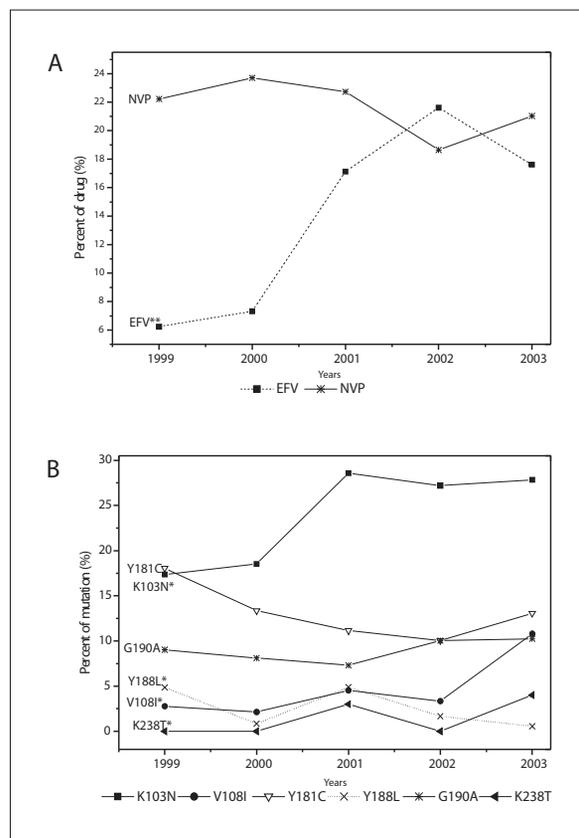


FIGURE 4 - NNRTI use and NNRTI-associated mutations over the years 1999-2003. (A) Percent of NNRTI use. (B) Frequency of NNRTI-associated mutations over the years 1999-2003. The NNRTI-associated mutations characterized by a frequency less than 6% over the time period analyzed, and whose difference between 1999 and 2003 was not statistically significant are not reported in B. Statistically significant differences were assessed by a chi-square test: *indicates $p<0.05$; ** $p<0.01$; *** $p\leq 0.001$.

particularly if used at the beginning of antiviral therapy) (Walmsley, *et al.*, 2002). The lower presence of resistance to all three drug classes may have positive implications in the management of HIV-infected patients, such as increasing the number of future therapeutic choices and decreasing the risk of death (Zaccarelli *et al.*, 2005).

A remarkable discrepancy regarding the prevalence of viruses resistant to all three drug classes was found in a recent study performed in France (Tamalet *et al.*, 2003), where, from 1999 to 2002, the proportion of patients harboring viruses resistant to all three drug classes remained stable around 25% overtime. Tamalet *et al.* (Tamalet *et al.*, 2003) also observed an overall higher prevalence (17.8%) of patients with no resistance-mutations, as noted in the United States (Rhee *et al.*, 2004), whereas we found a prevalence of 10% of patients with viruses without any resistance mutations. This difference may be related to a different prevalence of resistance to at least one NRTI (86.4% in our set versus the French 78.3%), which could reflect different patients' characteristics and therapeutic prescriptions (especially in the past). For instance, it should be noted that in our cohort the high prevalence of NRTI-resistance is also due to the high frequency of M184V mutation (in turn related to the increased use of 3TC).

If recent studies were mostly dedicated to the understanding of the general evolution of resistance over the years (Kagan *et al.*, 2004; Rhee *et al.*, 2004; Richman *et al.*, 2004; Scott *et al.*, 2004; Tamalet *et al.*, 2003), our main effort was to evaluate and characterize the dynamics of the single mutations over the years. Taken together, our data show that the prevalence of key mutations to NRTI and PI (drugs characterized by a high genetic barrier, particularly PI boosted by RTV) decreases over the years. Several major PI mutations (such as L90M, V82A, M46I, I54V, G73S, I84V, G48V) and several NRTI-associated NAMs (such as M41L, D67N, L210W, V118I) sharply decreased over the years. In contrast, the prevalence of resistance mutations to NNRTIs (drugs with low genetic barrier), such as K103N, V108I and K238T, increased over the years. This increase can be related to the specific increased use of EFV, and of the combination of NVP with AZT, that may favor the emergence of K103N

rather than Y181C (MacArthur *et al.*, Abstr 39th Intersci Conf on Antimicrob Agents and Chemother; Abstr 1171; Richman *et al.*, 1994). In addition, as shown for K103N (Nicastri *et al.*, 2003), these NNRTI mutations may confer limited fitness change, therefore they may remain in the viral genome even after NNRTI removal. Indeed, in our cohort about 50% of patients previously treated with NNRTI carry NNRTI-mutations even if their last therapy does not include these drugs.

In summary, our results confirm that mutations associated with drugs with low genetic barrier are the first to appear; and that genetic barrier remains a key factor regulating the appearance of mutations at the time of virological failure. Their early detection by GRT may represent a marker of early therapeutic failure that can lead to anticipated therapeutic change, thus preventing the later appearance of complex mutations, such as NAMs or PI mutations (other than D30N/N88D) more prone to induce high levels of cross resistance.

The remarkably reduced prevalence of major PI-resistance mutations in the overall population of treated patients should be first of all associated with a decreased use of PIs. In addition, in PI-treated patients we observed a decrease of PI-resistance in 2003 (after a median time of 595 days of PI exposure in the last regimen before GRT) with respect to 1999 (after a median time of 296 days of PI-exposure in the last regimen before GRT), suggesting a close relationship with ongoing changes (and improvements) in antiviral strategies, such as the increased use of boosted PIs in recent years.

The decline in the frequency of several NAMs can also be attributed to the decline of d4T use and increasing use of AZT in fixed combination with 3TC. Supporting this hypothesis, previous studies indicate that the M184V mutation arising from 3TC treatment can delay the development of AZT resistance-mutations (Larder *et al.*, 1995; Ait-Khaled *et al.*, 2002).

Furthermore, the immune and virologic parameters of patients undergoing their first GRT showed that its request occurs in recent years at the time of less advanced disease (higher CD4 cell count, lower plasma HIV RNA level, lower CDC-stage) and earlier in the therapeutic history (as confirmed by the recent decline of

patients at first GRT treated with >8 drugs). Similar results were obtained in a previous study performed on a larger cohort of 1,416 infected patients, including both naïve patients and those undergoing therapeutic interruption, in addition to treated patients analyzed here. For all these patients the first GRT requests over the years were associated with a progressive increase in CD4 cell-count and, at the same time, a progressive decrease of plasma HIV-1 RNA levels (Santoro *et al.*, 2004).

Overall, these results can be related to a better management of antiviral therapy by clinicians today. Indeed, this reflects the increased perception of the importance of GRT, as demonstrated by Ciancio *et al.* (2003) to a greater potency of new drugs (that decrease the number of mutations and viral load at failure), and to an increased number of therapeutic choices available in more recent years. Further studies, aimed to define which among these (and perhaps other) factors play a role in this recent change, may help in further improving the therapeutic strategies aimed at a longer and greater control of the progression of HIV-related disease and transmission of drug-resistant viruses from patients in whom resistance has developed during therapy.

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