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Comparative Analysis of Drug Resistance Among B and the Most Prevalent Non-B HIV Type 1 Subtypes (C, F, and CRF02_AG) in Italy

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Abstract

In recent years, increasing numbers of patients infected with HIV-1 non-B subtypes have been treated with modern antiretroviral regimens. Therefore, a better knowledge of HIV drug resistance in non-B strains is crucial. Thus, we compared the mutational pathways involved in drug resistance among the most common non-B subtypes in Italy (F, C, and CRF02_AG) and the B subtype. In total, 2234 *pol* sequences from 1231 virologically failing patients from Central Italy were analyzed. The prevalence of resistance mutations in protease and reverse transcriptase between non-B and B subtypes has been evaluated. Among patients treated with nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and with thymidine analogues (TA) experience, TAMs1 M41L and L210W were less prevalent in CRF02_AG, while TAMs2 T215F and K219E were more prevalent in the F subtype. In NRTI-treated patients having experience with abacavir, didanosine, tenofovir, or stavudine the K65R mutation was mostly prevalent in the C subtype. In non-NRTI (NNRTI)-treated patients infected by the C subtype the prevalence of K103N was lower than in patients infected with other subtypes, while the prevalence of Y181C and Y188L was higher compared to subtype B. The prevalence of Y181C was higher also in subtype F as compared to subtype B. In patients treated with protease inhibitors, L89V was predominantly found in CRF02_AG, while the TPV resistance mutation T74P was predominantly found in the C subtype. Some differences in the genotypic drug resistance have been found among patients infected with B, C, F, and CRF02_AG subtypes in relationship to treatment. These results may be useful for the therapeutic management of individuals infected with HIV-1 non-B strains.

Introduction

The human immunodeficiency virus type 1 (HIV-1) evolves rapidly due to high replication rates, changing selective pressures, and the error-prone reverse transcriptase (RT).

To date, in the viral M group nine subtypes (A, B, C, D, F, G, H, J, and K) have been described, together with many recombinant forms called, respectively, circulating recombinant form (CRF) and unique recombinant form (URF).[1,2] HIV-1 subtype B is the predominant variant in North America, the Caribbean, Latin America, Western and Central Europe, and Australia, while most HIV-1 infections worldwide are caused by subtype A (East Africa), subtype C (East and Southern Africa, Ethiopia, and India), CRF02_AG (West Africa), or CRF01_AE (Asia).[3–5]

However, several studies showed that non-B subtype HIV-1 infections have been rapidly increasing during the

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past years in previously subtype B homogeneous areas such as Europe and North America.[6–9] For example, in France and in Switzerland it is estimated that non-B infections constitute roughly 20% and 35% of HIV-1 infections,[6,8] with an increased prevalence of CRFs over the past 5 years. A similar situation has been observed in Italy, where HIV-1 non-B-infected patients increased from 0.3% in 1993 to 20% in recent years.[9] Several studies performed in this country have showed that the most prevalent non-B subtypes in this country are C, F, and the recombinant form CRF02_AG.[10–16]

In this regard, Italy is a good example of the way the spread of non-B subtypes is highly dependent upon several variables such as the demographics of local HIV-1 epidemics and their evolution over time: while the prevalence of non-B HIV-1 subtypes has remained low in South Italy, such as Sicily,[15] it has increased in North Italy, with a preponderance of C and F subtypes due to the increased number of immigrants from Africa and Eastern Europe.[14]

Although non-B infections are infrequent in North America, a study in New York identified non-B infections in a few U.S. citizens who never traveled abroad, suggesting that transmission of non-B subtype occurs in the United States independently of travel history.[7]

Due to the spread of non-B viruses and the introduction of antiretroviral drugs in developing countries (known for the largest assortment of non-B subtypes), further knowledge concerning the responsiveness to antiretroviral therapy and HIV-1 drug resistance in non-B strains is required. In this regard, it is known that different viral subtypes can be naturally less or more susceptible to specific drugs.[17,18] For example, the recombinant form CRF02_AG is more susceptible to nelfinavir (NFV) and ritonavir (RTV) than subtypes C and F, subtype G is more sensitive to tipranavir (TPV) and lopinavir (LPV) than other subtypes,[19] and subtype C has accelerated risk in developing resistance to tenofovir (TDF).[20–22] An explanation for the extreme variability of HIV-1 subtypes in the highly active antiretroviral therapy (HAART) response can be given by the presence of some polymorphisms, which can influence both the emergence of drug resistance mutations as well as the response to drugs. For example, polymorphisms at residues 20 and 36 of HIV-1 protease decrease the genetic barrier to TPV resistance in subtypes A, C, F, and G,[2] while nucleotide heterogeneity at 64 and 65 RT positions accelerated development of K65R in subtype C.[20,23]

However, comparative data about the pathways of resistance of these subtypes versus the B subtype are still incomplete in the European settings, where patients carrying B and non-B subtypes are treated with similar antiviral regimens.

Moreover, data about these issues are sometimes insufficient to set and validate drug resistance interpretation algorithms in populations infected with non-B variants.

Thus, the goal of this study was to compare the mutational pathways involved in resistance to nucleoside/nucleotide RT inhibitors (NRTIs), non-NRTI (NNRTIs), and protease inhibitors (PIs) among HIV-1 B and non-B (with particular attention to subtypes F and C and the recombinant form CRF02_AG) subtype-infected patients, with similar drug experiences, followed in clinical centers in Central Italy over the years 2001–2008.

Materials and Methods

Study population

The study included a total of 2234 *pol* sequences from 1231 virologically failing patients followed in several clinical centers in Rome and surroundings, undergoing a genotyping resistance test (GRT) over the years 2001–2008. Among these 1231 patients, 189 were infected by non-B strains. The most frequent HIV-1 non-B subtypes in our cohort were subtypes F (47, 24.9%), C (45, 23.8%), and the recombinant form CRF02_AG (36, 19.1%). At the time of GRT, all patients analyzed were under treatment and had a plasma viral load >50 copies/ml. Data for all patients were stored in a specifically designed anonymous database that included genotypic, demographic, immunologic, virologic, and therapeutic parameters.

HIV sequencing

HIV genotype analysis was performed on plasma samples by means of a commercially available kit (ViroSeq HIV-1 genotyping system; Abbott Laboratories). Briefly, RNA was extracted, retrotranscribed by murine leukemia virus RT, and amplified with Amplitaq-Gold polymerase enzyme by using two different sequence-specific primers for 40 cycles. *Pol*-amplified products (containing the entire PR and the first 335 amino acids of the RT open reading frame) were sequenced full-length in sense and antisense orientations by an automated sequencer (ABI 3100) by using seven different overlapping sequence-specific primers.[24] Sequences having a mixture of wild-type and mutant amino acid residues at single positions were considered to have the mutant(s) at that position.

Phylogenetic analysis

All HIV-1 *pol* sequences (1302 nucleotides) were aligned and compared with reference sequences for the major HIV-1 subtypes, available at http://hiv-web.lanl.gov/content/hivdb/ SUBTYPE_REF/align.html using CLUSTAL X.[25] The sequences were then manually edited with the Bioedit program,[26] and gaps were removed from the final alignment. All sequences were analyzed using the REGA HIV-1 subtyping tool.[27] Separate trees were then generated using F84 model of substitution with both neighbor-joining (NJ) and maximum likelihood (ML) tree building methods,[28] for both non-B pure subtypes and putative recombinant forms. Phylogenetic trees were performed with different evolutionary model according to the Hierarchical Likelihood Ratio Test (HLRT) implemented in the Model Test V3.0 software.[29] The statistical robustness within each phylogenetic tree was confirmed with a bootstrap analysis using 1000 replicates for the NJ tree. All calculations were performed with PAUP*4.0 software.[28]

Mutation prevalence

The prevalence of resistance mutations in the three most prevalent non-B subtypes (C, F, and CRF02_AG) and the 1042 B subtype-infected patients has been evaluated. For the analysis of drug resistance mutations, to avoid any relationship between the mutation prevalence in the different subtypes and drug pressure, the HIV-1-infected patients were divided into six different subgroups according to duration

and type of antiretroviral treatment: (1) patients treated with thymidine analogues (TAs) zidovudine (AZT) and/or stavudine (d4T) for the analysis of the prevalence of TA-related mutations (TAMs: M41L, D67N, K70R, L210W, T215F/Y, and K219E/Q); (2) patients treated with experience to d4T for the analysis of K65R[30–32]; (3) patients treated with abacavir (ABC), didanosine (ddI), and/or tenofovir (TDF) for the analysis of the prevalence of ABC/ddI/TDF-related mutations K65R, D67G, T69ins, K70E/G, and L74V[30,31]; (4) patients treated with lamivudine (3TC) and/or emtricitabine (FTC) for the analysis of M184V[30,31]; (5) patients treated with NNRTI for the analysis of the prevalence of NNRTI-related mutations (L100I, K101E/P, K103N/S, V106A/M, V108I, V179F, Y181C/I/V, Y188C/H/L, G190A/E/S, P225H, M230L, and K238T)[30,31]; and (6) patients treated with PI for the analysis of the prevalence of PI-related mutations (D30N, V32I, L33F, M46I/L, I47A/V, G48V, I50L/V, I54L/M, Q58E, T74P, L76V, V82A/F/L/S/T, I84A/C/V, N83D, N88D/S, L89V, and L90M).[30,31,33,34] For each subgroup one sequence per patient (the last one available under treatment) was analyzed.

Statistical analysis

To correctly compare rates of mutation prevalence among populations of different size (HIV-1 subtype B-infected patients vs. HIV-1 subtype non-B C, F, or CFR02_AG-infected patients), 50 sequences from the original HIV-1 subtype B-infected population were drawn randomly 500 times. The mutation prevalence found in each randomized HIV-1 B sample was then compared with that found in the subtypes C, F and CRF02_AG by Fisher exact test. p values < 0.05 were considered statistically significant for all tests utilized. Random selection of 500 samples was obtained by coding a specific Java program. Moreover, statistically significant differences in the prevalence of each drug resistance mutation and in the duration of therapy among the three most prevalent non-B subtype groups were assessed by a 3×2 Chi squared test and Kruskal–Wallis test, respectively. Again, p values < 0.05 were considered statistically significant. The calculations of all statistical test were performed by the R open source software.

Results

Patients characteristics

Demographic and therapeutic characteristics of HIV-1-infected patients stratified by HIV-1 subtype are reported in Table 1. At GRT, viral load, and CD4 cell count were comparable among patients infected by different HIV-1 subtypes ($p > 0.05$). Analyzing the antiretroviral treatment, patients infected by CRF02_AG had a median duration of therapy of 168 (interquartile range, IQR: 71–285) weeks, significantly lower than that observed for patients infected by C [188 (111–382) weeks], F [368 (226–551) weeks], or B subtype [415 (234–614) weeks] ($p = 0.03$).

Information about current and previous antiretrovirals used is shown both in Table 1 and Supplementary Table S1. (Supplementary Data are available online at www.liebertonline.com/aid.)

Mutation prevalence

NRTI resistance mutations. Among patients experienced with TA, the percentage of patients infected by CRF02_AG strains with TA resistance was quite less (4, 14.3%) than that observed for patients infected by C and F subtypes [9 (25.0%),

Table 1. Patients' Characteristics

Characteristic	*Subtypes*				
	Overall non-B	*C*	*F*	*AG*	*B*
Patients, *N*	189	45	47	36	1042
Male, *N* (%)	107 (58.4)	18 (42.8)	35 (74.5)	16 (45.7)	756 (72.8)
Italian, *N* (%)	31 (30.0)	4 (14.3)	16 (64.0)	3 (13.6)	634 (89.0)
HIV exposure, *N* (%)					
MSM	18 (13.1)	2 (6.5)	8 (22.2)	2 (7.7)	144 (18.1)
Heterosexual	107 (78.1)	29 (93.5)	23 (63.8)	23 (88.5)	271 (34.0)
IDU	6 (4.4)	0 (0.0)	2 (5.5)	1 (3.8)	357 (44.9)
Other	6 (4.4)	0 (0.0)	3 (8.3)	0 (0.0)	24 (3.0)
Age, years, median (IQR)	38.1 (32.7–45.3)	37.3 (32.5–42.2)	40.0 (35.4–46.6)	32.8 (29.1–39.7)	42.4 (38.1–46.9)
Viremia at GRT (log copies/ml), median (IQR)	3.3 (2.6–4.3)	3.2 (2.5–4.3)	3.4 (2.4–4.5)	3.2 (2.8–4.5)	3.8 (2.9–4.5)
CD4 cell count at GRT (cells/μl), median (IQR)	286 (152–476)	303 (154–420)	248 (114–483)	342 (247–509)	307 (160–506)
Year of ART initiation, *N* (%)					
≤ 2000	79 (42.0)	19 (42.2)	26 (55.3)	9 (25.8)	716 (69.4)
2001–2003	51 (27.1)	10 (22.2)	13 (27.7)	13 (37.1)	195 (18.9)
≥ 2004	58 (30.9)	16 (35.6)	8 (17.0)	13 (37.1)	121 (11.7)
Patients failing first line regimen, *N* (%)	28 (14.8)	8 (17.8)	5 (10.6)	15 (41.7)	168 (16.1)
Number of ART regimens, median (IQR)	3 (2–5)	2 (1–5)	4 (2–6)	2 (1–3)	3 (2–6)
ART duration,[a] weeks, median (IQR)	237 (85–418)	188 (111–382)	368 (226–551)	168 (71–285)	415 (234–614)

[a]ART duration indicates the time elapsed between the start of the first line regimen and the genotypic resistance test.
Prevalence was calculated on the samples with available data (range: 80% of all samples).
GRT, genotypic resistance test; AG, CRF02_AG; ART, antiretroviral therapy; IDU, injection drug users; MSM, males who have sex with males; IQR, interquartile range; *N*, number of patients.

14 (34.1%), $p = 0.178$]. In particular, TAMs1 M41L and L210W were less prevalent in patients with CRF02_AG compared to patients with F and C subtypes (Table 2); this difference was larger and statistically significant when compared with the B subtype ($p = 0.001$ and $p = 0.005$, respectively). Differently, TAMs2 T215F and K219E were more prevalent in patients infected with the F subtype compared with those infected with the C, CRF02_AG (completely absent in this viral strain), and B subtype ($p = 0.015$ and $p = 0.03$, respectively).

As previously reported, the prevalence of K65R was known to be significantly higher in patients infected with the C subtype and with experience with ABC, ddI, or TDF than those infected with F, CRF02_AG, and B subtypes.[20,21] Our results confirmed these data, but highlighted a higher prevalence of K65R in patients infected with subtype C and with experience with d4T ($p < 0.001$). In particular, as shown in Table 3, experience with d4T, as well as experience with ddI or TDF or ABC, influenced the presence of K65R at GRT only in subtype C-infected patients ($p = 0.01$).

NNRTI resistance mutations. In NNRTI-treated patients, the mutation K103N was present at lower frequency in subtype C compared with F, CRF02_AG, and with the B subtype ($p = 0.04$) (Table 2). This finding was confirmed also after stratifying the population for nevirapine (NVP) or efavirenz (EFV) experience (data not shown). Conversely, the mutations Y181C and Y188L were found with higher frequency in patients infected with subtype C compared to patients infected with subtype B ($p = 0.04$ and $p = 0.02$). The Y181C was also found with a higher frequency in subtype F when compared with subtype B ($p = 0.03$). As expected, this mutation was more prevalent in patients with NVP experience than in patients treated with EFV [NVP experience: C (66.7%), F (87.5%) vs. B (31.2%): $p = 0.07$ and $p = 0.002$, respectively; EFV experience: C (25.0%), F (26.3%) vs. B (8.6%): $p < 0.001$ and $p = 0.03$, respectively].

PI resistance mutations. In PI-treated patients, L90M was the most common mutation found in each subtype group (Table 2). The mutation D30N was completely absent in patients infected with CRF02_AG, subtype C, and subtype F while its prevalence reached 8.0% in patients infected with subtype B; this result was also confirmed when the analysis was performed only in NFV experienced patients (0% in non-B subtypes vs. 15.0% in B subtypes, $p = 0.02$, data not shown). Interestingly, the mutation L89V (potentially associated with resistance to fosamprenavir and, to a lesser extent, to darunavir and lopinavir) was predominantly found in CRF02_AG ($p = 0.004$), while the TPV resistance mutation T74P was predominantly found in subtype C ($p = 0.001$), even if only one patient was treated with TPV.

Discussion

The present study provides new information about resistance profiles occurring in patients infected with several HIV-1 non-B subtypes (C, F, and CFR02_AG) and the B subtype.

An improved knowledge of the significance of non-B subtypes for resistance evolution and interpretation is becoming mandatory today not only because antiretroviral therapy is being introduced in countries where non-B subtypes are driving the epidemic, but also because the number of infections by these variants is increasing sharply in several European countries.[6,8,9]

The results obtained in this study show that HIV-1 drug resistance does not emerge at the same rate among different HIV-1 subtypes, and that different pathways to resistance are taken in different proportions according to subtype under the same therapeutic pressure. For example, differences in the prevalence of some TAMs1 (M41L and L210W, but not T215Y) were observed among NRTI-treated patients with TA experience infected by B and C, F, and CRF02_AG strains. In particular in patients infected with the recombinant form CRF02_AG, the prevalence of M41L was lower than that observed for other subtypes, and L210W was completely absent. In this regard, the absence of this latter mutation in patients infected with CRF02_AG and its lower prevalence in patients infected with subtypes C and F in comparison to those infected with subtype B could be due to the increased genetic barrier of these subtypes toward the development of an L210W substitution. CRF02_AG, C, and F viral strains frequently contain either the CTG, CTA, or TTA codon at this position (all encoding for leucine), instead of TTG, the most common codon found in the B subtype (also encoding for leucine).[35] So, while in subtype B the L210W substitution required only one transversion (from TTG to TGG, total score = 2.5), in subtype non-B, because of the presence of other starting codons, this substitution required one transition and one transversion (total score = 1 + 2.5 = 3.5). Thus, this observation can suggest that the genetic barrier, based upon the different codon heterogeneities naturally present in different subtypes, may play a relevant role in the selection of mutations at failure.

It is important to note that even if in our cohort of patients, those infected by subtype C and CRF02_AG had a median duration of treatment lower than patients infected by subtypes F and B. The low prevalence of TAMs1 in CRF02_AG was also confirmed by analyzing a subgroup of NRTI-treated patients with TA experience and with similar therapy duration (data not shown). This finding was also confirmed in our resistance database including more than 8000 HAART-failing patients: patients infected by CRF02_AG failed their HAART regimen with fewer M41L and L210W mutations than patients infected with other HIV-1 subtypes (4.8% in CRF02_AG vs. 7.9% in C subtype vs. 20% in F subtype vs. 21% in B subtype, $p < 0.001$; M.M. Santoro, unpublished data). Other studies confirmed the lower prevalence of TAMs in the CRF02_AG-infected population when compared with B or other non-B subtypes.[18,36]

Similarly, the TAMs2 T215F and K219E were completely absent in the CRF02_AG population; however, these mutations were generally rare also in subtype B-infected and subtype C-infected patients (< 5%). Their prevalence reached 14% and 12% only in subtype F-infected patients. These patients also showed a high prevalence of other TAMs (in particular M41L, D67N, and K70R), suggesting an overall more rapid selection of TAMs during a TA regimen in patients infected with subtype F compared with other subtypes. Interestingly, the high prevalence of mutations at RT positions 67 and 70 in F subtypes was also recently found in a cohort of Brazilian HAART-treated patients, where these mutations reached 30%.[37] The molecular reasons for this selection in subtype F are still unknown, and it does not seem to be associated with a different genetic barrier at that position.[35]

Table 2. Prevalence of Drug Resistance Mutations in Patients Failing Highly Active Antiretroviral Therapy According to HIV-1 Subtype and Treatment

						p *value*[b] *(% of significance)*		
Mutation	*C*	*F*	*AG*	p *value*[a]	*B*	*C vs. B*	*F vs. B*	*AG vs. B*
NRTI-treated pts with TA exp, *N*	36	41	28		984			
M41L	8 (22.2)	10 (23.4)	1 (3.6)		299 (29.1)			0.001 (97.8)
D67N	5 (13.9)	8 (19.5)	3 (10.7)		198 (20.1)			
K70R	2 (5.5)	6 (14.6)	3 (10.7)		122 (12.4)			
L210W	3 (8.3)	5 (12.2)	0 (0.0)		185 (18.8)			0.005 (89.0)
T215Y	6 (16.7)	6 (14.6)	4 (14.3)		277 (28.2)			
T215F	1 (2.8)	6 (14.6)	0 (0.0)	0.03	46 (4.7)		0.015 (32.2)	
K219Q	2 (5.5)	1 (2.4)	1 (3.6)		49 (5.0)			
K219E	0 (0.0)	5 (12.2)	0 (0.0)	0.02	41 (4.2)		0.03 (11.2)	
NRTI-treated pts with d4T exp, *N*	25	25	11		717			
K65R	6 (24.0)	1 (4.0)	0 (0.0)		23 (3.2)	< 0.001 (60.8)		
NRTI-treated pts with ddI or TDF or ABC exp, *N*	35	41	18		734			
K65R	6 (17.1)	1 (2.4)	0 (0.0)	0.02	28 (3.8)	0.003 (42.0)		
D67G	0 (0.0)	1 (2.4)	0 (0.0)		0 (0.00)			
T69ins	1 (2.9)	1 (2.4)	0 (0.0)		1 (0.1)			
K70E	1 (2.8)	0 (0.0)	1 (5.6)		4 (0.5)			
K70G	0 (0.0)	0 (0.0)	0 (0.0)	NC	5 (0.7)			
L74V	1 (2.8)	7 (17.1)	0 (0.0)	0.03	69 (9.4)			
NRTI-treated pts with 3TC/FTC exp, *N*	43	44	31		1009			
M184V	19 (44.2)	22 (50.0)	14 (45.2)		545 (54.0)			
NNRTI-treated pts, *N*	20	23	10		335			
L100I	0 (0.0)	2 (8.7)	0 (0.0)		17 (5.1)			
K101E	2 (10)	0 (0.0)	0 (0.0)		30 (8.9)			
K101P	1 (5)	0 (0.0)	0 (0.0)		7 (2.09)			
K103N	5 (25)	14 (60.9)	4 (40)	0.06	168 (50.2)	0.04 (35.0)		
K103S	2 (10)	0 (0.0)	0 (0.0)		7 (2.1)			
V106A	0 (0.0)	0 (0.0)	0 (0.0)	NC	18 (5.4)			
V106M	1 (5)	0 (0.0)	0 (0.0)		3 (0.9)			
V108I	4 (20)	5 (21.7)	0 (0.0)		45 (13.4)			
Y181C	8 (40)	9 (39.1)	2 (20)		66 (19.7)	0.04 (22.2)	0.03 (46.6)	
Y181I	1 (5.0)	0 (0.0)	0 (0.0)		1 (0.1)			
Y188C	0 (0.0)	0 (0.0)	0 (0.0)	NC	1 (0.1)			
Y188L	3 (15)	2 (8.7)	1 (10.0)		9 (2.7)	0.02 (22.8)		
G190A	5 (25)	1 (4.3)	1 (10.0)		61 (18.2)			
G190E	0 (0.0)	0 (0.0)	0 (0.0)	NC	5 (1.5)			
G190S	1 (5)	1 (4.2)	1 (10)		15 (4.5)			
P225H	2 (10)	4 (16.7)	0 (0.0)		32 (9.6)			
M230L	0 (0.0)	0 (0.0)	0 (0.0)	NC	9 (2.7)			
K238T	0 (0.0)	2 (8.7)	0 (0.0)		28 (1.4)			
PI-treated pts, *N*	32	39	27		612			
D30N	0 (0)	0 (0)	0 (0)	NC	49 (8.0)			
V32I	1 (3.1)	3 (7.7)	0 (0.0)		30 (4.9)			
L33F	1 (3.1)	4 (10.3)	0 (0)		53 (8.7)			
M46I	6 (18.8)	6 (15.4)	1 (3.7)		114 (18.6)			
M46L	1 (3.1)	4 (10.3)	2 (7.4)		54 (8.8)			
I47A	1 (3.1)	2 (5.1)	0 (0)		1 (0.2)			
I47V	1 (3.1)	1 (2.6)	0 (0)		27 (4.4)			
G48V	0 (0)	0 (0)	0 (0)	NC	17 (2.8)			
I50L	0 (0)	1 (2.6)	0 (0)	NC	4 (0.6)			
I50V	1 (3.1)	1 (2.6)	0 (0)		8 (1.3)			
I54L	0 (0)	0 (0)	0 (0)	NC	15 (2.4)			
Q58E	1 (3.1)	3 (7.7)	0 (0)		26 (4.2)			
T74P	4 (12.5)	2 (5.1)	0 (0)		7 (1.1)	0.001 (51.0)		
L76V	0 (0)	2 (5.1)	1 (3.7)		10 (1.6)			
V82A	3 (9.4)	8 (20.5)	1 (3.7)		91 (14.9)			
V82F	0 (0.0)	0 (0.0)	0 (0.0)	NC	6 (1.0)			
V82L	0 (0.0)	0 (0.0)	0 (0.0)	NC	3 (0.5)			

(*Table continued* →)

Table 2. (Continued)

Mutation	C	F	AG	p value[a]	B	p value[b] (% of significance) C vs. B	F vs. B	AG vs. B
V82S	1 (3.1)	3 (7.7)	0 (0.0)		30 (4.9)			
V82T	0 (0)	0 (0)	0 (0)	NC	20 (3.3)			
I84A	0 (0)	0 (0)	0 (0)	NC	0 (0.0)			
I84C	0 (0)	0 (0)	0 (0)	NC	0 (0.0)			
I84V	1 (3.1)	3 (7.7)	0 (0)		55 (9.0)			
N88D	0 (0)	0 (0)	0 (0)	NC	36 (5.9)			
N88S	0 (0)	1 (2.6)	2 (7.4)		6 (1.0)			
L89V	3 (9.4)	1 (2.6)	4 (14.8)		13 (2.1)			0.004 (29.4)
L90M	8 (25)	9 (23.1)	4 (14.8)		150 (24.5)			

[a]Differences in the prevalence of each resistance mutation among the three non-B subtypes were assessed by the Chi square test (2×3 table). Only significant p values ($p < 0.05$) are reported.

[b]Differences in the prevalence of each drug resistance mutation between non-B subtypes and B subtype were assessed by the Fisher exact test. This test was repeated for each one of the 500 B subtype resamplings. Only the percentage of significant p values ($p < 0.05$) among the 500 resamplings are reported. The NNRTI mutations V179F, Y181V, and Y188H and the protease mutations I54M and N83D were completely absent in patients analyzed in the present study.

ABC, abacavir; AG, CRF02_AG; 3TC, lamivudine; ddI, didanosine; d4T, stavudine; Exp, experience; FTC, emtricitabine; 3TC, lamivudine; *N*, number of patients; NNRTI, nonnucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; PI, protease inhibitors; Pts, patients; TA, thymidine analogs; TDF, tenofovir; NC, not calculable.

Our study confirmed the finding that the K65R mutation in the RT occurs more frequently in patients infected with subtype C than in patients infected by other HIV-1 subtypes.[38] However, we found a high prevalence of this mutation in subtype C-infected patients exposed not only to TDF but also to an ABC, ddI, and d4T-containing regimen. This result suggests a role for K65R not only as a TDF resistance mutation,[39] but also as ABC, ddI, and d4T selected mutation, especially in patients infected with the C subtype.[32,40,41]

Regarding NNRTI mutations, our study showed that different resistant mutational pathways might characterize virologic failure in patients infected with subtypes B, C, F, and CRF02_AG. In particular, in patients infected with subtype C, a lower frequency of the K103N mutation and a higher prevalence of Y188L and Y181C than in patients infected with subtype B have been observed. While subtype C showed a low prevalence of K103N, as many as 60.9% of subtype F carried this mutation. Interestingly, we also found a higher prevalence of the NRTI mutation L74V in subtype F than in

Table 3. Prevalence of the K65R Mutation in Patients Failing Highly Active Antiretroviral Therapy According to HIV-1 Subtype and Treatment

NRTI-treated patients at GRT with	C	AG	F	p value[a]	B	p value[b] C vs. B	AG vs. B	F vs. B
Exp to ddI or TDF or ABC or d4T	21	18	26		405			
Therapy duration,[c] weeks, median (IQR)	68 (33–100)	47 (33–74)	67 (26–127)		68 (40–104)			
K65R prevalence, *N* (%)	4 (19.0)	0 (0.0)	0 (0.0)	0.03	17 (4.2)	0.01		
Exp to ddI or TDF or ABC, but no exp to d4T	17	8	22		141			
Therapy duration,[c] weeks, median (IQR)	57 (17–109)	37 (23–51)	27 (24–92)		49 (26–78)			
K65R prevalence, *N* (%)	1 (5.9)	0 (0.0)	0 (0.0)		6 (4.2)			
Exp to d4T, but no exp to ddI or TDF or ABC	2	3	3		28			
Therapy duration,[c] weeks, median (IQR)	72 (44–100)	42 (29–54)	77 (54–104)		73 (37–109)			
K65R prevalence, *N* (%)	1 (50.0)	0 (0.0)	0 (0.0)		1 (3.6)			

[a]Differences in the prevalence of K65R among the three non-B subtypes were assessed by the Chi square test (2×3 table).

[b]Differences in the prevalence of K65R between non-B subtypes and the B subtype were assessed by the Fisher exact test.

[c]Therapy duration indicates the total weeks under ddI, TDF, d4T, or ABC treatment.

The prevalence of the K65R mutation was calculated in groups of patients treated with NRTI and with a similar median therapy duration at the moment of the genotypic resistance test. Only significant p values ($p<0.05$) are reported in the table.

ABC, abacavir; AG, CRF02_AG; d4T, stavudine; ddI, didanosine; Exp, experience; GRT, genotypic resistance test; NRTI, nucleoside reverse transcriptase inhibitors; TDF, tenofovir.

subtype C and CRF02_AG. Moreover, four out of the seven HIV-1 subtype F-infected patients with L74V also carried K103N. The association of these two mutations can be explained by the compensatory role of L74V, known to restore the replication capacity impaired by the selection of K103N.[42,43]

Different results were obtained in the several studies comparing the prevalence of NNRTI-related mutations in different HIV-1 non-B subtypes. For example, the K103N mutation was relatively more frequent in subtype C-infected women failing NNRTI-based therapy than subtype A and D in a Uganda study.[44] The mutations G190A/S were seen as frequent polymorphisms in subtype C Israelian-infected patients, but not in Indian C-infected patients.[45,46] Finally, many studies confirmed that the V106M mutation is frequently seen in non-B subtypes (especially C and CRF02_AE) after therapy with EFV or NVP.[45–48] A higher prevalence of G190A/S and V106M was also observed in the present study in patients infected by the C subtype in comparison to other ones, even if these differences were not statistically significant. Overall, these differences may have clinical relevance, in view of the increasing use of second generation NNRTIs, such as etravirine or rilpivirine, whose efficacy is affected differently by the various mutations selected by first generation NNRTIs. In particular, K103N has no effect upon etravirine efficacy, while others, such as Y181C, Y188L, and G190A/S, are more relevant. Additional new studies will be necessary to better define the prevalence of these mutations, in view of the appropriate use of etravirine or rilpivirine in NNRTI-failing patients carrying non-B subtypes.

Regarding PI mutations, no significant differences in the prevalence of major resistance mutations were observed in patients infected with B, C, F, or CRF02_AG subtypes, with the exception of T74P and L89V. In particular, the TPV resistance mutation T74P was more frequently found in patients infected with subtype C, even if only one patient had experience with TPV, while mutation L89V was more frequently identified in patients infected with CRF02_AG. Of interest, the NFV mutation D30N was completely absent in non-B subtypes, even if around >35% of patients experienced this drug in the previous or current regimen, suggesting that this mutation is rarely selected in subtypes other than B.[49] All together, these findings may suggest that the different prevalence of drug resistance mutations in the different subtypes can be due to more intrinsic properties of the virus and not only to different pressures of antiretrovirals. For mutations T74P and L89V, their different prevalence is also supported by a different genetic barrier observed in different subtypes. Indeed, by analyzing 2357 HIV-1 *pol* sequences obtained from naive HIV-1-infected patients, more than 10.0% of drug-naive patients infected by HIV-1 subtype C showed the TCA codon (encoding serine) instead of the codon ACA (encoding threonine) at position 74. This serine codon displays a lower genetic barrier for the development of the resistance substitution proline (encoded by CCA, one transition, score = 1) compared to the threonine codon (encoded by ACA, one transversion, score = 2.5).

In the case of protease position 89, the wild-type codon in most of the non-B subtypes is the ATG (M), itself conferring resistance in most of the non-B subtypes,[33,50] in contrast to subtype B sequences, which show 89M in only 4.8% of cases, preferring the CTG (L) (present in 86.2% of subtype B). Genetic barrier analysis showed that the ATG codon has a lower resistance score to evolve into the resistance substitution GTG (V) (one transition, score = 1) compared to codon CTG (L) (one transversion, score = 2.5).

Interestingly, the CRF02_AG recombinant form was the non-B subtype with the highest frequency of codon ATG (M) at position 89 (95.4% of patients), if compared with F and C subtypes (F = 73.0% and C = 81.9%, respectively). Of note, these two protease positions 74 and 89 seem to be closely related to each other. As shown by Gonzales *et al.*, the protease mutation L89I/V is stabilized by the acquisition of T74S in subtype G.[51] It is conceivable that in subtype C, the mutation T74S, a frequent PR polymorphism in drug-naive patients, could favor the 89I/V selection under treatment. These findings suggest that some natural polymorphisms (such as T74S and L89M) should be considered with particular attention in the therapeutic management of patients infected by HIV-1 non-B subtypes. It should be remembered that they could favor the emergence of resistance mutations. For instance, it has been proposed that the presence of methionine at protease position 89 in non-B subtype viruses favors the selection of mutation I/V at position 89 and the PI major mutation L90M, rather than the lysine in subtype B.[33]

In conclusion, although the present exploratory analysis was limited to a small number of patients for each subtype and requires confirmation by further studies, these findings suggest that the genetic diversity within HIV-1 group M could play a role in the development of resistance to antiviral drugs. A tendency to a lower level of resistance to AZT was observed in patients infected with CRF02_AG HIV-1 variants, while a higher level of resistance to TDF and TAs was observed for subtype C and F, respectively. The therapeutic implications of these unique subtype differences in terms of long-term efficacy of different antiviral regimens are still unknown. They will be best explored in ad hoc studies, to be done in the future.

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References

1. Buonaguro L, Tornesello ML, and Buonaguro FM: Human immunodeficiency virus type 1 subtype distribution in the

worldwide epidemic: Pathogenetic and therapeutic implications. J Virol 2007;81:10209–10219.
2. Holguín A, Ramirez de Arellano E, Rivas P, and Soriano V: Efficacy of antiretroviral therapy in individuals infected with HIV-1 non-B subtypes. AIDS Rev 2006;8:98–107.
3. Peeters M, Aghokeng AF, and Delaporte E: Genetic diversity among human immunodeficiency virus-1 non-B subtypes in viral load and drug resistance assays. Clin Microbiol Infect 2010;16:1525–1531.
4. Hemelaar J, Gouws E, Ghys PD, and Osmanov S, and the WHO-UNAIDS Network for HIV Isolation and Characterisation: Global trends in molecular epidemiology of HIV-1 during 2000–2007. AIDS 2011;13:679–689.
5. Kantor R, Shafer RW, and Katzenstein D, and the HIV-1 Non-subtype B Workgroup: The HIV-1 Non-subtype B Workgroup: An international collaboration for the collection and analysis of HIV-1 non-subtype B data. MedGenMed 2005;23:71.
6. Barin F, Courouce AM, Pillonel J, *et al.:* Increasing diversity of HIV-1M serotypes in French blood donors over a 10-year period (1985–1995). Retrovirus Study Group of the French Society of Blood Transfusion. AIDS 1997;11:1503–1508.
7. Weidle PJ, Ganea CE, Irwin KL, *et al.:* Presence of human immunodeficiency virus (HIV) type 1, group M, non-B subtypes, Bronx, New York: A sentinel site for monitoring HIV genetic diversity in the United States. J Infect Dis 2000;181:470–475.
8. Yerly S, Vora S, Rizzardi P *et al.:* Acute HIV infection: Impact on the spread of HIV and transmission of drug resistance. AIDS 2001;15:2287–2292.
9. Lai A, Riva C, Marconi A, *et al.:* Changing patterns in HIV-1 non-B clade prevalence and diversity in Italy over three decades. HIV Med 2010;11:593–602.
10. Alteri C, Svicher V, Gori C, *et al.:* Characterization of the patterns of drug-resistance mutations in newly diagnosed HIV-1 infected patients naïve to the antiretroviral drugs. BMC Infect Dis 2009;9:111.
11. Bracciale L, Colafigli M, Zazzi M, *et al.:* Prevalence of transmitted HIV-1 drug resistance in HIV-1-infected patients in Italy: Evolution over 12 years and predictors. J Antimicrob Chemother 2009;64:607–615.
12. Riva C, Lai A, Caramma I, *et al.:* Transmitted HIV type 1 drug resistance and non-B subtypes prevalence among seroconverters and newly diagnosed patients from 1992 to 2005 in Italy. AIDS Res Hum Retroviruses 2010;26:41–49.
13. Giuliani M, Montieri S, Palamara G, *et al.:* Non-B HIV-1 subtypes among men who have sex with men in Rome, Italy. AIDS Res Human Retroviruses 2009;25:1–8.
14. Monno L, Brindicci G, Lo Caputo S, *et al.:* HIV-1 subtypes and circulating recombinant forms (CRFs) from HIV-infected patients residing in two regions of central and southern Italy. J Med Virol 2005;75:483–490.
15. Tramuto F, Bonura F, Perna AM, *et al.:* Group for HIV-1 Antiretroviral Studies in Sicily. Genetic diversity of HIV-1 non-B strains in Sicily: Evidence of intersubtype recombinants by sequence analysis of gag, pol, and env genes. AIDS Res Hum Retroviruses 2007;23:1131–1138.
16. Torti C, Lapadula G, Izzo I, *et al.:* Heterogeneity and penetration of HIV-1 non-subtype B viruses in an Italian province: Public health implications. Epidemiol Infect 2010;29:1–10.
17. Geretti AM, Harrison L, Green H, *et al.:* Effect of HIV-1 subtype on virologic and immunologic response to starting highly active antiretroviral therapy. Clin Infect Dis 2009;48: 1296–1305.
18. Martínez-Cajas JL, Pant-Pai N, Klein MB, and Wainberg MA: Role of genetic diversity among HIV-1 non-B subtypes in drug resistance: A systematic review of virologic and biochemical evidence. AIDS Rev 2008;10:212–223.
19. Marcelin AG, Masquelier B, Descamps D, *et al.:* Tipranavir-ritonavir genotypic resistance score in protease inhibitor-experienced patients. Antimicrob Agents Chemother 2008; 52:3237–3243.
20. Doualla-Bell F, Avalos A, Brenner B, *et al.:* High prevalence of the K65R mutation in human immunodeficiency virus type 1 subtype C isolates from infected patients in Botswana treated with didanosine-based regimens. Antimicrob Agents Chemother 2006;50:4182–4185.
21. Brenner BG, Oliveira M, Doualla-Bell F, *et al.:* HIV-1 subtype C viruses rapidly develop K65R resistance to tenofovir in cell culture. AIDS 2006;20:F9–F13.
22. Miller MD, Margot N, McColl D, and Cheng AK.: K65R development among subtype C HIV-1 patients in tenofovir DF clinical trials. AIDS 2007;21:265–266.
23. Coutsinos D, Invernizzi CF, Xu H, *et al.:* Template usage is responsible for the preferential acquisition of the K65R reverse transcriptase mutation in subtype C variants of human immunodeficiency virus type 1. J Virol 2009;83:2029–2033.
24. Ceccherini-Silberstein F, Erba F, Gago F, *et al.:* Identification of the minimal conserved structure of HIV-1 protease in the presence or absence of drug pressure. AIDS 2004;18:11–19.
25. Thompson JD, Higgins DG, and Gibson TJ: Clustal W: Improving sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 1994;22:4673–4680.
26. Hall TA: Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. Nucl Acids Symp Ser 1999;41:95–98.
27. De Oliveira T, Deforche K, Cassol S, *et al.:* An automated genotyping system for analysis of HIV-1 and other microbial sequences. Bioinformatics 2005;21:3797–3800.
28. Swafford KL: PAUP 4.0: Phylogenetic analysis with parsimony (and other methods), version 4,0b2a. Sinauer Associates Inc., Sunderland, MA, 1999.
29. Posada D and Crandall KA: MODEL TEST: Testing the model of DNA substitution. Bioinformatics 1998;14:817–818.
30. Johnson, VA, Brun-Vezinet F, Clotet B, *et al.:* Update of the drug resistance mutations in HIV-1: December 2010. Top HIV Med 2010;17:156–163.
31. Stanford HIV drug resistance, last updated on Jun 10, 2008. http://hivdb.stanford.edu/.
32. García-Lerma JG, MacInnes H, Bennett D, *et al.:* A novel genetic pathway of human immunodeficiency virus type 1 resistance to stavudine mediated by the K65R mutation. J Virol 2003;77:5685–5693.
33. Abecasis AB, Deforche K, Snoeck J, *et al.:* Protease mutation M89I/V is linked to therapy failure in patients infected with the HIV-1 non-B subtypes C, F or G. AIDS 2005;19:1799–1806.
34. McKeage K, Perry CM, and Keam SJ: Darunavir: A review of its use in the management of HIV infection in adults. Drugs 2009;69:477–503.
35. van de Vijver DA, Wensing AM, Angarano G, *et al.:* The calculated genetic barrier for antiretroviral drug resistance substitutions is largely similar for different HIV-1 subtypes. J Acquir Immune Defic Syndr 2006;41:352–360.
36. De Mendoza C, Garrido C, Poveda E, *et al.:* Changes in drug resistance patterns following the introduction of HIV type 1

non-B subtypes in Spain. AIDS Res Hum Retroviruses 2009;25:967–972
37. Munerato P, Sucupira MC, Oliveros MP, *et al.:* HIV type 1 antiretroviral resistance mutations in subtypes B, C, and F in the City of São Paulo, Brazil. AIDS Res Hum Retroviruses 2010;26(3):265–273.
38. Invernizzi CF, Coutsinos D, Oliveira M, Moisi D, Brenner BG, and Wainberg MA: Signature nucleotide polymorphisms at positions 64 and 65 in reverse transcriptase favor the selection of the K65R resistance mutation in HIV-1 subtype C. J Infect Dis 2009;200:1202–1206.
39. Miller MD, Margot N, Lu B, *et al.:* Genotypic and phenotypic predictors of the magnitude of response to tenofovir disoproxil fumarate treatment in antiretroviral-experienced patients. J Infect Dis 2004;189:837–846.
40. Winters MA, Shafer RW, Jellinger RA, Mamtora G, Gingeras T, and Merigan TC: Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxyinosine monotherapy for 1 to 2 years. Antimicrob Agents Chemother 1997;41:757–762.
41. Brenner BG and Coutsinos D: The K65R mutation in HIV-1 reverse transcriptase: Genetic barriers, resistance profile and clinical implications. HIV Ther 2009;3:583–594.
42. Kleim JP, Rösner M, Winkler I, *et al.:* Selective pressure of a quinoxaline nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on HIV-1 replication results in the emergence of nucleoside RT-inhibitor-specific (RT Leu-74→Val or Ile and Val-75→Leu or Ile) HIV-1 mutants. Proc Natl Acad Sci USA 1996;93(1):34–38.
43. Koval CE, Dykes C, Wang J, and Demeter LM.: Relative replication fitness of efavirenz-resistant mutants of HIV-1: Correlation with frequency during clinical therapy and evidence of compensation for the reduced fitness of K103NRL100I by the nucleoside resistance mutation L74V. Virology 2006;353:184–192.
44. Flys TS, Chen S, Jones DC, *et al.:* Quantitative analysis of HIV-1 variants with the K103N resistance mutation after single-dose nevirapine in women with HIV-1 subtypes A, C, and D. J Acquir Immune Defic Syndr 2006;42:610–613.
45. Deshpande A, Jauvin V, Magnin N, *et al.:* Resistance mutations in subtype C HIV type 1 isolates from Indian patients of Mumbai receiving NRTIs plus NNRTIs and experiencing a treatment failure: Resistance to AR. AIDS Res Hum Retroviruses 2007;23:335–340.
46. Grossman Z, Istomin V, Averbuch D, *et al.:* Genetic variation at NNRTI resistance-associated positions in patients infected with HIV-1 subtype C. AIDS 2004;18:909–915.
47. Hsu LY, Subramaniam R, Bacheler L, and Paton NI: Characterization of mutations in CRF01_AE virus isolates from antiretroviral treatment-naive and -experienced patients in Singapore. J Acquir Immune Defic Syndr 2005;38:5–13.
48. Sunpath H, France H, Tarin M, *et al.:* Prospective analysis of HIV-1 Drug Resistance After Virologic Failure on Antiretroviral Therapy (ART): Initial Results from a Paediatric Cohort Study from KZN, South Africa. 15th Conference on Retrovirus and Opportunistic Infections, Boston, MA, 2008.
49. Gonzalez, LMF, Brindeiro, RM, Aguiar, RS, *et al.:* Impact of nelfinavir resistance mutations on in vitro phenotype, fitness, and replication capacity of human immunodeficiency virus type 1 with subtype B and C proteases. Antimicrob Agents Chemother 2004;48:3552–3555.
50. Calazans A, Brindeiro R, Brindeiro P, *et al.:* Low accumulation of L90M in protease from subtype F HIV-1 with resistance to protease inhibitors is caused by the L89M polymorphism. J Infect Dis 2005;191:1961–1170.
51. Gonzalez A, Santos AF, Abecasis AB, *et al.:* Impact of HIV-1 protease mutations A71V/T and T74S on M89I/V-mediated protease inhibitor resistance in subtype G isolates. J Antimicrobial Chemother 2008;61:1201–1204.

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