A cluster of patients with a recombinant B/F HIV-1 infection: epidemiological, clinical and virological aspects.

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Table 1. Epidemiological aspects of patients infected with the B/F recombinant clade.

Subject	Age	Sex	Origin	CD4 at diagnosis	Transmission route	Year of diagnosis	CD4 at HAART initiation	CDC state at diagnosis
1	50	M	Eastern shore Lecco lake	823	Heterosexual	2007	214	B2
2	40	M	Lecco	402	Heterosexual	2006	182	A2
3	28	M	Brianza	669	Heterosexual	2008	146	A1
4	30	F	Brianza	1059	Heterosexual	2008	266	A1
5	49	M	Milan	254	Heterosexual	2002	292	B2
6	54	M	High Como lake	418	Heterosexual	2008	420	A2
7	55	F	High Como lake	367	Heterosexual	2008	302	B2
8	45	M	High Como lake	451	Heterosexual	2007	308	A2
9	31	F	Brazil	565	Heterosexual	2007	305	A1
10	46	M	Ivory Coast	509	Heterosexual	2008	//	A1
11	19	F	Kenya	536	Heterosexual	2007	//	A1
12	47	M	Bergamo	432	Bisexual	2007	//	B2
13	52	M	Western shore Lecco lake	712	Homosexual	2009	//	B1

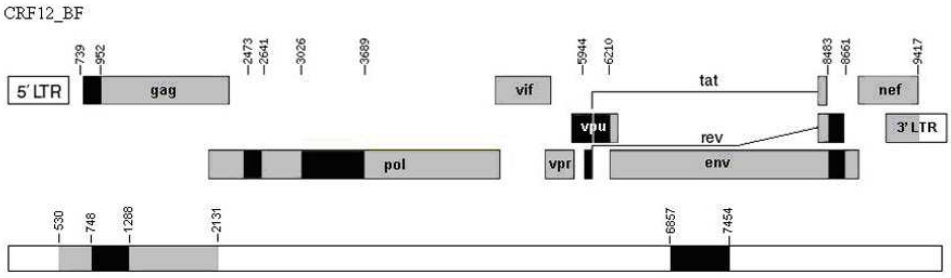
The Table shows epidemiological features of 13 subjects infected with a B/F recombinant strain. Aside from 3 individuals with African and South American origins (patient 9, 10 and 11), the others are Italian who live in the area of catchment of our general hospital. Even though their villages are not far from Lecco, the peculiar physical geography of the area, characterized by small alpine valleys and lakes, leads to a partial isolation of each mountain community, thus social contacts among them are generally uncommon. At diagnosis, they all have a good immunological condition (except patient 5) and there were no C stage conditions; heterosexual transmission was the main route of infection. All patients were diagnosed after 2006, except patient 5, who had an older infection, but he lived in Milan and moved to Lecco only later. CD4+ lymphocyte T cell count at HAART initiation was above 200 cell/mmc, with the exception of patient 2 (who refused to start treatment given the immuno-virological discordance) and patient 3 (who discovered to have a Non-Hodgkin lymphoma soon after the HIV diagnosis).

Table 2. Highly-Active Antiretroviral Therapy, mutational pattern and immuno-virological status at treatment beginning.

Subject	*rt* mutations	*pol* mutations	HAART regimen	CD4 cell count	HIV-RNA (log_{10})
1	399D	13V, 36I, 72T, 89M	FTC/TDF+**EFV**	214	3.47
2			FTC/TDF+**EFV**	182	4.61
3			*FTC/TDF+EFV*	*146*	*//*
4			FTC/TDF+**ATV/rtv**	266	6.51
5	44A	36I	3TC/ABC+**ATV/rtv**	292	5.48
6		13V, 36I, 77I	FTC/TDF+**EFV**	420	5.14
7			3TC/ABC+**NVP**	302	3.94
8		10I, 13V, 36I	FTC/TDF+**ATV/rtv**	308	4.18
9		16E, 36I	FTC/TDF+**NVP**	305	4.89
9	103N, 181C	16E, 36I	FTC/TDF+**ATV/rtv**	401	3.82

FTC/TDF: emtricitabine/tenofovir; EFV: efavirenz; ATV/rtv: atazanavri/ritonavir; 3TC/ABC: lamivudine/abacavir; NVP: nevirapine. Baseline genotype resistance test did not show any clinically relevant substitution; on the contrary, the second test performed by patient 9 after virological failure showed two typical NNRTI-related mutations (103N and 181C). CD4+ lymphocyte T cell count at HAART initiation was above 200 cell/mmc, with the exception of patient 2 (who refused to start treatment given the immuno-virological discordance) and patient 3 (who discovered to have a Non-Hodgkin lymphoma soon after the HIV diagnosis). A NNRTI-based regimen was started in 6 subjects (with either EFV and NVP), while a PI-based regimen was started in 4 (only with ATV/rtv).

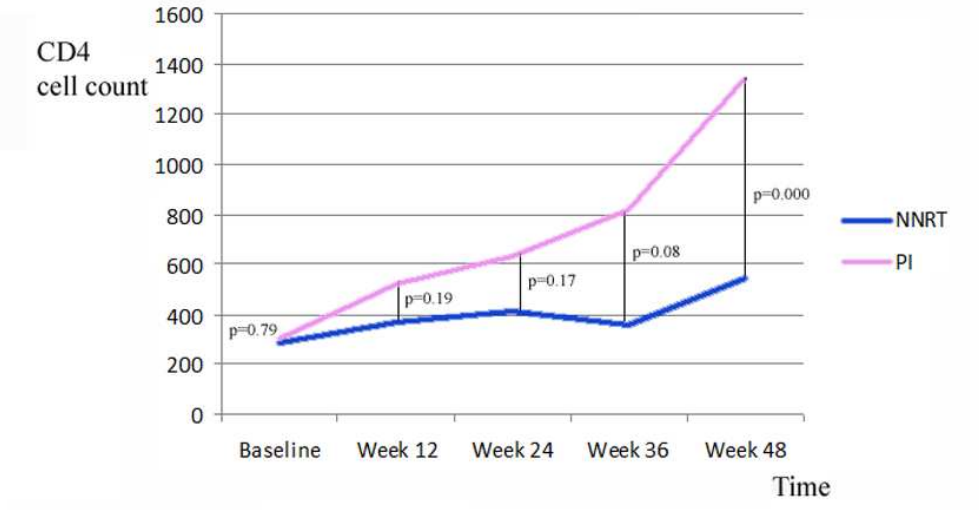
Figure 1. Genome structure of the 13 BF strains identified in this study and the reference CRF12_BF sequence.



Black: subtype B; grey: subtype F. All 13 B/F strains showed the same genomic structure. The sequences for the gag-protease and env regions were used for phylogenetic analysis and genotyping of the subjects. The subtyping function of NCBI showed that the gag-protease sequence was subtype F with a section of subtype B. This was confirmed by SimPlot software, which identified two B/F breakpoints: the first one located at nucleotide 748 (numbered on the basis of the HXB-2 strain [GenBank Accession Number M38432]) approximately 50 nucleotides upstream of the starting codon of the p17 protein; the second in the p24 coding region at nucleotide 1288. When previously characterized B/F recombinant HIV-1 sequences were included in the phylogenetic analysis, the sequence clustered closely with B/F recombinant sequences of various South American origins (88% bootstrap support). A relationship with South American CRF12_BF was suggested by the presence of the amino acid signature Val at Position 9 in p2gag.

156x134mm (150 x 150 DPI)

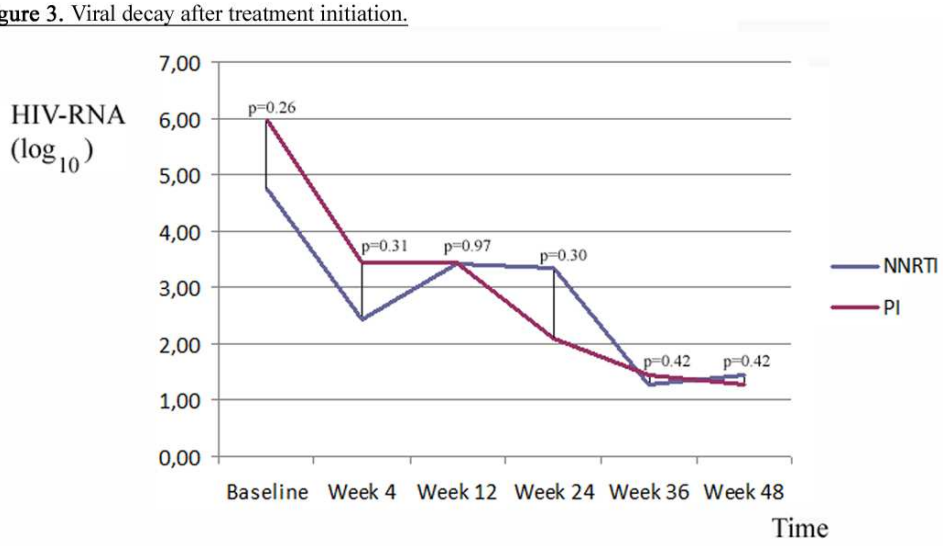
Figure 2. CD4+ lymphocyte T cell count.



The Figure shows the immunological recovery under treatment. Baseline mean values are similar (p=0.79); after the beginning of HAART, the mean CD4+ cell count increases in both groups but with a better response in the PI-based treatment arm. This difference is statistically significant from week 36 onwards (p=0.08). P: t-Student test.

143x113mm (150 x 150 DPI)

Figure 3. Viral decay after treatment initiation.



The Figure shows the viral decay after treatment initiation. Baseline mean HIV-RNA values are similar in PI-based and NNRTI-based treatment arms (p=0.26). Both groups showed a virologic response that was similar at each time point. After 4,12 and 24 weeks of treatment, HIV-RNA was still detectable with similar values (p=0.31, 0.97, 0.30 respectively).
The viral undectability (HIV-RNA<1.69 log10) was reached only at week 36 with either PIs and NNRTIs.
P: t-Student test.

184x137mm (150 x 150 DPI)

A cluster of patients with recombinant B/F HIV-1 infection: epidemiological, clinical and virological aspects.

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Abstract. Migratory processes have caused changes in HIV epidemiology and non-B subtypes are now playing an increasing role. In a cohort of 553 HIV-infected outpatients tested to identify non-B isolates, the largest group consisted of 13 subjects with a recombinant B/F form (prevalence 2.4%). Sequencing and phylogenetic analyses described a B/F recombinant clade with anomalous breakpoints that did not allow it to be classified as CRF12_BF. Viral load was not quantified efficiently because of a mismatch in the primers and probes used by commercial assays. An assessment of the clinical management, and epidemiological, immunological and virological characteristics of these patients, who were receiving non-nucleoside reverse transcriptase inhibitor (NNRTI)- or protease inhibitor (PI)-based regimens, showed that the immunological and virological mismatch delayed the start of treatment by a mean of 6.8 months. Therapy was started in nine patients. Both NNRTI- and PI-based regimens led to full virological suppression after a mean 36 weeks of treatment; the PI-based regimens proved to be more effective in terms of immunological recovery (1341 *versus* 544 CD4+ cells/mm^3). The spread of non-B subtypes is increasing throughout the world but their response to treatment is still unclear. PIs and NNRTIs are effective but further tests are needed to allow the more efficient recognition of these viral strains and establish how they should be treated.

Key words: HIV-1; non-B subtype; B/F recombinant form; response to HAART.

Introduction. The complex genetic evolution of human immunodeficiency virus (HIV) led to the development of different strains in different geographical regions throughout the world. HIV-1 includes three subgroups: M, N and O. The M group is divided into 11 subtypes and includes multiple circulating recombinant forms (CRFs) and unique recombinant forms (URFs).

Given their different biology, epidemiology and drug-susceptibility, every viral clade may give rise to particular difficulties in clinical management. HIV-1 subtype B is still the most common in Western countries, but immigration is changing the epidemiology of HIV and an increasing role is being played by non-B subtypes. The prevalence of non-B subtypes in Italy increased from 2.6% in the years 1980-1992 to 18.9% in 1993-2008 [Lai et al., 2010]. All of the subtypes (except clade K) and seven CRFs are currently circulating, and subtype F1 is the most prevalent. The prevalence of CRF12_BF is <2% in Northern Italy [Baldanti et al., 2008], whereas the B/F1 recombination is the most common of the URFs. This study was conducted in a general hospital in Lombardy, a Northern Italian region that has many immigrants mainly from West Africa. The prevalence of non-B subtypes among the new HIV diagnoses made over the last 12 months has been 10.3% (unpublished data). To date, there are few data concerning subtype F susceptibility to highly active antiretroviral treatment (HAART) and information about CRF12_BF and B/F URFs is even scarcer.

The case of a blood donor with HIV antibodies in whom it was difficult to detect HIV RNA using routine assays showed that problems can arise in identifying potentially infectious blood units [Foglieni et al., 2010], and a group of patients with discordant immunological and virological patterns (i.e. decreasing CD4+ T lymphocyte cell counts with HIV RNA viral load persistently below 2.6 $\log_{10}$) has also been described. The inefficient quantitation of the circulating virus was due to variations in the conserved *gag* region that reduced or abolished hybridisation with commercial assays. The *gag* sequence showed the presence of a number of sequence variations not belonging to a classic subtype B that hindered real-time PCR amplification.

When the HIV-infected outpatient cohort was tested for non-B subtypes, the non-B isolates included a B/F recombinant clade that was similar to CRF12_BF but had anomalous breakpoints. Epidemiological aspects and the clinical outcomes of the patients infected by this recombinant form were assessed during a follow-up period of 48 weeks.

Methods. The HIV-infected outpatient cohort was tested in parallel using routine and in-house quantitative real-time PCR (qRT-PCR) assays. The routine Roche qRT-PCR assays (COBAS Amplicor HIV1 Monitor v1.5 and COBAS AmpliPrep/COBAS TaqMan® (CAP/CTM) HIV-1 System v.1, Roche Diagnostics, Branchburg, NJ) target a specific sequence located in a highly conserved region of the HIV-1 *gag* gene that can be amplified using the primer pair SSK145_F/SKCC1B_R. The two in-house qRT-PCR-based assays were designed to avoid the regions targeted by the commercial assays and detect the *gag* and the 5'-*ltr* regions. The *env* and *gag* gene regions (including the sequence targeted by the Roche assays) were analysed phylogenetically.

Descriptive statistics were used to analyse the demographic, clinical, immunological and virological characteristics of the patients in order to evaluate possible epidemiological links, and assess the patients' clinical management.

After April 2009, detection was improved by the introduction of the new Roche qRT-PCR assay (CAP/CTM HIV-1 System v.2, Roche Diagnostics, Branchburg, NJ) based on the amplification of two different HIV regions. This assay quantified the actual viral load, and so HAART could be prescribed in accordance with the guidelines. The statistical analysis of the delay in starting HAART was made using only the data collected before the introduction of the new Roche assay.

As the analysis was partially retrospective and the patients were managed in accordance with the current guidelines, no ethical approval was required.

Results. In nineteen of the 553 patients in the HIV-infected outpatient cohort analysed by means of qRT-PCR (3.4%), the viral loads measured by the in-house assays were higher than those measured by the routine assays (data not shown). As the in-house assays targeted two different HIV-1 regions, it was possible to show that the failure of the commercial assays to quantify the actual viral load was due to the presence of base variations in the *gag* region that affected amplification.

Extensive sequencing analyses revealed a number of non-B subtypes, and phylogenetic analysis of the whole *gag* and C2V3 regions revealed the presence of a B/F recombinant strain in 13 subjects. The *env* sequence clustered with HIV-1 subtype B, and the *gag-protease* sequence belonged to subtype F with a section of subtype B, thus suggesting an intersubtype recombination (Fig. 1). Phylogenetic analysis of previously characterised B/F recombinant HIV-1 sequences showed that all of the sequences of the 13 subjects grouped in the same cluster as B/F recombinant sequences of various South American origins, and showed a close relationship with South American CRF12_BF [Foglieni et al., 2010]. Two recently described BF URFs from Luxemburg and Brazil (accession numbers EU170145 and AY455780 [Thomson et al., 2004]) were the closest to this strain. All of the sequences have been deposited in GenBank under accession numbers HQ834729-HQ834742.

Table 1 shows the characteristics of the patients with the recombinant form. Its prevalence in the cohort was 2.4%, and it was more frequent among heterosexuals (85% *versus* 15% homosexuals/bisexuals and no intravenous drug users). No epidemiological links could be established except for three identifiable couples (patients 3 and 4, 6 and 7, and 8 and 9). They belonged to different age and social groups, and some of them lived in small secluded mountain communities. It seems that the recombinant form penetrated the area recently as it was only found in subjects diagnosed after 2006 (excluding patient 5, who had an older infection but moved to Lecco in 2006). It has not yet been possible to identify any clear introductory event.

It is worth noting that patient 3 was diagnosed simultaneously with HIV infection and a non-Hodgkin lymphoma, and so HAART was started even though there was no detectable HIV RNA. Analysis of an older stored plasma sample made it possible to include his strain in the same recombinant B/F cluster but, as no other samples could be evaluated retrospectively, his immunological and virological status were not considered in the further analyses.

The mismatch between immunological and virological status led to an average 6.8 months' delay in starting HAART, at which time the patients had a mean CD4+ count of 229 cells/mm^3 (range 146-285). The delay was due to the need to repeat confirmatory tests and respect patient wishes (patient 2 refused to start therapy with an undetectable viral load despite his poor immunological status).

Baseline genotypic tests revealed a few mutations in the *rt* and *pol* regions. Nine patients started therapy with non-nucleoside reverse transcriptase inhibitor(NNRTI)-based or protease inhibitor(PI)-based regimens (Tab. 2). The NRTI backbone was emtricitabine/tenofovir (FTC/TDF) in seven patients and lamivudine/abacavir (3TC/ABC) in two. Patient 9 received the combination FTC/TDF + nevirapine (NVP) and showed virological failure: after six months of treatment, the viral load was 3.82 $\log_{10}$. A new genotypic resistance test revealed the development of two NNRTI-associated mutations (103N and 181C), and so NVP was replaced by atazanavir/ritonavir (ATV/rtv) and the patient slowly achieved full virological suppression.

Figure 2 shows the CD4+ cell counts. There were no significant differences between the two treatment groups at baseline, but the PI-based regimens led to greater improvements and, by week 48, there was a statistically significant between-group difference in mean CD4+ cell counts (1341 *vs* 544 cells/mm^3; $p<0.000$).

Figure 3 shows viral decay in the two treatment groups. Responses were slow, and viral load became undetectable in both groups after a mean 36 weeks of treatment, with no statistically significant difference at any of the timepoints. There was only one virological failure in the NNRTI

group (patient 9 described above); the presence of mutations in proviral DNA could not be excluded given the rapid development of the two substitutions typically associated with NNRTI-based regimens. No AIDS-related illnesses or other serious adverse events were recorded.

Discussion. The spread of non-B subtypes is increasing throughout the world, but their response to HAART is still unclear. B/F recombinants have spread from Asia [Leung et al., 2010] to Southern American regions outside Brazil [Espinosa et al., 2004]. The prevalence of the recombinant form detected in the outpatient cohort described above was slightly higher than that usually found in Italy, and probably reflects the high rates of immigration involving Lombardy. Three of the patients had extra-European origins, but came from different macro-areas (South America, East Africa and West Africa): subtype F is circulating in these areas, but it is unlikely that they brought such closely related viral strains from their own countries. It seems that this recombinant cluster has developed locally and that the mixing of non-B subtypes with native viruses is ongoing. Furthermore, the study findings confirmed that non-B subtypes are more common among heterosexuals, as has been found in both Italy [Balotta et al., 2007] and the UK [Booth et al., 2007].

The clinical management of these infections remains uncertain. There is a recent case report concerning a patient with an infection sustained by an underrated B/F recombinant virus who experienced a poor outcome [Bolivar et al., 2009]. Because of his low viral load, the patient was not treated until he developed an AIDS-defining illness. No AIDS-defining infections were observed in the cohort described in this report, although the CD4+ cell counts of two patients fell to below the threshold of 200 cells/mm^3. These experiences underline the risk that such recombinant viruses may affect the reliability of current virological methods, thus limiting correct clinical management and allowing a dangerous level of immune impairment.

The susceptibility of subtype F to the currently available drugs has not been studied in detail. Genotype resistance tests do not provide any strong information because their reliability is limited

by technical problems of amplifying and interpreting amino acid sequences, although there is no evidence that non-B subtypes develop different drug resistance mutations or have different phenotype susceptibility [Visco-Comandini and Balotta, 2003]. According to the International AIDS Society algorithm used in this study [Johnson, 2008], the amino acid substitutions may be clinically irrelevant, but it should be noted that the most frequent substitution (36I in the *pol* region, which has a prevalence of 85.7%) is one of the eight differences in protease protein between the subtype F and US subtype B consensus sequences [Waléria-Aleixo et al., 2008].

The choice of the best treatment remains difficult. Apetrei *et al*. [1998] have evaluated the *in vitro* effectiveness of a number of drugs against Romanian F strains, and found that their susceptibility to zidovudine, lamivudine, NVP, delavirdine, saquinavir and ritonavir was similar to what observed for subtype B, whereas they showed natural resistance to a TIBO derivative (R82913), a forerunner NNRTI that never entered clinical practice. A more recent *in vitro* study has shown that subtype F is less susceptible to amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir than subtype B viruses [Krauchenco et al., 2009], but the PI used in the present study (ATV) was not tested. Many of the drugs evaluated in these papers are no longer used in clinical practice in Western countries.

Waléria-Aleixo *et al.* [1998] described a higher genetic barrier for the development of thymidine-associated mutations (TAMs) in subtype F1, with no significant findings for PIs; it is worth noting that the NNRTI mutational pathway was slightly different from that of subtype B: there was a lower prevalence of the 190S/A substitution, with a preference for 190A, whereas 190S is more prevalent in subtype B. Another Brazilian study of virological failures in a large cohort of 2,474 patients in São Paulo found a lower prevalence of NNRTI-related mutations in subtype F, whereas subtype C harboured fewer PI-related mutations [Munerato et al., 2010]. A number of other studies have found different mutational pathways in subtype F [Cavalcanti et al., 2007; Calazans et al., 2005; Machado et al., 2004; Marconi et al., 2008; Abecasis et al., 2005], but these were mainly minor substitutions.

A recent meta-analysis has highlighted the lack of knowledge of subtype F [Martinez-Cajas et al., 2009].

In our experience, NNRTIs and PIs led to similar virological responses: both treatments achieved full virological control, although more slowly than in the case of subtype B viruses. Immunological recovery was better with PI-based regimens, as has been previously noted [Riddler et al., 2008; Daar et al., 2010].

The main limitation of the present study is the sample size and, as only a few patients started HAART, there was no randomization. Nevertheless, the two groups were homogeneous in terms of baseline immunological and virological status, and duration of infection. To the best of our knowledge, this is the first time that such a recombinant viral strain has been analyzed in terms of clinical outcomes, CD4+ cell count recovery and virological suppression for an evaluable follow-up period.

It is difficult to compare the clinical management of the clade described in this paper with that of the B/F strains found in South America: although recombinant forms are more frequent among heterosexuals in both settings, other factors are different, especially the drugs used, and the published studies do not exhaustively analyze PIs such as ATV or darunavir, which are commonly used in Western countries [Gomes da Silva et al., 2010]. Furthermore, the prevalence of resistance-associated mutations in Brazil is higher than that found in this cohort [de Sa-Filho et al., 2009]. Finally, data generated in countries in which recombinant forms are more common cannot be directly transferred to Western countries, where such forms arrived later.

The findings of this study suggest that, if good adherence is ensured, NNRTIs and PIs are both effective in treating recombinant B/F virus, and similar findings were made in a recent study of a large Italian resistance database including a number of non-B subtypes [Franzetti et al., 2010]. However, although the responses to HAART do not seem to be significantly different [Geretti,

2006], further analyses are needed to identify the biological differences among HIV-1 subtypes because the limited reliability of current diagnostic methods could be dangerous as there is a risk that HAART may be delayed, thus leading to more rapid clinical progression. PI- and NNRTI-based regimens may be effective but complete virological suppression was obtained slowly, and this needs to be taken into account when evaluating treatment responses. As antiretroviral drugs are developed for subtype B, it is important to determine whether sequence variability affects progression rates, drug susceptibility, treatment responses, and resistance pathways.

Conflict of interest: None of the authors has any conflict of interest to declare.

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