**Predominance of CXCR4 tropism in HIV-1 CRF14_BG strains from newly diagnosed infections**

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**Objectives:** R5-tropic viruses are associated with HIV-1 transmission and predominate during the early stages of infection. X4-tropic populations have been detected in ≈50% of patients with late-stage disease infected with subtype B viruses. In this study, we compared the frequency of X4 tropism in individuals infected with HIV-1 CRF14_BG viruses, which have a V3 loop of subtype B, with a control group of individuals infected with subtype B viruses.

**Methods:** Sixty-three individuals infected with HIV-1 CRF14_BG (n = 31) or subtype B (n = 32) were studied. Similar proportions of newly diagnosed and chronically infected individuals were included in the subtype B and CRF14_BG groups. V3 sequences were obtained and coreceptor tropism was predicted using the Geno2pheno[coreceptor] algorithm. V3 net charge and 11/25 rules were also used for coreceptor prediction.

**Results:** Overall, X4 tropism was more frequent among individuals infected with CRF14_BG viruses (87.1%) than subtype B viruses (34.3%), a difference that was statistically highly significant (P = 0.00001). Importantly, the frequencies among newly diagnosed individuals were 90% and 13.3%, respectively (P = 0.00007). Characteristic amino acids in the V3 loop (T13, M14, V19 and W20) were identified at higher frequencies in CRF14_BG viruses (54%) than subtype B viruses (0%; P < 0.000001).

**Conclusions:** CRF14_BG is the genetic form with the highest proportion of X4-tropic viruses reported to date in newly diagnosed and chronic infections. This suggests high pathogenicity for CRF14_BG viruses, potentially leading to rapid disease progression. CCR5 antagonists will be ineffective in most CRF14_BG-infected patients, even at early stages of infection.

**Keywords:** HIV, coreceptors, antiretroviral therapy

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**Introduction**

HIV-1 entry into host cells requires the interaction of gp120 with CD4 and a chemokine receptor (either CCR5 or CXCR4), which acts as coreceptor.1 The ability of HIV-1 to use CCR5, CXCR4 or both defines HIV-1 tropism as R5, X4 or R5X4 (dual tropic), respectively.2 The use of both coreceptors can be due to the presence of dual-tropic clones, to a mixture of pure R5-tropic and X4-tropic strains, or both. The V3 loop of gp120 is the major determinant of coreceptor specificity but other regions of gp120, such as V1V2, C4 and the bridging sheet,3,4 or even of regions of gp415,6 may be also implicated. Coreceptor tropism can be assessed with either phenotypic or genotypic approaches.7,8 Several bioinformatic methods have been developed to predict coreceptor use on the basis of V3 sequence data.9 The Geno2pheno[coreceptor] (G2P) algorithm has been evaluated for the V3 sequence of subtype B viruses in several cohort studies and retrospective analyses of clinical trials, showing that rescreening of tropism by the enhanced-sensitivity
Trofile assay (ESTA) and G2P predicted similar virological responses in subtype B infections.  

In general, R5-tropic viruses are associated with HIV-1 transmission and predominate during the early stages of infection. During disease progression, HIV-1 X4 populations have been detected in ~50% of patients infected with subtype B viruses. With regard to non-B genetic forms, the analysis of 498 individuals in late-stage disease showed a high frequency of X4 tropism for subtype A (60%), subtype D (61%), CRF01_AE (77%) and CRF02_AG (65%), but not for subtype C (15%). A low frequency of X4 tropism has been reported for subtype G viruses.  

Previous studies have reported high frequencies of X4 tropism for CRF14_BG viruses. In this respect, we have reported that all 14 CRF14_BG primary isolates studied, obtained from peripheral blood mononuclear cells from five patients at the chronic stage of infection, showed X4 tropism or a dual/mixed R5X4 phenotype. However, little is known about the frequencies of X4 viruses during the evolution of the infection in patients infected with other subtypes and circulating recombinant forms.

CRF14_BG consists of a subtype G genome with a unique fragment of subtype B that covers most of env (HXB2 positions: 6341–8270), including the V3 loop. It was initially identified in Galicia, north-west Spain, among intravenous drug users (IDUs), where it continues circulating among this population. Between 1999 and 2007, CRF14_BG strains were frequently found in Portugal where, in 2003, CRF14_BG prevailed over all other subtype B viruses used as control group (Figure1). Their assignment was shown to be infected with X4-tropic viruses. Moreover, the X4-tropic sequences were further analysed considering the other three FPR values that have been evaluated in clinical trials: ±2%, ±5.75% and ±10%. Additionally, V3 net charge and 11/25 simple rules based on the V3 loop amino acid sequence were also used. V3 net charge was calculated by subtracting positively charged residues [aspartic acid (D) and glutamic acid (E)] from positively charged ones [arginine (R) and lysine (K)] in the V3 loop. A V3 net positive charge ≥5 was considered to be predictive of X4 tropism, while a positive charge <5 was considered to be predictive of R5 tropism. According to the 11/25 rule, the presence of positively charged amino acids (R or K) at positions 11 or 25 of the V3 loop is considered to be predictive of X4 tropism. Nucleotide mixtures were resolved by sequence expansion. In these cases, both the highest and lowest FPRs were considered for tropism prediction.

### Methods

#### Study subjects

Sixty-three HIV-1-infected individuals were included in this study. Thirty-one were infected with CRF14_BG; 32 infected with subtype B viruses were used as a control group. Patients were diagnosed between 1986 and 2010. Samples taken during the first 3 months after HIV-1 diagnosis were classified as ‘newly diagnosed’. In this study, samples from chronically infected individuals were collected at least 2 years after HIV-1 diagnosis (intervals of 2–16 years (mean 7.4 years) and 3–26 years (mean 12.2 years) for CRF14_BG and subtype B, respectively). In order to avoid a bias with regard to the prevalence of X4-tropic viruses, and considering that during the course of HIV-1 infection X4-tropic viral variants may evolve, similar proportions of newly diagnosed and chronically infected individuals were included in the subtype B and CRF14_BG groups.

The study was approved by the Research Ethics and Animal Welfare Committee, Instituto de Salud Carlos III (CBB/A/ 2008 PI 322 and CEI PI 51_2011-v2). Signed statements of informed consent were obtained from all patients.

#### Viral RNA isolation, amplification, V3 sequencing and phylogenetic analysis

Viral RNA was extracted from plasma samples in all cases except three. In these, plasma was not available and RNA was obtained from 14 day supernatants from peripheral blood mononuclear cell cultures. In all cases, viral RNA was obtained using the NucleiSens® kit (bioMérieux, the Netherlands) according to the manufacturer’s instructions. Single-tube RT-PCR, followed by nested PCR amplification of an env region comprising HXB2 positions 7012–7648 (containing the V3 loop) was performed using primers and reaction conditions as previously described. Population sequencing was performed with the ABI Prism BigDye Terminator Cycle Sequencing Kit and the ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were assembled with SeqMan 6.1 (DNAStar), edited with BioEdit v7.0 and aligned with MAFFT v.6.9.8 for subsequent phylogenetic analysis with maximum likelihood trees using RAxML.  

### Tropism prediction algorithms

Tropism prediction based on the V3 loop nucleotide sequence was performed using the G2P algorithm available at http://coreceptor.bioinf.mpi-inf.mpg.de/index.php. The result is given with a quantitative value – the false positive rate (FPR) – that defines the probability of classifying an R5 virus falsely as an X4 virus. Following the European guidelines on the clinical management of HIV-1 tropism testing, a conservative FPR of 20% was used, by which patients with an FPR <20% were considered to be infected with X4-tropic viruses. Moreover, the X4-tropic sequences were further analysed considering the other three FPR values that have been evaluated in clinical trials: ±2%, ±5.75% and ±10%. Additionally, V3 net charge and 11/25 simple rules based on the V3 loop amino acid sequence were also used. V3 net charge was calculated by subtracting negatively charged residues [aspartic acid (D) and glutamic acid (E)] from positively charged ones [arginine (R) and lysine (K)] in the V3 loop. A V3 net positive charge ≥5 was considered to be predictive of X4 tropism, while a positive charge <5 was considered to be predictive of R5 tropism. According to the 11/25 rule, the presence of positively charged amino acids (R or K) at positions 11 or 25 of the V3 loop is considered to be predictive of X4 tropism. Nucleotide mixtures were resolved by sequence expansion. In these cases, both the highest and lowest FPRs were considered for tropism prediction.

### Statistical analysis

Differences among categorical variables were analysed by the χ² test with Yates’ correction when at least 20% of expected frequencies were <5, using the statistical program available at http://quantpsy.org/chiq/chiq.htm.

#### Results

The epidemiological and immunological data from HIV-1 patients infected with CRF14_BG and subtype B strains are shown in Table 1. The use of intravenous drugs was the most frequent transmission route (86.3%) among CRF14_BG-infected individuals diagnosed between 1997 and 2003 and, conversely, sexual transmission was more frequent (87.5%) than intravenous drug use (12.5%) among CRF14_BG-infected individuals diagnosed between 2004 and 2010. The phylogenetic analysis of the env V3 region confirmed the monophyletic clustering of CRF14_BG viruses, separated from the subtype B viruses used as control group (Figure 1). Their assignment as CRF14_BG viruses was also supported by the clustering of partial pol sequences with the subtype G variant circulating in Portugal and Spain (E. Delgado, Y. Vega, T. Cuevas, A. Fernández-García, M. M. Thomson and L. Pérez-Álvarez, unpublished data) and by

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V3 amino acid sequences of CRF14_BG and subtype B viruses, with their corresponding tropism predictions, FPR values and V3 net charges, are shown in Figure 2. X4 tropism was detected in 27 (87.1%) of 31 and in 11 (32.4%) of 34 individuals infected with CRF14_BG and subtype B viruses, respectively ($P = 0.00001$). Importantly, among newly diagnosed infections the difference in the percentage of X4-tropic viruses between individuals infected with CRF14_BG (90%) and subtype B (13.3%) was also statistically significant ($P = 0.0007$). Moreover, the difference in the percentage of X4-tropic viruses among chronically infected and newly diagnosed patients was statistically significant ($P = 0.01$) in patients infected with subtype B but not those infected with CRF14_BG.

Among CRF14_BG infections, X4 tropism was predicted in 19 (90.5%) of 21 IDUs and 8 (80%) of 10 individuals infected by sexual transmission, while among subtype B infections, X4 tropism was predicted in 3 (75%) of 4 IDUs and 5 (21.7%) of 23 sexually transmitted infections.

Although the prediction of X4 tropism was made using an FPR ≤20%, it should be noted that 25 (92.6%) of the X4-tropic sequences predicted with this FPR were also predicted as X4 tropic using an FPR ≤10%, 24 (88.9%) using an FPR ≤5.75%, and even 14 (51.9%) using an FPR <2%. The differences in the frequency of X4 tropism between CRF14_BG and B subtypes remained significant across all these FPR values used in clinical trials.

Table 1. Epidemiological and immunological data

<table>
<thead>
<tr>
<th></th>
<th>CRF14_BG, n (%)</th>
<th>Subtype B, n (%)</th>
<th>$P$ value</th>
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<tr>
<td>Patients, n</td>
<td>31</td>
<td>32</td>
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</tr>
<tr>
<td>Year of diagnosis</td>
<td>1997 - 2010</td>
<td>1986 - 2012</td>
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<tr>
<td>Sex</td>
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<td>male</td>
<td>23 (74.2)</td>
<td>25 (78.1)</td>
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<td>female</td>
<td>8 (25.8)</td>
<td>7 (21.9)</td>
<td>0.66</td>
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<tr>
<td>Patient group</td>
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<tr>
<td>newly diagnosed</td>
<td>10 (32.3)</td>
<td>15 (46.8)</td>
<td>0.27</td>
</tr>
<tr>
<td>IDUs</td>
<td>4 (12.9)</td>
<td>1</td>
<td>0.03</td>
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<tr>
<td>sexual transmission</td>
<td>6 (19.4)</td>
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<tr>
<td>no data</td>
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<td>1 (3.1)</td>
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<tr>
<td>chronically infected</td>
<td>21 (67.7)</td>
<td>17 (53.1)</td>
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<td>IDUs</td>
<td>16 (51.6)</td>
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<td>sexual transmission</td>
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<td>9 (28.1)</td>
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<td>3 (9.4)</td>
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<td>17 (54.8)</td>
<td>7 (21.9)</td>
<td>0.007</td>
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Figure 1. Maximum likelihood phylogenetic tree of the env V3 region of samples analysed in this study. Subtype B and CRF14_BG references are underlined. Only bootstrap values ≥80% are shown.
**Figure 2.** V3 amino acid sequence alignment and tropism prediction for (a) CRF14_BG and (b) subtype B viruses. FPR indicates the false positive rate of R5 viruses being classified as X4, determined using G2P. X4 tropism is predicted when the FPR is <0.20. For sequences with nucleotide mixtures, the FPR value obtained from the mixture is indicated, followed by the nucleotide composition. CRF14_BG signature amino acids are highlighted in bold in the alignment. Amino acid positions 11 and 25 are shaded. Positively charged amino acids are boxed. CRF14_BG signature amino acids are highlighted in bold in the alignment. Amino acid positions 11 and 25 are shaded. Samples from newly diagnosed individuals.
Figure 2. Continued
The analysis of V3 sequences by simple rules showed that 14 (51.9%) of the 27 CRF14_BG sequences predicted to be X4 tropic had arginine (R) or lysine (K) at position 11 and only three had these amino acids at position 25.

Overall, a high correlation (88.8%) was observed between a net positive charge ≥5 and X4 tropism among CRF14_BG sequences, and a net positive charge <5 and R5 tropism in subtype B viruses (95.2%). However, this correlation failed in three of four R5-tropic CRF14_BG sequences that had a net charge of <5. For subtype B, all five X4 sequences with a V3 net positive charge <5 had FPR values between 10% and 20%.

A comparison between CRF14_BG and subtype B V3 loop amino acid sequences showed that threonine (T) at position 13, methionine (M) at position 14, valine (V) at position 19 and tryptophan (W) at position 20 were more frequently detected in CRF14_BG viruses than subtype B viruses. These differences were statistically highly significant (P<0.000001).

Discussion

CRF14_BG, the first circulating recombinant form reported to have originated in Western Europe, has been estimated by molecular dating to have emerged in Portugal in the early 1990s, soon after the beginning of the HIV-1 epidemic in the country, and to have spread to Spain in the late 1990s as a consequence of the migration of IDUs.22

In the current study, we report a decrease in the proportion of CRF14_BG-infected individuals infected via intravenous drug use from 1997 to 2003 and a predominance of sexual transmission cases since 2004, which is related to a temporal shift observed in the epidemiological characteristics of CRF14_BG viruses with respect to their transmission route (E. Delgado, Y. Vega, T. Cuevas, A. Fernández, M. M. Thomson and L. Pérez Alvarez, unpublished data). This shift was concomitant with a progressive decrease in intravenous drug use as a risk factor associated with HIV-1 infection in Spain from 20.2% in 2004 to 5.9% in 2010.27

The CRF14_BG viruses analysed in this study are representative of the CRF14_BG infections diagnosed in our laboratory, since 31 (79.5%) of 39 newly diagnosed individuals were included, comprising 23 (79.3%) of 29 diagnosed between 1997 and 2003 and 8 (80%) of 10 individuals diagnosed between 2004 and 2010.

Although we characterized 11 full-length genomes, all but one corresponded to infections in IDUs diagnosed before 2004 and only one to a sexually transmitted infection diagnosed after 2004. It would be interesting to analyse other full-length genomes from the latter period, which would allow the analysis of potential genetic changes associated with the shift in the predominant transmission route of CRF14_BG in recent years.

The high prevalence (90.5%) of X4 tropism among CRF14_BG-infected IDUs detected in the current study could partly reflect a greater prevalence of X4 viruses among IDUs than sexually infected subjects, as previously reported for subtype B infections.31,32 Due to the low number of subtype B-infected IDUs in this study it was not possible to perform a comparative statistical analysis of X4 tropism among IDUs infected with CRF14_BG or subtype B viruses. However, considering that the prevalence of X4 tropism previously reported among chronically infected IDUs was 52.6%,32 and among IDUs with recent B subtype HIV seroconversion was 35.7%,33 the high frequency (90.5%) of X4 viruses detected in this study among CRF14_BG-infected IDUs is remarkable.

Although R5-tropic viruses are usually predominant during the early stages of infection,15,16 X4-tropic viruses have been found in individuals with high CD4 cell counts or recent infection in proportions of 6% to 19%,34–38 and between 4% and 38% in cohorts of antiretroviral drug-naive individuals.39,40 The difference between these proportions may be influenced by the different characteristics of the populations studied, the methodology used for tropism determination or the inclusion of late-diagnosed individuals in the antiretroviral drug-naive cohorts. Studies including non-B subtypes found a low frequency of CXCR4-using viruses among patients at the time of primary infection.33,41–45 In a recent study, significantly more frequent CXCR4 use was predicted among recently diagnosed individuals infected with CRF01_AE viruses (17%) than among those infected with CRF02_AG (8%), subtype A (7%) or subtype C (5%) strains.46 The HIV-GRADE network47 has reported differences in the distribution of coreceptor tropism among different subtypes48 by using G2P, showing a significant deviation compared with the mean distribution for subtype D and CRF01_AE towards more frequent X4 prediction and for subtypes A1 and G towards more frequent R5 prediction.

In this study we report a large overall predominance (87.1%) of X4 tropism determined using G2P among CRF14_BG viruses, both in newly diagnosed individuals (90%) and in chronically infected patients (85.7%). To our knowledge, this is the highest proportion of X4-tropic viruses ever reported in newly diagnosed and chronic infections for any HIV-1 genetic form,17 even higher than the 60% X4-tropic subtype D viruses recently detected among patients from Kenya39 and also higher than the 30%–70% X4 tropism detected among antiretroviral drug-naive and -experienced patients infected with subtype D viruses in Uganda.48 The predominance of X4 tropism among CRF14_BG-infected individuals, both during chronic infection and soon after diagnosis, is consistent with the predominant CXCR4 coreceptor use previously determined in CRF14_BG primary isolates49–52 and with the X4 tropism genotypic prediction determined using G2P in CRF14_BG sequences from Portuguese isolates.5

The use of multiple algorithms for the genotypic prediction of tropism is convincing, although evidence from phenotypic or other in vitro tropism assays would be the ultimate proof of CXCR4 tropism. In this regard, in vitro coreceptor usage was previously determined in GHOST cells for 14 genotypically predicted X4-tropic CRF14_BG primary isolates, all of which were X4 tropic in the phenotypic assay.51 However, it would also be necessary to know the correlation between genotype and phenotype for CRF14_BG R5 viruses. Other gp120 regions outside the V3 loop (such as V1, V2 and C4), and even the gp41 transmembrane protein, are involved in coreceptor binding.52–58 Consequently, the inclusion of genetic markers outside the V3 loop could improve the accuracy of the predictions for coreceptor usage. In this regard, a supervised Bayesian classification approach employing specific nucleotide positions selected from the complete env sequence has been used to predict HIV-1 coreceptor tropism for B and non-B subtypes, showing higher accuracy and specificity than G2P.52

It has recently been reported that G2P prediction of X4 tropism using an FPR ≤2% defines far better than an FPR ≤10% a viral population associated with low CD4+ T cell counts and with more advanced disease.53 In our study, 51.9% of X4-tropic CRF14_BG
sequences had an FPR <2%. Moreover, CD4+ T lymphocyte counts were <200 cells/mm³ in 54.8% of newly diagnosed individuals infected with CRF14_BG viruses. Altogether, the combination of high-frequency X4 tropism and low CD4+ cell counts in newly diagnosed individuals with CRF14_BG viruses was associated with more rapid disease progression than in those with subtype B infections,54–59 suggesting high pathogenicity for CRF14_BG.

Characteristic amino acids in the V3 loop of CRF14_BG were identified – T13, M14, V19 and W20 – whose frequencies were significantly higher in CRF14_BG than subtype B viruses ($P < 0.0001$). In order to provide better genotypic characterization, it would be interesting to study the presence of other CRF14_BG-specific polymorphisms in other segments of gp120.

Finally, predominant X4 tropism in CRF14_BG viruses also has important implications regarding antiretroviral treatment. CCR5 antagonists are a new class of anti-HIV-1 drugs that specifically inhibit the entry of CCR5-tropic HIV-1 strains into the target cells charged residues in the HIV type 1 gp120 V2 domain without fixed positions, elongation, or relocated N-linked glycosylation sites. AIDS Res Hum Retroviruses 1995; 11: 1169–75.  

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Transparency declarations
None to declare.


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