Prevalence of R5 and X4 HIV variants in antiretroviral treatment experienced patients with virologic failure

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ABSTRACT

Background: Antiretroviral therapy (ART) inhibits virus replication. Nevertheless, ART has the disadvantage of generate selective resistance and adverse events. Coreceptor antagonists are a family of antiretroviral drugs that are used with the prior knowledge of patients HIV tropism.
Objectives: The purpose of this work was to estimate the prevalence of R5 and X4 variants among Chilean patients under antiretroviral therapy and virological failure and investigate variables such as plasma viral load (pVL) and CD4 cell count in the population studied.
Study design: HIV RNA or proviral DNA was extracted from 454 consecutives patients and tropism testing was performed using a genotypic method performed with Geno2pheno setting a cutoff value for FPR 5.75%.
Results: Among 454 individuals analyzed, 299 (66%) harbouring exclusively R5 variants. They not displayed a better clinical profile than individuals harbouring X4 strains (22%). For R5 patients the median of pVL and CD4 cell count were 268,000 copies/mL, and 223 cells/μL respectively. For X4 samples the values were 368,000 copies/mL and 214 cells/μL [$P > 0.05$]). Only, 53 patients (12%) could not be analyzed and were categorized as non-reportable.
Conclusions: The genotypic method confirmed that R5 strains were more prevalent despite the fact that patients were treatment-experienced for several years. The genotypic strategy proved to be a faster and cost-effective option as compared to phenotypic assays. According to our results, two of every three patients under antiretroviral therapy and with virologic failure harbour R5 strains, and may be candidates for use of a CCR5 antagonist.

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1. Background

Official data indicate that in Chile exist 26,740 people living with HIV [1]. The strategy to inhibit virus replication consists on providing antiretroviral therapy (ART) to the total of patients of the country [2]. ART has permitted longer survival and a better quality of life for patients [3,4]. Nevertheless, ART has the disadvantage of generate selective resistance and adverse events that entail a continuous quest for new drugs to solve such drawbacks. Due to this, new drugs based on coreceptor inhibition have emerged over the last years [5]. Two of such HIV coreceptors are CCR5 and CXCR4, which are fundamental for viral entry into the host cell. Viral preference for the use of any of such two coreceptors is defined as HIV viral tropism. When the virus uses exclusively CCR5 coreceptor, the virus is named R5, and when it uses CXCR4 coreceptor it is named X4. A virus may sometimes use both coreceptors. In such case the virus is said to have dual tropism and is designated as R5/X4, mixed tropism occurs when both X4 and R5 variants co-exist in a patient [5,6]. CCR5 antagonist drugs exclusively inhibit the replication of HIV variants that have CCR5 tropism. Therefore, the use of a CCR5 antagonist requires previous knowledge of viral tropism [7,8]. Currently the only CCR5 antagonist approved by the FDA (Food and Drug

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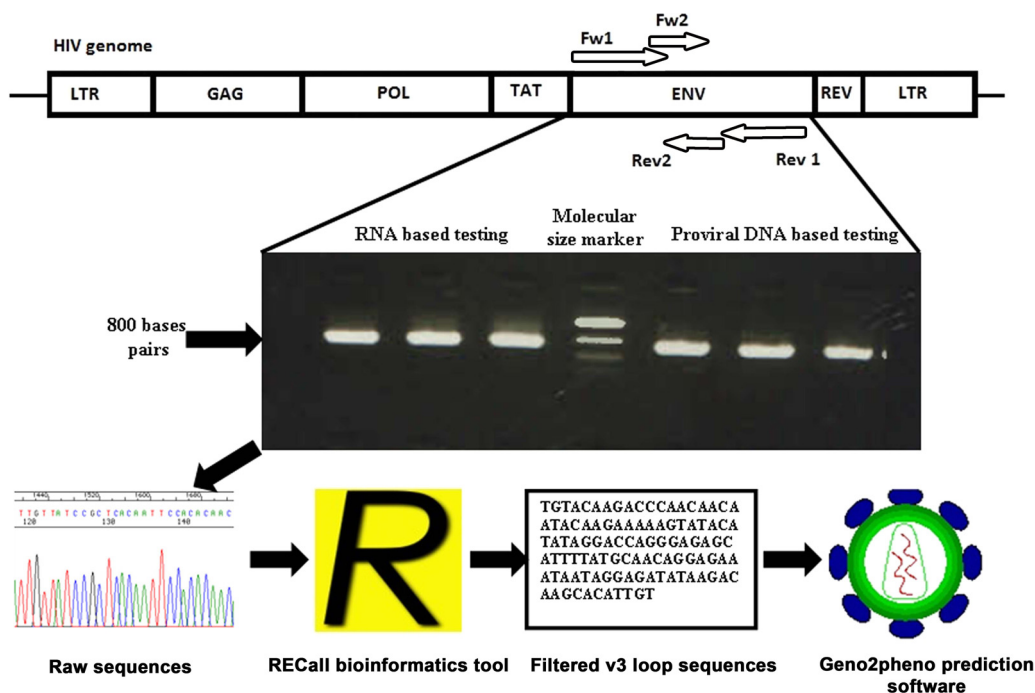cape@med.puc.cl (C.M. Pérez), aafani@vtr.net (A. Afani).

Fig. 1. Genotipic and bioinformatics HIV tropism prediction. Using the primers Fw 1 with Rev 1 and Fw2 with Rev2, RNA or DNA HIV-1 V3 loop was analyzed in triplicate through reverse transcription (omitted for proviral DNA) and nested PCR, resulting in the generation of three PCR products of 800 pair bases each. Then amplicons were sequenced with the Sanger's traditional sequencing method obtaining raw sequences. The raw sequences were assembled using the bioinformatics tool RECall. Only the filtered sequences by RECall of 105 nucleotides were used to obtain the genotypic tropism using the prediction tool geno2pheno (G2P).

Administration) is Maraviroc (MVC) although other CCR5 inhibitors have been developed such as Vicriviroc and Aplaviroc [9]. MVC was initially approved for the treatment of patients in virologic failure as a result of resistance developed to other antiretroviral drug families. Subsequently, its use was approved in ART-naive patients in the United Sates [7,10].

Viral tropism assessment can be achieved through either phenotypic or genotypic testing. Phenotypic testing is typically based on an automated assay involving recombinant virus coreceptors [11]. However, it has important logistic and technical limitations responsible for the low uptake of its use in clinical practice in South America. Genotypic assays are based on the analysis of the amino acids in the V3 (loop) region of gp120 glycoprotein of HIV, which is closely related to viral tropism. Therefore, genotypic testing represents a more clinically realistic option in Chile, since tests are faster to perform, more cost-effective and with a wider availability in HIV laboratories [5,6].

2. Objectives

In the present work we addressed the study of HIV viral tropism to determine how many Chilean patients under ART and with virologic failure might include in their new therapeutic regime a CCR5 inhibitor.

3. Study design

Four hundred and fifty-four consecutives treatment-experienced patients with virologic failure who needed a change in their ART regime were included in this study. They were chosen according to the following inclusion criterion: under ART and having at least one virologic failure which was defined when one patient had two consecutives viral load values over 1000 copies/mL. The patients included were diagnosed between 1988 and 2011. The genotypic tropism test was carried out between July 2011

and April 2013. Plasma viral load (pVL) and CD4 cell count from patients were reported in the medical order. Non-parametric Mann–Whitney Rank Sum Test was used to statistics analysis.

3.1. Genotypic and bioinformatics tropism test

Viral RNA was extracted from plasma and total DNA was obtained from peripheral blood mononucleated cells. RNA or DNA HIV V3 loop was analyzed in triplicate with the use of a previously described protocol [12,13], through reverse transcription (omitted for proviral DNA) and nested PCR, resulting in the generation of three 800 bp PCR products [14]. Then the amplicons obtained were sequenced with the Sanger's traditional sequencing method. The sequences were assembled using the bioinformatics tool RECall [15]. Filtered sequences by RECall were used to obtain the genotypic tropism using the prediction tool geno2pheno (G2P) (Fig. 1). G2P is based on the FPR (False Positive Rate) concept, defined as the probability of wrongly classifying an R5 virus as X4. FPR has significance levels from 1% to 20% and the FPR value chosen as cutoff is set in accordance with the patient's therapeutic alternatives. Bioinformatics G2P software, allow to select how conservative the detection of CXCR4 usage should be [6,16]. Three predictions were obtained for each sample and the lowest FPR value was considered for prediction of viral tropism. HIV-1 viral subtype was determined in all the study patients using G2P.

4. Results

Four hundred fifty-four samples were analyzed patients. Epidemiological and clinical features are shown in Table 1. A total of 411 (90.5%) had viral load above 1000 copies/mL. Forty-three samples (9.5%) had a viral load under 1000 copies/mL and were tested by proviral DNA. The concordance between proviral DNA and RNA had been previously compared, with >95% concordance [12,17]. The V3 loop amplification from proviral DNA for patients with viral load under 1000 copies/mL showed an amplicon with a similar size to

Table 1
Patients epidemiological and clinical features (*n* = 454).

Age (range)	44 (18:70)[a]
Gender (male:female)	(363:91)
CD4 count (cells/μL)	232 (5–1162)[a]
Plasma viral load (log RNA copies/mL)	3.79 (1.28–6.23)[a]
Tropism (R5:X4)	(299:155)
Viral subtype B	99.3%
Risk factor Sexual/IVDU[c]	454/0
Others viral subtypes	0.7%[b]

[a] Median and range.
[b] others subtypes detected were A, C, F, and G.
[c] IVDU: intravenous drug use.

the observed when we used viral RNA (Fig. 1). In the present work, FPR was set in 5.75%, since this value showed best correlation with virological outcome of the MVC therapy [6,18]. A value under 5.75% was predictive of an X4 virus, while a value equal or above 5.75 indicated an R5 virus. After analyzing and assembling sequences in RECall, three consensus sequences per patient were obtained. Three predictions were obtained for each sample and the worst prognosis, (lowest FPR), was considered as the tropism for the virus of the patient. Thus, a patient was reported as R5 only when the three FPR values were higher than the cut-off FPR value. Conversely, a patient was reported as X4 when at least one of the three values was under the cutoff value. In such case, the sample was considered as incompatible with the use of a CCR5 inhibitor.

According to this G2P showed that 299 (66%) patients had a virus with R5 tropism, 102 (22%) samples had X4 tropism and 53 patients (12%) were non-reportable.

When we analyzed if X4 and R5 viruses have difference in the time lapsed between the date of HIV diagnostic and date of tropism test, we were unable to find significant differences. Although this information (time lapsed) was available for only 34 samples. The median CD4 lymphocyte count among X4 patients was 214 and for the R5 group was 223 cells/μL. This difference was not statistically significant ($p > 0.05$). The median viral load among X4 patients was 368,000 copies/mL and for the R5 group was 268,000 copies/mL; which being stronger than the difference between CD4 cell counts, such difference did not reach statistical significance either ($p > 0.05$). (Table 2). In the non-reportable samples obtained by genotyping method we found that fourteen had a viral load median of 2070 copies/mL (210–5200) with CD4 median of 406 (109–611) cells/μL and thirty-nine had a viral load median of 218,000 copies/mL (15,000–760,000) with CD4 median of 205 (27–391) cells/μL.

We detected the subtype B in the 99.3% of the studied samples, in other three patients we found four subtypes different to B: in two patients with R5 tropism were found C and F or B/F subtypes; in a third patient with X4 tropism, A or A/G subtypes were identified.

5. Discussion

In this study we reported the total number of HIV tropism tests that have been performed in Chile. To our knowledge, this is the first study of HIV coreceptor usage conducted in our country, either using a phenotypic or a genotypic method. According to our results, two out of three persons in virologic failure appear to harbour R5 virus. This data is relevant since MVC and others CCR5 inhibitors as Vicriviroc and Aplaviroc are a new therapeutic option available for these patients.

Chilean guideline for treatment of patient with HIV-1 establishes that asyntomatics patients must begin ART when median CD4 counts be equal or lower than 350 cells/μL. In addition to this, the national guidelines recommend start the ART when median

CD4 counts range between 350 and 500 cells/μL, considering the projection of adherence each patients individually [19]. The high percentage of R5 strains detected is in according with Brumme's results about the distribution of R5/x4 HIV-1, stratified by basaline CD4 cell count [20]. To this respect, a limitation of our study is that information on baseline or nadir CD4 count is not available for each patient nor is data on the duration of therapy before tropism testing started.

Interestingly, in a Spanish study about tropism with Trofile involving 865 patients, 83% of which were in virologic failure, the prevalence found for R5 strains was 66%, an identical value to that obtained in our study where the 100% of the patients were in virologic failure [21].

When comparing the prevalence of X4 strain (22%) detected in Chile with the Spanish study (34%) [21], it can be see that our prevalence is lower than the European. This may be due to the different epidemiological and clinical characteristics of the samples, the length and the type of antiretroviral therapy and the viral subtype.

Another work carried out in Italy with 53 patients, of which 52 were under ART, studied viral tropism through a genotypic method. The prevalence of R5 strain was 69.6% and the prevalence of the X4 strain was 38.9% [22]. In this case, the difference in X4 prevalence is a result mainly of the way in which the genotypic tropism test was carried out. The Italian study the genotypic test was realized using proviral DNA for all samples, and they obtained the tropism prediction with a single test, setting the cutoff of FPR value in 20% and 10% subsequently. Proviral DNA and FPR value used like cutoff are two factors known to impact directly on the sensitivity to detect X4 species [22,23]. Moreover, it is important to mention that the therapeutic options available for the patient also have an impact on the setting of the cutoff value of G2P. For example, if the patient has few therapeutic alternatives then the cutoff of FPR value cannot be fixed with FPR values higher [23]. In our case, all the patients analyzed were ART-experienced patients with at least one virologic failure, and thus we considered adequate a cut-off FPR of 5.75 conformed it has been recommended in the European guidelines and by other authors when the test is performed in triplicate and using RNA [14,18,23,24].

From the perspective of the clinical laboratory, one of the main advantages found in our work is that we obtained only 12% of non-reportable samples. It was lower than values published previously [17]. Other advantages of genotypic methods over phenotypic methods are the time spend and cost of the assays. The genotypic strategy was faster and cost-effective option as compared to phenotypic assays. This makes the genotypic method an excellent alternative for developing countries.

Although the number of samples that were non-reportable was low, it represents a limitation of our work. In this respect is important consider that that not all the non-reportable results were due to low or undetectable viral loads. This happened due to deficiencies in amplification and sequencing reactions which generated sequences with low quality that were not approved by RECall. In our opinion the reverse transcription followed by the two rounds of amplification and subsequent sequencing could not detect all HIV genetic variants. Namely, using HIV RNA and Sanger's sequencing, the genotyping method has a low sensitivity to detect species that have a representation under 10%. Therefore, in the future, to further increase the sensitivity of the genotypic method, we recommend explore new cell compartments such as HIV DNA inserted in the infected cell's genome. The latter together with deep sequencing will enable finding HIV variants that are represented in levels as low as 1% [25,26]. Similar values of non-reportable samples have been previously reported in 14% of the analyzed samples by proviral DNA and in 10% of samples analyzed by RNA, and failures have not necessarily been related to low viral loads [24]. In that

Table 2
Relationship between R5 and X4 strains and CD4 cell counts and viral load values.

Tropism	% FPR (median)	CD4 cells/μL [Median (Range)]	VL copies/mL [median (range)]
R5	37.2 (6.4–99.3)	223 (71–600)	268,150 (19–6,600,000)
X4	1.9 (0–4.7)	214 (19–569)	368,347 (19–9,000,000)

cases the explanation for such limitations were deficiencies in RNA and proviral DNA amplification and sequencing reactions due to primers mismatches.

In our analysis, the number of samples analyzed was increased (~10%) due to the use of proviral DNA in those patient with viral loads <1000 copies/mL or undetectable. The latter was possible thanks to the fact that a tropism assay using HIV proviral DNA had been previously validated and statistically significant correlation was found between the RNA-based and the proviral DNA-based assays [17].

In the present work individuals harbouring exclusively R5 variants not displayed a better clinical profile than individuals harbouring CXCR4-using HIV as might be expected, namely, were not found lower viral loads and higher CD4 cell counts in patients infected with R5 strains than in patients with X4 variants. Such result may be due to the fact that the size of the study sample is too small to detect the correlation that would exist between viral load, CD4 cell count and viral tropism parameters. Nevertheless, several clinical studies on correlation between tropism, viral load and CD4 cell count have widely reported that lower CD4 cell counts and higher viral load are commonly found in patients harbouring X4 variants [27–29]. Therefore, relating X4 strains to a poorer prognosis in HIV infection [30]. The X4 variants are considered more pathogenic viral species than R5 strains [31]. In this sense, detection of X4 strains might be considered in the future as a potentially novel marker to initiate ART and to guide the clinical management of the patient over the course of the disease. Another argument supporting such consideration is the discovery of X4 strains and not R5, as capable to infect hematopoietic stem cells. The latter might explain the low CD4 cell counts and the poor prognosis related to X4 strain [32].

The main circulating viral subtype in Chile is B. Upon reviewing the literature, it can be seen that other subtypes that have been published previously in our country are F and B/F mixture subtypes [33]. In the present work we have demonstrated that A, A/G and C HIV viral subtypes are circulating in the country.

In conclusion, the genotypic method confirmed that R5 strains were more prevalent despite the fact that patients were treatment-experienced for several years. The genotypic strategy proved to be a faster and cost-effective option as compared to phenotypic assays. According to our results, two of every three patients under antiretroviral therapy and with virologic failure harbour R5 strains, and may be candidates for use of a CCR5 antagonist.

Competing interests

No conflict of interest.

Ethical approval

This work was approved by the Hospital Clínico de la Universidad de Chile Ethics Committee and was under the Hospital Research Project Registry number OAIC 559/12. Certificate of Approval, Number 43 (Sep/2012).

References

[1] MINSAL. Informe Nacional "Evolución VIH SIDA, Chile 1984–2011". Ministerio de Salud de Chile; 2012.

[2] MINISTERIO DE SALUD CNDS. Evaluación del impacto de una política pública: Ampliación del acceso a terapia antiretroviral en Chile. Rev Chil Infect 2003;20:277–84.

[3] Volberding PA, Deeks SG. Antiretroviral therapy and management of HIV infection. Lancet 2010;376:49–62.

[4] Adolescents PoAGfAa. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services; 2010.

[5] Ferrer P, Rodríguez C, Tordecilla R, Guzmán M, Afani A. Antagonistas de CCR5 en la infección por virus de la inmunodeficiencia humana (VIH): aspectos generales y tropismo viral. Rev Hosp Clín Univ Chile 2012;23:346–53.

[6] Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R. Bioinformatics prediction of HIV coreceptor usage. Nat Biotechnol 2007;25:1407–10.

[7] Gilliam BL, Riedel DJ, Redfield RR. Clinical use of CCR5 inhibitors in HIV and beyond. J Transl Med 2011;9(Suppl. 1):S9.

[8] Poveda E, Paredes R, Moreno S, Alcami J, Cordoba J, Delgado R, et al. Update on clinical and methodological recommendations for genotypic determination of HIV tropism to guide the usage of CCR5 antagonists. AIDS Rev 2012;14:208–17.

[9] Strizki JM, Tremblay C, Xu S, Wojcik L, Wagner N, Gonsiorek W, et al. Discovery and characterization of vicriviroc (SCH 417690), a CCR5 antagonist with potent activity against human immunodeficiency virus type 1. Antimicrob Agents Chemother 2005;49:4911–9.

[10] Lieberman-Blum SS, Fung HB, Bandres JC. Maraviroc: a CCR5-receptor antagonist for the treatment of HIV-1 infection. Clin Ther 2008;30:1228–50.

[11] Whitcomb JM, Huang W, Fransen S, Limoli K, Toma J, Wrin T, et al. Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob Agents Chemother 2007;51:566–75.

[12] Ferrer P, Montecinos L, Tello M, Tordecilla R, Rodriguez C, Ferres M, et al. HIV-1 tropism: a comparison between RNA and proviral DNA in routine clinical samples from Chilean patients. Virol J 2013;10:318.

[13] McGovern RA, Harrigan PR, Swenson LC. Genotypic inference of HIV-1 tropism using population-based sequencing of V3. J Vis Exp 2010;46, http://dx.doi.org/10.3791/2531. Dec 27. pii: 2531.

[14] McGovern RA, Thielen A, Mo T, Dong W, Woods CK, Chapman D, et al. Population-based V3 genotypic tropism assay: a retrospective analysis using screening samples from the A4001029 and MOTIVATE studies. Aids 2010;24:2517–25.

[15] Woods CK, Brumme CJ, Liu TF, Chui CK, Chu AL, Wynhoven B, et al. Automating HIV drug resistance genotyping with RECall, a freely accessible sequence analysis tool. J Clin Microbiol 2012;50:1936–42.

[16] Beerenwinkel N, Daumer M, Oette M, Korn K, Hoffmann D, Kaiser R, et al. Geno2pheno: estimating phenotypic drug resistance from HIV-1 genotypes. Nucleic Acids Res 2003;31:3850–5.

[17] Verhofstede C, Brudney D, Reynaerts J, Vaira D, Fransen K, De Bel A, et al. Concordance between HIV-1 genotypic coreceptor tropism predictions based on plasma RNA and proviral DNA. HIV Med 2011;12:544–52.

[18] Skrabal K, Low AJ, Dong W, Sing T, Cheung PK, Mammano F, et al. Determining human immunodeficiency virus coreceptor use in a clinical setting: degree of correlation between two phenotypic assays and a bioinformatic model. J Clin Microbiol 2007;45:279–84.

[19] MINSAL. Guía clinica AUGE, Sindrome de la Inmunodeficiencia adquirida ViH/SIDA; 2013.

[20] Brumme ZL, Goodrich J, Mayer HB, Brumme CJ, Henrick BM, Wynhoven B, et al. Molecular and clinical epidemiology of CXCR4-using HIV-1 in a large population of antiretroviral-naive individuals. J Infect Dis 2005;192:466–74.

[21] Arribas JR, Rivero A, Leal M, Sanchez-de la Rosa R. Human immunodeficiency virus (HIV) type 1 with R5 tropism among HIV-1 antiretroviral-experienced patients in Spain. Enferm Infecc Microbiol Clin 2010;28:559–60.

[22] Svicher V, Alteri C, Montano M, D'Arrigo R, Andreoni M, Angarano G, et al. Performance of genotypic tropism testing on proviral DNA in clinical practice: results from the DIVA study group. New Microbiol 2012;35:17–25.

[23] Vandekerckhove LP, Wensing AM, Kaiser R, Brun-Vezinet F, Clotet B, De Luca A, et al. European guidelines on the clinical management of HIV-1 tropism testing. Lancet Infect Dis 2011;11:394–407.

[24] Symons J, Vandekerckhove L, Paredes R, Verhofstede C, Bellido R, Demecheleer E, et al. Impact of triplicate testing on HIV genotypic tropism prediction in routine clinical practice. Clin Microbiol Infect 2012;18:606–12.

[25] Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, et al. Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 2012;30:434–9.

[26] Swenson LC, Dong WW, Mo T, Demarest J, Chapman D, Ellery S, et al. Use of cellular HIV DNA to predict virologic response to maraviroc: performance of population-based and deep sequencing. Clin Infect Dis 2013;56:1659–66.

[27] Raymond S, Delobel P, Mavigner M, Cazabat M, Encinas S, Souyris C, et al. CXCR4-using viruses in plasma and peripheral blood mononuclear cells during primary HIV-1 infection and impact on disease progression. Aids 2010;24:2305–12.

[28] Waters L, Mandalia S, Randell P, Wildfire A, Gazzard B, Moyle G. The impact of HIV tropism on decreases in CD4 cell count, clinical progression, and subsequent response to a first antiretroviral therapy regimen. Clin Infect Dis 2008;46:1617–23.

[29] Frange P, Galimand J, Goujard C, Deveau C, Ghosn J, Rouzioux C, et al. High frequency of X4/DM-tropic viruses in PBMC samples from patients with primary HIV-1 subtype-B infection in 1996–2007: the French ANRS CO06 PRIMO Cohort Study. J Antimicrob Chemother 2009;64:135–41.

[30] Almeida FJ, Zaparoli MS, Moreira DH, de Souza Cavalcanti J, Rodrigues R, Berezin EN, et al. Association of X4 tropism with disease progression in antiretroviral-treated children and adolescents living with HIV/AIDS in Sao Paulo, Brazil. Braz J Infect Dis 2013, http://dx.doi.org/10.1016/j.bjid.2013.10.002. Nov 22. pii: S1413-8670(13)00266-3.

[31] Saracino A, Monno L, Scudeller L, Bruno G, Ladisa N, Punzi G, et al. X4 viruses are frequently archived in patients with long-term HIV infection but do not seem to influence the "inflamm-aging" process. BMC Infect Dis 2013;13: 220.

[32] Carter CC, McNamara LA, Onafuwa-Nuga A, Shackleton M, Riddell Jt, Bixby D, et al. HIV-1 utilizes the CXCR4 chemokine receptor to infect multipotent hematopoietic stem and progenitor cells. Cell Host Microbe 2011;9: 223–34.

[33] Ríos M, Villanueva C, San Martín C, Ramírez E. Identification of B and F human immunodeficiency virus subtypes in Chilean patients. Rev Med Chil 2003;131:711–8.