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Genotypic Determination of HIV Tropism - Clinical and Methodological Recommendations to Guide the Therapeutic Use of CCR5 Antagonists

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Abstract

The approval of maraviroc (Selzentri®), the first CCR5 antagonist, with specific antiviral activity against CCR5 (R5)-tropic HIV variants, has promoted the determination of HIV coreceptor usage in the clinical setting. The phenotypic assay Trofile™, which is based on recombinant virus technology, has been the most widely used diagnostic test, given that it was the only assay which provided tropism information in the pivotal maraviroc clinical trials. However, this method displays logistical and technical limitations that make it far from convenient as a diagnostic test in clinical practice. Genotypic methods based on V3 genotyping represent a more feasible alternative and progressively are replacing phenotypic assays. Even though their sensitivity to detect X4-tropic variants is lower compared to Trofile™, recent studies have demonstrated that specific genotypic tools (geno2pheno and PSSM) are comparable to Trofile™ and ES-Trofile™ in predicting virologic response to maraviroc. This review summarizes clinical and methodological recommendations for the genotypic determination of HIV tropism to guide therapeutic decisions with CCR5 antagonists in HIV therapeutics. **(AIDS Rev. 2010;12:135-48)**

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Key words

HIV tropism. CCR5 antagonists. V3 genotyping. Maraviroc.

Introduction

Maraviroc is the first CCR5 antagonist approved for the treatment of HIV-1 infection[1] that exclusively inhibits the replication of R5 viruses[2]. Consequently, the determination of HIV coreceptor usage is required before recommending treatment with CCR5 antagonists. Several assays have been developed to determine HIV tropism in clinical samples. The Trofile™ phenotypic assay (Monogram Biosciences, California, USA) which is based on recombinant virus technology, has been extensively used to provide tropism information in clinical trials, showing good correlation with virologic outcomes, and accordingly it has been the most widely used to date[3]. This assay has been replaced since June 2008 by the Enhanced Sensitivity Trofile Assay (ESTA or ES-Trofile™), which is 10- to 100-fold more sensitive for detecting X4 minor populations[4]. However, the Trofile™ assay remains far from perfect as a diagnostic test for clinical purposes. It is labor intensive, expensive, and specimens must be

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shipped to the unique reference laboratory in the USA. Genotypic methods, based on the analysis of the third variable region (V3) of the HIV envelope, represent a cheaper and more rapid alternative, widely available among laboratories specializing in HIV diagnosis[5]. The reliability of genotypic tools in determining HIV tropism in clinical samples compared with phenotypic assays has been examined in multiple studies[6]. Overall, these studies highlighted the low sensitivity of the genotypic assays to detect X4 variants in comparison with phenotypic methods[7-9]. Subsequently, different strategies were designed to improve the sensitivity of genotypic assays to detect X4 variants. These approaches included simple modifications in the interpretation algorithms, or the combination of the results given by different genotypic algorithms. However, validation of genotypic tropism prediction methods ultimately requires not just evidence of perfect concordance with the Trofile™ assay (or ESTA), but rather evidence of a similar ability to correctly identify patients who will benefit from the use of CCR5 antagonists and experience a good virologic response. In this context, recent results from retrospective analyses from the maraviroc clinical trials (MOTIVATE and MERIT) have shown that the use of specific genotypic tools has a similar ability to that Trofile™ and ES-Trofile™ to predict virologic response to maraviroc[10,11]. These data support the reliability of genotypic tools for determination of HIV tropism in clinical practice.

In March 2010, the Spanish Group for the Genotypic Determination of HIV Tropism was constituted, composed of clinicians and virologists with recognized experience in the field of HIV infection, to reach a consensus for the genotypic determination of HIV coreceptor usage in the clinical setting. This review summarizes clinical and methodological recommendations proposed by this panel for the genotypic determination of HIV tropism to guide therapeutic decisions with CCR5 antagonists.

CCR5 antagonists: mechanism of action and efficacy in clinical trials

During the HIV entry process, the interaction between the CD4 receptor and the envelope glycoprotein gp120 induces conformational changes in the viral envelope that expose a chemokine receptor binding site and consequently allows the CD4-gp120 complex to interact with a chemokine coreceptor, typically CCR5 or CXCR4. The CD4-gp120 complex binds to either coreceptor through interactions with the V3 region of gp120, although other HIV gp120 regions such as V1/V2, and C4 are also involved[12]. The V3 region of gp120 is considered the major determinant in the choice of CCR5 or CXCR4 coreceptors. According to coreceptor use, HIV isolates are classified as CCR5-tropic (R5), CXCR4-tropic (X4), or dual/mixed-tropic. The term dual/mixed-tropic is used to refer to isolates that may contain true dual-tropic viruses (those that can use both chemokine coreceptors) or mixtures of viruses that exclusively use CCR5 and others that use CXCR4[13].

Maraviroc is the first CCR5 antagonist and the only oral HIV entry inhibitor approved by the US Food and Drug Administration (FDA) in June 2007 for the treatment of HIV infection[1]. It is an allosteric inhibitor of the CCR5 chemokine coreceptor, orally bioavailable. Maraviroc binds to the transmembrane coreceptor cavity, within the 2, 3, 6, and 7 helix[2]. Following binding, CCR5 coreceptor conformational changes occur, especially in the ECL2 region, which ultimately inhibits the interaction of the ECL2 with the V3 region of gp120, and consequently the HIV entry process.

The MOTIVATE 1 and 2 (Maraviroc plus Optimized Therapy in Viremic Antiretroviral Treatment Experienced Patients) trials demonstrated the safety and efficacy of maraviroc at doses of 150 or 300 mg once-daily (QD) or twice-daily (BID) versus placebo combined with an optimized background regimen in triple-class-resistant patients exclusively harboring R5-tropic viruses[14]. The MERIT trial evaluated the safety and efficacy of maraviroc versus efavirenz, each in combination with co-formulated zidovudine and lamivudine, in drug-naive HIV-1 patients. The trial initially failed to demonstrate non-inferiority of either QD or BID maraviroc arms compared to efavirenz using the attainment of plasma HIV RNA < 50 copies/ml at week 48 as the primary endpoint[15]. A re-analysis of the MERIT trial using ES-Trofile™, more sensitive for the detection of X4 variants, reclassified as dual/mixed-tropic nearly 15% of viruses from samples originally scored as having R5 by Trofile™. Following this new assignment, the proportion of patients achieving < 50 HIV RNA copies/ml at 48 weeks was the same (68%) in patients treated with maraviroc than in those treated with efavirenz[16]. More recently, in an *ad hoc* re-analysis of the MERIT trial using V3 genotyping by bulk sequencing to determine HIV tropism, maraviroc remained non-inferior to efavirenz[11].

Recently, data from the phase III trials of the CCR5 antagonist vicriviroc, known as VICTOR E3 and E4, have been presented. These studies compared vicriviroc

Table 1. Main technical characteristics of recombinant tropism assays

	VIRalliance Phenoscript[25]	Xtrack^{C}/PhenX-R In Pheno AG[26]	Virco NH2-V4 gp120[27]	Monogram Biosciences ESTATrofile™[4]	Univ. Toulouse Toulouse Tropism Test (TTT)[28]	ISCIII-FISPE Tropitest[29]
Amplicon/ vector backbone	V1-V3 gp120/ pNL4-3	VI-V3/pNL4-3ΔV1-V3	NH2-V4/ pHXB2D-ΔNH2-V4-eGFP	gp160/ pCXA-envelope expression gene plus RTV1.F-lucP. CNDOΔU3	gp160/ pNL43Δ*env*-Luc2 vector	gp160/ pNL4-3Δ*env*-lacZ
Construction of vector	Recombination	Clonal technology	Recombination	Clonal technology	Recombination	Clonal technology
Target cells	U373-CD4-CCR5 U373-CD4-CXCR4	CXCR4 CCR5/CXCR4	U87.CD4.CCR5 U87.CD4.CXCR4	U87.CD4.CCR5 U87.CD4.CXCR4	U87.CD4.CCR5 U87.CD4.CXCR4	U87/Ghost/ PBMc CD4.CCR5
Report gene	β-galactosidase	β-galactosidase	GFP	Luciferase	Luciferase	Luciferase
Virus stocks	Competent replication	Defective replication	Competent replication	Defective replication	Competent replication	Competent replication
Sensitivity	5-10%	1%	5-10%	0.3-1%	0.5%	1%

GPF: green fluorescent protein.

(30 mg once-daily) to placebo in combination with an optimized background regimen in which at least two fully active antiretroviral drugs were required. At week 48 of treatment, the proportions of patients with HIV RNA < 50 copies/ml were similar for the vicriviroc and placebo arms (64 vs. 62%, respectively). The results obtained might be explained by the good background combinations taken with vicriviroc in these trials, which make it harder to sort out how much the CCR5 antagonist contributes to the virologic response[17].

Currently, there are other CCR5 antagonists under clinical development, for most part orally bioavailable such as INCB9471[18], SHC 532706[19], and TBR-652[20], or through subcutaneous or endovenous injection as PRO-140[21].

HIV tropism determination in the clinic: phenotypic and genotypic assays

The antiviral activity of CCR5 antagonists is limited to HIV R5-tropic variants[2]. The presence of detectable dual/mixed-tropic or X4-tropic viruses has been associated with therapeutic failure using CCR5 antagonists[15,22]. Therefore, assessment of HIV-1 tropism is required before recommending treatment with CCR5 antagonists. Several phenotypic and genotypic assays have been developed to determine viral tropism[23,24].

Phenotypic assays

Phenotypic assays are mainly based on recombinant viruses' technology. Briefly, the HIV *envelope* gene is amplified by polymerase chain reaction (PCR) from plasma samples. Subsequently, recombinant virions are generated by clonal or genetic recombination. The recombinant virus particles are used to infect cell lines expressing the CD4 receptor and either CCR5 or CXCR4. Some key methodological characteristics of several phenotypic recombinant method[25-29] developments in the last years to assess HIV tropism are summarized in table 1.

The phenotypic assay Trofile™ has been extensively used to provide tropism information in the maraviroc clinical trials, and accordingly it has been the most widely used to date. The Trofile™ assay identifies X4 strains with a sensitivity of 10% when using clonal mixtures[3]. Monogram Biosciences has developed an enhanced sensitivity tropism assay (ESTA), which is 10- to 100-fold more sensitive for detecting X4 minor populations. ES-Trofile™, with a sensitivity to detect X4-variants of around 0.3%, has been available since June 2008 and has replaced the original Trofile™ assay used in the pivotal clinical trials[4].

Although Trofile™ is the most extended assay, phenotypic testing can be performed by other methods.

Table 2. Genotypic rules and algorithms for determining viral tropism

Methodology	Principle
Rules and algorithms	
– 11/25 rule[30]	R or K at position 11 and/or 25 is associated with an X4-tropic phenotype.
– 11/24/25 rule[32]	R or K at positions 11, 24, or 25 is associated with an X4-tropic phenotype.
– Net charge[33]	K+R – (D+E) ≥ 5 is associated with an X4-tropic phenotype.
– Wetcat[37] (http://genomiac2.ucsd.edu:8080/wetcat/v3.html) – Geno2pheno[38]$_{coreceptor}$ (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php) – WebPSSM[39] (http://indra.mullins.microbiol.washington.edu/webpssm) – Fortinbras PSSM (http://fortinbras.us/cgibin/fssm/fssm.pl)	HIV tropism predictions are inferred from genotypic/phenotypic paired dataset employing statistical methods. These algorithms for HIV tropism interpretation are freely available on websites.
Deep sequencing	Detects minority HIV variants by sequencing hundreds of thousands of clones within a single sample. It is sophisticated, expensive, with limited availability.

R: arginine; K: lysine; D: aspartic acid; E: glutamic acid.

Cloning or recombination of PCR-amplified sequences encompassing partial regions of the gp160 are proposed by three different approaches. HIV-1 Phenoscript Env™ (VIRalliance, Paris, France)[25] amplifies V1-V3 sequences by PCR. Subsequently, the amplicons generated are cloned in the pNL4-3 backbone in which the V1-V3 region has been deleted. PhenoX-R (InPheno AG, Basel, Switzerland)[26], combines two methodologies: probe hybridization (X-TrackC) for a rapid discrimination between R5- and X4-variants and a phenotypic assay (PhenX-R) for dual/mixed-tropic variants. In the latter, V1-V3 regions are amplified and cloned in NL4-3 vector for the generation of recombinant viruses. Virco laboratories have developed a platform (Virco® type HIV-1)[27] to assess HIV-1 tropism combining genotypic and phenotypic information. The phenotypic approach is based on the generation of chimeric viruses through *in vitro* recombination of the NH2-V4 amplicon with the vector pHXB2D-ΔNH2V4-eGFP. Finally, two recently published assays performed direct cloning of the full length gp160 in HIV vectors, TTT (Toulouse Tropic Test, University of Toulouse)[28] and TropiTest (Instituto de Salud Carlos III-Fundación FIPSE)[29], allowing the assessment of all potential regions determining HIV tropism. They present a major advantage in comparison with Trofile™ based on the generation of fully competent viruses carrying luciferase reporter genes, an approach that increases sensitivity in the detection of minority variants to 1%. Both tests have been validated and compared with ES-Trofile™, showing > 90% of concordant results.

Genotypic assays

Genotypic assays represent a more feasible alternative to phenotypic assays since they are more rapid, cheaper, and broadly available among laboratories specializing in HIV diagnosis. Since the early 1990s, several rules and algorithms have been developed to predict HIV coreceptor usage based on V3 sequences (Table 2). Many of them are now freely available via publicly accessible websites[5].

The "11/25 rule" was the earliest algorithm developed for viral tropism interpretation and remained one of the most popular until recent times. It is based on the fact that viruses presenting basic amino acids such as arginine (R) or lysine (K) at positions 11 and/or 25 are often associated with an X4-tropic phenotype. Conversely, the absence of R or K in these positions is associated with R5-tropic viruses[30]. Although this rule shows high specificity (80-90%), it may suffer from low sensitivity (30-40%) in identifying X4-tropic viruses in

comparison with phenotypic assays. Recently, a modification of the 11/25 rule has been proposed that improves the predictive value for viral tropism[31]. It is known as the 11/24/25 rule and considers variants as X4-tropic when positions 11, 24, or 25 harbor any basic amino acid; otherwise the virus is classified as R5-tropic[32].

The "net charge rule" is a simple interpretation algorithm that estimates the global net charge of the V3 region according to the following formula: (K+R) – (aspartic acid [D] + glutamic acid [E]). If the result is ≥ 5, the virus is classified as X4-tropic; otherwise it is R5-tropic. There is an alternative rule for calculating the net charge that includes the basic amino acid histidine (H); this is as follows: (K+R+H) – (D+E). However, this alternative method is less accurate than the rule that does not consider H (79 vs. 49%). Similar to the 11/25 rule, the net charge rule shows high specificity, but suffers from low sensitivity in identifying X4 variants[33,34].

Over the last decade, efforts have been made to identify residues within the V3 domain that are involved in determining viral tropism. The natural variability of the V3 region has been examined in multiple HIV isolates phenotypically classified as R5- and X4-tropic. Consequently, new residues and specific patterns of amino acids have been recognized as influencing viral tropism. No single change seems to be responsible for the tropism; rather, several clusters of genotypes appear to largely determine viral tropism. Employing statistical methods (support vector machines [SVM] or position-specific scoring matrices [PSSM]), these data have been analyzed and used as a basis for the development of more sophisticated algorithms that can be used for viral tropism determination[35,36]. Some of these algorithms are freely available on websites such as Wetcat, Geno2pheno$_{coreceptor}$, Web PSSM, and Fortinbras PSSM. Their main characteristics are described in more detail below.

Wetcat

Wetcat is a web service developed and maintained by the University of California at San Diego, USA[37]. It provides the possibility of using several classifiers to generate predictions: net charge, C4.5, C4.5 p8 and p12, PART, and SVM. Viral tropism estimations are obtained using V3 amino acid sequences that are manually translated into a specific format, which is described on the website. Information must fit a particular format in order to perform the subsequent alignment, taking a consensus sequence as a reference. This requirement represents the main disadvantage of the tool, as it is time-consuming and prone to errors. The main benefit of Wetcat is that it allows batch predictions in a single run. It bases predictions on a set of 292 V3 sequences (43% are non-B subtypes): 168 from R5-tropic, 103 from X4-tropic, and 21 from R5X4 dual/mixed-tropic viruses, all of which are recorded at the Los Álamos HIV sequence database (Los Álamos National Laboratory, USA).

Geno2pheno$_{coreceptor}$

This has been developed by researchers at the University of Cologne, Germany, and the Max-Planck Institute (Munich, Germany)[38]. Its predictions are based on the statistical method SVM. They can be performed from FASTA-formatted nucleotide or amino acid sequences containing the V3 region. The V3 sequences can be copied and pasted into a text field or uploaded from a file. The server does not allow batch predictions of V3 sequences; therefore, V3 sequences must be introduced independently. The Geno2pheno$_{coreceptor}$ database comprises a total of 1,100 V3 sequences from 332 patients: 769 from R5-tropic, 210 from X4-tropic, and 131 from R5X4-tropic viruses, mainly obtained from the Los Álamos database. The majority of V3 sequences included belong to HIV-1 clade B, but there are some from non-B subtypes. The server allows configuration to different user's requirements by varying the settings for significance levels (i.e. to minimize the false-positive rate as required). The latest version of Geno2pheno$_{coreceptor}$ has the option of including additional clinical parameters, such as plasma HIV RNA levels, CD4$^+$ cell counts, and the presence of the Δ32 deletion in the *CCR5* gene; this may improve viral tropism predictions. It is one of the most accepted and used algorithms at this time for the genotypic determination of viral tropism.

WebPSSM

WebPSSM was originally developed by the University of Washington (Seattle, USA). It predicts coreceptor usage from V3 sequences given in amino acid FASTA format using PSSM as the statistical method[39]. WebPSSM is an easy and rapid bioinformatic method for viral tropism estimation as V3 sequences do not require further manipulation. Alignment of the tested sequence with the consensus sequence is automatically completed by the server, using the Smith-Waterman algorithm for scoring. A score is given to a sequence by summing the cells in the matrix that

correspond to the particular residue present in the sequence at each V3 position. It is possible to select different matrices to perform the predictions: the R5X4 matrix or SINSI (SI/NSI) matrix may be used for B subtypes, whereas only the SINSI matrix can be used for subtype C. The R5X4 matrix uses a set of 213 V3 sequences from HIV-1 clade B: 168 from R5-tropic, 17 from X4-tropic, and 28 from R5X4 dual/mixed-tropic phenotypes. The SINSI matrix bases its predictions on a set of 257 V3 sequences for subtype B: 70 from SI and 187 from NSI phenotypes. For subtype C viruses, there is a SINSI matrix based on 279 V3 sequences: 228 identified as NSI and 51 as SI[40]. The R5 threshold values are –6.96 for the R5X4 matrix and –5.4 for the SINSI matrix. Thus, according to the matrix used to perform the predictions, V3 sequences will be considered as being R5 when the score is less than the R5 threshold. Similar to Wetcat, WebPSSM allows the analysis of multiple (up to 1,000) V3 sequences in the same run.

Fortinbras PSSM

Fortinbras PSSM, written by the original WebPSSM developer, is a recently available website for providing HIV coreceptor genotyping based on PSSM (as described above). It is intended to deliver the same predictions as for the original WebPSSM, but also to provide other matrices as desired by the user, and to respond quickly to user questions and suggestions. For example, Fortinbras PSSM allows predictions using threshold values developed by Poveda, et al.[8], with the aim of increasing the sensitivity of detecting X4-tropic variants. The R5 threshold values are –8 and –6.4 for the R5X4 and SINSI matrices, respectively. Fortinbras PSSM has several advantages over WebPSSM. For example, nucleotide sequences may be uploaded directly and sequences do not have to be trimmed to the V3 loop beforehand. Moreover, V3 sequences with nucleotide mixtures are allowed, and the user has the capability of obtaining scores for all possible non-ambiguous sequences or to record the simple average score of all possible sequences.

Combinatorial Methods

This approach combines the results given by different genotypic algorithms to produce a "pooled" X4-sensitive tropism prediction. In some cases, these strategies also add clinical parameters such as plasma viremia or length of infection in their predictions. These approaches allow improving the sensitivity to detect X4 variants around 80-90%[7,9]. However, we consider their performance too laborious to be introduced as first-line tools for routine use.

New technologies: massive pyrosequencing by 454

The use of deep-sequencing technology has allowed investigation of whether improvements in prediction of X4 variants can be achieved by searching a larger number of genomes in comparison with the use of conventional ("bulk") sequencing. This technology may provide a unique opportunity to enhance the sensitivity for identification of minority variants, including those from X4-tropic viruses[41-43]. Currently, 454 (454 Life Sciences/Roche Diagnostics) is the best adapted platform of massive sequencing for determining viral tropism. This technology has recently demonstrated to be comparable to Trofile™ and ES-Trofile™ to predict virologic response to maraviroc in naive and antiretroviral-experienced patients[44].

Deep sequencing, however, is a sophisticated and very expensive method that is only available in a few research facilities. Moreover, interpretation of the large amount of sequencing data generated by each sample remains challenging. These technical limitations might be solved very soon with the coming generations of this technology, such as 454 Junior (www.454.com), and the development of new tools for viral tropism interpretation as geno2pheno-454 (http://g2p-454.bioinf.mpi-inf.mpg.de/index.php).

Limitations of phenotypic and genotypic methods

Both approaches show technical and interpretation challenges. Regarding phenotypic assays, there are three steps that induce less efficiency and loss of sensitivity of the system for the detection and generation of recombinant viruses from HIV minority variants:

- The reverse transcription process has an efficiency ≤ 10%. Therefore, in a context of low HIV RNA viral load there is a low probability for the retro transcription of minority variants and consequently for their detection. This limitation is shared with genotypic assays.
- The efficiency and sensitivity to detect minority variants during the generation of the plasmid is higher using clonal technology than using homologous recombination.

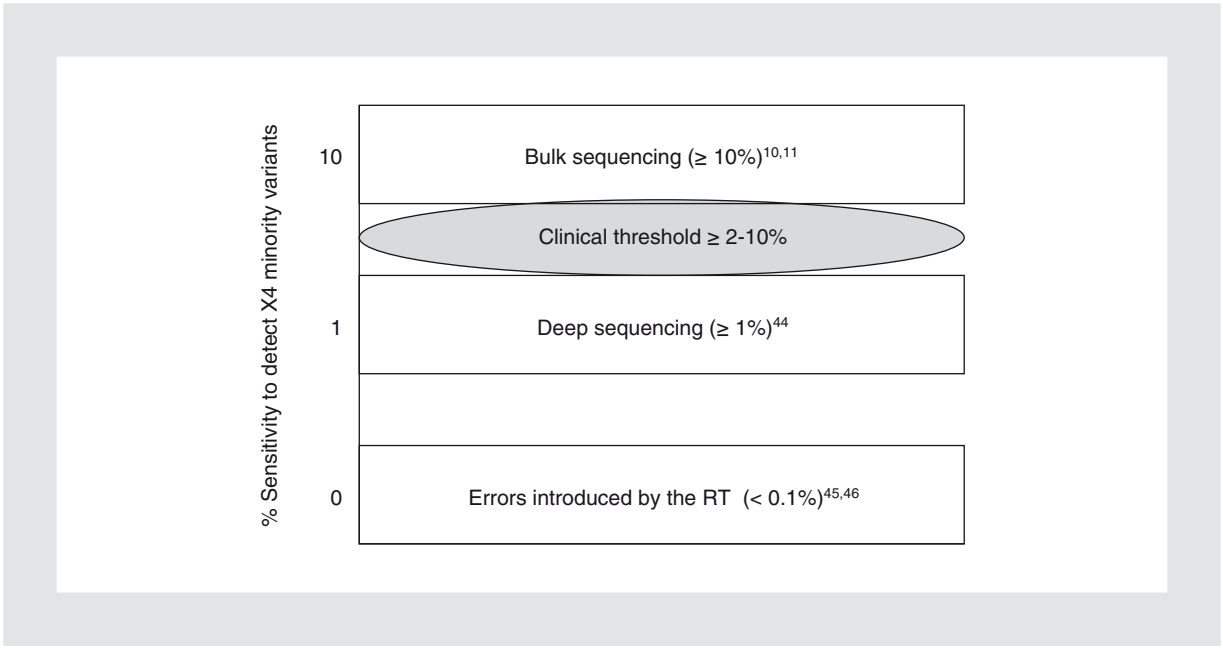

Figure 1. *Sensitivity of genotypic assays to detect X4 minority variants: bulk vs. deep sequencing. RT: retro transcriptase.*

– The generation of recombinant viruses using a multiple cycle system increases the sensitivity to detect minority variants compared to a single cycle system since cycles of infection/reinfection allow the amplification of minority variants.

Regarding interpretation, the clinical threshold for the detection of X4 variants by phenotypic assays, which predicts virologic response to CCR5 antagonists, remains challenging. Data from the MOTIVATE trials have shown that a sensitivity to detect X4 variants of 10% using the original Trofile™ assay predicts virologic response to maraviroc in antiretroviral-experienced patients[14]. Subsequently, the re-analysis of the MERIT trials using ES-Trofile™ with sensitivity to detect X4-variants around 0.3% reclassified as dual/mixed-tropic nearly 15% of viruses from samples originally scored as having R5 by the Trofile™ assay. However, approximately 43% of patients reclassified as dual/mixed-tropic using ES-Trofile™ had achieved HIV RNA viral load < 50 copies/ml[16]. Therefore, a higher sensitivity to detect X4 viruses using ES-Trofile™ may not increase the power of the assay to discriminate between responders and nonresponders to maraviroc. A recent re-analysis of the MERIT trial using 454 technologies had demonstrated a similar ability of deep sequencing to predict virologic response to maraviroc compared with ES-Trofile[44]. In this analysis, a sample was classified as X4 if ≥ 2% of the V3 sequences generated by deep sequencing were labeled as X4-tropic. According to the data reported to date, it can be established that the clinical threshold for the detection of X4 variants might range between 2-10%[10,11,14,44-46] (Fig. 1).

In addition, the phenotypic assays such as Trofile™ are labor intensive, expensive, and require special laboratory facilities and expertise. They are not widely available, and in the case of Trofile™, specimens must be shipped to the reference laboratory in the USA. Moreover, up to 15% of specimens are non-reportable, even when testing samples with plasma HIV RNA > 1,000 copies/ml[7,8]. Phenotypic assays must also face the challenge for determining HIV tropism in patients with HIV RNA viral loads < 1,000 copies/ml or even < 50 copies/ml, in which maraviroc may be considered as part of simplification strategies to avoid drug toxicities[47].

Genotypic assays using bulk sequencing present the follow limitations:

– The low efficiency during the retro transcription process diminished the probability to detect minority variants (shared with phenotypic assays).
– Sequencing technologies have limited sensitivity for the detection of minority variants, typically in the range of 10-20%. This represents the main difference in terms of sensitivity compared to phenotypic assays, the latter being more sensitive mainly due to the use of reporter genes (β-galactosidase, luciferase, or enhanced green fluorescent protein) as detection systems.

Table 3. Concordance of the genotypic algorithms geno2pheno and PSSM with Trofile™ and ES-Trofile™ in predicting virologic response to maraviroc

Genotypic algorithm	Genotype vs. Trofile™		
	Sensitivity† (%)	Specificity (%)	% of patients reaching < 50 copies/ml after start a maraviroc-based therapy (%)
Geno2pheno[38] coreceptor FPR *(false positive rate)*			
5%	63	91	42 vs. 42 (MOTIVATE)[10]
5.75%	55	93	66 vs. 68 (MERIT)*[11]
10%	65	71	
20%	76	58	
PSSM[39]			
Matrix R5X4	59	89	41 vs. 42 (MOTIVATE)[10]
Matrix SINSI	61	87	
Matrix R5X4[8]	93	69	
Matrix SINSI[8]	93	70	

*Phenotypic results were assessed by ES-Trofile™.
†Sensitivity/specificity rates could slightly range depending on the cohort of patients considered. This table shows the results obtained in the study designed by Poveda, et al., except for the results obtained during the re-analysis of the MOTIVATE[10] and MERIT[11] trials.

Moreover, the algorithms for HIV tropism interpretation currently in use are based on paired genotypic/phenotypic databases constituted by 100-1,100 V3 sequences with paired phenotypic data. This represents a relatively low number of genotypic/phenotypic paired results compared with current HIV drug resistance databases (Standford, VIRCOtype, REGA, or ResRIS), which are based on more than 50,000[24,36]. An increased number of V3 sequences with paired phenotypic results more likely improve the sensitivity and specificity to detect X4 variants of these algorithms. Finally, genotypic interpretation of viral tropism is exclusively based on the analysis of the V3 region, which is considered the main determinant of HIV coreceptor usage. However, other regions within gp120 (V1, V2, and C4) may have an impact on viral tropism that may be underestimated for the algorithms current in use[12].

Clinical validation of phenotypic and genotypic assays

The phenotypic assay Trofile™ is the only clinically validated assay for viral tropism determination to guide the use of maraviroc. The MOTIVATE and MERIT trials demonstrated the ability of Trofile™ to identify responders and nonresponders to a maraviroc-based therapy, but also revealed its limitations for the detection of minority X4-tropic variants associated with virologic failure to maraviroc[10,11,15,22]. ES-Trofile™, the current version of Trofile™ to determine viral tropism, has been retrospectively validated in the MERIT trial. ES-Trofile™ reclassified as dual/mixed-tropic nearly 15% of viruses from samples originally scored as having R5 at baseline by the original Trofile™. However, as previously explained, a detailed analysis of the results showed that in spite of the higher sensitivity of the new version to detect minority X4 variants, ES-Trofile™ seems not improve the ability of the assay to discriminate between responders and nonresponders to maraviroc, since nearly 43% of patients reclassified as dual/mixed-tropic had reached HIV RNA < 50 copies/mll at week 48, even harboring detected X4 variants[28].

The validation of genotypic prediction methods, do not requiere perfect concordance with the Trofile™ (or ESTA) assay, but rather evidence of a similar ability to correctly identify patients who will benefit from the use of maraviroc. In this context, recent studies have evaluated the reliability of genotypic tropism prediction tools to guide the therapeutic use of CCR5 antagonists[48-51].

The retrospective analyses of the MOTIVATE trials[10] have demonstrated that specific genotypic tools and the Trofile™ assay are comparable in predicting virologic response to maraviroc, although the sensitivity to detect X4 variants of the genotypic algorithms used, geno2pheno (false-positive rate 5%) and PSSM, was 63 and 59%, respectively, compared with Trofile™ (Table 3). Likewise, the re-analysis of the MERIT trial can demonstrate the availability of geno2pheno (false-positive rate 5.75%) to distinguish between responders and nonresponders to maraviroc similarly to ES-Trofile™, even when the sensitivity to detect X4 variants was 55% compared with ES-Trofile™.

Recent reports show results from prospective studies performed in different European cohorts in which the virologic response to maraviroc has been evaluated based on a genotypic determination of viral tropism. Overall, the results obtained have shown rates of virologic response to maraviroc of up to 85% in those patients in which HIV variants were classified genotypically as R5-tropic viruses[48-51].

Moreover, the contribution of the drugs administered together with maraviroc to achieve viral suppression has been highlighted. Valdez, et al. showed that a weighted optimized background treatment susceptibility score, rather than low-level X4 viruses at baseline, was the strongest predictor of virologic response at 48 weeks in the MOTIVATE trials[52]. It is the activity of the accompanying drugs that may enable maraviroc to benefit patients with a low proportion of X4 variants. In the contemporary therapeutic context, with new and potent drugs available to administer together with maraviroc, the presence of X4 variants most likely might have a relative impact on the virologic response.

Considering the aforementioned, different guidelines for HIV infection management, such as the Spanish (http://www.gesida.seimc.org)[53], British (http://www.bhiva.org/Tropism.aspx)[54], and European (www.europehivresistance.org)[55] guidelines, include within their recommendations the use of genotypic methods to guide the clinical use of CCR5 antagonists.

Clinical recommendations for V3 genotyping in the clinical setting

Drug-naive HIV-infected patients (CIII)

To date, there is no data to extend the recommendation for HIV tropism determination in patients who are going to start antiretroviral therapy. However, although maraviroc is not recommended as a first-line regimen[56], there are special clinical situations in which maraviroc could be considered as a good therapeutic option in drug-naive patients (i.e. presence of primary resistance or toxicity to drugs included in first-line therapy, or tuberculosis) and therefore viral tropism determination must be considered. Alternatively, there is the possibility to store (–80ºC) patient's plasma samples before starting an antiretroviral therapy in case viral tropism determination could be required in the future[57,58].

Antiretroviral-experienced patients (AIII)

Assessment of HIV tropism is recommended in all patients who experience virologic failure. Viral tropism information should be available together with each drug resistance test to facilitate the design of an optimal rescue therapy.

In HIV-infected patients under suppressive antiretroviral therapy, in which a simplification therapy based on maraviroc is planned, HIV tropism could be performed from peripheral blood mononuclear cells (PBMC)[47,58]. Although there is as yet scarce data regarding the clinical validation of this therapeutic strategy, the results reported to date are very promising[57].

Other specific clinical situations (AIII)

Once X4 variants are detected, a subsequent determination of viral tropism is not recommended. HIV tropism information must be clearly recorded in the medical history. In those patients with limited therapeutic options, the quantification of X4 variants could be considered, since their presence between 10-30% has been associated with a viral load reduction > 1.5 log[44]. HIV tropism determination during transiently detectable viremia (blips) is not recommended.

Technical and methodological recommendations for V3 genotyping in the clinical setting

Table 4 records the main technical and methodological recommendations that are detailed below.

Plasma volume (AII)

It is recommended to use a minimum plasma volume of 500 µl. Lower volumes might not properly allow the detection of minority variants, regardless of the methodology used (genotypic or phenotypic assays).

Number of reverse transcriptase polymerase chain reaction (AII)

It is recommended to perform three reverse transcriptase polymerase chain reaction (RT-PCR) assays since this was the methodology used in the re-analysis of the MOTIVATE and MERIT trial in which V3 genotyping was clinically validated to discriminate between responders and nonresponders to maraviroc[10,11]. The performance of three RT-PCR has demonstrated to increase the sensitivity to detect X4 variants from 4 to 8% compared with the performance of one single PCR[60-62]. However, in recent studies in which the feasibility of genotypic tools to identify patients as responders to

Table 4. Clinical and methodological recommendations for determining HIV tropism in the clinic

Topic	Specific recommendation	Recommendation grading	Comments
Patients	Antiretroviral-naive	CIII	Only consider in special clinical situations (presence of primary resistance or drug toxicities).
	Treatment-experienced	AIII	Recommended for each treatment failure.
Report	R5 tropism/X4 tropism	AIII	Genotypic assays based on bulk sequencing cannot distinguish between dual/mixed-tropic or X4-tropic variants.
	In parallel together with the resistance test for RT, PR, integrase and fusion inhibitors	AIII	CCR5 antagonist might be considered similarly to other drugs in rescue therapies.
Interpretation	Geno2pheno 5-5.75%, PSSM X4R5/SINSI	AII	These were the thresholds established for detecting X4 variants in the re-analysis of the MOTIVATE and MERIT trials.
	Geno2pheno 10-20%	BII	There are promising results in several European cohorts of patients.
	Plasma or PBMC	CIII	The criterion for tropism interpretation is the same regardless of the type of sample used.
Plasma volume	≥ 500 µl	AII	Increase the sensitivity to detect X4-tropic variants.
Number of RT-PCR	Triplicate	AII	Triplicate V3 sequencing was performance during the re-analysis of the MOTIVATE and MERIT trials.
	Single	BII	There are promising results in several European cohorts of patients.
Proviral DNA	Whether HIV RNA viral load is ≤ 500 copies/ml	CIII	There are promising results in several European cohorts of patients.
Sequence analysis	It is indicated to expand the V3 sequence in the case of nucleotide mixtures in all possible permutations	AIII	Increase the sensitivity to detect X4-tropic variants.
	If the V3 sequence has ≥ 8 nucleotide mixtures, do not consider it for subsequent analysis.	AIII	A heterogeneous V3 sequence might cause errors during interpretation.
Non-B subtypes	To advise regarding the lower sensitivity to non-B compared with B subtype	AIII	To perform a phenotypic assay in non-subtypes different to CRF02_AG, G, or C might be considered.

Strength of recommendation. A: strong recommendation for the statement; B: moderate recommendation for the statement; C: optional recommendation for the statement.
Quality of evidence for recommendation. I: One or more randomized trials; II: One or more well-designed, nonrandomized trials; III: Expert opinion.
RT: reverse transcriptase; PR: protease; PBMC: peripheral blood mononuclear cell.

maraviroc were assessed, V3 amplicons were obtained by a unique RT-PCR, reaching rates of virologic response to maraviroc of up to 85%[48-51]. Moreover, studies in which the virologic response to maraviroc had been compared depending on the number of RT-PCR performed are lacking. For this reason, the use of a unique RT-PCR (BII) to obtain V3 amplicons is also recommended. In this case, it is suggested to increase the sensitivity of the algorithm of interpretation used to detect X4 variants. For example, for geno2pheno it is recommended to use a false-positive rate (FPR) higher than 5.75%. Three RT-PCR are strongly recommended in samples with HIV RNA < 500 copies/ml.

Proviral DNA (CIII)

Genotypic determination of HIV tropism from proviral DNA is indicated in patients with undetectable viremia

or HIV RNA < 500 copies/ml in which RNA amplification from plasma samples are not available. V3 genotyping can be performed from blood or from PBMC. Current data agree that viral tropism determination from proviral DNA is more sensitive for the detection of X4 variants compared to that performed from plasma RNA[63,64]. Data regarding the rates of virologic response to maraviroc in patients in which viral tropism have been carried out from proviral DNA are scarce[48,59].

Quality of V3 sequences (CIII)

It is recommended to discard the analysis of V3 sequences with more than eight nucleotide mixtures. In these cases, it is suggested to repeat both V3 amplification and sequencing.

Expansion of V3 sequences (AIII)

To increase the sensitivity for the detection of X4 variants, V3 sequences with nucleotide mixtures (considering a nucleotide mixture when the second highest peak in the electropherogram was above 25%) need to be expanded into all possible amino acid permutations. Specimens will be considered as harboring R5 viruses only when all permutations excluded X4-tropic strains. The use of this strategy leads to an increase in the sensitivity to detect X4 variants of up to 10%.

Choosing an algorithm for viral tropism interpretation (AII)

Viral tropism interpretation is based on a V3 nucleotide or amino acid sequence. Although there are several rules and algorithms available for viral tropism interpretation, geno2pheno and PSSM are considered the most appropriate to use in the clinical setting. Table 3 represents the rate of concordance between geno2pheno and PSSM compared with the Trofile™ and ES-Trofile™ assays for genotypic interpretation of viral tropism and to predict clinical response to maraviroc. For each algorithm it is possible to obtain different rates of sensitivity to detect X4 variants depending on the FPR used[38] or the matrix selected for interpretation in the case of PSSM[8,39]. An increase in sensitivity for the detection of X4 variants is accompanied by a loss in specificity. For geno2pheno, it is recommended to use an FPR of 5 or 5.75% since both have demonstrated to be comparable to the original Trofile™ and ES-Trofile™ assays, respectively, in predicting virologic response to maraviroc, even though their sensitivities to detect X4 variants was ≤ 63%[10,11] compared to Trofile™. Recent reports show prospective data regarding clinical response to maraviroc in which HIV tropism was determined genotypically using geno2pheno with an FPR of 10 and 20%, with rates of virologic response of up to 85%[48,51]. The use of these FPR is especially indicated when V3 genotyping has been obtained with a single RT-PCR (BII). In the case of PSSM, the R5X4 matrix was used for the re-analysis of the MOTIVATE trials and demonstrated to be comparable to Trofile™, although its sensitivity to detect X4 variants compared with Trofile™ was 59%. The PSSM matrices R5X4 and SINSI have shown to be similar in terms of sensitivity and specificity compared with Trofile™; therefore, both matrices are suitable to use for viral tropism determination.

The PSSM interpretation matrices proposed by Poveda, et al. have shown an improved sensitivity to detect X4 variants of up to 93%[8]. These matrices have been validated in an independent cohort of patients showing a negative predictive value of 97%: that is, the possibility of misclassified X4 variants as R5 using Trofile™ is < 3%. However, its positive predictive value is 50%, which means half of V3 sequences classified as X4 would be classified as R5 using Trofile™, representing an overestimation of X4 variants. These new matrices are freely available at the Fortinbras PSSM website (http://fortinbras.us/cgibin/fssm/fssm.pl).

The combinatorial algorithms for HIV-1 tropism interpretation proposed by Sánchez, et al.[9] and Chueca, et al.[7], which increased the sensitivity and specificity for X4 variants detection up to 90%, are considered too complex to be used as first choice in routine clinical practice.

Interpretation in non-B HIV subtypes

Overall, the sensitivity of genotypic assays to identify X4 variants is lower for non-B HIV-1 subtypes than for B-subtypes. In a recent study published by Seclén, et al.[64], the sensitivity to identify X4 variants was 94% for B subtypes and 63% for non-B subtypes using geno2pheno (FPR 20%) compared with the phenotypic assay HIV-1 Phenoscript Env (ViRalliance, Paris, France). Similarly, in the same set of samples, the sensitivity to detect X4 variants using PSSMr5x4 was 89% for clade B and 58% for non-B subtypes[64] (Table 5). The feasibility of genotypic tools was also evaluated for specific HIV-1 subtypes. The CRF02_AG and subtype G are the most prevalent in Spain (47%)[65] and several European countries[66,67]. The sensitivity of geno2pheno (FPR 10%) and PSSMr5x4 to detect X4 variants in specimens from patients infected with CRF02_AG and

Table 5. Sensitivity and specificity of the genotypic algorithms geno2pheno and PSSM for the detection of X4 variants compared with phenotypic assays in specimens from patients infected with non-B HIV-1 subtypes

		Genotype vs. Phenotype*	
Genetic subtype (n)	**Algorithm**	**Sensitivity (%)**	**Specificity (%)**
CRF02_AG (52)[68]	Geno2pheno	40	90
	PSSMx4r5	80	76
	PSSMsinsi	70	90
C (73)[70]	Geno2pheno (FPR- 10%)	87	89
	PSSMx4r5 (matriz B)	80	93
	PSSMsinsi (matriz C)	93	82
CRF02_AG+G (37)[64]	Geno2pheno (FPR- 10%)	71	80
	PSSMx4r5	71	90
	PSSMsinsi	57	93
Non-B subtypes (75)[64]	Geno2pheno (FPR- 10%)	58	84
	PSSMx4r5	58	87
	PSSMsinsi	50	90
B subtypes (75)[64]	Geno2pheno (FPR- 10%)	94	51
	PSSMx4r5	89	86
	PSSMsinsi	89	81

G was 71%. Raymond, et al. obtained similar results for CRF02_AG specimens using the PSSM matrices R5X4 and SINSI, although the sensitivity found using geno2pheno was 40%[68]. In the case of patients infected with subtype C, the most prevalent worldwide[67], PSSM has developed a specific matrix showing a sensitivity of 93%[70].

There is scarce data regarding the feasibility of genotypic tools to predict clinical response to maraviroc in patients infected with non-B subtypes. A recent report from a small cohort of HIV-infected patients has shown that the clinical response to maraviroc was comparable between B and non-B subtypes using geno-2pheno (FPR 20%)[48]. In the case of patients infected with non-B subtypes a phenotypic assay to determine viral tropism may be considered[4,25-29].

Conclusions

Genotypic methods represent a rapid, reliable, and widely available approach for determining HIV tropism to guide the therapeutic use of CCR5 antagonists. The use of specific genotypic tools such as geno2pheno and PSSM have demonstrated, in retrospective analyses of the MOTIVATE and MERIT trials, their ability to predict virologic responses to a CCR5 antagonist-based therapy, even though their sensitivity to detect X4 variants is low compared with Trofile™. Although more prospective results from studies in large cohorts of patients are required, the data available to date supports the feasibility of V3 genotyping for HIV tropism determination in the clinical setting. The clinical and methodological recommendations recorded in this review may be useful for a proper performance of genotypic HIV tropism determination.

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