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Concordance between Two Phenotypic Assays and Ultradeep Pyrosequencing for Determining HIV-1 Tropism▽†

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There have been few studies on the concordance between phenotypic assays for predicting human immunodeficiency virus type 1 (HIV-1) coreceptor usage. The sensitivity of ultradeep pyrosequencing combined with genotyping tools is similar to that of phenotypic assays for detecting minor CXCR4-using variants. We evaluated the agreement between two phenotypic assays, the Toulouse tropism test (TTT) and the Trofile assay, and ultradeep pyrosequencing for determining the tropism of HIV-1 quasispecies. The concordance between the TTT and Trofile assays was assessed for 181 samples successfully phenotyped by both assays. The TTT was 86% concordant with the standard Trofile assay and 91.7% with its enhanced-sensitivity version. The concordance between phenotypic characterization of HIV-1 tropism and ultradeep pyrosequencing genotypic prediction was further studied in selected samples. The HIV-1 tropism inferred from ultradeep pyrosequencing of 11 samples phenotyped as X4 and dualtropic and 12 phenotyped as R5-tropic agreed closely with the results of phenotyping. However, ultradeep pyrosequencing detected minor CXCR4-using variants in 3 of 12 samples phenotyped as R5-tropic. Ultradeep pyrosequencing also detected minor CXCR4-using variants that had been missed by direct sequencing in 6 of 9 samples phenotyped as X4-tropic but genotyped as R5-tropic by direct sequencing. Ultradeep pyrosequencing was 87% concordant with the Trofile and TTT phenotypic assays and was in the same range of sensitivity (0.4%) than these two phenotypic assays (0.3 to 0.5%) for detecting minor CXCR4-using variants. Ultradeep pyrosequencing provides a new way to improve the performance of genotypic prediction of HIV-1 tropism to match that of the phenotypic assays.

Human immunodeficiency virus type 1 (HIV-1) enters its target cells by binding first to the CD4 receptor and then to one or both of the chemokine receptors CCR5 and CXCR4 (5, 7, 9). Some virus clones bind to CCR5 (R5 variants), others to CXCR4 (X4 variants), and still others to both (R5X4 or dual variants) (2). Virus populations that use both coreceptors are termed dual/mixed, since they may contain a mixture of the three variants.

The recent development of drugs that block the entry of HIV-1, such as CCR5 antagonists, has made it necessary to determine accurately HIV-1 coreceptor usage. Maraviroc, the first CCR5 antagonist approved for clinical use (6), is currently used to treat only patients harboring R5-tropic viruses. Hence, the presence of CXCR4-using viruses must be investigated prior to using any CCR5 antagonist-based therapy (8, 12).

Phenotypic and genotypic assays have been developed to assess HIV-1 coreceptor usage (29). Most phenotypic assays are based on recombinant viruses that bear the challenged envelope glycoprotein. These are the gold standard method for predicting HIV-1 coreceptor usage (22, 33, 34). Clinical trials of maraviroc have relied on the phenotypic determination of HIV-1 tropism with a commercial assay (Trofile assay; Monogram Biosciences) (12). A few alternative phenotypic assays are currently available, but little is known about their concordance (11, 16, 22, 31). The Toulouse tropism test (TTT) detects minor CXCR4-using variants down to 0.5% of the virus population, a sensitivity close to that of the enhanced-sensitivity version of the Trofile assay (0.3%) (22, 32). Genotypic assays can also infer HIV-1 tropism using algorithms from the amino acid sequence of the V3 *env* region. This region is where the main genotypic determinants governing receptor use are located (10, 13–15, 30). These assays are simpler, faster, and less expensive than phenotypic assays, but the direct sequencing of V3 is not very sensitive for detecting CXCR4-using variants when they are present at below 10 to 20% (4, 17, 21, 24). The recent development of ultradeep pyrosequencing can increase the sensitivity of genotypic assays to levels similar to that of the ultrasensitive phenotypic assays (1, 18, 28). The 454 Life Sciences Technology, available on the Genome Sequencer FLX and Junior systems (Roche), can be used for ultradeep pyrosequencing of PCR products with a read length sufficient for sequencing the V3 *env* region.

We assess here the concordance between two phenotypic assays, the Trofile and the TTT assays, and ultradeep pyrosequencing for determining the tropism of virus quasispecies with a high sensitivity in detecting minor CXCR4-using variants.

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MATERIALS AND METHODS

Patients and samples. The patients studied were screened for inclusion in the maraviroc expanded access program (EAP) in France between 2007 and 2008. Among 236 patients screened, 189 were successfully phenotyped by the Trofile assay (Monogram Biosciences) and genotyped in parallel by direct sequencing of V3. Coreceptor usage could not be determined for the other 47 patients because of a lack of PCR amplification or a failure of the recombinant virus assay. HIV-1 receptor usage was determined by using either the standard or the enhanced-sensitivity version of the Trofile assay, depending on the date of inclusion. The French agency for AIDS Research (ANRS) GenoTropism study was nested within the maraviroc EAP (26). Patients harboring R5-tropic viruses as phenotyped by the Trofile assay received maraviroc; 68% of these patients reach a plasma viral load below 50 copies/ml at month 6 (26). Plasma samples taken at screening were sent to the Laboratory of Virology of Toulouse University Hospital, Toulouse, France, for determining HIV-1 coreceptor usage with the TTT phenotypic assay, in parallel with the Trofile assay. The TTT successfully phenotyped 181 of the 189 patients. The assay failed for the other eight patients. Informed consent was obtained from all patients regarding their participation in the maraviroc EAP and the ANRS GenoTropism study.

Phenotypic characterization of HIV-1 coreceptor usage with the Trofile assay. The Trofile assay and its enhanced-sensitivity version were used to determine HIV-1 coreceptor usage in plasma samples as described elsewhere (27, 34). Briefly, the entire gp160 *env* gene was amplified by reverse transcription-PCR (RT-PCR) and cloned into the pCXAS-PXMX expression vector. The latter was cotransfected into HEK293 cells with the HIV genomic RTV1.F-lucP.CNDOΔU3 vector harboring the luciferase gene. The resultant pseudoviruses were used to infect U87 indicator cells bearing CD4 and either CCR5 or CXCR4. Viruses were classified as R5-tropic when only the $CD4^+$ $CCR5^+$ U87 cells were infected, X4-tropic when only the $CD4^+$ $CXCR4^+$ U87 cells were infected, and R5X4-tropic when both indicator cell lines were infected.

Phenotypic characterization of HIV-1 coreceptor usage with the TTT. We determined HIV-1 coreceptor usage using the TTT recombinant virus assay as described elsewhere (22). Briefly, a fragment encompassing the gp120 and the ectodomain of gp41 was amplified by nested RT-PCR using HIV-1 RNA isolated from plasma samples. Two amplifications were performed in parallel from each sample, and the resulting materials were pooled to prevent sampling bias of the assessed virus population. The phenotype of HIV-1 coreceptor usage was determined by using a recombinant virus entry assay with the pNL43-Δ*env*-Luc2 vector. 293T cells were cotransfected with NheI-linearized pNL43-Δ*env*-Luc2 vector DNA and the product of the nested *env* PCR obtained from the challenged HIV-1-containing sample. The chimeric recombinant virus particles released into the supernatant were used to infect U87 indicator cells bearing CD4 and either CCR5 or CXCR4. Virus entry was assessed by measuring the luciferase activity as relative light units. R5, X4, and R5X4 viruses were defined as in the Trofile assay.

Direct sequencing of V3 *env*. V3 was sequenced directly from the bulk *env* PCR products obtained for the TTT, as previously described (24). Samples were sequenced in both directions by the dideoxy chain termination method (BigDye Terminator, v3.1; Applied Biosystems) on a ABI 3130 DNA sequencer, and the results were analyzed with Sequencher (Genecodes), blinded to the phenotype. We used two algorithms to infer the virus tropism from the V3 amino acid sequence; one was the combined 11/25 and net charge rule, and the other was the Geno2pheno tool (4, 24, 30). The combined 11/25 and net charge rule requires one of the following criteria for predicting CXCR4 coreceptor usage: (i) an R or K at position 11 of V3 and/or a K at position 25; (ii) an R at position 25 of V3 and a net charge of at least +5; and (iii) a net charge of at least +6. Geno2pheno was used with a false-positive rate of 5.75% (19).

Ultradeep pyrosequencing of V3 *env*. Ultradeep pyrosequencing was performed on a 454 GS Junior. A 415-nucleotide fragment encompassing the V3 *env* region was generated by nested RT-PCR. The RT-PCR was carried out in duplicate as previously described (22). The nested PCR was performed with the Expand High Fidelity Plus PCR system (Roche Diagnostics), with the following conditions: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min; followed by a final extension at 72°C for 7 min. The amplified PCR products were purified by using Agencourt Ampure PCR purification beads (Beckman Coulter, Brea, CA) and quantified with the Quant-iT Picogreen dsDNA assay kit (Invitrogen) on a LightCycler 480 (Roche). Pooled PCR products were clonally amplified on capture beads in water-in-oil emulsion microreactors. A total of 500,000 enriched-DNA beads were deposited in the wells of a full GS Junior Titanium PicoTiterPlate device and pyrosequenced in both forward and reverse directions. The 200 nucleotide cycles were performed in a 10-h sequencing run.

TABLE 1. Concordance between TTT and Trofile phenotypic assays

Assay	Variant	TTT assay (no. of isolates tested)		Concordance (%)
		R5	R5X4/X4	
Trofile	R5	104	8	86.0
	R5X4/X4	14	31	
Trofile ES	R5	17	1	91.7
	R5X4/X4	1	5	

Genotypic prediction of HIV-1 coreceptor usage from ultradeep pyrosequencing data. The sequence reads of the V3 *env* regions were quantified by using GS amplicon variant analyzer (AVA) software, version 2.5p1 (Roche). The AVA software assigns each read to the proper amplicon and sample using multiplex identifiers. The sequence reads were aligned with the BaL consensus sequence (GenBank accession no. AY426110), and sequence alignments were manually edited to correct for insertions or deletions in homopolymeric regions that would result in a frameshift. The virus tropism of each clone was inferred from the V3 amino acid sequence by the combined 11/25 and net charge rule and the geno2pheno tool (false-positive rate = 5.75%).

Sensitivity of ultradeep pyrosequencing for detecting minor V3 *env* variants. We assessed the frequency of errors resulting from V3 amplification and GS Junior pyrosequencing by analyzing the pyrosequencing data from a panel of 10 plasmid clones of *env* previously sequenced by the Sanger method. The mean error rate of pyrosequencing was 0.000853 (99% confidence interval [CI99], 0.000322 to 0.001384). The upper confidence limit of the error rate was used to calculate the sensitivity of pyrosequencing for a given number of reads. At least 1,600 V3 reads reliably detected minor V3 variants when they accounted for over 0.5% of the quasispecies. Figure S1 in the supplemental material shows the detection threshold as a function of the read number.

Statistical analysis. A Poisson distribution was used to distinguish authentic variants from artifactual V3 sequences resulting from errors arising during PCR amplification and ultradeep pyrosequencing. P values of <0.001 were considered statistically significant.

RESULTS

Patient characteristics. The 189 patients studied had a median age of 45.3 years (interquartile range [IQR], 41.8 to 50.7), a median $CD4^+$ T cell count of 247 cells/μl (IQR, 110 to 396), a median nadir $CD4^+$ T cell count of 75 cells/μl (IQR, 14 to 160), and a median plasma HIV-1 RNA load of 4.2 $\log_{10}$ copies/ml (IQR, 3.5 to 4.8). Most (88.7%) of the patients were infected with subtype B HIV-1.

Concordance between the TTT and Trofile assays. The concordance between the TTT and Trofile assays was assessed for 181 of the 189 samples successfully phenotyped by both assays. These 181 samples included 157 that were tested with the standard version of the Trofile assay and 24 tested with its enhanced-sensitivity version (Trofile ES). The TTT was 86% concordant with the standard version of the Trofile assay and 91.7% with its enhanced-sensitivity version (Table 1).

Concordance between the phenotypic characterization of HIV-1 tropism and the genotypic prediction by ultradeep pyrosequencing. We assessed the agreement between the phenotype and the genotype inferred from ultradeep pyrosequencing data for 23 patients. These patients were selected on the basis of (i) sample availability, (ii) a plasma virus load of >400 copies/ml, and (iii) virus tropism (R5X4/X4 for 11 of them and R5 for the other 12, as characterized by both phenotypic assays). These 23 patients had a median plasma virus load of 50,154 copies/ml (IQR, 6,461 to 180,408). An average of 3,687

TABLE 2. Ultradeep pyrosequencing of samples phenotyped as CXCR4 and CCR5-using

Patient	HIV-1 subtype	Plasma HIV-1 RNA (log_{10} copies/ml)	Phenotypic assay		Ultradeep pyrosequencing on GS Junior				
					No. of reads	Geno2pheno 5.75		Combined 11/25 and net charge rule	
			TTT	Trofile		% CXCR4-using clones	Tropism	% CXCR4-using clones	Tropism
1	B	3.38	X4	R5X4	1,712	85.57	X4-containing	85.57	X4-containing
2	D	4.78	X4	R5X4	4,858	100	X4-containing	87.94	X4-containing
3	B	4.70	R5X4	R5X4	2,884	22.68	X4-containing	22.68	X4-containing
4	B	6.34	X4	R5X4	2,892	100	X4-containing	93.05	X4-containing
5	B	2.78	R5X4	X4	3,268	100	X4-containing	100	X4-containing
6	B	2.88	R5X4	R5X4	5,704	4.59	X4-containing	4.59	X4-containing
7	B	3.83	R5X4	R5X4	3,178	10.89	X4-containing	10.89	X4-containing
8	B	3.82	R5X4	R5X4	2,875	3.20	X4-containing	0	Pure R5
9	B	5.08	X4	R5X4	2,807	100	X4-containing	100	X4-containing
10	B	4.46	R5X4	R5X4	3,150	39.81	X4-containing	38.98	X4-containing
11	B	4.75	R5X4	R5X4	2,689	3.05	X4-containing	10.52	X4-containing
12	B	5.81	R5	R5	1,478	2.50	X4-containing	2.50	X4-containing
13	B	4.66	R5	R5	4,162	18.07	X4-containing	0.38	X4-containing
14	B	3.80	R5	R5	5,023	0.58	X4-containing	0	Pure R5
15	B	5.32	R5	R5	5,048	0	Pure R5	0	Pure R5
16	B	4.74	R5	R5	5,271	0	Pure R5	0	Pure R5
17	B	5.75	R5	R5	4,532	0	Pure R5	0	Pure R5
18	B	5.19	R5	R5	3,637	0	Pure R5	0	Pure R5
19	B	5.33	R5	R5	4,785	0	Pure R5	0	Pure R5
20	B	2.70	R5	R5	3,559	0	Pure R5	0	Pure R5
21	B	4.55	R5	R5	3,369	0	Pure R5	0	Pure R5
22	B	5.77	R5	R5	2,575	0	Pure R5	0	Pure R5
23	B	2.62	R5	R5	5,349	0	Pure R5	0	Pure R5

analyzable reads of V3 per sample was obtained by ultradeep pyrosequencing. This number of reads could reliably detect minor V3 variants representing 0.4% of the quasispecies (see Fig. S1 in the supplemental material). This sensitivity is close to that of the TTT (0.5%) and of the enhanced-sensitivity Trofile assay (0.3%) for detecting minor CXCR4-using variants (22, 32). The genotypic algorithms (the combined 11/25 and net charge rule and the Geno2pheno 5.75 tool) were used to predict the virus tropism from the V3 clonal sequences obtained by ultradeep pyrosequencing. All 11 samples containing CXCR4-using variants as assessed by the TTT and Trofile assays were correctly identified by ultradeep pyrosequencing when using the Geno2pheno 5.75 tool, and all but one sample were correctly identified using the combined 11/25 and net charge rule. The discordant case (patient 8) was due to two minor clones (2.12 and 1.08%) being wrongly predicted as R5-tropic by the combined 11/25 and net charge rule but correctly predicted as X4-tropic by Geno2pheno 5.75. Ultradeep pyrosequencing plus the Geno2pheno 5.75 tool correctly identified 9 of the 12 samples shown to contain CCR5-using variants by the TTT and Trofile assays, and 10 of the 12 samples when used with the combined 11/25 and net charge rule. Minor X4-tropic viruses were detected in samples from patients 12, 13, and 14 by ultradeep pyrosequencing but not by the phenotypic assays (Table 2). Globally, the ultradeep pyrosequencing results were 87% (20/23) concordant with those of the phenotypic assays.

Detection by ultradeep pyrosequencing of minor CXCR4-using variants missed by direct sequencing. We analyzed 9 discordant samples (CXCR4-using viruses found by the TTT but only CCR5-using viruses predicted by direct sequencing) by ultradeep pyrosequencing to determine whether ultradeep pyrosequencing could accurately detect minor CXCR4-using variants missed by direct sequencing. These 9 patients had a median plasma virus load of 29,000 copies/ml (IQR, 4,519 to 57,839). An average of 3,301 analyzable reads of V3 was obtained per sample, which should enable minor V3 variants accounting for 0.4% of the quasispecies to be detected (see Fig. S1 in the supplemental material). Ultradeep pyrosequencing plus Geno2pheno 5.75 revealed minor CXCR4-using variants in 6 of the 9 samples, and in 4 of the 9 when used with the combined 11/25 and net charge rule (Table 3).

DISCUSSION

CCR5 antagonists are new drugs for treating HIV-1 infection that are unique in their mechanism of action as they target the host cells rather than the virus. Since some HIV-1 strains can use CXCR4 as an alternative coreceptor to CCR5 for entry, the presence of CXCR4-using viruses must be investigated prior to using any CCR5 antagonist-based therapy.

Recombinant virus phenotypic entry assays have been fundamental for assessing coreceptor usage when CCR5 antagonists were first used to treat HIV-1 infection. The commercial Trofile phenotypic assay has been used in the clinical trials of maraviroc, the first-in-class CCR5 antagonist (8, 12). A few other assays have been developed recently, but only limited data on their concordance with the Trofile assay are available (16, 22, 31, 33, 34). The phenotypic assays have been optimized to improve their ability to detect minor CXCR4-using variants among virus quasispecies, reaching sensitivities below 1% (22, 32). However, technical and cost limitations have led to phe-

TABLE 3. Ultradeep pyrosequencing of discordant samples phenotyped as CXCR4-using but as CCR5-using by direct sequencing

Patient	HIV-1 subtype	Plasma HIV-1 RNA (log_{10} copies/ml)	Phenotype (TTT)	Genotype by direct sequencing	Ultradeep pyrosequencing on the GS Junior				
					No. of reads	Geno2pheno 5.75		Combined 11/25 and net charge rule	
						% CXCR4-using clones	Tropism	% CXCR4-using clones	Tropism
6	B	2.88	R5X4	R5	5,704	4.59	X4-containing	4.59	X4-containing
8	B	3.82	R5X4	R5	2,875	3.20	X4-containing	0	Pure R5
11	B	4.75	R5X4	R5	2,689	3.05	X4-containing	10.52	X4-containing
24	B	5.50	R5X4	R5	2,641	5.11	X4-containing	5.11	X4-containing
25	B	3.31	R5X4	R5	3,476	5.84	X4-containing	5.84	X4-containing
26	B	4.93	R5X4	R5	2,357	8.70	X4-containing	0	Pure R5
27	B	4.78	R5X4	R5	2,881	0	Pure R5	0	Pure R5
28	B	5.83	R5X4	R5	2,665	0	Pure R5	0	Pure R5
29	B	3.34	R5X4	R5	4,425	0	Pure R5	0	Pure R5

notypic assays being increasingly replaced in routine clinical practice by simpler genotypic assays that infer HIV-1 coreceptor usage from the V3 *env* amino acid sequence. However, direct sequencing of V3 at a population-based level is not sensitive enough to detect minor variants in the quasispecies (4, 17, 21, 24). The recent development of ultradeep pyrosequencing has made genotypic assays capable of sensitivities similar to those of the phenotypic assays (1, 18, 28). Our present findings indicate an excellent concordance between the TTT and Trofile assays, both in its standard and enhanced-sensitivity versions. Ultradeep pyrosequencing also performed well, providing a good concordance with the results of the phenotypic assays. It was also better than direct sequencing at detecting minor CXCR4-using viruses. The 454 technology could thus fill the gap between recombinant virus phenotypic entry assays and direct sequencing of V3.

There may be several reasons why the results of the assays for determining HIV-1 tropism differ. First, it is critical to avoid sampling bias of the virus quasispecies in the initial amplification of the *env* gene, which is common to both phenotypic and genotypic assays. Sampling bias could result from unequal amplification of the variants in the quasispecies by the primers or from too few copies of the HIV-1 genome in the sample assessed. We used highly conserved primers to amplify the fragment of *env* subsequently used in the TTT. This assay performs well for various HIV-1 subtypes, although subtype-specific primer pairs may sometimes be required (22, 23, 25). We used degenerate primers for ultradeep pyrosequencing of V3 to take into account some variations in the targeted sequence. The median virus load in our plasma samples was of 54,714 copies/ml. Moreover, we performed RT-PCR and nested PCR amplifications in duplicate and pooled the products before further processing in both the phenotypic and the genotypic assays to ensure adequate representation of the quasispecies composition. Others have reported that triplicate PCR amplification before direct sequencing of V3 provides a better detection of CXCR4-using variants than a single PCR amplification (20).

The differences between the results of HIV-1 tropism assays could also be due to intrinsic differences in the abilities of the assays to detect minor CXCR4-using variants. Direct sequencing only detects variants that account for over 10 to 20% of the virus population (4). In contrast, ultrasensitive phenotypic assays, such as the enhanced-sensitivity version of Trofile and the TTT, can detect amounts of minor variants as low as 0.3 to 0.5% (22, 32). Ultradeep pyrosequencing can be similarly sensitive when a sufficient number of reads are analyzed. However, ultradeep pyrosequencing is prone to artifactual errors. We measured the sequence error rate resulting from PCR amplification and ultradeep pyrosequencing by comparing the GS junior reads to the Sanger sequences of 10 plasmid clones. Artifactual insertions or deletions in homopolymeric regions of V3 that result in frameshifts were manually corrected in sequence alignments. The residual mean error rate was 0.000853 substitutions per base (CI99, 0.000322 to 0.001384). We used the upper 99% confidence limit of the error rate to calculate the frequency of artifactual sequences for a given number of reads and the Poisson distribution to estimate the number of reads above which V3 sequences found at low frequencies were authentic rather than artifactual. Only those variants whose frequency of occurrence yielded a P value of <0.001 according to the Poisson model were considered authentic. We found that our assay on the GS Junior can detect minor variants that account for only 0.5% of total virus if the number of V3 reads is above 1,600. Thus, ultradeep pyrosequencing enabled us to accurately detect minor CXCR4-using variants that had been missed by direct sequencing.

Lastly, differences between the results of phenotypic and genotypic assays could be due to inadequate prediction of HIV-1 tropism from the V3 amino acid sequence. The prediction of tropism from both direct sequencing and ultradeep pyrosequencing relies on algorithms built from genotype-phenotype correlations in V3 amino acid sequence datasets (4, 14, 24, 30). The performance of these algorithms is thus critical for accurate predictions. We have assessed the concordance between direct sequencing and virus coreceptor usage determined by the TTT for various HIV-1 subtypes (23–25). We found that the combined 11/25 and net charge rule and Geno2pheno provided the best tropism predictions. Geno2pheno 10 was initially used, but it lacks specificity. Recent versions now use a more adequate 5.75% false-positive rate. We have also shown that the performance of the combined 11/25 and net charge rule are excellent at a clone-based level, suggesting that most of the discordances observed between direct sequencing and phenotypic assays when assaying virus populations are due to sampling bias or to direct sequenc-

ing not being sensitive enough to detect minor CXCR4-using variants (4). Genotypic algorithms are thus well suited for predicting HIV-1 tropism by ultradeep pyrosequencing since it consists of multiple clonal reads. Although the geno2pheno algorithm appears to be slightly better on this data set, the combined 11/25 and net charge rule has been shown to be better at predicting the CXCR4 usage of some non-B subtypes, particularly CRF02-AG (23). However, further studies are needed to address this point.

Ultra-deep pyrosequencing may provide an alternative to evaluate HIV-1 tropism compared to the phenotypic approaches. Amplifying by PCR a short fragment of *env* encompassing V3 could result in a lower rate of technical failure for genotypic assays than amplifying the whole *env* gene since it is required for the phenotypic assays. The clinically relevant threshold above which minor CXCR4-using viruses might significantly influence the virological response to CCR5 antagonists remains unclear. However, reanalysis of the MERIT and AIDS Clinical Trials Group A5211 studies with the enhanced-sensitivity Trofile assay revealed that taking into account minor CXCR4-using viruses allows better prediction of the virological response to CCR5 antagonists (3, 32).

In conclusion, we have shown that the TTT and Trofile phenotypic assays are in excellent agreement when determining HIV-1 coreceptor usage. Ultradeep pyrosequencing provides a new way to improve the performance of genotypic prediction of HIV-1 tropism to match that of the phenotypic assays. Prospective clinical trials are now needed to confirm this new approach in routine practice. Quantitative assessment of the frequencies of CCR5 and CXCR4-using variants in the quasispecies by ultradeep pyrosequencing will also enable us to determine the clinically relevant cutoff above which minor CXCR4-using variants significantly influence the virological response to CCR5 antagonists.

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