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Performance of Geno2Pheno[coreceptor] to infer coreceptor use in human immunodeficiency virus type 1 (HIV-1) subtype A

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ABSTRACT

Background: Assessment of human immunodeficiency virus type 1 (HIV-1) coreceptor usage is required prior to treatment with the CCR5 antagonist maraviroc to exclude the presence of CXCR4-using (X4) strains. Genotype-based interpretation systems are mostly designed on subtype B and have been reported to be less accurate for subtype A/CRF02_AG.

Objectives: To evaluate the performance of the widely used Geno2Pheno[coreceptor] (G2P[c]) algorithm for prediction of coreceptor usage with subtype A/CRF02_AG vs. subtype B.

Study design: Co-receptor tropism of 24 subtype A/CRF02_AG and 24 subtype B viruses was measured phenotypically by a homebrew single-cycle assay and genotypically by using G2P[c]. Samples with discrepant genotype-phenotype results were analyzed by next generation sequencing (NGS) and interpreted by the NGS Geno2Pheno algorithm (G2P[454]).

Results: At 10% false positive rate (FPR), the G2P[c]/phenotype discordance rate was 12.5% (n = 3) for subtype A/CRF02_AG and 8.3% (n = 2) for subtype B. Minority X4 species escaping detection by bulk sequencing but documented by NGS explained the two subtype B and possibly one subtype A/CRF02_AG discordant case. The other two subtype A/CRF02_AG miscalled by G2P[c] could be explained by X4 overcalling at borderline FPR and/or by algorithm failure.

Discussion: Our study did not demonstrate relevantly higher G2P[c] inaccuracy with subtype A/CRF02_AG with respect to subtype B. Genotype/phenotype discordances can be due to different reasons, including but not limited to, algorithm inaccuracy. Very large genotype/phenotype correlation panels are required to detect and explain the reason for any consistent difference in genotypic tropism prediction for subtype A/CRF02_AG vs. subtype B.

1. Background

Delivery of the human immunodeficiency virus type 1 (HIV-1) nucleocapsid into host cells is mediated by the interaction between the gp120 envelope glycoprotein and the CD4 receptor and coreceptors (mostly CCR5 or CXCR4) [1]. The third hypervariable loop domain (V3) of HIV-1 gp120 is the major determinant of coreceptor tropism and viral strains are classified as R5 when using the CCR5 coreceptor for viral entry, X4 when using CXCR4, and dual tropic or mixed (DTM) when using both coreceptors. R5 viruses are prevalent in the early stage of HIV-1 infection and are the most frequently transmitted variants, while X4 strains often become dominant at late stage of infection and are associated with expanded cell tropism, an increased virus replication rate and faster disease progression [2].

With the advent of maraviroc, the only approved anti-HIV-1 drug targeting CCR5, assessment of coreceptor usage has become necessary before drug administration, to exclude the presence of maraviroc-insensitive X4 or DTM variants. Viral tropism can be predicted through genotypic assays, based on sequencing of the V3 loop domain followed by interpretation algorithms, or directly measured in vitro by phenotypic systems based on infection of reporter cell lines with virus vectors expressing the gp120 of interest [3,4]. Various commercial phenotypic tropism services were indeed developed following licensing of maraviroc, with the Enhanced Sensitivity Trofile Assay (ESTA; Monogram Biosciences, San Francisco, California, USA) established as the reference method allowing detection of X4 virus from plasma RNA or

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proviral DNA down to 0.3% of the virus population [5]. While phenotypic assessment of coreceptor use remains the gold standard, indirect genotype-based assays, particularly Geno2Pheno[coreceptor] (G2P[c]) [6], have been implemented in clinical routine due to wider availability and lower cost and turnaround time compared to phenotyping [7] and are recommended by European guidelines [8]. However, minority X4 variants representing less than 15–20% of the total viral quasispecies escape detection by standard bulk sequencing yet can outcompete the majority R5 species under maraviroc pressure [9]. This limitation can be overcome by next generation sequencing (NGS) analysis of the V3 quasispecies, mostly in advanced laboratories, resulting in sensitivity comparable to the best phenotyping methods [10].

A potential issue with G2P[c], as well as with other genotypic systems, is that the training data sets correlating genotype with phenotype and genotype with clinical outcome of maraviroc therapy are largely biased towards subtype B, which is most common in high income countries but accounts only for an estimated 11% of HIV-1 burden worldwide [11]. Indeed, disagreement between genotypic algorithms and phenotypic methods appears to be larger with non-B subtypes [12–15]. Subtype-specific algorithms [16–20] or adjustment of the G2P [c] cut-off discriminating R5 from non-R5 viruses [21–23] have been proposed for a few subtypes, yet the generic G2P[c] is still the most widely used coreceptor prediction system, irrespective of HIV-1 subtype. Subtype A and CRF02_AG, also comprising a subtype A *env* gene, have been most often indicated as variants where G2P[c] lacks accuracy, particularly overestimating X4 virus [13,15,23].

This study compared the performance of G2P[c] in the determination of coreceptor usage in two panels of plasma samples harbouring subtype A/CRF02_AG and subtype B viruses. The same samples were analyzed with an in-house phenotypic single-cycle tropism assay (SCTA) based on the generation of virus pseudoparticles expressing patient derived *env* amplicons and infection of CCR5- and CXCR4-expressing target cells. Samples with discrepant genotype-phenotype results were analyzed by NGS and interpreted by the NGS specific Geno2Pheno algorithm.

2. Objectives

To evaluate the performance of the G2P[c] algorithm in the prediction of coreceptor usage in 24 samples carrying the subtype A *env* with respect to an equal number of subtype B controls.

3. Study design

3.1. Clinical samples

Access to residual anonymized plasma samples derived from clinical practice was initially obtained through patient informed consent as approved by the local Ethics Committee at the University Hospital of Siena. A total of 48 viremic plasma samples, previously tested genotypically for HIV-1 coreceptor tropism and drug resistance, were selected on the basis of the G2P[c] version 2.5 (http://coreceptor.geno2pheno.org/index.php) False Positive Rate (FPR, defined as the likelihood that an R5 virus is misclassified as X4) and subtype information as obtained from analysis of the *pol* gene through the REGA Subtyping tool version 3.0 (http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool/). Four groups, composed of 12 consecutive samples each, were generated: A_high (FPR > 60, subtype A), A_low (FPR < 20, subtype A), B_high (FPR > 60, subtype B) and B_low (FPR < 20, subtype B). In addition, another eight viremic plasma samples, previously classified by ESTA as four DTM, two R5 and two X4 viruses were collected as controls for the SCTA assay.

3.2. Development and performance of the SCTA

Phenotypic coreceptor usage was determined by SCTA as previously described by Lin et al. [24], with minor modifications (Supplementary Fig. 1 and Supplementary Table 1). Briefly, two millilitres of plasma were centrifuged at 20,000 *g* for 1 h and 30 min to concentrate the virus. RNA was extracted from the bottom 400 µl of plasma using the EZ1 automatic system and the DSP Virus Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, resuspended in a final volume of 60 µl and stored at −80 °C for later use. The reverse transcription and first-round PCR were performed using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen). To improve the sensitivity of assay at low plasma viremia, the original 4733-base pairs (bp) *env* target region [24] was shortened to 3779 bp, excluding the vif coding region while maintaining the whole env (gp120 plus gp41). The human cytomegalovirus promoter was attached to the *env* amplicon by overlapping PCR as in the original protocol. However, the Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA; USA) was used to reduce the DNA extension time (30 s per kilobase) and to increase the fidelity of amplification. The resulting PCR amplicons were sequenced using primers spanning the V3 region to confirm the original sequence and FPR. Virus pseudoparticles expressing the *env* population from patient samples were produced by co-transfecting into 293 T Lenti-X cells (Takara Bio USA, Mountain View, CA, USA) 700 ng of promoter PCR product together with 2 µg of pNL4-3.Luc.R-.E- (NIH AIDS Reagent Program), an *env* defective HIV-1 backbone plasmid expressing luciferase. A reaction including only pNL4-3.Luc.R-.E- was used in each transfection as negative control and the corresponding supernatant was used in the subsequent infection of target cells to define the signal background. Coreceptor usage was determined by assessing pseudoparticles entry into U87 cells expressing CD4 and either CXCR4 (U87.CD4.CXCR4) or CCR5 (U87.CD4.CCR5) (National Institute for Biological Standards and Control, Ridge, UK) in the presence and absence of a coreceptor antagonist (maraviroc and AMD3100 for CCR5 and CXCR4, respectively). U87.CD4.CXCR4 and U87.CD4.CCR5 were infected with virus pseudoparticles in quadruplicate (two infections with and two without the coreceptor antagonist) and relative luminescence units (RLUs) were measured after 72 h in white 96-well plate with the GloMax Discover reader (Promega, Madison, WI, USA). A pseudoparticle was defined as X4 or R5 when the mean of the RLU duplicate was at least two-fold the negative control background and the X4 or R5 coreceptor antagonist reduced RLUs of at least 0.3 log, in U87.CD4.CXCR4 or U87.CD4.CCR5 cells, respectively. The pseudoparticle was classified as DTM when both criteria were satisfied in both cell lines.

The performance of the modified SCTA was assessed by (i) testing serially diluted HIV-1 RNA input to determine the lower limit of amplification with plasma samples, (ii) testing in three independent experiments mixtures of plasmids carrying X4 and R5 *env* at predefined ratios to determine the threshold of detection of minority X4 viruses and (iii) testing in two independent experiments plasma samples previously analyzed with ESTA to verify SCTA accuracy.

3.3. NGS and phylogenetic analysis

NGS was performed on samples with discordant coreceptor tropism assignment based on SCTA and G2P[c]. The same PCR products obtained for SCTA were used to prepare the library for Illumina deep sequencing by using the Nextera XT DNA Sample Preparation and Index kit (Illumina, San Diego, California, USA) according to the manufacturer's manual. The library concentration was determined with the Quant-iT Picogreen dsDNA assay (Invitrogen, Carlsbad, CA, USA), then

the libraries were normalized and pooled for subsequent sequencing on an Illumina MiSeq platform using the 2 × 150 cycle paired-end sequencing protocol. Results were mapped and aligned to the HXB2 gp120 V3 reference. The consensus sequence for the reads obtained from individual samples was generated by Geneious software, version 9.1.5 (http://www.geneious.com). Coreceptor tropism was predicted by using Geno2Pheno[454] (G2P[454], https://454.geno2pheno.org/index.php), following sequence editing for the pre-processing program as indicated by the authors [25]. The system returns the percentage of X4 viruses at predefined FPR cut-offs (20%, 15%, 10%, 5%, 3.75%, 2.5%, 1%), with 5% being the value closest to the 5.75% proposed as the best compromise between sensitivity and specificity of detection of clinically relevant X4 species [26].

To compare Sanger bulk sequences with the NGS consensus for the individual SCTA/G2P[c] discordant samples, Bayesian phylogenetic trees were constructed using a general-time reversible + invariant sites + gamma distribution (GTR + I + G) model of nucleotide substitution with MrBayes software [27]. A Markov Chain Monte Carlo search was made until convergence was reached by sampling the tree every 100th generation with a burn-in fraction of 50%. Statistical support for specific clades was obtained by calculating the posterior probability of each monophyletic clade, and a posterior consensus tree was generated after a 50% burn-in. Tree figures were rendered using FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

4. Results

4.1. Performance of SCTA

Based on probit analysis, the SCTA lower limit of amplification was 195 and 324 HIV-1 RNA copies/ml at 50% and 95% hit rate (Supplementary Table 2). Based on analysis of X4 and R5 plasmid mixtures, the assay consistently detected X4 variants present at proportion as low as 1% in samples containing at least 10,000 copies/ml and the dose-response curve correlating RLU values with X4 proportion was linear over the 1–16% range evaluated (Supplementary Fig. 2). Seven of 8 (87.5%) samples previously analyzed with ESTA gave concordant tropism results with SCTA in duplicate experiments (Supplementary Table 3). The only discordant sample had an FPR value of 40% and was scored as DTM by ESTA and R5 by SCTA.

4.2. Phenotypic and genotypic analysis of subtype A vs. Subtype B

The comparison between the FPR values originally obtained with the clinical samples and those derived from the larger SCTA amplicon showed minimal or no variation, compatible with stochastic differences in the V3 region amplification [28], confirming in all cases the previously assigned coreceptor usage and predefined FPR range (< 20 or > 60) (Supplementary Table 4). Also, the *env* sequence confirmed the original subtype assigned by *pol* sequencing (Fig. 1).

As shown in Fig. 2 and Table 1, coreceptor tropism assignment by G2P[c] and SCTA was discordant with 3 (12.5%) subtype A and 2 (8.3%) subtype B isolates. NGS analysis of these five discordant samples indicated high homology between the NGS consensus and the Sanger sequence informing G2P[c] tropism interpretation (Fig. 3). Taking 5% FPR as the cut-off for classification of X4 by NGS, the two subtype B G2P[c] R5/SCTA DTM discordant samples (B_high32 and B_high33) could be explained by minority X4 species occurring at 20.0–21.7% of the viral population and escaping detection by bulk sequencing. The X4 minority detected by NGS in the third sample with G2P[c] R5/SCTA DTM discordance (A_high3) was present at much lower prevalence (1.6% in the < 3.75% to < 20% FPR range), thus less likely to explain the DTM SCTA result. On the other hand, the prevalence of NGS reads with an FPR below the reference < 5% FPR cut-off for sample A_low17 (0.4%) was below the threshold usually employed to discriminate true sequence data from background NGS noise and in agreement with the R5 SCTA result, highlighting the uncertainty of the X4 assignment by G2P[c] at borderline FPR (9%). Finally, sample A_low24 also had a borderline G2P[c] FPR value (8.5%) but NGS consistently detected a majority species at 60.9% prevalence across the < 1% to < 20% FPR range, in sharp contrast with the R5 SCTA result.

5. Discussion

In this study, the rate of disagreement between genotypic and phenotypic coreceptor tropism testing was comparable on two panels of plasma samples carrying subtype A and B viruses (3/24 and 2/24, respectively). G2P[*c*]/SCTA discrepancies appeared to be attributable to the general issue of underestimation of X4 virus due to failure to detect X4 minority species by bulk Sanger sequencing [29], for both the two subtype B cases (B_high32 and B_high33) and possibly for one of the three subtype A cases (A_high3). However, the X4 species detected in the latter case was present only as 1.6% of the virus population, which is borderline with the 2% threshold suggested for V3 NGS analysis [30]. The sample was highly viremic (> 1 million copies HIV-1 RNA per millilitre, data not shown), supporting the phenotypic relevance of the minority X4 species, however the possibility remains that A_high3 was actually miscalled as R5 by a faulty subtype A G2P[c] prediction. Incidentally, the G2P[c] R5/G2P[454] X4 discordance detected with these three samples in the high FPR panel challenges the previous suggestion that samples with FPR > 60 by Sanger sequencing do not harbour minority X4 species detectable by NGS [31]. On the other hand, samples A_low17 and A_low24, classified as R5 by SCTA and X4 by G2P[c] with an FPR close to the 10% threshold, may well reflect X4 overcalling by G2P[c] as previously suggested by other studies of subtype A [15,23,32] as well as of subtype C [33,34]. However, sample A_low24 was unique in having a majority sequence labelled as X4 at FPR < 1% by NGS, suggesting a likely X4 miscalling by the G2P[c] interpreter. A potential cause for X4 overcalling in subtype A could be the role of the V2 domain in determining coreceptor tropism [32]. As a general caution, G2P[c] and all genotypic assays indeed consider only the V3 domain sequence while phenotypic assays encompass the whole *env* region including potentially relevant domains outside the V3 loop [35–38].

Overall, our study could not consistently demonstrate subtype A-specific issues with the current G2P[c] version. G2P[*c*]/SCTA discordances can be due to different reasons, including but not limited to, algorithm inaccuracy. As a consequence, very large genotype/phenotype correlation panels are required to detect any possible systematic difference between subtype B and A. The same probably applies to other non-B subtypes, with the possible exception of subtype C where G2P[c] seems to predict coreceptor tropism as accurately as with subtype B [13,15,39]. Although maraviroc use is nowadays mostly restricted to a small number of patients under deep salvage therapy, novel CCR5 ligands are being explored [40] and one promising monoclonal antibody is successfully completing clinical evaluation [41]. In addition, maraviroc is being investigated as a possible HIV latency reversal agent, a novel feature which could revive interest in this small molecule CCR5 antagonist [42,43]. Thus, perspectives in antiretroviral therapy, together with the intrinsic role of coreceptor use in HIV pathogenesis [2], would still benefit from clarifying the limitations of and possibly improving coreceptor tropism prediction systems.

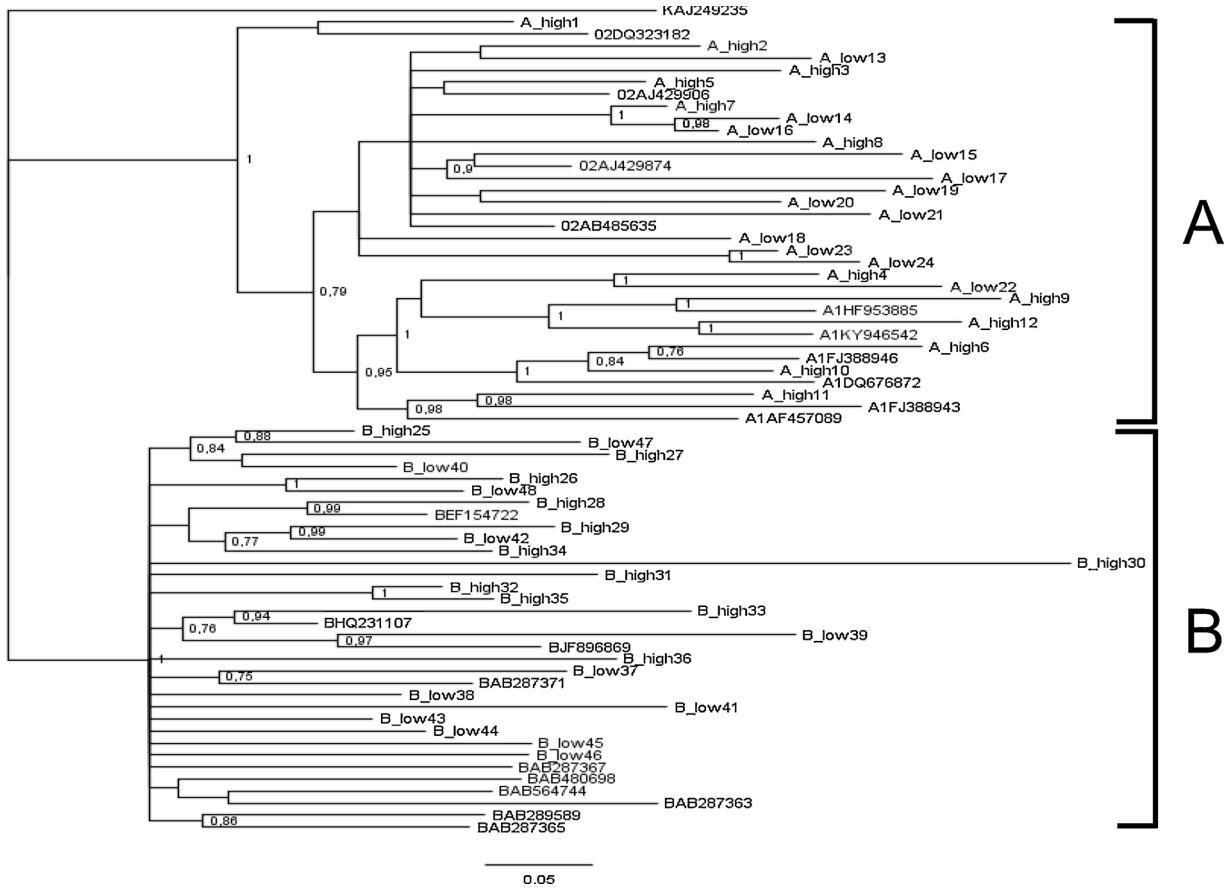

Fig. 1. Maximum clade credibility tree of V3 HIV-1 sequences sequenced with Sanger reconstructed using MrBayes. The numbers on the branches represent posterior probabilities (> 0.8).

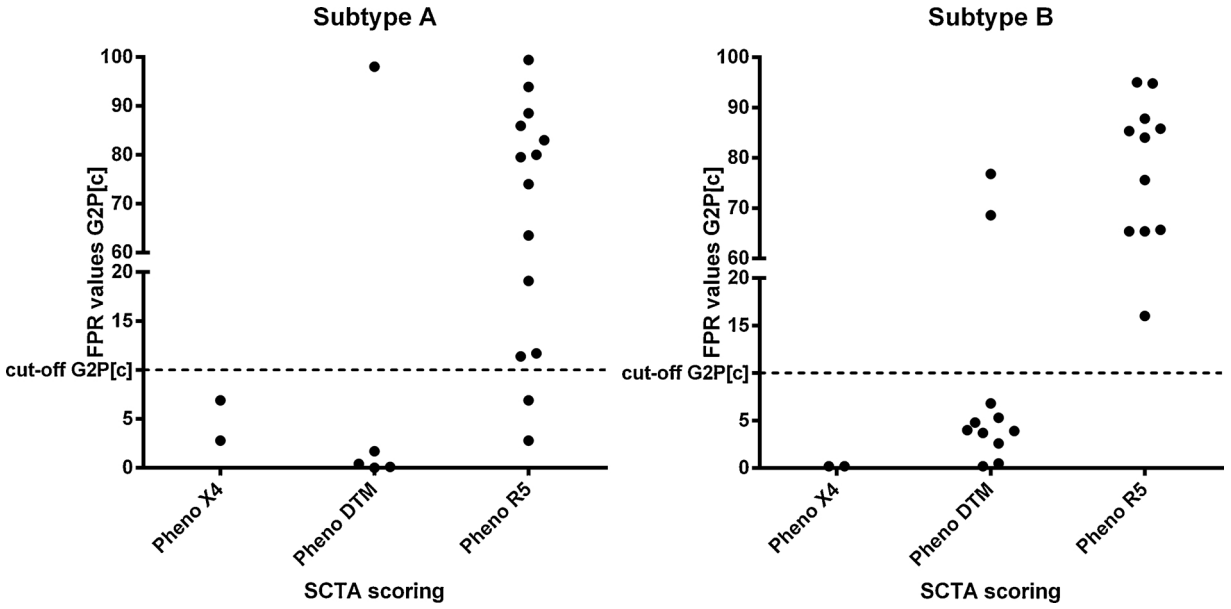

Fig. 2. Coreceptor tropism assignment by G2P[c] as determined by FPR values and SCTA in whole case study of subtype A and subtype B isolates.

Table 1
Tropism testing results in the five samples with discordant genotypic and phenotypic coreceptor tropism assignment.

Sample code	Subtype	G2P[c]		SCTA	% predicted X4 species at G2P[454] predefined FPR cut-offs						
		FPR	tropism	tropism	1%	2.5%	3.75%	5%	10%	15%	20%
A_high3	CRF02_AG	98.0	R5	DTM	0.0	0.0	1.6	1.6	1.6	1.6	1.6
A_low17	CRF02_AG	9.0	X4	R5	0.0	0.1	0.3	0.4	3.7	62.3	77.3
A_low24	A	8.5	X4	R5	60.9	60.9	60.9	60.9	60.9	60.9	60.9
B_high32	B	60.5	R5	DTM	3.1	13.6	18.0	21.7	31.3	35.3	38.9
B_high33	B	68.6	R5	DTM	0.0	20.0	20.0	20.0	20.0	40.0	40.0

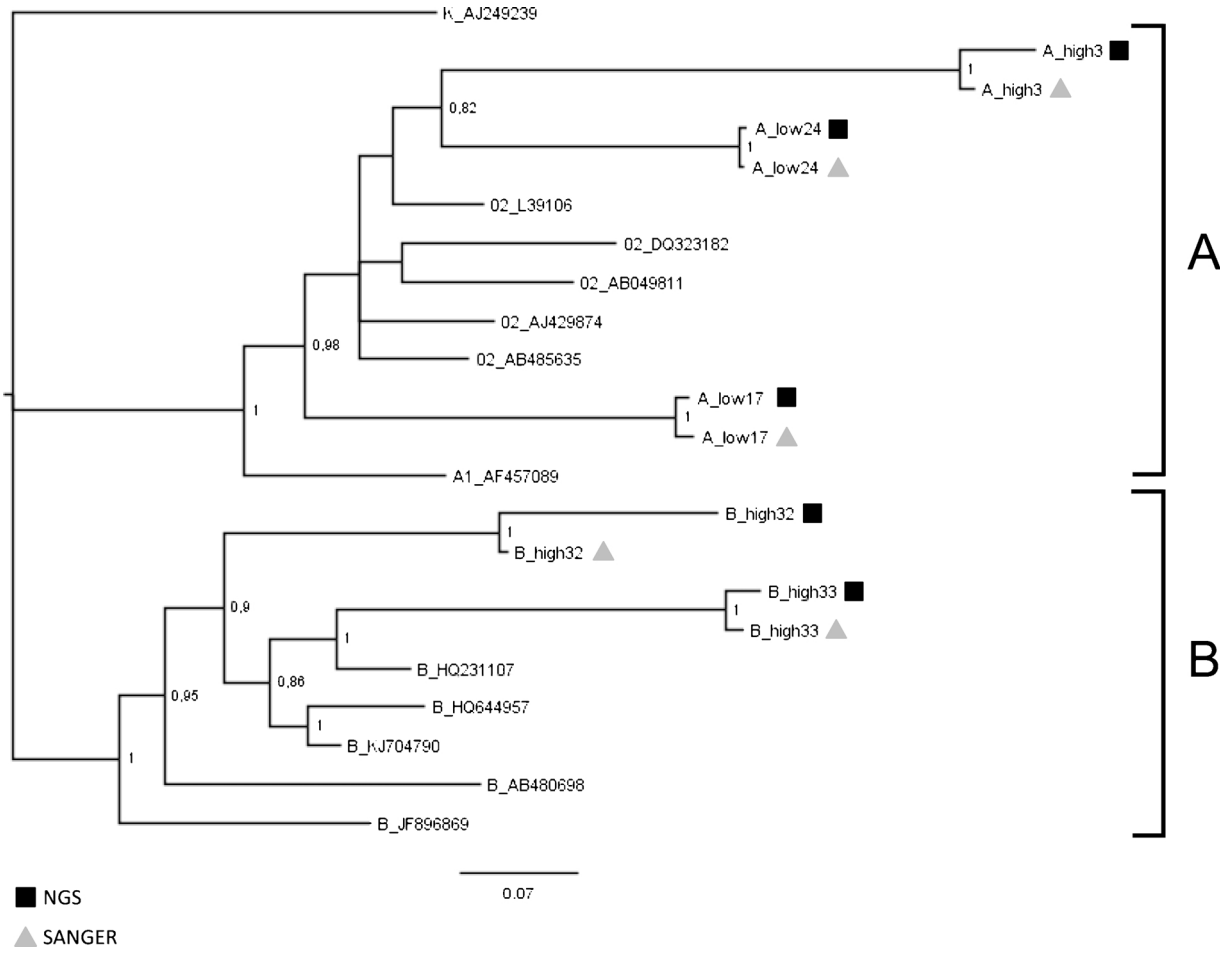

Fig. 3. Maximum clade credibility tree of V3 HIV-1 sequences sequenced with Sanger and NGS reconstructed using MrBayes. The numbers on the branches represent posterior probabilities.

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Competing interests

MZ declares personal fees from Janssen-Cilag and ViiV Healthcare and grants from Gilead Sciences and ViiV Healthcare outside the submitted work. The other authors declare no conflict of interest.

Ethics approval

Access to residual anonymized plasma samples derived from clinical practice was initially obtained through patient informed consent as approved by the local Ethics Committee at the University Hospital of Siena.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2018.12.007.

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