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Andre Minhoto Lanca¹, Jeova Keny Baima Collares², João Leandro de Paula Ferreira³, Danielle Malta Lima², Luís Fernando de Macedo Brígido¹/², Rosangela Rodrigues¹, Benedito Antonio Lopes da Fonseca³

¹Laboratório de Retrovirus, Centro de Virologia, Instituto Adolfo Lutz, São Paulo, SP, Brasil ²Universidade de Fortaleza, Fortaleza, CE, Brasil ³Centro de Pesquisa em Virologia, Faculdade de Medicina, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

While human immunodeficiency virus (HIV)-1 chemokine co-receptors 5 tropism and the GWGR motif in the envelope third variable region (V3 loop) have been associated with a slower disease progression, their influence on antiretroviral response remains unclear. The impact of baseline V3 characteristics on treatment response was evaluated in a randomized, double blind, prospective cohort study with patients initiating highly active antiretroviral therapy with lopinavir or efavirenz plus azithromycin/3TC (1:1) over 48 weeks. Similar virological and immunological responses were observed for both treatment regimens. The 43 individuals had a mean baseline CD4 T cell count of 119 cells/mm³ (standard deviation (SD) = 99) and a mean viral load of 5.09 log₁₀ copies/mL (SD = 0.49). The GWGR motif was not associated with a CD4 T cell response, but predicted R5 tropism by the geno2pheno [clinical] algorithm correlated with higher CD4 T cell levels at all monitoring points (p < 0.05). Moreover, higher false-positive rates (FPR) values from this analysis revealed a strong correlation with CD4 T cell recovery (p < 0.0001). Transmitted drug resistance mutations, documented in 3/41 (7.3%) cases, were unrelated to the assigned antiretroviral regimen and had no impact on patient outcomes. In conclusion, naïve HIV-1 R5 infected patients exhibited higher CD4 T cell counts at baseline; this difference was sustained throughout therapy. The geno2pheno [clinical] option FPR positively correlated with CD4 T cell gain and may be useful in predicting CD4 T cell recovery.

Key words: HIV-1 - disease progression - CD4 T lymphocytes - viral tropism - diversity - Brazil

Since the identification of human immunodeficiency virus (HIV)-1 as the aetiologic agent of acquired immune deficiency syndrome (AIDS), several studies have associated different viral molecular characteristics, especially the diversity of the envelope gene, with disease progression (Potts et al. 1993, Santoro-Lopes et al. 2000). Most of these studies are focused on the envelope third variable region, or V3 loop, which is involved in HIV-1 binding to the chemokine co-receptors 5 (R5) and/or CXCR4 (X4). This step is necessary for viral fusion to the host cell (Shioda et al. 1991). A change in viral tropism has been associated with different phases of HIV-1 infection, with a predominance of R5 tropic virus observed at the initial stages of infection. X4-tropic viruses emerge at advanced disease stages in about half of all cases and this emergence is correlated with clinical progression and CD4 T cell depletion (Richman & Bozzette 2004, Goetz et al. 2009). The pathogenic potential of X4 virus is highlighted by its capacity to induce syncytia formation (Richman & Bozzette 2004). Several bioinformatics tools have been used to determine viral genotypic tropism, such as the website geno2pheno and the 11/25 rule. Additionally, amino acid signatures at the V3 loop tip tetramer, which commonly harbour a proline at codon 16 (16 GPGR18), show a substitution to tryptophan (W) at this position in some Brazilian clade B envelopes and are referred to as B’ or Brazilian-B (Shioda et al. 1991, Potts et al. 1993, Pinto et al. 2008). The GWGR signature has a prevalence of 20-50% in Brazil and has been associated with a slower progression to AIDS (Santoro-Lopes et al. 2000, Casseb et al. 2002, 2004, de Brito et al. 2006). However, the actual relevance of this molecular diversity on treatment response is not yet clear. To expand the understanding of the potential role of genotypic viral tropism, false-positive rate (FPR) values obtained at geno2pheno and the presence of the GWGR motif in patients on antiretrovirals (ARV), the correlations of these characteristics with viral loads (VLs) and CD4+ T lymphocyte counts were assessed in patients during 48 weeks of first-line highly active antiretroviral therapy (HAART).

PATIENTS, MATERIALS AND METHODS

Study population - HIV-1-infected individuals not exposed to antiretroviral therapy (ART), but with indications for treatment according to the Brazilian Clinical Guideline, were invited and those eligible were randomly assigned in a 1:1 ratio to receive either lopinavir or efavirenz with azithromycin (AZT)/3TC backbone. Clinical follow-up was conducted at the HIV Clinic, Medicine School Hospital, Ribeirão Preto, São Paulo University to evaluate the virological and immunological responses of the patients to HAART during the 48 weeks of follow-
up. Data from patients who died before completing the follow-up were included in the baseline evaluation and the individuals who did not complete all visits were included in an intention to treat (ITT) analysis. A written informed consent was obtained from all patients and both Institutional Review Boards approved the study.

**Laboratory and molecular analysis** - CD4 T cell counts and percentages (flow cytometry, BD, USA) and VLs ( Branched DNA, Siemens, USA) were determined at baseline and at weeks 6, 12, 24, 36, and 48. CD4 T cell gain was calculated as the absolute cell count from each week of observation minus the baseline CD4 T cell count. HIV-1 RNA and genomic DNA were extracted using the QIAamp® RNA/DNA extraction kit (Qiagen, Germany) according to manufacturer instructions and were stored at -70°C until used. Synthesis of complementary DNA (cDNA) was performed by reverse transcription with Superscript III (Invitrogen, USA). Polymerase chain reaction amplification was performed on cDNA (pol) and proviral DNA (env) using a nested protocol as previously described (Ferreira et al. 2008). Amplicons were sequenced directly using BigDye and were resolved with an ABI 3100 Genetic Analyzer (Applied Biosystems, USA). Sequences of partial env and pol genes were manually edited with Sequencher 4.14 (GeneCodes, USA) software. Multiple sequence alignments were performed using the CLUSTALW algorithm with a reference set obtained from the Los Alamos Sequence Database. HIV subtyping was screened using NCBI Genotyping and was confirmed by phylogenetic methods using the software PAUP* version 4.0b10 (Sinauer Associates, USA). Transmitted drug resistance mutations (tDRM) were defined according to Calibrated Population Resistance v.6 (HIV Stanford Database) and the International AIDS Society (IAS) 2010 mutation list. Viral tropism was evaluated using the website geno2pheno [co-receptor] bioinformatics tool (Sing et al. 2007) and both clonal and clinical options were used to obtain FPR values. Cut-offs of 5.75% (MOTIVATE option) and 20% (Vanderkerckhove et al. 2011) were employed to predict X4 tropism using clonal and clinical data. To obtain the FPR using the clinical option, baseline (absolute and percentage) CD4 T cells counts and VLs were added to the model. These predictions were analysed both as categorical variables (X4 or R5) and as continuous variables. V3 loop amino acid composition was determined by analysis of alignments with the HXB2 reference sequence from the Los Alamos Sequence Database (accession K03455).

**Statistical analysis** - Categorical variables were tested using Fisher’s exact test or the Yates corrected test (two-tailed), as appropriate. Continuous variables were tested using the Mann-Whitney test, two-way analysis of variance (ANOVA) (non-parametric), the Spearman correlation and linear regression. Epinfl6 (Centers for Disease Control and Prevention) and GraphPad Prism 5.0 software were used in the statistical analysis. Virological and CD4 T cell outcomes were evaluated as ITT, missing equals failure (ITT, M = F) and as treated (AT), missing equals exclusion (AT, M = E).

**RESULTS**

The 43 patients enrolled from 2004-2006 were mostly male (70%) and had a mean age of 41 years [standard deviation (SD) 15 years], a mean baseline CD4 T cell count of 119 cells/mm³ (SD = 99 cells/mm³) and a mean VL of 5.09 log₁₀ c/mL (SD = 0.49). CD4 T cell counts and VLs are depicted in Table. Three patients died and two abandoned therapy before their first return at week 6 of the follow-up. One patient discontinued treatment before 24 weeks and three left between 24-36 weeks of the follow-up. All three deaths occurred before concluding the clinical follow-up and these patients had baseline CD4 T lymphocyte counts below 50 cells/mm³.

Patients were randomly assigned in a 1:1 ratio to receive either lopinavir (48.8%) or efavirenz (51.2%) with AZT/3TC backbone. The tDRM frequency at baseline (available after treatment initiation) was 7.3%, all mutations were observed in the protease gene (mutations V82L, I85V and G73S) and the predicted loss of ART susceptibility was not relevant to the assigned ARV regimen. V82L and I85V substitutions were observed in patients assigned to receive lopinavir; these individuals exhibited a similar response to those without detectable mutations. The G73S mutation was observed in a patient receiving efavirenz who died two weeks after enrolment without an evaluation of the ARV response.

Virological and immunological outcomes after HAART on both regimen arms were comparable, with no differences between lopinavir and efavirenz response except for a higher occurrence of hypertriglycerideremia in the lopinavir group (data not shown). Viral suppression was achieved in most patients by week 24, when 89% of individuals had viremia below detection limits. After 48 weeks of HAART, only two individuals (6%) had a detectable VL (> 50 copies/mL), with one on each regimen arm (AT, M = E). Further genotyping test analysis of one of these cases, RP16 on lopinavir + AZT/3TC therapy with several adherence issues, revealed the emergence of the mutation M184V, which is predicted to reduce 3TC susceptibility.

V3 env region sequencing was performed for the 43 patient samples, with 42 sequences validated; one sequence for each sample was obtained. All HIV-1 envelopes were clade B, except for one subtype F that was excluded from the Brazilian-B GWGR analysis. HIV-1 predicted tropism to CXCR4 co-receptor prevalence was 17/42 (40%) when using geno2pheno [co-receptor] and 24/42 (57%) when using geno2pheno [clinical] and 6/42 (14%) when using the MOTIVATE option (Table). Prediction of three sequences classified as X4 and 10 sequences classified as R5 by a clonal algorithm were discordant when clinical data were incorporated into the algorithm. When sequences were classified by the V3 loop motif, 4/9 (44%) of GWGR, 4/12 (33%) of GPGR and 9/20 (45%) of “other motifs” were predicted to be X4 by geno2pheno [co-receptor]. Moreover, among “other motif” isolates, 6/20 had basic amino acids at positions 11 or 25 and 3/20 had a GWGR motif. No GPGR or GWGR isolate had basic amino acids at positions 11 or 25 and 3/20 had a GWRR motif. The six viruses harbouring basic amino acids at positions 11 and 25 had clonal and clinical FPR values below 20%, except for one sequence. This case had
a 30% FPR value when we used the clinical algorithm, despite presenting an arginine (R) at position 25. The V3 loop alignment and tropism prediction using clonal data, clinical data and the 11/25 rule are shown in Fig. 1.

CD4 T cell counts at each interval were lower for patients with X4-tropic viruses and were significantly different when tropism was determined by geno2pheno [20%] (Fig. 2A, B). CD4 T cell counts and VLs according to different tropism prediction criteria are described in Table. CD4 T lymphocyte cell counts throughout the 48 weeks (Fig. 2C) and VLs (Fig. 2D) were comparable for patients infected with either GWGR or GPGR variants. Taking into consideration the FPR values generated using geno2pheno [clonal20%] for each isolate from all cases (ITT analysis), the CD4 T cell gain showed a positive, significant correlation with FPR values at all observation weeks. This was observed by both linear regression ($p < 0.0001$) (Fig. 3) and the Spearman linear correlation ($p < 0.01$). A similar trend was also observed when only those completing week 48 of follow-up were evaluated (“as treated”, data not shown). Baseline CD4 T cell counts and geno2pheno [clonal20%] FPR values were not significantly correlated with CD4 T cell gain ($p = 0.3$ and $0.2$, respectively).

**DISCUSSION**

This study evaluated the potential role of the molecular characteristics of the HIV-1 envelope on CD4 T lymphocyte gains in patients initiating their first HAART regimen from a structured cohort study performed to compare two first-line regimens. Viral tropism was determined by bioinformatics tools, using the geno2pheno algorithm with different criteria and inspecting the amino acid composition of the V3 loop of env (11/25 rule). The geno2pheno clinical option incorporated VL, CD4 T lymphocyte cell counts and percentages to generate an FPR value in which the predicted tropism was weighted by these parameters. Sequences were also evaluated using the MOTIVATE option, which is the most specific criterion for detection of X4-tropic viruses, as it uses a more stringent FPR cut-off. The results exhibited some discordance in tropism interpretation when different algorithms were
used. Interestingly, most patients had advanced disease at the beginning of treatment, with a mean CD4 T cell count of 119 cells/mm³; this could explain the tropism disparities when clinical data were incorporated.

To assess the impact of viral tropism on clinical evolution, both VL suppression and CD4 T cell recovery were followed-up for one year. Treatment, tDRM, V3 loop motif and viral tropism did not impact VL suppression. Lower numbers of CD4 T cells were observed at both the baseline and the follow-up weeks in patients infected with X4 virus using both clinical and clonal algorithms. Furthermore, CD4 T cell numbers were significantly lower in patients harbouring X4 variants when prediction used the geno2pheno\([\text{clinical20%}]\) option (Fig. 2A). The use of baseline CD4 T cell counts on the clinical algorithm could be responsible for this stronger association. However, baseline CD4 T cell counts did not show a correlation with CD4 T cell gain (p = 0.3). Although CD4 T cell counts of R5-infected patients remained higher at most determinations, CD4 T cell recovery was similar for both groups. In other words, despite the higher CD4 T cell counts in R5-infected patients, the response to HAART was comparable for both R5 and X4-infected individuals.

Values of FPR as continuous variables were also evaluated to assess its association to CD4 T cell gain. Using the geno2pheno\([\text{clinical}]\) option, envelope sequences FPR values exhibited a strong correlation with CD4 T cell gain (p < 0.0001) (Fig. 3). Again, although CD4 T cell count is used in this model to determine geno2pheno\([\text{clinical}]\) FPR, neither CD4 T cell baseline counts (p = 0.3) nor the clonal FPR (p = 0.2) per se revealed a significant correlation with CD4 T cell gain. This suggests that FPR generated by the geno2pheno clinical algorithm may constitute a potential predictor for CD4 T cell gain after therapy. The usefulness of this option has also been proposed in previous studies (Prosperi et al 2010).

Neither the GWGR nor the GPGR V3 loop motif had an association with CD4 T cell gain, despite the fact that CD4 T cell numbers were slightly higher at baseline in the former group (Fig. 2C). An important
prevalence of X4 variants in GWGR viruses was observed using both the geno2pheno\textsubscript{clinical} (44%) and the geno2pheno\textsubscript{clonal} (67%) algorithms, contrary to previous studies, which suggested that GWGR viruses are rarely X4 (da Silva 2006, Leal et al. 2008). This difference could be due to the algorithm used, as most studies use the 11/25 rule as a prediction criterion. To clarify this matter, the presence of basic amino acids at positions 11 and 25 was evaluated in our sequences; none of the GWGR or GPGR isolates had basic amino acids at these codons. Interestingly, three V3 tip sequences of non-GWGR tryptophan-harboung viruses, classified here as “other motifs”, were predicted as X4. These viruses had a G→R polymorphism in the V3 loop tip at position 17 (GWRR\textsubscript{17}), two had basic amino acids at codons 11 or 25 and one had a G→R substitution at position 28, a highly conserved codon (Leal et al. 2008, Franca et al. 2011). GWRR seems to be a very rare motif previously documented in the state of Rio de Janeiro, Brazil and Paraguay and its biological relevance is yet to be understood (Cabello et al. 1995, Tanuri et al. 1999)

The small number of samples in this study limits the strength of these observations and it is conceivable that these outcomes may be different if a larger cohort was sampled and a longer follow-up period was employed. Further studies may provide support for the use of geno2pheno\textsubscript{clinical} analysis of V3 envelope sequences as a surrogate marker for CD4 T cell gain after ART.

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