A Very Low Geno2pheno False Positive Rate Is Associated with Poor Viro-Immunological Response in Drug-Naïve Patients Starting a First-Line HAART

Daniele Armenia¹, Cathia Soulie²-³-⁴, Domenico Di Carlo¹, Lavinia Fabeni⁵, Caterina Gori⁵, Federica Forbici⁵, Valentina Svicher¹, Ada Bertoli¹-⁶, Loredana Sarmati⁷, Massimo Giuliani⁸, Alessandra Latini⁸, Evangelo Boumis⁹, Mauro Zaccarelli⁹, Rita Bellagamba⁹, Massimo Andreoni⁷, Andrea Antinori⁹, Francesca Cicercherini-Silberstein¹, Carlo-Federico Perno¹-⁵-⁶, Maria Mercedes Santoro¹*¹

¹Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, Rome, Italy, ²Unité Mixte de Recherche en Santé (UMR_S) 1136 Pierre Louis Institute of Epidemiology and Public Health, Université Pierre et Marie Curie (UPMC) University Paris 06, Paris, France, ³UMR_S 1136 Pierre Louis Institute of Epidemiology and Public Health, Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France, ⁴Laboratoire de Virologie, Assistance Publique-Hôpitaux de Paris (AP-HP), Groupe hospitalier Pitié Salpêtrière, Paris, France, ⁵Antiviral Drug Monitoring Unit, Istituto Nazionale delle Malattie Infettive (INMI) Lazzaro Spallanzani, Rome, Italy, ⁶Molecular Virology, University Hospital Tor Vergata, Rome, Italy, ⁷Infectious Disease Unit, University Hospital Tor Vergata, Rome, Italy, ⁸Department of Infectious Dermatology, San Gallicano Hospital, Rome, Italy, ⁹Infectious Diseases Division, Istituto Nazionale delle Malattie Infettive (INMI) Lazzaro Spallanzani, Rome, Italy

Abstract

Background: We previously found that a very low geno2pheno false positive rate (FPR ≤2%) defines a viral population associated with low CD4 cell count and the highest amount of X4-quasispecies. In this study, we aimed at evaluating whether FPR ≤2% might impact on the viro-immunological response in HIV-1 infected patients starting a first-line HAART.

Methods: The analysis was performed on 305 HIV-1 B subtype infected drug-naïve patients who started their first-line HAART. Baseline FPR (%) values were stratified according to the following ranges: ≤2; 2–5; 5–10; 10–20; 20–60; >60. The impact of genotypically-inferred tropism on the time to achieve immunological reconstitution (a CD4 cell count gain from HAART initiation ≥150 cells/mm³) and on the time to achieve virological success (the first HIV-RNA measurement <50 copies/mL from HAART initiation) was evaluated by survival analyses.

Results: Overall, at therapy start, 27% of patients had FPR ≤10 (6%, FPR ≤2; 7%, FPR 2–5; 14%, FPR 5–10). By 12 months of therapy the rate of immunological reconstitution was overall 75.5%, and it was significantly lower for FPR ≤2 (54.1%) in comparison to other FPR ranks (78.8%, FPR 2–5; 77.5%, FPR 5–10; 71.7%, FPR 10–20; 81.8%, FPR 20–60; 75.1%, FPR >60; p = 0.008). The overall proportion of patients achieving virological success was 95.5% by 12 months of therapy. Multivariable Cox analyses showed that patients having pre-HAART FPR ≤2% had a significant lower relative adjusted hazard [95% C.I.] both to achieve immunological reconstitution (0.37 [0.20–0.71], p = 0.003) and to achieve virological success (0.50 [0.26–0.94], p = 0.031) than those with pre-HAART FPR >60.

Conclusions: Beyond the genotypically-inferred tropism determination, FPR ≤2% predicts both a poor immunological reconstitution and a lower virological response in drug-naïve patients who started their first-line therapy. This parameter could be useful to identify patients potentially with less chance of achieving adequate immunological reconstitution and virological undetectability.

Citation: Armenia D, Soulie C, Di Carlo D, Fabeni L, Gori C, et al. (2014) A Very Low Geno2pheno False Positive Rate Is Associated with Poor Viro-Immunological Response in Drug-Naïve Patients Starting a First-Line HAART. PLoS ONE 9(8): e105853. doi:10.1371/journal.pone.0105853

Copyright: © 2014 Armenia et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was financially supported by the European Commission Framework 7 Programme (CHAIN, the Collaborative HIV and Anti-HIV Drug Resistance Network, Integrated Project no. 223131), and by the European AIDS Treatment Network (NEAT, contract number LSHT/CT/2006/037570); Italian Ministry of Health (CUP: EB1I10000000001, Ricerca Corrente and Progetto AIDS grant no. n. 40H78); and by an unrestricted grant from AVIRALIA foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Introduction

Despite the great progress in treating HIV-1 infection, in some patients starting their first treatment the effectiveness of highly active antiretroviral therapy (HAART) is still not sufficient, with consequent virological failures [1–4]. Furthermore, although antiretroviral therapy improves immune response, some patients infected with human immunodeficiency virus type 1 (HIV-1) present unsatisfactory CD4 T cell recovery despite achieving viral suppression, resulting in increased morbidity and mortality [4–10]. In this regard, an increase in CD4 cell count in the range of 50 to 150 cells/mm³ per year (generally with an accelerated response in the first 3 months of treatment) is considered an adequate CD4 response for most patients starting their first-line regimen [11–12]. It has been shown that the use of the CXCR4 co-receptor is generally seen in more advanced stages of disease, and has been associated with an increased severity of HIV disease, higher viral load, and a decreased CD4 cell count [13–16]. In the absence of antiretroviral therapy, CXCR4-using viruses (X4), detected by phenotypic or genotypic assays, are associated with faster CD4 cell count decreases, regardless of baseline CD4 cell count or viral load [14,15,17–19]. However, in the presence of antiretroviral therapy, this issue has still been poorly investigated, and the available results are controversial [20–23].

Nowadays, especially after the introduction of CCR5 antagonists in clinics, the determination of HIV-1 tropism is beginning to be routinely performed. For this reason, the classical phenotypic assays, such as Trofile [24], are taken over by more cost-effective genotypic tests in the large majority of countries.

One of the most widely used tools for tropism determination is geno2pheno [coreceptor] (G2P) [25]. By using the genetic information contained in the sequence of HIV-1 gp120 V3-loop, this web-tool gives a percentage score (false positive rate [FPR]) that allows us to estimate the probability of having CCR5-using virus (R5). G2P has been shown to have good concordance with classical phenotypic tests [26]. So far, the FPR cut-off of 10% is recommended by European guidelines to discriminate R5- and X4-tropic patients using G2P system [27]. However, there is evidence indicating that this system can provide reliable discrimination between R5 and X4 sequences even when FPR is set at lower values [26,28,29].

Notably, beyond the crude tropism determination, some recent studies (including ours) provided new important information about the relevance of FPR in terms of association with viro-immunological parameters and X4-tropic intra-patient quasispecies prevalence. Indeed, by a cross-sectional study we demonstrated that within the first 3 months of treatment) is considered an adequate CD4 cell count and viral load measurements available in the time-window from 3 months before to 1 week after HAART initiation; iv) at least one CD4 cell count and viral load measurement available after 6 months of therapy. In patients with more than 1 viral load or CD4 cell count before HAART started, the last measurement was considered as pre-HAART value.

Ethics statement

Approval by Ethics Committee was deemed unnecessary because, under Italian law, such an approval is required only in the hypothesis of clinical trials on medicinal products for clinical use (art. 6 and art. 9, leg. decree 211/2003). This research was conducted on [samples and] data already available, and not collected for this study. CD4 cell counts were previously determined for each patient only for clinical reasons and not for research. All samples and data used in the study were previously anonymized, according to the requirements set by Italian Data Protection Code (leg. decree 196/2003) and by the General authorizations issued by the Data Protection Authority.

CD4 cell count and HIV-RNA quantification

Flow cytometry from whole blood was used to determine CD4 cell counts at each study visit. Depending on methodologies available at the different clinical centers participating in this study, plasma viremia was determined using three different assays: the Roche Cobas CA/CTM version 2.0 (Mannheim, Germany), the Abbott RealTime HIV-1 (Chicago, Illinois), and the VERSANT HIV-1 Version 3.0 (Bayer Corporation, Diagnostics Division, Tarrytown, New York) [32,33]. Previous studies demonstrated that even if there was not a uniform approach regarding the HIV-1 viral load detection, the results obtained by these assays correlated very well, only a few samples having a difference of more than 0.5 log10 copies/mL [34,35]. For 304/305 patients using species detected by 454-pyrosequencing. In particular, at very low FPR (≤2%) by population sequencing the highest prevalence of X4-species by ultra-deep pyrosequencing was observed [31].

In view of all these considerations, the aim of this longitudinal study was to evaluate whether FPR ≤2% at the moment of starting HAART might be associated with viro-immunological responses of the first-line regimen.

Materials and Methods

Patients

HIV-1-infected patients starting a first-line regimen not including CCR5-antagonists in several clinical centres from Italy and France were selected on the basis of the following criteria: i) B subtype infected; ii) age ≥18 years; iii) V3-genotyping test available at therapy starting (in the time-window from 6 months before to the moment of therapy initiation); iv) pre-HAART CD4 cell count and viral load measurements available in the time-window from 3 months before to 1 week after HAART initiation; v) at least one CD4 cell count and viral load measurement available after 6 months of therapy. In patients with more than 1 viral load or CD4 cell count before HAART started, the last measurement was considered as pre-HAART value.
Table 1. Characteristics of 305 HIV-1 B subtype infected patients starting their first-line HAART.

| Characteristics                              | Categories     | Overall (N = 305) |
|----------------------------------------------|----------------|-------------------|
| Gender, n (%)                                | Male           | 252 (82.6)        |
| Age, Median (IQR)                            | Years          | 41 (34–46)        |
| Pre-HAART CD4 (cells/mm³), n (%)             | ≤50            | 48 (15.7)         |
|                                              | 51–100         | 24 (7.9)          |
|                                              | 101–200        | 48 (15.7)         |
|                                              | 201–350        | 117 (38.4)        |
|                                              | >350           | 68 (22.3)         |
| CDC stage, n (%)                             | A              | 107 (35.1)        |
|                                              | B              | 91 (29.8)         |
|                                              | C              | 68 (22.3)         |
|                                              | Unknown        | 39 (12.8)         |
| Pre-HAART HIV-RNA (copies/mL)a, n (%)        | ≤30,000        | 82 (26.9)         |
|                                              | 30,001–100,000 | 66 (21.6)        |
|                                              | 100,001–300,000| 82 (26.9)        |
|                                              | 300,001–500,000| 23 (7.5)         |
|                                              | 500,001–1,000,000| 32 (10.5)     |
|                                              | >1,000,000     | 20 (6.6)          |
| Genotypic determination of HIV tropismb, n (%)| X4             | 84 (27.5)         |
|                                              | R5             | 221 (72.5)        |
| Risk factor, n (%)                           | Heterosexual   | 80 (26.2)         |
|                                              | Homosexual     | 107 (35.1)        |
|                                              | Drug addiction | 15 (4.9)          |
|                                              | Sexual         | 32 (10.5)         |
|                                              | Other or unknown| 71 (23.3)        |
| Coinfection, n (%)                           | Hepatitis C    | 24 (7.9)          |
| Transmitted drug resistancec, n (%)          |                | 24 (7.9)          |
| HAART initiation, Median (IQR)               | Year           | 2010 (2009–2011) |
| NRTI backbone, n (%)                         | TDF+FTC        | 258 (84.6)        |
|                                              | AZT+3TC        | 17 (5.6)          |
|                                              | Otherd         | 30 (9.8)          |
| Third drug, n (%)                            | NNRTI          | 112 (36.7)        |
|                                              | Ritonavir boosted PI | 161 (52.8) |
|                                              | Raltegravirm   | 32 (10.5)         |
| More than 3 drugs used, n (%)                |                | 21 (6.9)          |
| Adherence levelf, n (%)                      | High           | 266 (87.2)        |
|                                              | Median         | 19 (6.2)          |
|                                              | Low            | 18 (5.9)          |
|                                              | Unknown        | 2 (0.7)           |
| CD4 cell count measurements, Median (IQR)    | Number per patient | 6 (4–10)      |
| Time of CD4 cell count follow-up from starting HAART, Median (IQR) | Months | 12 (8–19) |
| Viral load measurements, Median (IQR)        | Number per patient | 9 (6–12)     |
| Time of HIV-RNA follow-up from starting HAART, Median (IQR) | Months | 19 (13–27) |

a. Viremia was not quantifiable above 500,000 copies/mL only for one patient. We arbitrarily included this patient in the viremia level 500,001–1,000,000 copies/mL; b. Geno2pheno false positive rate set at 10%; c. At least 1 mutation associated with resistance to protease inhibitors, nucleos(t)ide reverse transcriptase inhibitors and/or non-nucleoside reverse transcriptase inhibitors, according to surveillance list from Bennett et al. 2009 [39]; d. ABC+3TC (n = 11); TDF+3TC (n = 3); DDI+3TC (n = 1); NRTI sparing (n = 15); e. Patients treated with raltegravir were considered as independent category regardless the other drugs included in the same regimen; f. Data about adherence levels were retrieved from physicians’ reports. ABC: Abacavir. AZT: Zidovudine. DDI: Didanosine. FTC: Emtricitabine. IQR: Interquartile range. HAART: Highly active antiretroviral therapy. NRTI: Nucleos(t)ide reverse transcriptase inhibitor. NNRTI: Non-NRTI. PI: Protease inhibitor. TDF: Tenofovir. 3TC: Lamivudine.

doi:10.1371/journal.pone.0105853.t001
(99.7%) viremia measurements were quantifiable above 500,000 copies/mL.

Genotyping
Sequencing of HIV-1 pol gene (containing the entire protease and the first 240/335 amino acids of the reverse transcriptase open reading frame) and of HIV-1 gp120 V3-loop was performed using plasma samples collected from the patients before their first-line therapy. For about 90% (N = 274) of plasma samples, pol genotypic tests used in this analysis were performed by means of a commercially available kit (The ViroSeq HIV-1 Genotyping System, Abbott Molecular, Des Plains, Illinois, USA) according to the manufacturer’s recommendations [36]. HIV-1 gp120 V3 loop sequencing for these 274 samples was performed by using a well validated diagnostic-use protocol, based on commercially available RNA-extraction (QIAamp RNA Viral Mini kit, Qiagen), reverse-transcription and amplification (SuperScript One-Step RT-PCR for Long Templates – Invitrogen) and genotyping (BigDye terminator version 3.1 cycle sequencing kit, Applied-Biosystems, Foster City, CA) kits [37]. Amplified gp120 V3 products were full-length sequenced in sense and antisense orientations by an automated sequencer (ABI 3130 XL) by using four different overlapping sequence-specific primers to ensure the coverage of the V3 sequence by at least two sequence segments [37].

For the remaining 31 plasma samples, HIV-1 pol and gp120 V3 loop sequencing were performed by using the technique of ANRS (French National Agency for AIDS Research, described on the web site HIV French resistance: http://www.hivfrenchresistance.org/) [38].

To estimate the prevalence of transmitted drug resistance at the start of HAART, the list of mutations reported by Bennett et al., 2009 [39] was used. Subtype has been determined by using the phylogenetic approach, as previously described [40]. The genotypic susceptibility score (GSS) for optimized therapy was also calculated according to Rega algorithm (version: v8.0.2; http://regaweb.med.kuleuven.be/software/rega_algorithm/), based on the sum of genotype sensitivities to all drugs prescribed in the HAART. GSS for single drugs was scored as 0 (resistant virus), 0.5 (virus with intermediate resistance) and 1 (susceptible virus).

Genotypic prediction of viral tropism
HIV-1 co-receptor usage was determined from the V3 nucleotide sequence by using the G2P algorithm available at the following website http://coreceptor.bioinf.mpi-inf.mpg.de/ [41]. G2P was set at FPR of 10%, as recommended by current guidelines [27]. To evaluate the impact of the burden HIV-1 CXCR4-using species on immunological and virological response, FPR values were further stratified according to the following 6 FPR (%) ranges: ≤2; 2–5; 5–10; 10–20; 20–60; >60, as previously described [31]. In this categorization all 6 ranges are left-open and right-closed (e.g. ≤2; >2 and ≤5; >5 and ≤10; >10 and ≤20; >20 and ≤60; >60).

Statistical analysis
All the analyses were performed using the statistical R open source environment (version 3.0.2) and the software package SPSS (version 19.0) for Windows (SPSS Inc., Chicago, Illinois).

Survival analyses
To estimate the time to achieve immunological reconstitution and viral load undetectability, Kaplan-Meier curves were used. Log-rank test for trend was implemented for FPR values stratified.

To estimate the predictive impact of genotypically-inferred tropism on immunological reconstitution and virological success, Cox proportional hazard models were used. Immunological reconstitution was defined as a CD4 cell count gain from HAART initiation of at least 150 cells/mm³ [11–12]. Virological success
Low False Positive Rate Is Associated with Poor Response to HAART

Prevalence of patients infected with X4-tropic viruses at first-line HAART start

V3 genotypic tropism test was performed in a median (Interquartile Range, IQR) time of 20 (5–47) days before HAART start. The proportions of patients infected with X4- or R5-tropic viruses according to different FPRs are represented in Figure 1. In particular, 82 of 305 (27%) patients showed X4-using viruses at the time of genotypic tropism testing with FPR set at 10% (FPR ≤ 2% = 6%; FPR 2–5% = 7%; FPR 5–10% = 14%). Among 221 patients infected by R5-tropic viruses, 31% had FPR >60% (corresponding to 23% of the overall population analyzed).

Survival analyses for the evaluation of immunological reconstitution

In the overall population, the median time to achieve immunological reconstitution, described as in the Materials and Methods, was 4.4 (95% confidence interval, CI): 3.1–5.6 months. By 12 months of treatment, the probability of achieving immunological reconstitution was 75.5%.

Among patients who achieved immunological reconstitution, the 92.9% reached a CD4 cell count gain ≥150 cells/mm^3 during the viremia drop or under virological suppression.

Stratifying patients by using the classical 10% FPR cut-off, only a trend of difference in the rates of immunological reconstitution was observed in X4-infected patients (72.3%) compared to R5-infected patients (77.5%) (p = 0.064, Figure 2 Panel A). By contrast, a further FPR stratification that quantitatively reflects the burden of X4 quasispecies [31], the rate of immunological reconstitution by 12 months was significantly lower for FPR ≤2% (54.1%) in comparison to other FPR [%] ranks (FPR = 2–5: 78.8%; FPR = 5–10: 77.5%; FPR = 10–20: 71.7%; FPR = 20–60: 81.8%; FPR >60: 75.1%, p = 0.008) (Figure 2, Panel B).

Both uni- and multivariable Cox models showed that the relative hazard to achieve immunological reconstitution significantly decreased in X4-infected patients with the lowest FPR rank. In particular, by univariable analysis, X4-infected patients having pre-HAART FPR ≤2% had a significantly lower relative hazard compared to R5-infected patients with pre-HAART FPR >60% (relative hazard [95% CI]: 0.51 [0.28–0.91], p = 0.024) (Table 2). By Cox multivariable analysis, these results were confirmed with stronger significance after adjusting for age, gender, risk factor, pre-HAART CD4 cell count, pre-HAART viremia, hepatits C coinfection, year of starting treatment, transmitted drug resistance, third drug administered (NNRTI vs. PI/r vs. raltegravir), NRTI backbone used, number of drugs being used (≤3 vs. >3 drugs).

Results

Patients’ characteristics at HAART initiation

Overall, 305 patients satisfying all criteria were included in the present analysis. Baseline characteristics are summarized in Table 1. The prevalent mode of transmission was the homosexual route (107, 35.1%), 77.7% of patients had CD4 cell count <350 cells/mm^3. About half of patients showed HIV-1 viral load >100,000 copies/mL; in particular, 10.5% and 6.6% of patients had viremia 500,001–1,000,000 and >1,000,000 copies/mL, respectively. Twenty-four (7.9%) patients were coinfected with hepatitis C virus.

Nearly all patients were treated with a modern genotype-tailored HAART, including currently recommended drugs; 93% (N = 285) of patients started their first antiretroviral regimen after 2008, and 95% (N = 290) were treated with at least 2 NRTIs (most NRTIs combination used: tenofovir + emtricitabine, 238 [84.6%]) in combination with either an NNRTI (N = 112 patients, 94.5% of them treated with efavirenz) or a ritonavir-boosted PI (N = 161 patients, mainly treated with darunavir [35.4%] or lopinavir [29.8%] or atazanavir [29.2%]). Thirty-two patients (10.5%) were treated with raltegravir, mainly administered in combination with 2 NRTIs +1 ritonavir-boosted PI (25 patients, 78%). No significant correlation between the third drug used and the different FPR levels was observed (data not shown). The majority of patients attended a high compliance level (87.2%). Transmitted drug resistance was found in around 8% of patients.

Nearly all patients (99%) have been treated with effective therapy with GSS ≥3.
Table 2. Relative hazard to achieve viro-immunological response according to baseline FPR ranks in HIV-1 infected patients starting their first-line HAART (Cox Models).

| FPR | Relative hazard to reach immunological reconstitution | Relative hazard to reach virological success |
|-----|------------------------------------------------------|---------------------------------------------|
|        | (CD4 cell count gain $\geq 150$ cell/mm$^3$)         | (HIV-RNA < 50 copies/mL)                    |
| crude | (95% C.I.)                                           | (95% C.I.)                                  |
|       | P value                                              | P value                                    |
| Adjusted* | (95% C.I.)                                          | Adjusted* (95% C.I.)                        |
|       | P value                                              | P value                                    |
| $\leq$2% | 1.04 (0.74–1.46)                                    | 1.04 (0.74–1.46)                            |
|        | 0.94 (0.57–1.07)                                    | 0.94 (0.57–1.07)                            |
| >2% – 60% | 1.01 (0.79–1.30)                                    | 1.01 (0.79–1.30)                            |
|        | 0.99 (0.79–1.27)                                    | 0.99 (0.79–1.27)                            |
| 20–60% | 1.06 (0.43–2.13)                                    | 1.06 (0.43–2.13)                            |
|        | 0.97 (0.48–1.95)                                    | 0.97 (0.48–1.95)                            |
| 10–20% | 1.15 (0.52–2.53)                                    | 1.15 (0.52–2.53)                            |
|        | 0.99 (0.51–1.98)                                    | 0.99 (0.51–1.98)                            |
| 5–10%  | 1.61 (0.79–3.28)                                    | 1.61 (0.79–3.28)                            |
|        | 1.21 (0.61–2.41)                                    | 1.21 (0.61–2.41)                            |
| 2–5%   | 2.09 (0.53–8.38)                                    | 2.09 (0.53–8.38)                            |
|        | 1.70 (0.36–8.70)                                    | 1.70 (0.36–8.70)                            |

* Adjusted for age, gender, risk factor, pre-HAART CD4 cell count, pre-HAART HIV-RNA, hepatic C infection, year of starting treatment, transmitted drug resistance, third drug administered (NNRTI vs. PI/r vs. raltegravir), NRTI backbone used, number of drugs administered (3 vs. >3 drugs), b. Reference group (dummy). Boldface indicates the geno2pheno false positive rate (FPR) ranks that were significantly associated with viro-immunological response. C.I.: confidence interval.

Low False Positive Rate Is Associated with Poor Response to HAART

Survival analyses for the evaluation of virological success

By Kaplan-Meier estimates, overall the median time (95% C.I.) to achieve virological success was 4.0 (3.7–4.5) months. The overall probability of achieving virological success was 95.5% at 12 months. No difference in terms of rates of virological suppression was observed between X4- and R5-infected patients regardless of FPR levels (data not shown). However, by univariable Cox analysis, patients with FPR $\leq$2% showed a significantly lower hazard to achieve virological success compared to patients with FPR $>$60% (relative hazard [95% C.I.]: 0.31 (0.29–0.90); p = 0.019) (Table 2). By multivariable analysis, adjusting for age, gender, risk factor, pre-HAART CD4 cell count, pre-HAART viremia, hepatic C infection, year of starting treatment, transmitted drug resistance, third drug administered (NNRTI vs. PI/r vs. raltegravir), NRTI backbone used and more than 3 drugs administered, this result was confirmed (relative hazard [95% C.I.]: 0.50 (0.26–0.94); p = 0.031) (Table 2).

Repeating all the analyses by excluding patients with FPR $\leq$ 2%, all the associations between genotypically-inferred assessed tropism and virological response were lost (data not shown).

Among the other confounders, very high pre-HAART viremia (>500,000 copies/mL) was significantly associated with poorer virological response, as previously observed [32] (Table S1). Of note, the lowest hazard of virological undetectability was found in patients with pre-HAART viremia >1,000,000 copies/mL. The use of raltegravir was associated with a higher hazard to achieve virological success compared with an NNRTI or a PI/r based regimen.

Discussion

By this longitudinal study we found that FPR $\leq$2% is an independent predictor of both a poor immunological reconstitution and a lower virological response in HIV-1 B subtype infected patients who have initiated their first-line antiretroviral regimen. Repeating our analyses by excluding patients with FPR $\leq$2%, all the associations between genotypically-inferred assessed tropism and viroimmunological response were lost, confirming the crucial role of FPR $\leq$2%.

These results reinforce our hypothesis that the highest intra-patient prevalence of X4 variants is found in patients with very low FPRs [30,31], and that, in this particular situation, high prevalence of X4-species might influence the CD4 recovery after therapy start. Furthermore, it was recently observed that failures of maraviroc-containing regimens select only viruses with an extremely low FPR, implying that FPR $\leq$2% could indicate the presence of pure-X4, really insensitive to anti-CCR5 antagonists [44].

The analysis described in the results section refers to a time frame of 12 months. By extending our analysis to 36 months of treatment, more than 90% of patients achieved a gain of CD4 cell count $\geq 150$ cells/mm$^3$ (data not shown). Also in this case, patients with FPR $\leq$2% showed the lowest probability (83.4%) compared to those having higher FPR ranks. Thus, these findings suggest that the negative effect of a very low FPR is maintained in the long term in patients starting first HAART, and its predictive value could be relevant to identifying patients with a blunted increase in their CD4 cell counts. Ad hoc studies to clarify the role of tropism in long term suppressed HIV-1 patients are needed to confirm these results.
Findings about immunological reconstitution are reported in the ArTEN and ANRS 130 APOLLO studies: genotypically-assessed viral tropism (with FPR set at 10% and 5.75%) did not seem to impact on the extent of CD4 cell count recovery on antiretroviral therapy [22,23]. These results are in line with our observations, showing that only FPR ≤2% is related to immune reconstitution, while FPR values >2 are not associated with this parameter [23]. Our data also show a relationship between FPR ≤2% and virological response. Indeed, by both uni- and multivariable Cox analyses we found a lower hazard to achieve virological suppression for this FPR range if compared with the others, suggesting that patients carrying a pure X4 tropic virus are potentially in a compromised immunological status and consequently might have a lower and/or delayed virological suppression after first-line HAART. These findings are in agreement with the results obtained in the ArTEN study in which HIV-1 B subtype X1-infected patients showed a lower virologic response compared to those R5-infected at 48 weeks from their first-line HAART [22].

This study may have some limitations. First, tropism was inferred by the analysis of only V3 sequences. Indeed, it is known that other residues outside of V3 loop within gp120 and gp41 could be relevant for viral coreceptor usage [45–47]. In addition, our cohort includes only B subtype infected patients. While this has been done on purpose to perform cleaner analyses, at the same time the results cannot be extrapolated to non-B infected patients. The accuracy of genotypic tools to assess viral tropism is reported to be so far lower with non-B subtypes than with clade B variants [16,48,49], therefore any extrapolation to non-B subtypes must be done with great caution. Furthermore, we did not evaluate the potential role of therapy compliance on viro-immunological response. It is known that less adherent patients have a higher risk of death and of inadequate CD4+ count recovery [50]. In this study we did not find any correlation between adherence level and immunological recovery (data not shown). On the other hand, as expected in a population achieving a high rate of virological success, around 90% of patients attended high compliance to therapy regardless FPR levels; thus we decided not to consider adherence level as potential confounder in the Cox analyses. Moreover, we decided not to take account of the reason/timing of potential therapy switches during the observation of patients. Indeed, only 5% of patients changed therapy before the achievement of virological success. These few patients changed their regimen within 3 months showing a similar rate of immunological reconstitution compared to those who never changed therapy (data not shown).

Finally, it would be interesting to evaluate the relationship between immunological reconstitution and genotypically-inferred tropism in patients with acute infection. However, this category of patients could not be assessed in this analysis, since in clinical practice the diagnosis is frequently made after a time of infection that cannot be quantified.

In conclusion, our findings show that FPR ≤2% defines patients carrying a viral population significantly associated with both poorer immunological reconstitution and lower and/or delayed virological response in HIV-1 B infected patients starting their first-line therapy. These data reinforce a previous suggestion that FPR ≤2% may not only identify those patients whose virus is insensitive to CCR5-inhibitors, but can also be useful to identify patients potentially with less chance of achieving adequate viro-immunological response.

Supporting Information

Table S1 Factors related to immunological and virological response in HIV-1 infected patients starting their first-line HAART (Cox Models).

Acknowledgments

We gratefully thank Andrea Biddittu, Massimiliano Bruni, Luca Carioti, Fabio Continenza, Alberto Gianetti, Anna Pacifici, Daniele Pizzi and Marzia Romani for sequencing and data management.

The manuscript was presented in part at the following meetings: 9th European Workshop on HIV and Hepatitis Treatment Strategies and Antiviral Drug Resistance, 23–25 March 2011, Paphos, Cyprus (Reviews in Antiviral Therapies, Vol 2, pag 30, Abstract O_27); 10th European Meeting on HIV & Hepatitis Treatment Strategies & Antiviral Drug Resistance, March 28–30 2012, Barcelona, Spain (Reviews in Antiviral Therapy & Infectious Diseases 2/2012 # P_31 pp 52–53).

Author Contributions

Conceived and designed the experiments: DA MMS CFP FCS. Performed the experiments: CS LF CG AB FF. Analyzed the data: DA CS LF CG VS FCS. Contributed reagents/materials/analysis tools: DDC MG AL EB MZ RB MA AGM VC AA.

References

1. Robbins GK, Daniels B, Zheng H, Chueh H, Meigs JB, et al. (2007) Predictors of antiretroviral treatment failure in an urban HIV clinic. J Acquir Immune Defic Syndr 44: 30–37.

2. Robbins GK, De Gruttola V, Shafer RW, Smeaton LM, Snyder SW, et al. (2003) Comparison of sequential three-drug regimens as initial therapy for HIV-1 infection. N Engl J Med 349: 2293–2303.

3. Lucas GM, Chaisson RE, Moore RD (1999) Highly active antiretroviral therapy in a large urban clinic: risk factors for virologic failure and adverse drug reactions. Ann Intern Med 131: 81–87.

4. Moore DM, Hogg RS, Chan K, Tyrall M, Yip B, et al. (2006) Disease progression in patients with virological suppression in response to HAART is associated with the degree of immunological response. AIDS 20: 371–377.

5. Kauffman GM, Farehr H, Ledergerber B, Perzin L, Opaza M, et al. (2005) Characteristics, determinants, and clinical relevance of CD4+ T cell recovery to <500 cells/microL in HIV type 1-infected individuals receiving potent antiretroviral therapy. Clin Infect Dis 41: 561–572.

6. Baker JV, Pong G, Rapkin J, Kranos D, Reilly C, et al. (2008) Poor initial CD4+ recovery with antiretroviral therapy prolongs immune depletion and increases risk for AIDS and non-AIDS diseases. J Acquir Immune Defic Syndr 48: 541–546.

7. Moore RD, Gheb KA, Lucas GM, Kerdly JC (2008) Rate of comorbidities not related to HIV infection or AIDS among HIV-infected patients, by CD4 cell count and HAART use status. Clin Infect Dis 47: 1102–1104.

8. Gazzola L, Tincati G, Bellisi GM, Monforte A, Marchetti G (2009) The absence of CD4+ T cell count recovery despite receipt of virologically suppressive highly active antiretroviral therapy: clinical risk, immunological and therapeutic options. Clin Infect Dis 48: 329–337.

9. Paley CT, Weiss L, Thomas F, Mohamed AS, Belc HS, et al. (2001) Long-term clinical outcome of human immunodeficiency virus-infected patients with discordant immunologic and virologic responses to a protease inhibitor-containing regimen. J Infect Dis 183: 1329–1335.

10. Tan R, Westfall AO, Willig JH, Magaven MJ, Saag MS, et al. (2008) Clinical outcome of HIV-infected antiretroviral-naive patients with discordant immunologic and virologic responses to highly active antiretroviral therapy: J Acquir Immune Defic Syndr 47: 553–558.

11. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. May 2014; Available: http://aidsinfo.nih.gov/contentfiles/Adultandadolescent.pdf. Accessed 2014 May 29.

12. Thompson MA, Aberg JA, Hove TF, Teleni A, Benson C, et al. (2012) Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel. JAMA 308: 387–402.

13. Dour IS, Kesler KL, Petropoulos CJ, Huang W, Bates M, et al. (2007) Baseline HIV type 1 coreceptor tropism predicts disease progression. Clin Infect Dis 45: 643–649.
31. Svicher V, Cento V, Rozera G, Abbate I, Santoro MM, et al. (2013) The impact of HIV tropism on decreases in CD4 cell count, clinical progression, and subsequent response to a first antiretroviral therapy regimen. Clin Infect Dis 46: 1617–1623.

29. Poveda E, Paredes R, Moreno S, Alcami J, Cordoba J, et al. (2012) Update on CXCR4-tropic viruses in plasma and peripheral blood mononuclear cells during primary HIV-1 infection and impact on disease progression. AIDS 24: 2305–2312.

27. Nozza S, Canducci F, Galli I, Cozzi-Lepri A, Capobianchi MR, et al. (2012) Viral tropism by geno2pheno as a tool for predicting CD4 decrease in HIV-1-infected naive patients with high CD4 counts. J Antimicrob Chemother 67: 1224–1227.

25. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591–595.

23. Feinberg MB, Miedema F, Moore JP, Schuitemaker H (2002) HIV-1 tropism and CD4+ T-cell depletion. Nat Med 8: 537.

21. Brummel ZZ, Dong WW, Yip B, Wynshoven B, Hoffman NG, et al. (2004) Clinical and immunological impact of HIV envelope V3 sequence variation after starting initial triple antiretroviral therapy. AIDS 18: F1–9.

19. Waters L, Mandala S, Randell P, Wildfire A, Azzam E, et al. (2008) The impact of HIV tropism on decreases in CD4 cell count, clinical progression, and subsequent response to a first antiretroviral therapy regimen. Clin Infect Dis 46: 1617–1623.

17. Coakley E, Reeves JD, Huang W, Mangas-Ruiz M, Maurer I, et al. (2009) Population-based sequencing of the V3-loop can predict the virological response of HIV coreceptor usage (2007). Nat Biotechnol. 25: 1407–1410.

15. Goetz MB, Leduc R, Kostman JR, Labriola AM, Lie Y, et al. (2009) High sequence conservation of human immunodeficiency virus type 1 reverse transcriptase in non-B subtypes. AIDS Res Hum Retroviruses 29: 979–984.

13. Ceccherini-Silberstein F, Gago F, Santoro M, Gori C, Svicher V, et al. (2005) Clinical and immunological impact of HIV envelope V3 sequence variation after starting initial triple antiretroviral therapy. AIDS 18: F1–9.

11. Sing T, Low AJ, Beerenwinkel N, Sander O, Cheung PK, et al. (2007) Predicting HIV coreceptor usage on the basis of genetic and clinical covariates. Antivir Ther 12: 1097–1106.

9. Viladiu M, Soileau S, Vahlen MA, Forouzi S, Simon A, et al. (2011) Historical HIV RNA-resistance test results are more informative than proviral DNA genotyping in cases of suppressed or residual viraemia. J Antimicrob Chemother 66: 709–712.

7. Bennett DE, Canacho RJ, Oteola D, Kuritzkes DR, Fleury H, et al. (2009) Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PLoS One 4: e4724.

5. Altei R, Svicher V, Gori C, D’Arrigo R, Cioccozzi M, et al. (2009) Characterization of the patterns of drug-resistance mutations in newly diagnosed HIV-1-infected patients naïve to the antiretroviral drugs. BMC Infect Dis 9: 111.

3. Carrillo A, Ratner L (1996) Human immunodeficiency virus type 1 tropism profiles in clinical samples by the Trolle and MT-2 assays. Antimicrob Agents Chemother 53: 4636–4640.

1. Seclen E, Garrido C, Gonzalez Mdel M, Gonzalez-Lahoz J, de Mendoza C, et al. (2010) High sensitivity of specific genotypic tools for detection of X4 variants with limited effect on the total viral set point. J Antimicrob Chemother 66: 2007–2014.

34. Sire JM, Vray M, Merzouk M, Plantier JC, Pavie J, et al. (2011) Comparative performance of genotypic tropism testing in clinical practice using the enhanced sensitivity version of Trofile as reference assay: results from the OSCAR Study Group. New Microbiol 33: 195–206.

36. Cecccherini-Silberstein F, Gago F, Santoro M, Gori C, Svicher V, et al. (2005) High sequence conservation of human immunodeficiency virus type 1 reverse transcriptase under drug pressure despite the continuous appearance of mutations. J Virol 79: 10718–10729.

38. Wirden M, Soulie C, Valantin MA, Fourati S, Simon A, et al. (2011) Comparative evaluation of the VERSANT HIV-1 RNA 1.0 kinetic PCR molecular system (kPCR) for the quantification of HIV-1 plasma viral load. J Clin Virol 49: 297–301.

39. Sing T, Low AJ, Beerenwinkel N, Sander O, Cheung PK, et al. (2007) Predicting HIV coreceptor usage on the basis of genetic and clinical covariates. Antivir Ther 12: 1097–1106.

32. Soutis C, Charpentier G, Flandre P, Sino C, Carcelain G, et al. (2012) Natural evolution of CD4+ cell count in patients with CD4 >350 or >500 cells/mm3 at the time of diagnosis according to HIV-1 coreceptor tropism. J Med Virol 84: 1853–1856.

30. Lee GQ, Harrigan PR, Dong W, Poon AF, Heera J, et al. (2013) Comparison of the patterns of drug-resistance mutations in newly diagnosed HIV-1-infected patients naïve to the antiretroviral drugs. BMC Infect Dis 9: 111.

28. Seclen E, Garrido C, Gonzalez Mdel M, Gonzalez-Lahoz J, de Mendoza C, et al. (2010) High sensitivity of specific genotypic tools for detection of X4 variants in antiretroviral-experienced patients suitable to be treated with CCR5 antagonists. J Antimicrob Chemother 65: 1486–1492.

26. Carrillo A, Ratner L (1996) Human immunodeficiency virus type 1 tropism for T-lymphoid cell lines: role of the V3 loop and C4 envelope determinants. J Virol 70: 1301–1309.

24. Connor RI, Sheridan KE, Gerardin D, Choo S, Landau NR (1997) Change in coreceptor use correlates with disease progression in HIV-1–infected individuals. J Exp Med 183: 621–628.

22. Ceccherini-Silberstein F, Gago F, Santoro M, Gori C, Svicher V, et al. (2005) Clinical and immunological impact of HIV envelope V3 sequence variation after starting initial triple antiretroviral therapy. AIDS 18: F1–9.

20. Weiser B, Pilpott S, Klimkait T, Burger H, Kilchen C, et al. (2008) HIV-1 coreceptor usage and CXCR4-specific viral load predict clinical disease progression during combination antiretroviral therapy. AIDS 22: 469–479.

18. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591–595.

16. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591–595.

14. Goetz MB, Leduc R, Kostman JR, Labriola AM, Lie Y, et al. (2009) Relationship between HIV coreceptor tropism and disease progression in persons with untreated chronic HIV infection. J Acquir Immune Defic Syndr 56: 259–266.

12. Raymond S, Delobel P, Mavigner M, Cazabat M, Encinas S, et al. (2010) CXCR4-tropic viruses in plasma and peripheral blood mononuclear cells during primary HIV-1 infection and impact on disease progression. AIDS 24: 2305–2312.

10. Nozza S, Canducci F, Galli I, Cozzi-Lepri A, Capobianchi MR, et al. (2012) Viral tropism by geno2pheno as a tool for predicting CD4 decrease in HIV-1-infected naive patients with high CD4 counts. J Antimicrob Chemother 67: 1224–1227.

8. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591–595.

6. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591–595.

4. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591–595.

2. Wilkin TJ, Su Z, Kuritzkes DR, Hughes M, Flexner C, et al. (2007) HIV type 1 chemokine coreceptor use among antiretroviral-experienced patients screened for a clinical trial of a CCR5 inhibitor: AIDS Clinical Trial Group A5211. Clin Infect Dis 44: 591–595.