Major Article

Correlating HIV Tropism With Immunological Response Under cART

Coreceptor Use and Disease Progression

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Abstract

Background: Patients infected with the human immunodeficiency virus type 1 (HIV-1) may experience only suboptimal CD4 cell recovery while treated with combination therapy (cART). Little is known about whether viral properties such as cell tropism could play a role for such incomplete immune response. Thus this study was designed to follow the evolution of the viral tropism during periods of suppressive cART.

Methods: Virus from two distinct patient groups, a “steady” and an “incomplete” CD4-responder group after five years of suppressive cART, were genotypically analyzed for HIV tropism at baseline and at the end of the study period.

Results: Patients with CCR5-tropic viruses at baseline tended to maintain this tropism to the study end. In the incomplete CD4-responder group viruses of CXCR4-tropism at baseline were overrepresented after therapy. Overall, however, and somewhat unexpectedly, the great majority of all patients presented with CCR5-tropic viruses at follow-up.

Conclusion: Our data lend support to the hypothesis that tropism determination can be used as parameter for disease progression even if analyzed years prior to the establishment of an incomplete immune response. Moreover, the observed lasting predominating CCR5-tropism under virus suppression suggests the involvement of cellular mechanisms preferentially reducing CXCR4-tropic viruses during cART.

[198 words]

Keywords: HIV, tropism, cART, CXCR4, CCR5, immune response

Introduction

Whereas it is well established that sustained CD4-recovery is a key determinant of therapy success, much less is known about viral properties during disease progression or immunological recovery phases of the infection.

On the molecular level successful HIV infection critically depends on high-affinity binding of the virus to the cellular receptors CD4 and a chemokine receptor serving as viral coreceptor. Prior to cell entry conformational changes in the HIV envelope enable an interaction of variable loop 3 (V3) and either chemokine receptor CCR5 (R5) or CXCR4 (X4) [1]. Although there is no free choice for the virus, the key determinants, mainly located in V3, dictate, which chemokine receptor can be bound.

Numerous reports have shown that early after infection most HIV-1 isolates use the R5 receptor (up to 85% of cases). Accordingly, X4-tropic virus isolates remain rare early after infection and are more frequently isolated at later disease stages [2-5]. Only for subtype D a greater percentage of X4-using variants has been reported [6]. The drivers of this “tropism switch” from R5 to X4 in up to 50% of patients are not well understood. Nevertheless, earlier reports have established that HIV-1 infections utilizing the X4 receptor associate with faster disease progression and a more rapid decline of CD4 cell counts [7-9]. Whether herein X4 tropism is cause or consequence is still under debate. As there are currently no entry inhibitors clinically available that target the X4 receptor, and with maraviroc (Celsentri, Selzentry) as the only modality acting on the R5 receptor [10], a better understanding of the mechanisms that drive viral infection via the X4 receptor is of central relevance. Reliable genotypic tropism tests have been established to identify the viral tropism and dissect mixed virus populations [11].

A reported paradox is that HIV-infected patients, although fully responding to antiretroviral treatment, may experience massively delayed, insufficient CD4 cell

recovery. In 2005 Kaufmann et al. [12] had studied such profiles of CD4 cell recovery over time during fully suppressive cART and assigned patients to either an immunologically responding or a non-responding group. The only significant determinants in that study were advanced stage at diagnosis and age at time of infection. Considering that in other studies X4 tropism associated with poorer disease outcome, we hypothesized that an impaired CD4 cell response could directly be affected by properties of the Env protein. We set out to assess in detail viral tropism both, at the time of cART initiation and after extended periods of successful therapy. Tropism was analyzed for a sup-population of the above-mentioned study, i.e. for virus from patients with continuous versus transient or poor immune response. Aside from this CD4-response aspect our study followed viral tropism also over time, comparing samples from pre-cART and after five years of therapy. For this free plasma virus as well as proviral DNA in PBMC were examined.

Methods

Study design: Plasma and cell samples from 110 patients in the Swiss HIV Cohort Study were included. Eighty-three samples stemmed from the study by Kaufmann et al. and 27 further samples from SHCS patients matching the inclusion and outcome criteria from the Kaufmann et al. study. In agreement with the involved ethical boards and guidelines all patients in the SHCS have given their informed written consent.

Samples were grouped according to the patients' CD4 recovery after five years on cART, into "responders" (n=60) with largely steady responses to >550 CD4 cells/µL or "incomplete responders" (n=50) where CD4 cells stayed below 550 cells/µL. Tropism analysis was performed on plasma and on PBMC near the time of cART initiation, and five years later on PBMC's. For tropism determination two methods were available: Geno2Pheno (Max-Planck-Institute, Saarbrücken, Germany) and XTrack (InPheno AG, Basel, Switzerland). All patients had experienced a rapid viral load decline after cART initiation. Viral loads stayed below 500 copies/mL without blips for the entire study period. cART was defined as any combination of ≥3 antiretroviral drugs, either 2 nucleosidic reverse transcriptase inhibitors (NRTI) combined with one or two protease inhibitors or with one non-nucleosidic reverse transcriptase inhibitor (NNRTI). None of the patients ever received R5 antagonists for therapy.

RNA and DNA extraction was performed with chemagen Prepito (Perkin Elmer, Baesweiler, Germany) using the NA Body Fluid Kit according to protocol. For amplification of the V3 loop a validated in house protocol was used. In brief, RNA was reverse transcribed using the Affinity Script One-Step RT-PCR Kit (Agilent Technologies, Basel, Switzerland). The product was cleaned with illustra Exostar 1-

Step (GE Healthcare, Glattbrugg, Switzerland), and nested PCR was performed using PfuUltra II Fusion HS (Agilent). PCR products were sequenced on an ABI 3130 Genetic Analyzer, and tropism was assigned using the online tool Geno2Pheno(coreceptor) version 2.5 with a FPR cut-off value of either 5% or 10%. For tropism assessment with XTrack the kit protocol was followed.

Statistical analysis: Categorical data were compared by means of Chi-square test, whereas continuous data were compared by Mann-Whitney-Wilcoxon test.

Results

Patient characteristics. Epidemiological analysis could be performed for a total of 94 patients (81 plasma and 13 PBMC). The patient population had a mean age of 54.4 ± 10.7 years. 76% were male and 90% were white; 46% were men-who-have-sex-with-men (MSM). Mean duration of infection was 19.2 ± 5.7 years [13] and 75% of the patients had started with cART prior to the year 2000. Baseline CD4 cell count was 181 cells/µL, baseline CD8 cell count 841 cells/µL, and baseline viral load was 5.42 $\log_{10}$ copies/mL. Based upon analysis at the follow-up time point 18% (17/87; eight not analyzable) of the patients had a Δ32 heterozygous genotype. Baseline characteristics are listed in table 1.

In the incomplete responder group patients had initiated cART at a lower CD4 cell count 101 cells/µL vs. 244 cells in the responder group [$p<0.001$]. It was key to rule out any CD4-based bias. Therefore a sub-analysis of incomplete responders with high CD4 nadir vs. complete responders with low CD4 nadir was performed, showing that a low CD4 nadir is not the primary cause of incomplete response.

CD8 cell count at baseline for incomplete responders was 840 vs. 843 cells/µL [$p = 0.952$], and viral loads were 5.59 $\log_{10}$ copies/mL vs. 5.21 $\log_{10}$ copies/mL [$p=0.005$].

Demographic characteristics revealed a longer duration of infection [13] for the incomplete responders with 20.3 vs. 18.4 years [p = 0.010], and more cases with CDC stage C disease: 17 vs. 9 cases [p = 0.024]. The occurrence of a Δ32 heterozygous genotype was similar in both groups (9 and 8 cases, respectively [p = 0.558]).

There was no statistically significant difference in the first line regimen between the two patient groups. For 90% the primary regimen included one protease inhibitor with an NRTI backbone. Most frequently used drugs were combinations of Combivir with indinavir or nelfinavir (table 2).

Sample analysis – Tropism analysis at baseline was successful for 82 plasma and 53 PBMC samples. Corresponding baseline data sets of plasma virus and provirus (PBMC) were available for 40 patients. Tropism analysis was largely concordant for virus and provirus at baseline with both G2P and XTrack in 88% (35/40).

Regarding CD4 response a total of 95 BL profiles were available for comparison (82 BL-PL, 13 BL PBMC). Overall 52 were responders and 43 were incomplete responders according to the patients' CD4 cell counts after five years of follow-up. Eighty-eight samples of the 95 (93%) had concordant results between G2P and XTrack. For following an evolution of the viral tropism proviral data from PBMCs at BL (BL-PBMC) were compared with PBMCs at follow-up (5Y-PBMC), where 51 sets were available. For comparison of the 5y-PBMC samples with their corresponding baseline plasma samples (BL-Plasma) 65 sample sets were available.

CD4-response - correlating baseline tropism with immunological outcome

The mean time between baseline analysis and time point of follow-up was 5.2 years. For 91 patients a tropism assignment was successful using the online tool Geno2Pheno$_{tropism}$ (G2P). The “False Positive Rate” cutoff was set to 5%. For three patients G2P yielded no result due to the presence of a mixed virus population; and for one patient G2P gave an unknown error. G2P predicted 74 (81.3%) patients to carry an R5-tropic virus and 17 (18.7%) to carry an X4-tropic virus, considering that around 90% of samples had subtype B determined by G2P. 70.6% (12/17) of patients with X4-tropic viruses and 39.2% (29/74) with R5-tropic viruses were in the incomplete responder group ($p = 0.019$). For G2P FPR adjustment to 10% the univariate analysis still resulted in a significant p-value ($p = 0.044$); data are shown in table 3.

With the XTrack analysis thirty two percent (17/53) of the patients with R5-tropic viruses, 60.7% (17/28) with a mixed virus population and 64.3% (9/14) of patients with X4-tropic viruses belonged to the incomplete responder group ($p = 0.015$), as shown in table 3. Disregarding the 28 patients with mixed virus populations, Geno2pheno and XTrack yielded concordant results in 89.6% (60/67).

Multivariate logistic regression analysis including age, duration of infection, CDC stage, baseline viral load, baseline CD4, and tropism interpretation revealed that only baseline CD4 cell count (($p < 0.001$) was an independent predictor of an insufficient CD4 cell response. When excluding baseline CD4 from the multivariate model, the tropism interpretation reached the significance level ($p < 0.05$).

Tropism evolution in provirus at baseline and after therapy

For the comparison of PBMC samples at baseline and five years after (BL-PBMC/5Y-PBMC) a complete data set from 51 patients (G2P and XTrack) was available. Seven patients had no G2P result due to mixed viral sequences. With 38 of 44 analyzed samples the vast majority of patients (86.4%) carried R5-tropic virus at baseline. Thirty-two of these 38 patients (84.2%) still had an R5-tropic virus at follow-up, in the remaining six patients the tropism had changed to X4 use. From the six patients with X4-tropic virus at baseline four (66.7%) changed to R5 tropism at follow-up. Data summarized in figure 1.

The XTrack system was used to address especially mixed virus populations: Among the 28 patients with initially R5-tropic HIV four cases (14.3%) presented with a mixed virus population at follow-up. For seven patients with a mixed population at baseline the mixed tropism remained stable in two cases (28.6%) after 5 years. A tropism change from mixed to R5 occurred in three (42.9%) patients, and in two (28.6%) cases the later samples revealed solely X4-tropic virus. For the nine patients with X4-tropic virus at baseline, only one (11.1%) changed to a mixed tropism. Data summarized in figure 2.

"Tropism evolution" – tropism of plasma virus at baseline and provirus after treatment

From the initial data set, 65 patients had a G2P and XTrack baseline plasma tropism result available as well as a corresponding follow-up PBMC tropism result. Among the 65 patients, eight had no G2P result due to mixed viral sequences. At baseline we identified R5-tropic viruses in 48 (84.2%) of 57 patients samples, of which 42 (87.5%) were still R5-tropic at follow-up. Among the nine patients with X4-tropic virus

at baseline the tropism had changed in seven (77.8%) patients to R5 at follow-up; data summarized in figure 1.

XTrack was used also here to examine mixed virus populations. Among the 37 (64.9%) patients with an R5 tropism at baseline four (10.8%) changed to a mixed population. For 12 patients with a mixed virus population at baseline HIV changed to become R5-tropic in seven (58.3%) cases, one (8.3%) became X4-tropic, and in four (33.3%) cases the viral tropism remained mixed. Among the eight patients with an X4-tropic virus at baseline one (12.5%) changed to a mixed population. The results are shown in figure 2.

Considering the excellent concordance that was observed between baseline plasma and PBMC samples (in 88% of all cases) we grouped all samples changing from an X4 tropism at baseline to R5 at follow-up, regardless of their origin, plasma or PBMC. Thus overall 73.3% (11/15) of all patients with an X4 tropism at baseline had changed to R5 at follow-up considering G2P results.

Analysis of viruses with tropism switch. For the majority of X4-viruses that underwent a switch to R5 tropism several nucleotide changes in the V3 loop sequence were responsible for altering the tropism according to the G2P algorithm. In contrast, for most R5-viruses switching tropism to CXCR4 use, a single nucleotide change was sufficient to yield the X4 tropism. Herein most frequent were G to A transitions, which are typically associated with an amino acid change, mostly from Serine, Glycine, or Glutamine to Arginine. Those occurred at positions 11, 24, or 25 of the V3 loop. Also about half of the affected viruses lost at least one potential N-linked glycosylation

site. Analysis with Hypermut 2.0 (www.hiv.lanl.gov) did not detect accumulations of hypermutations in any of the sequences.

Discussion

Impaired CD4 gain despite successful cART has previously been described, largely in the absence of known plausible causes [12]. This study addressed, whether a tropism determination prior to cART initiation would be able to predict a later poorer immune response. We observed that more than 70% of all patients with X4-tropic viruses at therapy initiation would experience only a partial immune recovery during the following 5 years (<550 CD4 cells/µL). In addition, analysis of proviral HIV sequences at this later time point during therapy revealed that in a striking majority of patients X4 tropic BL-HIV changed to R5. Contrasting this most patients with R5 tropism at baseline retained R5-tropic viruses. We further observed that typically a single nucleotide change was sufficient for the change to X4 tropism, whereas a tropism change from X4 to R5 required several point mutations. This lends further support to the notion that tropism changes did not occur as consequence of spontaneous, isolated mutations. It supports the concept that R5 variants evolved from mixed virus populations already pre-existing in the patient. Most single nucleotide changes occurred at positions 11, 24, or 25 of the V3 loop, mostly as G to A transitions. It has been reported before that APOBEC3 triggers such G to A mutations and might thus be directly responsible for or involved in viral tropism changes [14]. Moreover, in half of the patients a change to X4-tropism coincided with the loss of a potential N-linked glycosylation site. This is in good concordance with earlier findings that glycosylation patterns change during the disease course, more often with a loss of sites in late virus isolates and a correlation with X4 tropism [15-17].

Based on the data of this study it appears unlikely that a Δ32 genotype, which would potentially favor X4-tropism, impacted on our findings. The identified 18.1% patients with Δ32 heterozygosity were equally distributed between both groups and had no influence on the viral tropism under therapy. Overall the Δ32 prevalence was not different from other studies on Caucasian populations [18].

Although the small sample size of this study poses certain limitations, a clear trend is seen a) for the association of X4 tropism with CD4 deterioration under cART and b) towards a strong reduction of X4-tropic variants under successful therapy. A possible confounder in our study is the principally lower BL CD4 cell counts in the non-responder group. Yet both groups overlapped significantly and some patients with low CD4 <200/µL belonged to the responders, whereas others with BL CD4 >200/µL still only reach a plateau. Alternatively to this being suggestive of a poor prognosis for CD4-recovery, this might indicate higher levels of inflammation and immune stimulation by X4-tropic HIV. It has been reported that such activation can favor the elimination of cells infected with X4-tropic HIV [19].

The finding that most (11/15) viruses with X4 tropism at baseline had changed to R5-tropic viruses during the period of successful cART argues against the driving role of persistently replicating X4-tropic HIV in the CD4 deterioration. It rather provides evidence for a greater proviral stability of R5-tropic HIV during suppressive therapy and may suggest that R5 variants represent the main targets for therapy.

The intriguing observation that during suppressive cART X4-tropic (proviral) HIV appears to decrease over time invites to hypothesize that under successful cART and good CD4 cell recovery the immune system manages to preferably reduce and potentially eliminate X4-tropic HIV.

Further support comes from reports that a superior immunological recognition of X4–tropic HIV associates with a tropism-sensitive overstimulation of the immune system and associated CD4 cell death [19]. Other studies had suggested that antiretroviral therapy itself could lead to a diminution of X4 tropic HIV-1 [20, 21]. Those data are in line with observations from the MOTIVATE studies of patients on salvage regimens, where the majority of these heavily pre-treated patients presented at baseline with R5-tropic virus despite long infection- and therapy episodes [22]. A striking finding in the same studies was that, upon discontinuation of maraviroc, X4 tropic virus variants rapidly disappeared with the prompt re-emergence of R5 tropic HIV [23], similar to emerging virus in failing first-line cART [24].

In line with some [20, 21] and in contrast to other reports [25] we observed in our study a reduction of X4-tropic variants during cART, and our data do not support the claim of an over-representation of X4-tropic virus in proviral samples compared to plasma. This was also observed by others, who, however, reached a different interpretation by suggesting that proviral tropism testing should not be used due to an obviously false under-representation of X4 in the proviral reservoir [26]. Yet our observation is fully in line with their primary data: Also Swenson et al. saw over time a higher proportion of R5-tropic virus sequences in the cellular compartment. We, however, interpret these findings in a way that the lower representation is real and rather reflects an immunological shift during cART and a loss of X4-harboring cells.

It will therefore be of interest to further investigate, whether a tropism-dependent mechanism of virus control can consistently be exerted during cART. Should it be possible to preferentially control X4-tropic HIV variants by strengthening immune functions, e.g. under maximal pressure of combination therapy and while CD4 function is still intact (>500 cells/µL), R5-coreceptor antagonists might gain a new role in therapy [19].

Further studies with larger sample size will be needed to clinically confirm our results, but these observations could have bearing for new strategies of controlling HIV in immunologically active body compartments.

[3031 words]

Conflict of interest

None of the authors declared any conflicts of interest for the conduct of this work.

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Table 1. Baseline characteristics for 94 HIV-1 infected, treatment-naïve patients

Characteristic	All patients (n=94)	All patients, by CD4 response		P
		Complete responder (n=52)	Incomplete responder (n=42)	
Sex				
Male	71 (75.5)	37 (71.2)	34 (81.0)	0.391
Female	23 (24.5)	15 (28.8)	8 (19.0)	
Age, mean years ± SD	54.4 ± 10.7	52.2 ± 10.9	57.1 ± 10.1	0.013
Ethnicity				0.561
White	84 (89.4)	45 (86.5)	39 (92.9)	
Black	6 (6.4)	4 (7.7)	2 (4.8)	
Hispanic	2 (2.1)	2 (3.8)		
Asian	2 (2.1)	1 (1.9)	1 (2.4)	
HIV transmission				0.539
Blood	1 (1.1)	1 (1.9)		
MSM	43 (45.7)	23 (44.2)	20 (47.6)	
HET	34 (36.2)	21 (40.4)	13 (31.0)	
IDU	14 (14.9)	7 (13.5)	7 (16.7)	
Other	1 (1.1)		1 (2.4)	
Not available	1 (1.1)		1 (2.4)	
Age at infection, mean years ± SD	35.7 ± 11.7	34.6 ± 12.3	37.1 ± 10.9	0.217
Age at cART start, mean years ± SD	41.2 ± 11.03	39.5 ± 11.42	42.9 ± 10.46	0.096
Duration of infection, mean years ± SD	19.2 ± 5.7	18.4 ± 6	20.3 ± 5.1	0.010
CDC Stage				
C	26 (27.7)	9 (17.3)	17 (40.5)	0.024
cART initiation				
before 2000	71 (75.5)	40 (76.9)	31 (73.8)	0.727
after 2000	23 (24.5)	12 (23.1)	11 (26.2)	
Baseline HIV RNA load, log_{10} copies/mL	5.42 (0-6.39)	5.21 (0-6.3)	5.59 (1.36-6.39)	0.005

Baseline CD4 T cell count, cells/μL	181 (7-497)	244 (12-497)	101 (7-304)	<.0001
Baseline CD8 T cell count, cells/μL	841 (142-2408)	840 (165-2408)	843 (142-2264)	0.952
Delta 32 genotype				
heterozygous	17 (18.1)	8 (15.4)	9 (21.4)	0.391
wild type	70 (74.5)	41 (78.8)	29 (69.0)	

Data are presented as No. (%), mean or min/max values unless otherwise indicated. Categorical data were compared by means of Chi-square test, whereas continuous data were compared by Mann-Whitney-Wilcoxon test. One patient in incomplete responder group is missing due to no data availability. Abbreviations: CDC, Centers for Disease Control and Prevention.

Table 2. Initial combination antiretroviral therapy regimen for 94 HIV-1 infected, treatment naïve patients

cART Regimen	All patients (n=94)	All patients, by CD4 response		P
		Complete responder (n=52)	Incomplete responder (n=42)	
PI				
Indinavir ≥ 2NAs	33 (35.1)	17 (32.8)	16 (38.1)	0.585
Nelfinavir ≥ 2NAs	25 (26.6)	15 (28.8)	10 (23.8)	0.583
Ritonavir ≥ 2NAs	11 (11.7)	6 (11.5)	5 (11.9)	1
other PI or NNRTI	19 (20.2)	10 (19.2)	9 (21.4)	0.791
boosted PI	6 (6.4)	4 (7.7)	2 (4.8)	0.563
NRTI comb.				
Azidothymidine-Lamivudine	56 (59.6)	27 (51.9)	29 (69.1)	0.093
Stavudine-Lamivudine	17 (18.1)	12 (23.1)	5 (11.9)	0.162
Stavudine-Didanosine	4 (4.2)	1 (1.9)	3 (7.1)	0.213
Other NA's	17 (18.1)	12 (23.1)	5 (11.9)	0.162

Data are presented as No. (%) of patients. Data were compared by means of Chi-square test. One patient in incomplete responder group is missing due to no data availability. Abbreviations: PI, Protease inhibitor; NA, nucleoside analogue; NNRTI, nonnucleoside analogue reverse-transcriptase inhibitor; NRTI, nucleoside analogue reverse-transcriptase inhibitor.

Table 3. Tropism analysis at baseline for 95 HIV-1 infected, treatment naïve patients

Method	All patients (n=91)	All patients, by CD4 response		P
		Complete responder (n=50)	Incomplete responder (n=41)	
Geno2Pheno (FPR 5%)				
R5 tropic	74 (81.3)	45 (90)	29 (70.7)	0.019
X4 tropic	17 (18.7)	5 (10)	12 (29.3)	
Geno2Pheno (FPR 10%)				
R5 tropic	69 (75.8)	42 (84)	27 (65.9)	0.044
X4 tropic	22 (24.2)	8 (16)	14 (34.1)	

Method	All patients (n=95)	All patients, by CD4 response		P
		Complete responder (n=52)	Incomplete responder (n=43)	
XTrack				
R5 tropic	53 (55.8)	36 (69.2)	17 (39.5)	0.015
X4 tropic	14 (14.7)	5 (9.6)	9 (20.9)	
Mixed tropic	28 (29.5)	11 (21.2)	17 (39.6)	

Data are presented as No. (%) of patients. Data were compared by means of Chi-square test.

Figure 1. Geno2Pheno (FPR 5%) results comparing tropism between the proviral compartments (n=44) and free virus (plasma) at baseline with provirus (n=57) after five years of therapy for patients with R5- (left panel) or X4-tropic (right panel) HIV at baseline. Abbreviations: PBMC, peripheral blood mononuclear cell; R5, R5 tropic HIV; X4, X4 tropic HIV.

Figure 2. XTrack results showing the tropism comparison between the proviral compartments (n=44) or from free virus (plasma) with provirus (n=57) after five years of therapy for patients with R5- (left panel), mixed- (middle panel) or X4-tropic HIV (right panel) at baseline. Abbreviations: PBMC, peripheral blood mononuclear cell; BL, baseline; R5, R5 tropic HIV; X4, X4 tropic HIV.

Figure 1.

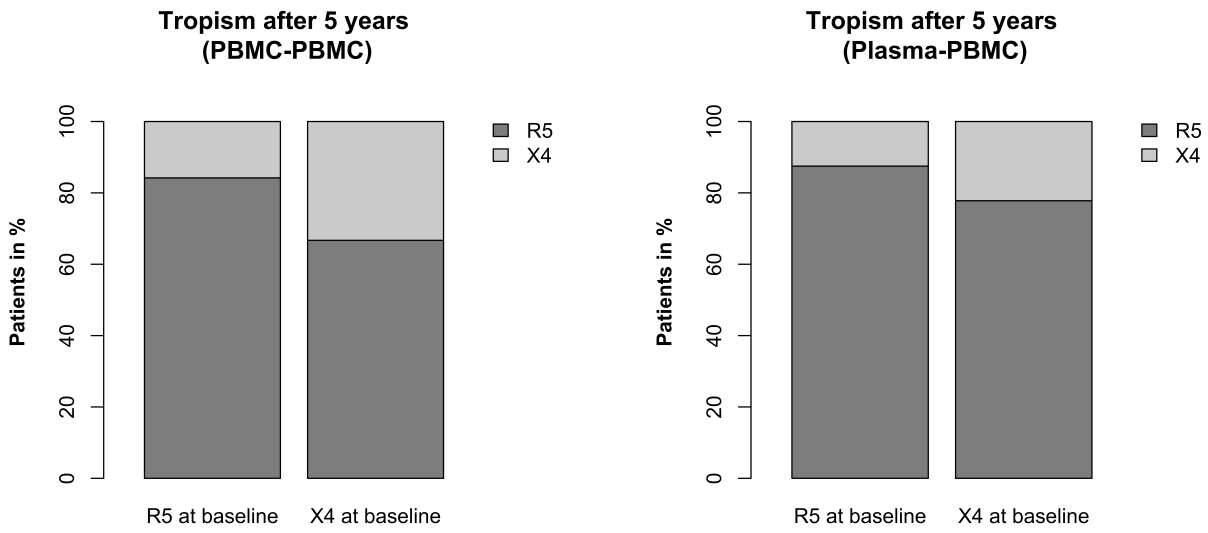
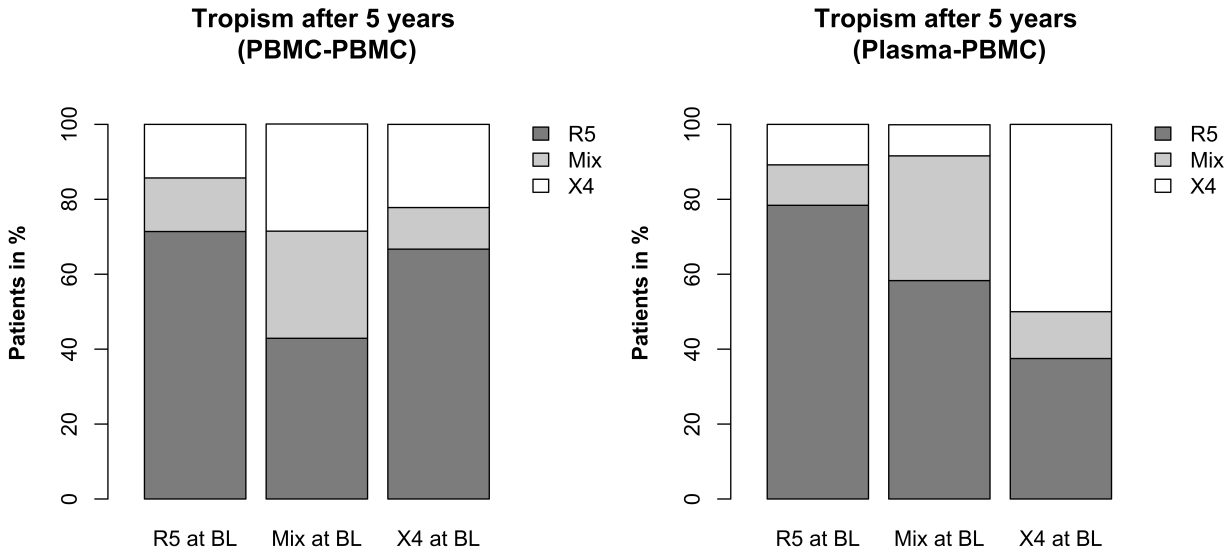

Tropism after 5 years
(PBMC-PBMC)
Patients in %
0
20
40
60
80
100
R5
X4
R5 at baseline
X4 at baseline
Tropism after 5 years
(Plasma-PBMC)
Patients in %
0
20
40
60
80
100
R5
X4
R5 at baseline
X4 at baseline

Figure 2.


Tropism after 5 years
(PBMC-PBMC)
Patients in %
0
20
40
60
80
100
R5 at BL
Mix at BL
X4 at BL
R5
Mix
X4
Tropism after 5 years
(Plasma-PBMC)
Patients in %
0
20
40
60
80
100
R5 at BL
Mix at BL
X4 at BL
R5
Mix
X4