Dynamics of HIV-1 Tropism During Immune Recovery Driven by Combination Antiretroviral Therapy

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Joëlle Bader
aus Holderbank SO

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Prof. Dr. Markus Affolter, Prof. Dr. rer. nat. Thomas Klimkait und Dr. Marcel Stöckle

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Prof. Dr. Jörg Schibler
Dekan der Philosophisch-
Naturwissenschaftlichen Fakultät

"To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science."

Albert Einstein

For Joël,
for my mother and father,
for my sister and brother.

AIDS	Acquired immunodeficiency syndrome
APOBEC3G/F	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 3G/F
BL	Baseline
C	Cysteine
cART	Combination antiretroviral therapy
CCR5	CC-motive chemokine receptor 5
CD	Cluster of differentiation
CXCR4	CXC-motive chemokine receptor 4
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
FPR	False positive rate
G	Glycine
G2P	Geno2Pheno
GALT	Gut associated lymphoid tissue
gp41	Glycoprotein 41
gp120	Glycoprotein 120
GPCR	G-protein coupled receptor
HET	Heterosexual
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-DR
IDU	Injecting drug use
kb	Kilo base
LTR	Long terminal repeat
MIP-1α	Macrophage inflammatory protein-1α
MIP-1β	Macrophage inflammatory protein-1β
MOTIVATE	Maraviroc versus Optimized Therapy in Viremic Antiretroviral Treatment-Experienced Patients
MSM	Men who have sex with men
MWM	Molecular weight marker
N	Asparagine
NGS	Next generation sequencing

NNRTI	Non-nucleoside reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PEP	Post-exposure prophylaxis
PI	Protease inhibitor
PrEP	Pre-exposure prophylaxis
R	Arginine
R5	Chemokine receptor CCR5
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RT-PCR	One-step reverse transcriptase PCR
S	Serine
SD	Standard deviation
SDF-1	Stromal derived factor-1
SHCS	Swiss HIV Cohort Study
SIV	Simian immunodeficiency virus
START	Strategic Timing of Antiretroviral Therapy
TBE	Tris borate EDTA
Tris	Trishydroxymethylaminomethane
V3	Variable loop 3
VL	Viral load
X4	Chemokine receptor CXCR4
%X4	Frequency of X4-tropic HIV variants

I. Abstract

HIV is characterized by the infection of CD4 T-cells and the accompanied attack of the immune system. Successful infection thus requires the binding to the CD4 receptor and a chemokine receptor, either CCR5 or CXCR4. In the early phase of infection the virus almost exclusively uses the CCR5-receptor. Only later during disease progression CXCR4-tropic viruses are found as majority in up to 50% of all patients. They associate with a more rapid CD4 T-cell loss and accelerated disease progression. The underlying causes of this change in the viral tropism are not known. As a significant percentage of patients only achieve a suboptimal CD4 T-cell recovery while treated, the viral tropism might play a role for such an incomplete immune response. The aim of this thesis was to follow the viral tropism dynamics, especially the abundance of CXCR4-tropic cell-associated viruses during phases of immune recovery by combination antiretroviral therapy with the intention to understand and help improving the immune situation during therapy. Patient samples from the Swiss HIV Cohort Study were used to asses the viral tropism before and after several years of treatment initiation. As during therapy free plasma virus was fully suppressed, analysis focus laid on integrated viral genomes. This work found that CXCR4-tropic viral variants are not inevitably associated with an impaired CD4 T-cell recovery as most of the viruses with a pre-treatment CXCR4 tropism presented a CCR5-tropism during therapy. Furthermore, it identified that the frequency of CXCR4-tropic viruses under therapy generally decreases and that therefore the proviral reservoir mostly persists of CCR5-tropic viruses. Based on the fact that all free viral variants were equally suppressed these findings suggest the involvement of the immune system, which appears to selectively target CXCR4-tropic infected cells. If a competent immune system were capable of preferentially controlling CXCR4-tropic viruses, this would also explain why these variants mostly appear later during disease when the damage of the immune system is accelerated. As a consequence, the findings discussed in this thesis support early therapy initiation as strategy to prevent the later occurrence of CXCR4-tropic HIV. Furthermore, remaining CCR5-tropic viruses may most effectively be suppressed by additional therapy options of CCR5-receptor antagonists.

II. Introduction

II. I. STATUS QUO ON HIV

With currently around 36.9 million people infected and 1.2 million HIV related deaths by the end of 2014 the burden of HIV in the world is still huge[1]. Nevertheless, the tremendous efforts that have been undertaken and have led to 15 million patients treated with combination antiretroviral therapy (cART) by the year 2015[1] have to be acknowledged. These improvements are not only owed to a more reliable availability of diagnostic testing and drug distribution worldwide but also to one of the main focuses in recent years – treatment as prevention[2]. Prevention mainly includes pre-exposure prophylaxis (PrEP), post-exposure prophylaxis (PEP) and cART for pregnant and breastfeeding women[2]. PrEP supports the daily intake of antiviral drugs for uninfected people at high risk for an infection and has been shown to protect against infection in different risk populations (e.g. serodiscordant couples or men who have sex with men (MSM))[2-4]. PEP includes the immediate intake of antivirals after exposure to HIV, protection against the establishment of an infection has been shown in studies including health care workers or victims of sexual assaults[5-7]. By the end of June the World Health Organization announced that Cuba, as one of the first countries worldwide, has eliminated mother-to-child transmission of HIV[8]. Together with other methods of prevention like male circumcision these all have contributed to prevent HIV transmission[2]. Despite these advances, however, the situation in developing countries, mainly in sub-Saharan Africa, looks different than in the Western countries. In Switzerland for example, data from 2012 show that over 90% of patients in care received cART and over 90% of those receiving cART were suppressed[9]. In contrast, a recently published survey from Kenya reported that only close to 40% of the enrolled patients received cART, and that of those also only 40% had a viral load (VL) below 1000 copies/mL[10]. In terms of HIV care cascades efforts have to improve substantially. This is also the reason why the United Nations initiated the goal of 90-90-90 by 2020[11]. Briefly, 90% of all infected people worldwide should know their status, 90% of them should have access to treatment, and 90% of these treated patients should be able to reach suppression[11]. While working towards this goal, which focuses more on the clinical side, the leading term now promoted on the research side is "eradication". HIV eradication appears to be extremely difficult to achieve, and to date only one case is known worldwide where indeed HIV seems to be cleared from a patient. In this special case known as "the Berlin patient" the person infected with HIV needed a stem cell

transplantation due to his leukemia[12]. A donor was searched possessing the rather rare homozygous delta32 mutation (<1% in the Caucasian population[13]) in the gene coding for one of the co-receptors HIV needs to infect a cell. After successful bone marrow transplantation the patient was not only cured from leukemia but is now lacking any sign of HIV or infected cells for years[12,14]. Aside from the special circumstances that probably allowed for a cure in this single case of HIV, two main features make it so hard to reach a cure. First, HIV integrates its own genome into the host T-cell genome, and secondly reverse transcriptase lacks proof reading, which results in a massive viral variability that cannot be controlled by the immune system of the host[15-17]. Therefore, to date most strategies for eradication include either mechanisms to activate latent infected cells and kill them, to excise the HIV genome of the host genome, or to develop broadly neutralizing antibodies that can cope for a large variety of virus variants[18-21]. A huge effort lies in all these research fields to contribute to a cure of HIV infection that not yet seems reachable. Additional ideas and maybe also different ways of looking at known paradigms are needed to highlight possible new ways of HIV control.

II.II. BACKGROUND ON HIV CO-RECEPTORS

HIV infection depends on the binding of the virus to the cell surface receptor CD4 and, in addition, a chemokine receptor[22]. This "co-receptor" is in most cases either the CCR5 (R5) or the CXCR4 (X4) receptor[23,24]. "CCR5" stands for CC-motif chemokine receptor 5 and "CXCR4" for the CXC-motif chemokine receptor 4[25,26]. Their names derive from the cysteine motif of the chemokine that can activate the receptor for signaling, both receptors belong to the family of the G-protein coupled receptors (GPCRs)[24-26]. The R5-receptor is stimulated by the chemokines MIP-1α (macrophage inflammatory protein-1α), MIP-1β (macrophage inflammatory protein-1β) and RANTES (regulated on activation, normal T-cell expressed and secreted), they all are involved in the attraction of leukocytes to sites of inflammation[26]. The ligands for the X4-receptor are SDF-1 (stromal derived factor-1) and MIF (macrophage migration inhibitory factor), again both involved in cell recruitment to inflammatory sites[25,27].

II.III. CHARACTERISTICS OF HIV CO-RECEPTORS IN DISEASE

HIV variants that infect cells via the R5-receptor (R5-tropic/R5-tropism) are macrophage tropic and are also called "non-syncytium inducing virus[28,29]. If, on the other hand, the virus uses the X4-receptor (X4-tropic/X4-tropism) it is either T-cell or macrophage tropic, and also called "syncytium inducing"[28-30]. A virus might also have the properties to infect a cell via both receptors (dual tropism), or there is furthermore the possibility that a host harbors different viruses that have different tropisms (mixed tropism)[28]. The most critical region in the HIV genome to bind to the co-receptor is called the variable loop 3 (V3) located in the glycoprotein 120 (gp120)[31]. A schematic drawing of how the virus binds to its receptor is shown in FIGURE 1. The V3 loop is generally 35 amino acids long, positions 11, 24, and 25 in the loop are especially critical as positively charged amino acids at these positions are strongly associated with an X4-tropism[32]. Also the loss of a glycosylation site within the loop is a determinant for X4-tropism[33]. It was recognized early that X4-tropic viruses tend to associate with a faster cell infection rate, show higher cytopathic effects, and induce extended cell fusion events (syncytia), whereas the R5-tropic viruses show slower progression rates and less cytopathology[34]. Therefore X4-tropic viruses were associated in many studies with an accelerated decline in CD4 T-cells and a faster disease progression[35,36]. However, whether X4-tropic viruses are the cause or the consequence of this disease state still needs to be elucidated.

FIGURE 1 Schematic drawing of receptor ligand interaction.

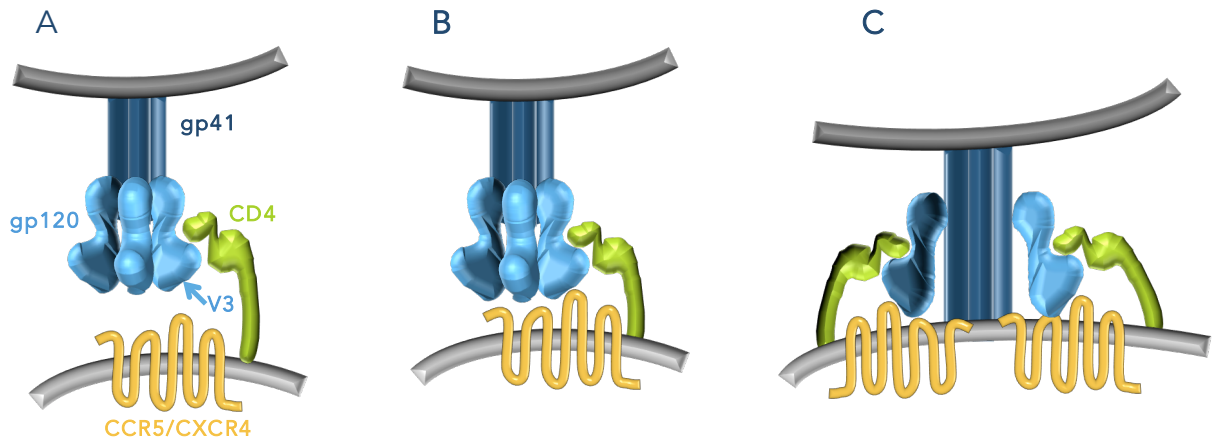

FIGURE 1 Schematic drawing of the binding of the CD4 receptor to gp120 and the V3 loop to the co-receptor. A) Binding of gp120 to the CD4 receptor. B) The V3 region binds to the co-receptor. C) Gp41 provides fusion of HIV with the cell membrane. Abbreviations: gp120, glycoprotein 120; gp41, glycoprotein 41; V3, variable loop 3.

Adapted from Moore et al. 2003[37]

Currently no drugs are approved for HIV therapy that target the X4-tropic viruses or the X4-receptor. There is, however, a drug on the marked called "maraviroc" that blocks the human R5-receptor[38,39]. Around one percent of the Caucasian population has a homozygous deletion in the gene coding for the R5-receptor, these individuals cannot be infected with an R5-tropic virus[13]. Additionally, up to 20% of this population have a heterozygous deletion that is associated with decelerated disease progression[13,40]. Blocking the R5-receptor, however, does not prevent the infection with X4-tropic viruses[13]. The consequences for the immune system if the R5-receptor is missing are not clear, however, it has been associated with a higher susceptibility to West-Nile Virus infection[41,42]. Maraviroc was approved only for the use on patients with R5-tropic viruses[43], therefore testing systems to determine the "tropism" of HIV were needed. Test systems, like Geno2Pheno, were developed to determine the tropism via genotyping by using the sequence information of the V3 loop[44]. Another possibility to determine the tropism is via a hybridization based approach to a probe of known tropism, this technique was developed in our lab and is called XTrack[45].

II.IV. ROLE OF CHEMOKINE RECEPTORS IN HIV INFECTION

Transmission of HIV mostly occurs through an R5-tropic virus[46], and X4-tropic virus variants are only rarely found during the early stages of disease[47,48]. The reason for the selective infection advantage of R5-tropic viruses is not entirely clear up to date and is rather seen as interplay between different barriers[49]. For example, one of these barriers is the female genital tract, cervical tissue is known to express mainly the R5- and not the X4-receptor[50]. Another example is the presence of SDF-1 (ligand for the X4-receptor) in the mucosal tissue that leads to a continuous down regulation of the X4-receptor and may therefore prohibit infection via this route[51]. There are also reports that suggest a more readily elimination of X4-tropic variants by the neutralizing antibody response based on their fewer glycosylation sites and lack of the so called glycan shield, which protects R5-tropic variants better from the recognition of the immune system[33,52-54]. R5-tropic variants have been shown to have a selective escape advantage during chronic infection as the viruses were glycosylated at specific sites known to prohibit antibody recognition[55]. Although the early dominance of infection via the R5-receptor, in up to 50% of patients X4-tropic variants appear later in disease, and most important in the absence of treatment[30,56,57]. The underlying mechanisms of such a change in the viral preferences is not well understood and most of the

observations on an increase in X4-tropic viruses were observed in treatment free situations[30,56,57]. However, how the viral tropism behaves in a treated situation is not well studied. Although the emergence of X4-tropic variants associates with a faster disease progression[35,36], the majority of patients still progress to immunodeficiency without showing an increase in X4-tropic variants[58]. Interestingly, there are studies that determined the viral tropism to identify patients eligible for R5-receptor antagonist treatment[59,60]. These patients were long-term treated, under a failing therapy regimen and the majority of them presented R5-tropic viruses at screening[59,60]. The exact contribution of the viral tropism, especially the X4-tropism, to the disease dynamics during antiviral therapy remains controversial. In the absence of clinically available X4-receptor antagonists for directly targeting X4-tropic viruses a better understanding is needed of whether and how X4-tropic viruses contribute to the progression of the disease.

II.V. LINK BETWEEN TROPISM AND CD4 T-CELL RESPONSE

It has been reported that even patients under potent combination antiretroviral therapy and fully suppressed free virus experience suboptimal CD4 T-cell recovery[61]. Determinants for such a CD4 T-cell impairment during fully suppressive therapy have been studied, and only higher age at time of infection as well as an advanced disease stage at diagnosis have been identified as significant associates[61]. As therapy was successful and did not associate with the different CD4 T-cell outcome either, it may be suggested that other factors are involved, such as viral properties and/or the immune system of the host. Viral properties such as the tropism have not been addressed in this context, but are known to initiate different immune responses mainly based on their distinctive glycosylation patterns[33,54,55]. As X4-tropism is associated with disease progression and a more rapid CD4 T-cell loss[35,36], it was the aim of this work to investigate the dynamics of the viral tropism during treatment and to address if the impaired CD4 T-cell response in some patients under therapy is affected by the viral tropism.

III. Results

III.I. TROPISM AND DISEASE PROGRESSION

III.I.I. AIM OF THE STUDY

HIV infection via chemokine receptor X4 correlates with faster disease progression, a more rapid decline in CD4 T-cells, and therefore earlier signs of AIDS related illnesses[35,36]. Furthermore, it has been clinically observed that in a substantial number of HIV infected individuals, even under suppressive cART, CD4 T-cell recovery is often not adequate[61]. The hypothesis is that this impaired CD4 T-cell response during therapy may associate with the viral tropism, considering that X4-tropism seems to associate with a poorer outcome of the disease[35,36]. The aim of this study was to substantiate a link between viral tropism and immunological outcome.

III.I.II. STUDY DESIGN AND SAMPLE SELECTION

To assess a possible association of viral tropism with immunological outcome patients from the Swiss HIV Cohort Study (SHCS) who had been under cART for more than five years and had no virological failure (VL during study period below 50 copies/mL) were included. Samples were grouped according to the increase in CD4 T-cells from therapy initiation (baseline, BL) to after five years on cART, into "responders" (n=44) with a delta above 400 CD4 T-cells or "incomplete responders" (n=44) where the CD4 T-cell delta remained below 400 cells. It was possible to choose a high CD4 T-cell delta since all patients were long-term infected and therefore had a low baseline CD4 T-cell count (median 174 cells/µL). Tropism analysis was performed on plasma near the time of cART initiation. All patients responded well to the antiviral treatment as all had a rapid viral load decline after cART initiation. Viral loads then remained below 50 copies/mL with no viral rebound for the entire study period (VL was routinely measured every quarter), which indicated that patients were adherent and the virus did not acquire resistances. cART was defined as a combination of at least three antiretroviral drugs, consisting of two nucleosidic reverse transcriptase inhibitors (NRTI) combined with either one or two protease inhibitors or with one non-nucleosidic reverse transcriptase inhibitor (NNRTI). None of the patients ever received CCR5 antagonists for therapy. For genotypic tropism determination Geno2Pheno (G2P; Max-Planck-Institute, Saarbrücken, Germany) and XTrack (InPheno AG, Basel, Switzerland) were used (see Methods for more details).

III.I.III. PATIENT CHARACTERISTICS

The patient population (n=88) had a mean age of 54.5 ± 10.9 years, 76% were male and 90% were white, 49% were MSM. The mean duration of infection was 18.7 ± 4.7 years [62], and 76% of the patients had started with cART prior to the year 2000. Baseline median CD4 T-cell count was 174 cells/µL, baseline CD8 cell count was 787 cells/µL, and baseline viral load was 5.0 $\log_{10}$ copies/mL. Based upon analysis at the follow-up time point 21% of the patients (17/81; seven were not analyzable) had a delta32 heterozygous genotype, none was homozygous.

In the incomplete responder group patients had overall initiated cART at a lower median CD4 T-cell count of 139 cells/µL vs. 220 cells/µL in the responder group (p=0.039). Thus, it was key to rule out a CD4-based bias in the findings. Therefore, a sub-analysis (n=38) of incomplete responders with high CD4 T-cell baseline count vs. complete responders with low CD4 T-cell baseline count was performed, indicating that a lower CD4 T-cell baseline count was not the primary cause of incomplete response (p=0.073).

Median CD8 cell count at baseline for incomplete responders was 861 cells/µL vs. 764 cells/µL (p=0.780) and median viral loads were 5.0 $\log_{10}$ copies/mL vs. 4.8 $\log_{10}$ copies/mL (p=0.152). Demographic characteristics revealed a longer but not significant duration of infection[62] for the incomplete responders with 19.6 vs. 17.8 years (p=0.106). The relative occurrence of a delta32 heterozygous genotype was similar in both groups (10 and 7 cases, respectively (p=0.446)). Baseline characteristics are summarized in TABLE 1.

There was no statistically significant difference in the first line regimen between the two patient groups, for 90% the primary regimen consisted of a protease inhibitor with an NRTI backbone.

TABLE 1 Baseline characteristics for 88 HIV-1 infected, treatment-naïve patients.

Characteristic	All patients (n=88)	All patients, by delta CD4 T-cell response		P
		Responders (n=44)	Incomplete responders (n=44)	
Sex				
Male	67 (76.1)	30 (68.2)	37 (84.1)	0.080
Female	21 (23.9)	14 (31.8)	7 (15.9)	
Age, mean years ± SD	54.5 ± 10.9	52.1 ± 10.9	56.9 ± 10.5	0.019
Ethnicity				0.239
White	79 (89.8)	39 (88.6)	40 (90.9)	
Black	5 (5.7)	3 (6.8)	2 (4.6)	
Hispanic	2 (2.3)	2 (4.6)		
Asian	2 (2.3)		2 (4.6)	
HIV transmission				0.610
Blood	1 (1.1)	1 (2.3)		
MSM	43 (48.9)	20 (45.5)	23 (52.3)	
HET	31 (35.2)	17 (38.6)	14 (31.8)	
IDU	11 (12.5)	6 (13.6)	5 (11.4)	
Other	1 (1.1)		1 (2.3)	
Not available	1 (1.1)		1 (2.3)	
Age at infection, mean years ± SD	36.1 ± 11.7	34.5 ± 12.4	38 ± 10.6	0.073
Duration of infection, mean years ± SD	18.7 ± 4.7	17.8 ± 4.7	19.6 ± 4.7	0.106
CDC Stage				
C	24 (27.3)	12 (27.3)	12 (27.3)	1.000
cART initiation				
before 2000	67 (76.1)	32 (72.7)	35 (79.5)	0.453
after 2000	21 (23.9)	12 (27.3)	9 (20.5)	
Baseline HIV RNA load, log_{10} copies/mL	5.0 (4.3-5.3)	4.8 (4.2-5.2)	5.0 (4.5 -5.5)	0.152
Delta CD4 T-cell count, cells/μL	399 (235-579)	579 (499-705)	235 (192-308)	<0.001
Baseline CD4 T-cell count, cells/μL	174 (91-259)	220 (125-282)	139 (56-234)	0.039
Baseline CD8 T-cell count, cells/μL	787 (459-1132)	764 (473-1126)	861 (444-1132)	0.780
Delta32 genotype				
heterozygous	17 (21.0)	10 (24.4)	7 (17.5)	0.446
wild type	64 (79.0)	31 (75.6)	33 (82.5)	

Data are presented as No. (%), median or IQR values unless otherwise indicated. Categorical data were compared by means of Chi-square test, whereas continuous data were compared by Mann-Whitney-Wilcoxon test. Abbreviations: SD, standard deviation; MSM, men who have sex with men; HET, heterosexual; IDU, injecting drug users; CDC, Centers for Disease Control and Prevention.

III.I.IV. SAMPLE ANALYSIS

A total of 88 baseline (BL) profiles were available for comparison. Overall 44 were responders and 44 were incomplete responders according to the patients' delta CD4 T-cell count after five years of follow-up. Eighty-one of the 88 samples (92%) had concordant results between G2P and XTrack.

III.I.V. CD4-RESPONSE - CORRELATING BASELINE TROPISM WITH IMMUNOLOGICAL OUTCOME

The mean timespan between baseline analysis and time point of follow-up was 5.2 ± 0.6 years. For 84 patients a tropism assignment was successful using the online tool G2P. The "False Positive Rate" cutoff was set to 5%. For four patients G2P yielded no result due to the presence of a mixed virus population. This is the case when the sequencing electropherogram shows peaks that mask each other and give no clear result at a given position. G2P predicted 69 patients (82.1%) to carry an R5-tropic virus and 15 (17.9%) to carry an X4-tropic virus, considering that 88% of samples had subtype B determined by G2P. 60% of the patients (9/15) with X4-tropic viruses and 46.4% (32/69) with R5-tropic viruses were in the incomplete responder group (p=0.339). Data are shown TABLE 2. In addition, XTrack assigned mixed virus populations in a given specimen. In the incomplete responder group 42% of the patients (21/50) harbored R5-tropic viruses, 64% (16/25) a mixed virus population, and 53.8% (7/13) X4-tropic viruses (p=0.190), as shown in TABLE 2.

TABLE 2 Tropism analysis at baseline for 88 HIV-1 infected, treatment naïve patients.

		All patients, by CD4 T-cell response		
Method	All patients (n=84)	Responders (n=43)	Incomplete responders (n=41)	P
Geno2Pheno (FPR 5%)				
R5-tropic	69 (82.1)	37 (86.0)	32 (78.0)	0.339
X4-tropic	15 (17.9)	6 (14.0)	9 (22.0)	
Geno2Pheno (FPR 10%)				
R5-tropic	65 (77.4)	34 (79.1)	31 (75.6)	0.705
X4-tropic	19 (22.6)	9 (20.9)	10 (24.4)	

		All patients, by CD4 T-cell response		
Method	All patients (n=88)	Responders (n=44)	Incomplete responders (n=44)	P
XTrack				
R5-tropic	50 (56.8)	29 (65.9)	21 (47.7)	0.190
X4-tropic	13 (14.8)	6 (13.6)	7 (15.9)	
Mixed tropic	25 (28.4)	9 (20.5)	16 (36.4)	

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

III.I.VI. CONTRIBUTION OF THE DELTA32 GENOTYPE

According to G2P of the analyzed 17 patients (21%) with a heterozygous delta32 genotype two (11.8%) had a mixed viral sequence, nine (52.9%) had an R5-tropism and six (35.3%) an X4-tropism at baseline (see FIGURE 2). The portion of X4-tropic viruses in this subpopulation of patients with a delta32 genotype was higher than in the general patient population (p=0.0135), but patients with R5-tropic viruses still accounted for the majority with a heterozygous delta32 deletion.

FIGURE 2 *Tropism distribution for patients with heterozygous delta32 genotype.*

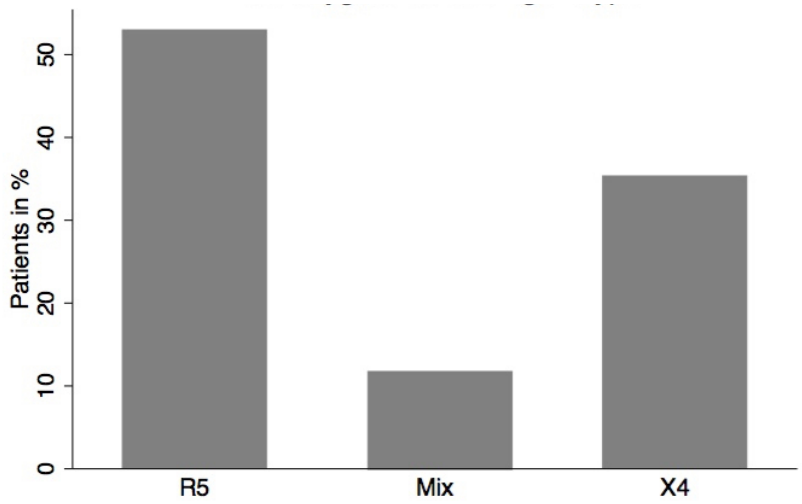

FIGURE 2 G2P *tropism analysis for the 17 patients with a heterozygous delta32 genotype. Abbreviations: R5, R5-tropic HIV; Mix, mixed sequence; X4, X4-tropic HIV.*

III.II. PREFERENTIAL SUPPRESSION OF CXCR4-TROPIC HIV-1 UNDER CART

III.II.I. AIM OF THE STUDY

In the previous study a possible association of tropism and immunological outcome was assessed. As the tropism determination was years before the impaired CD4 T-cell recovery was established and X4-tropic variants did not significantly associate with this impairment, a subsequent study was needed to assess the tropism in the proviral reservoir at the time point where the CD4 T-cell impairment was established. Aim of this subsequent study was to compare the tropism determination in free plasma virus with provirus over time and to investigate if the viral tropism is constant during therapy.

III.II.II. STUDY DESIGN AND SAMPLE SELECTION

To study the tropism dynamics during the time of therapy the same study population as described in chapters III.I.III and TABLE 1 was used. Since all patients were under successful treatment they had no free virus in the plasma, rendering its analysis at this later time point during therapy impossible. Therefore, tropism analysis had to be done from integrated virus (provirus) on peripheral blood mononuclear cells (PBMC). The sample set was extended by inclusion of PBMC samples from the same patients at the time point they had been on treatment for five years. This was the same time point from which also the data on CD4 T-cell counts for grouping of the patients in the first study stemmed. It was assumed that a bias would be introduced by comparing data from plasma at baseline and PBMCs at follow-up. Therefore, also PBMC samples from the baseline time point were included to compare the viral tropism in the different compartments. Genotypic tropism determination was again performed with Geno2Pheno (Max-Planck-Institute, Saarbrücken, Germany) and XTrack (InPheno AG, Basel, Switzerland) (see Methods for more details).

III.II.III. SAMPLE ANALYSIS

Corresponding baseline data sets of plasma virus (PL) and provirus (PBMC) were available for 39 patients. Tropism analysis with G2P was largely concordant for virus and provirus at baseline in 90% of them (35/39). In order to follow a possible evolution of the viral tropism from before therapy to five years after therapy initiation, proviral tropism data from PBMCs at baseline (BL-PBMC) were compared with tropism data from PBMCs at follow-up (5Y-PBMC) (n=50 sample sets). For comparison, the tropism

data from the five year follow-up PBMC samples (5Y-PBMC) were compared with the corresponding baseline plasma samples (BL-Plasma) (n=61 sample sets). See FIGURE 3 for clarification.

FIGURE 3 *Sample set distribution.*

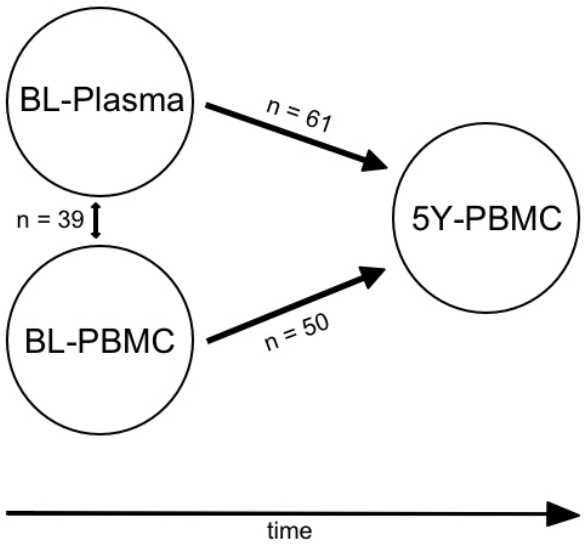

FIGURE 3 Distribution of the samples between different compartments and time points. *Abbreviations: BL, baseline; PBMC, peripheral blood mononuclear cells; 5Y, after five years of therapy.*

III.II.IV. PROVIRAL TROPISM AT BASELINE AND ITS EVOLUTION AFTER FIVE YEARS OF THERAPY

For the comparison of proviral deoxyribonucleic acid (DNA) in PBMC at baseline and five years after (BL-PBMC/5Y-PBMC) a complete data set (G2P and XTrack) was available from 50 patients. Five patients had no G2P result due to mixed viral sequences and one had no XTrack result due to an inconclusive result. With 39 of 45 analyzed samples (86.7%), the majority of patients carried R5-tropic viruses at baseline. Thirty-three of these 39 patients (84.6%) still had an R5-tropic virus at follow-up, and in the remaining six patients the viral tropism had changed to X4. Of the six patients with X4-tropic virus at baseline, four (66.7%) had changed to R5-tropism at follow-up. G2P data are summarized in FIGURE 4A.

The XTrack analysis was used to address especially mixed virus populations: Among the 28 patients with initially R5-tropic HIV, four cases (14.3%) presented a mixed virus population at follow-up. Among the nine patients with a mixed population at baseline, the mixed tropism remained stable in two cases (22.2%) after 5 years. A tropism change to R5 occurred in four patients (44.5%), and in three cases (33.3%) the later

samples revealed solely X4-tropic virus. For the 12 patients with X4-tropic virus at baseline, only one (8.3%) changed to a mixed tropism. Data are summarized in FIGURE 5A.

FIGURE 4 G2P analyzed tropism changes during therapy.

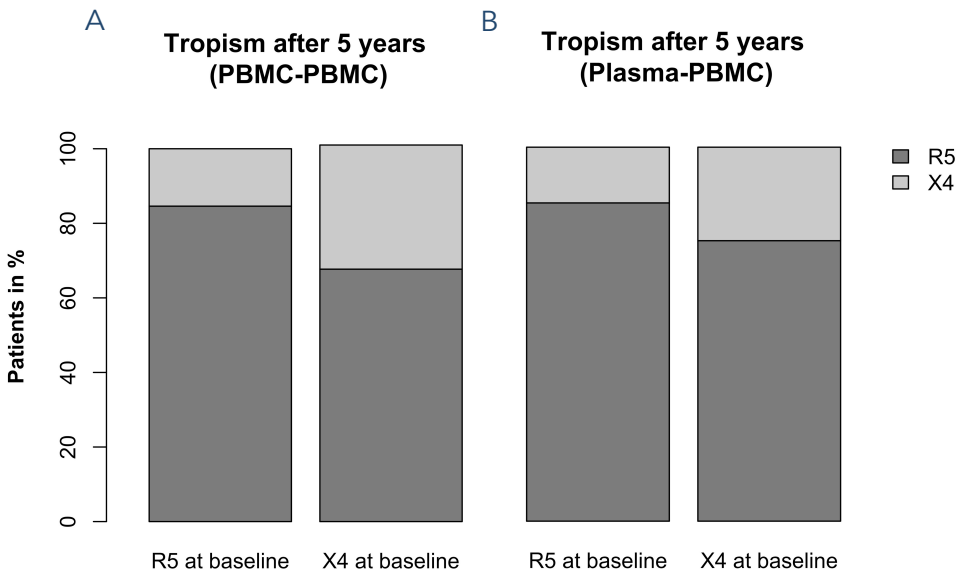

FIGURE 4 Geno2Pheno analysis between compartments and time points. Geno2Pheno (FPR 5%) results comparing tropism between (A) the proviral compartments (n=45) and (B) free virus (plasma) at baseline with provirus (n=55) after five years of therapy for patients with R5- (left panel) or X4-tropic (right panel) HIV at baseline. Abbreviations: PBMC, peripheral blood mononuclear cell; BL, baseline; R5, R5-tropic HIV; X4, X4-tropic HIV.

III.II.V. TROPISM IN PLASMA VIRUS AT BASELINE AND COMPARISON TO PROVIRAL TROPISM AFTER FIVE YEARS OF THERAPY

In the data set, 61 patients had a G2P and XTrack baseline plasma tropism result available as well as a corresponding follow-up proviral tropism result from PBMC. The comparison of the two different compartments was possible due to the observed good concordance of 90% between plasma and PBMC samples at baseline (see chapter III.I.IV). Six had again no G2P result due to mixed viral sequences, and one had no XTrack result due to inconclusive results. At baseline R5-tropic viruses were identified in 47 of the remaining 55 patient samples (85.5%), of which 40 (85.1%) were still R5-tropic at follow-up. Among the eight X4-tropic virus samples at baseline the tropism had changed in six patients (75%) to R5 at follow-up. G2P data are summarized in FIGURE 4B. XTrack was again used to examine mixed virus populations, among the 36 R5-tropic samples (60%) from baseline, four (11.1%) changed to a mixed population. For 17 patients with a mixed virus population at baseline, HIV changed to become R5-

tropic in nine cases (52.9%), two (11.8%) became X4-tropic, and in six (35.3%) cases the viral tropism remained mixed. Among the seven patients with an X4-tropic virus at baseline, one (14.3%) changed to a mixed population. The results are shown in FIGURE 5B.

Considering the excellent concordance observed between baseline plasma and PBMC samples (in 90% of all cases) the mean percentage of samples changing from an X4-tropism at baseline to R5 at follow-up was calculated, regardless of the origin, plasma or PBMC. Thus in 70.9% of all samples with an X4-tropism at baseline the tropism changed to R5 at follow-up in the G2P analysis, whereas only 15.2% changed from R5 to X4-tropism.

FIGURE 5 XTrack analyzed tropism changes during therapy.

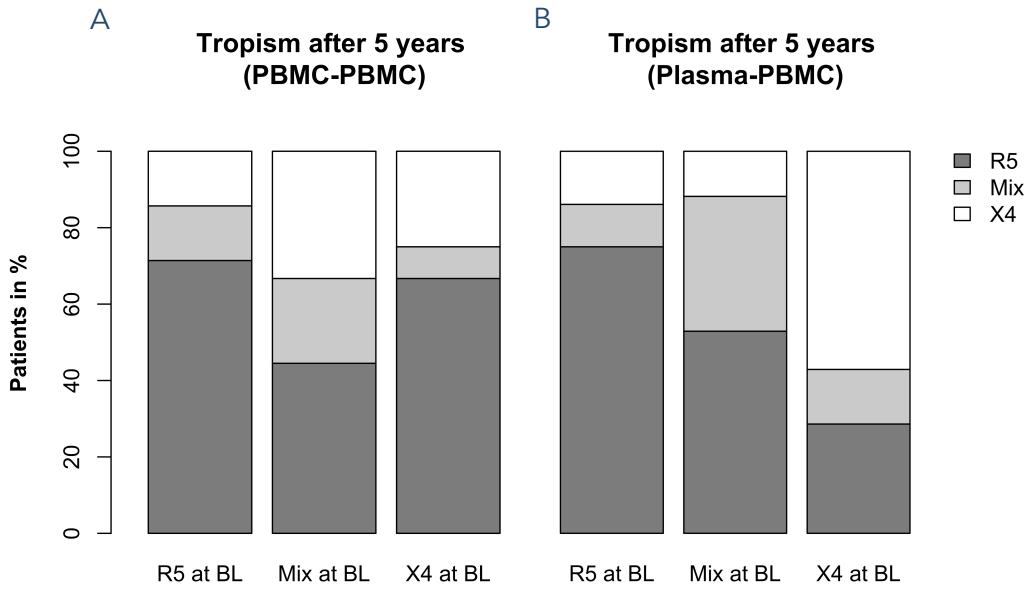

FIGURE 5 XTrack analysis between compartments and time points. XTrack results comparing tropism between (A) the proviral compartments (n=49) and (B) free virus (plasma) with provirus (n=60) after five years of therapy for patients with R5- (left panel), mixed- (middle panel) or X4-tropic HIV (right panel) at baseline. Abbreviations: PBMC, peripheral blood mononuclear cell; BL, baseline; R5, R5-tropic HIV; X4, X4-tropic HIV.

III.II.VI. BULK SEQUENCING ANALYSIS OF VIRUS ENVELOPES WITH TROPISM DIFFERENCES OVER TIME

For the majority of X4-tropic viruses that presented an R5-tropism determination after five years several random nucleotide changes in the V3 loop sequence were observed. In contrast, for most R5-tropic viruses switching to X4-tropism, one or two nucleotide changes were sufficient to yield the X4-tropism. Most frequent were amino acid

changes from Serine (S), Glycine (G), or Glutamine (E) to Arginine (R). Those occurred at positions 11, 24, or 25 of the V3 loop. Also about half of the affected viruses lost a potential N-linked glycosylation site. Representative amino acid sequences of the V3 loop for a patient that changed the viral tropism from R5 to X4-tropism and vice versa are shown in FIGURE 6.

FIGURE 6 *AMINO ACID CHANGES IN THE V3 LOOP DURING THERAPY.*

FIGURE 6 Amino acid sequence of the V3 loop for two patients. Patient A had an R5-tropic variant before therapy that changed to an X4-tropic variant under therapy. Patient B changed from an X4-tropic variant before therapy to an R5-tropic variant under therapy. Glycosylation sites are marked in orange, changed amino acids in the follow-up sequence are marked in yellow. X4-tropic variants are known often to have a charged amino acid (like R) at positions 11 and/or 24/25, these positions are framed by a black box. *Abbreviations: R5, R5-tropic HIV; X4, X4-tropic HIV.*

III.III. cART DRIVEN IMMUNE RECOVERY FAVORABLY TARGETS CXCR4-TROPIC HIV-1

III.III.I. AIM OF THE STUDY

In early HIV infection the majority of patients carry HIV strains with R5-tropism[46]. Only later in the disease, along with a deterioration of the immune situation, the proportion of X4-tropic virus isolates in the circulation starts to rise[30,56,57]. Reasons for this change in the viral preferences are not known. The previous findings on bulk sequence analysis identified that most patients with an X4-tropism before therapy changed to R5-tropism five years later, which suggests a preferential targeting of X4-tropic variants under therapy. The aim of this study was to monitor in detail the frequency of X4-tropic variants during phases of immune recovery by administration of cART through next generation sequencing (NGS).

III.III.II. STUDY DESIGN AND SAMPLE SELECTION

Seventy PBMC samples from 35 patients from the Swiss HIV Cohort Study prior to and 4 years post cART initiation were included. Patients had to be in the chronic infection phase with low CD4 T-cell count at therapy initiation (median CD4 T-cell count 180 cells/µL). Patients needed to present throughout undetectable virus load under therapy (measured quarterly) with a good CD4 T-cell response (delta CD4 T-cells above 200 cells/µL in four years of therapy). The first line regimen consisted of a combination of at least three antiretroviral drugs, either three NRTIs or two NRTI combined with one or two protease inhibitors, or with one NNRTI. None of the patients ever received R5-receptor antagonists for therapy.

Tropism analysis was performed on PBMC samples with Geno2Pheno$_{454}$ (Max-Planck-Institute, Saarbrücken, Germany) on sequences obtained by a MiSeq benchtop sequencer (Illumina, San Diego, California) (see Methods for more details).

III.III.III. PATIENT CHARACTERISTICS

Thirty-five patients were included in the study with a total of 70 proviral samples. The patient population had a mean age of 49.6 ± 8.7 years, 68.6% were male, and 91.4% were white; 45.7% were MSM. Median baseline CD4 T-cell count was 180 cells/µL, and the median delta CD4 T-cell count was 459 cells/µL. Median viral load at baseline was 5.1 log_{10} copies/mL. Four of the 35 patients (11.4%) had a delta32 heterozygous genotype. Baseline characteristics are summarized in TABLE 3.

First line regiment included an NRTI backbone either with one protease inhibitor (n=19, 54.3%), two protease inhibitors (n=8, 22.9%), one NNRTI (n=7, 20%), or was exclusively NRT inhibitor based for one patient.

TABLE 3 Baseline characteristics for the 35 HIV-1 infected, treatment-naïve patients.

		All patients, by %X4		
Characteristics	All patients (n=35)	%X4 decrease (n=28)	%X4 increase (n=7)	P
Sex				
Male	24 (68.6)	19 (67.9)	5 (71.4)	0.856
Female	11 (31.4)	9 (32.1)	2 (28.6)	
Age, mean years ± SD	49.6 ± 8.7	49.5 ± 8.8	50 ± 8.8	0.741
Ethnicity				0.210
White	32 (91.4)	26 (92.8)	6 (85.7)	
Black	1 (2.9)		1 (14.3)	
Hispanic	1 (2.9)	1 (3.6)		
Asian	1 (2.9)	1 (3.6)		
HIV transmission				0.535
MSM	16 (45.7)	12 (42.9)	4 (57.1)	
HET	15 (42.9)	12 (42.9)	3 (42.9)	
IDU	4 (11.4)	4 (14.2)		
Baseline HIV RNA load, log_{10} copies/mL	5.1 (3.8-6.4)	5.0 (4.2-6.0)	5.5 (5.3 -6.4)	0.016
Baseline proviral load, log_{10} c/10^6 PBMCs	3.8 (2.4-5.5)	3.8 (2.4-5.1)	4.1 (3.7-5.5)	0.137
Delta CD4 T-cell count, cells/µL	459 (222-853)	434 (222-853)	463 (243-659)	0.550
Baseline CD4 T-cell count, cells/µL	180 (7-511)	187 (9-511)	91 (7-242)	0.174
Baseline CD8 T-cell count, cells/µL	756 (165-1595)	843 (165-1595)	652 (266-1288)	0.403
Delta32 genotype				
heterozygous	4 (11.4)	4 (14.3)		0.288
wild type	31 (88.6)	24 (85.7)	7 (100.0)	

Data are presented as No. (%), median (min-max) values unless otherwise indicated. Abbreviations: %X4, frequency of X4-tropic HIV variants; SD, standard deviation; MSM, men who have sex with men; HET, heterosexual; IDU, injecting drug users; PBMC, peripheral blood mononuclear cells.

III.III.IV. SAMPLE CHARACTERISTICS

For the 70 samples the median read size was 45'833 reads per sample with a median variant count per sample of 354.

At an FPR of 3.5% X4-tropic HIV-1 variants were identified in all samples except one. This sample contained X4-tropic variants with an FPR threshold of 5%, which is considered a reasonable cut-off for X4-tropic viruses using bulk sequencing. By applying the cut-off of 2%, as suggested by Swenson et al.[63], all samples with a percentage of X4-tropic viruses below 2% were categorized as R5-tropic. With this rule 40 samples (57.1%) were solely R5-tropic, and 30 (42.9%) contained at least small fractions of X4-tropic viruses. The determined proportion of X4-tropic viruses with NGS in this patient population was higher than in the previous one ($p<0.001$) (compare with TABLE 2). The mean percentage of X4-tropic variants in the 40 samples with a sole R5-tropism assignment was 0.13% (min: 0%, max: 0.91%). This low percentage is in good accordance with observations by Swenson et al., suggesting that by ultra deep sequencing X4-tropic viruses can be found in virtually every patient[63]. This is important because only patients with a tropism test negative for X4-tropic viruses get access to an R5-receptor antagonist[43]. However, also this NGS analysis has shown that X4-tropic variants are present in almost all samples. In contrast the mean percentage of R5-tropic variants in the 30 mainly X4-tropic samples assigned as X4-tropic was 47.25% (min: 0%, max: 97.89%) ($p\leq0.001$). The max value of 97.89% means that in this case the 2% cut-off rule led to an X4-tropism assignment although 97.89% of all variants in this patient were R5-tropic according to the FPR.

III.III.V. X4-TROPIC HIV FREQUENCY CHANGES UNDER THERAPY

With regards to relative frequencies the proviral X4-tropic HIV-1 variants decreased or remained stable over time in the majority of patients (28 of the 35 patients, 80%, $p<0.001$). It increased in 7 patients (20%). For every one of these seven patients with an increase in the frequency of X4-tropic HIV variants (%X4) over time, one single provirus variant emerged in cells during suppressive therapy, and eventually became solely responsible for the increase in %X4. In six out of seven cases the emerging variant had been present as a minority already prior to therapy with a mean abundance of 1.95% (min: 0.02%, max: 6.99%) of the total proviral population. Only in one case the emerging X4 variant had not been detectable prior to therapy initiation.

When applying more stringent FPR thresholds 74.3% (for FPR 5%) and 71.4% (for FPR 10%) of the patients showed decreasing or stable frequencies of proviral X4-tropic HIV-1 variants over time. Interestingly, in patients who experienced a decline of viral X4 populations, this reduction occurred in a slow gradual process (mean change of 28.33%), whereas in all cases with X4 persistence the increase of X4-tropic viruses occurred more rapidly and towards exclusive X4 representation in the viral population (mean change 62.09%, $p<0.001$).

The univariate analysis of the baseline characteristics, which could have an influence on the increase in %X4, revealed that the only associating parameter was a higher viral load ($p=0.016$) at therapy initiation for patients with an increase in %X4 (see TABLE 3).

III.III.VI. ASSOCIATION WITH THE DELTA32 DELETION IN THE R5-GENE

Thirty-one patients were homozygous for the wild type R5 gene, four patients (11.4%) carried a heterozygous delta32 genotype, and none was homozygous for the delta32 mutation. The virologic outcome did not differ between these groups: All patients with increasing %X4 carried the wild type R5 gene ($p=0.288$).

III.III.VII. ASSOCIATION WITH PROVIRAL DNA LOAD

Proviral DNA loads were assessed for each sample to confirm the previously reported provirus decline under successful antiretroviral therapy[64]. In 80% of the patients in this study decreasing proviral loads were noted under therapy (see FIGURE 7). Furthermore, an increase in proviral load under therapy was not associated with a simultaneous increase in %X4 ($p=0.343$).

FIGURE 7 *Proviral load changes during therapy.*

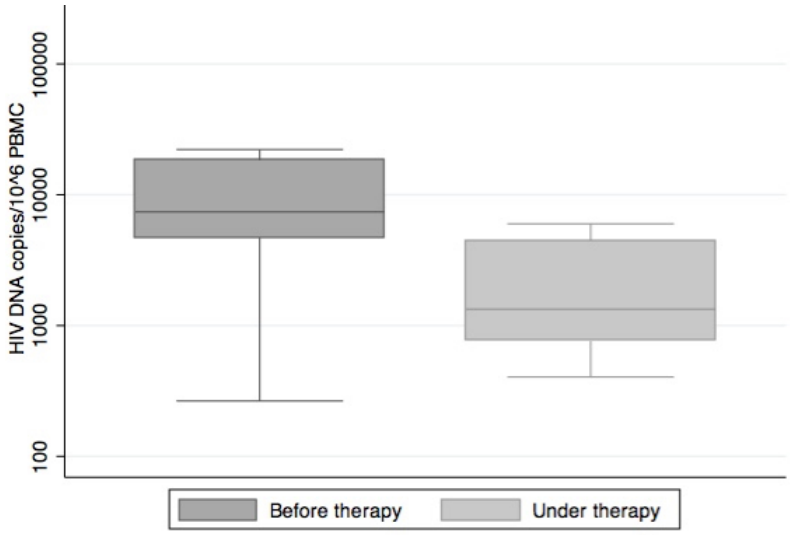

FIGURE 7 HIV proviral load in copies/10^6 PBMC for the patient population before and under therapy. *Abbreviations: PBMC, peripheral blood mononuclear cell; BL, baseline; R5, R5-tropic HIV; X4, X4-tropic HIV.*

III.III.VIII. MOLECULAR CHARACTERISTICS - GLYCOSYLATION PATTERNS, EVOLUTION AND DIVERSITY OVER TIME

The mean percentage of the most prevalent variant of all the present variants in a sample was 52.7% for those samples where an X4-tropic variant was most prevalent, and 61.2% for samples with a most prevalent R5-tropic variant (p=0.148).

A sample with X4-tropism assignment contained on average five viral variants with a frequency above 2% of the total viral population compared to four variants in the R5-tropic samples (p=0.037). The samples with either tropism contained on average two variants with a frequency higher than 10% (p=0.183). In 60% of cases with an R5-tropism the most prevalent variant did not change in the follow-up sample. This was different in samples with X4-tropism, for which 73.3% presented a different most prevalent variant in the follow-up sample (p=0.050).

Considering only the predominant variants in samples with decreasing %X4 it was found that 67% of these samples did not have an N-linked glycosylation site. In samples with increasing %X4, 57% lacked an N-linked glycosylation site (p=0.697).

During therapy more than half of the patients (57.1%) showed evidence for a marked sequence evolution in their proviruses, and in 21 patients (60%) the virus developed a markedly greater diversity over time and therapy. In contrast, an increase in %X4 was not associated with a similar increase in diversity (p=0.490) or evolution (p=0.523). Phylogenetic trees were used for visualizing the genetic dynamics under therapy.

FIGURE 8 shows a representative phylogenetic tree for the patient group with decreasing %X4 and decreasing diversity. FIGURE 9 depicts a typical virus profile for a patient with an increase in %X4 that is characterized by the emergence of a single virus variant. As for some patients NGS data were also available for an intermediate time point (see chapter III.III.IX), the included bar chart in FIGURE 8 and FIGURE 9 shows the tropism frequency change over the years with an additional time point for the better understanding of the tropism dynamics over the years with therapy.

III.III.IX. ANALYSIS ON EIGHT LONGITUDINALLY FOLLOWED PATIENTS

For eight patients NGS data at three consecutive time points before therapy initiation and at three time points thereafter were analyzed. They were included as a control group to observe if an increase in %X4 before therapy can be confirmed as reported in literature[30,56,57]. In six of them (75%) the proviral load increased before therapy initiation. Four patients with initially R5-tropic viral variants (%X4 below 2%) had the same viral tropism over all follow-up time points. Three of the four patients (75%) with a majority of X4-tropic viral variants experienced an increase in %X4 before therapy as expected.

FIGURE 8 Phylogenetic trees for all detected V3 variants in a patient with decreasing %X4.

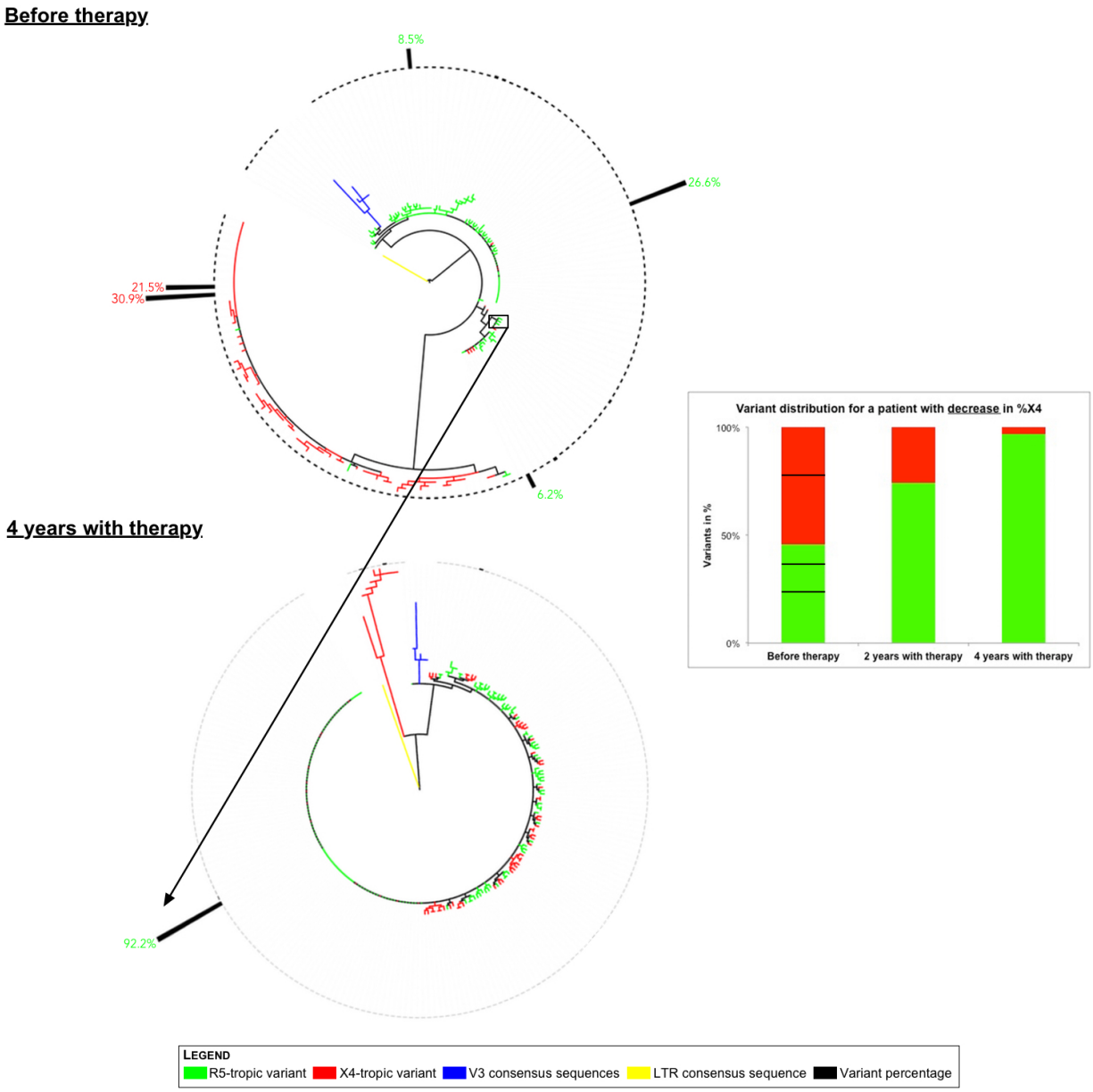

FIGURE 8 Phylogenetic trees with percentage of variant frequency before and with therapy for all present V3 variants for a representative patient with decreasing %X4 and decreasing diversity. The green leaves represent R5-tropic HIV-variants and the red leaves X4-tropic variants. Blue leaves represent the consensus V3 sequences of all subtypes, subtype A, B and C. The individual yellow leaf represents the consensus HIV-1 subtype B LTR sequence used for rooting. The height of the black bars indicates the frequency percentage of a variant in the whole sequence pool. Main variants are marked with a percentage number, colored according to their tropism. The variant that represents the majority under therapy is marked with a square before therapy. The separate bar chart represents the change in tropism frequencies counting in all variants over time. Abbreviations: V3, variable loop 3; LTR, long terminal repeat.

FIGURE 9 Phylogenetic trees for all detected V3 variants in a patient with increasing %X4.

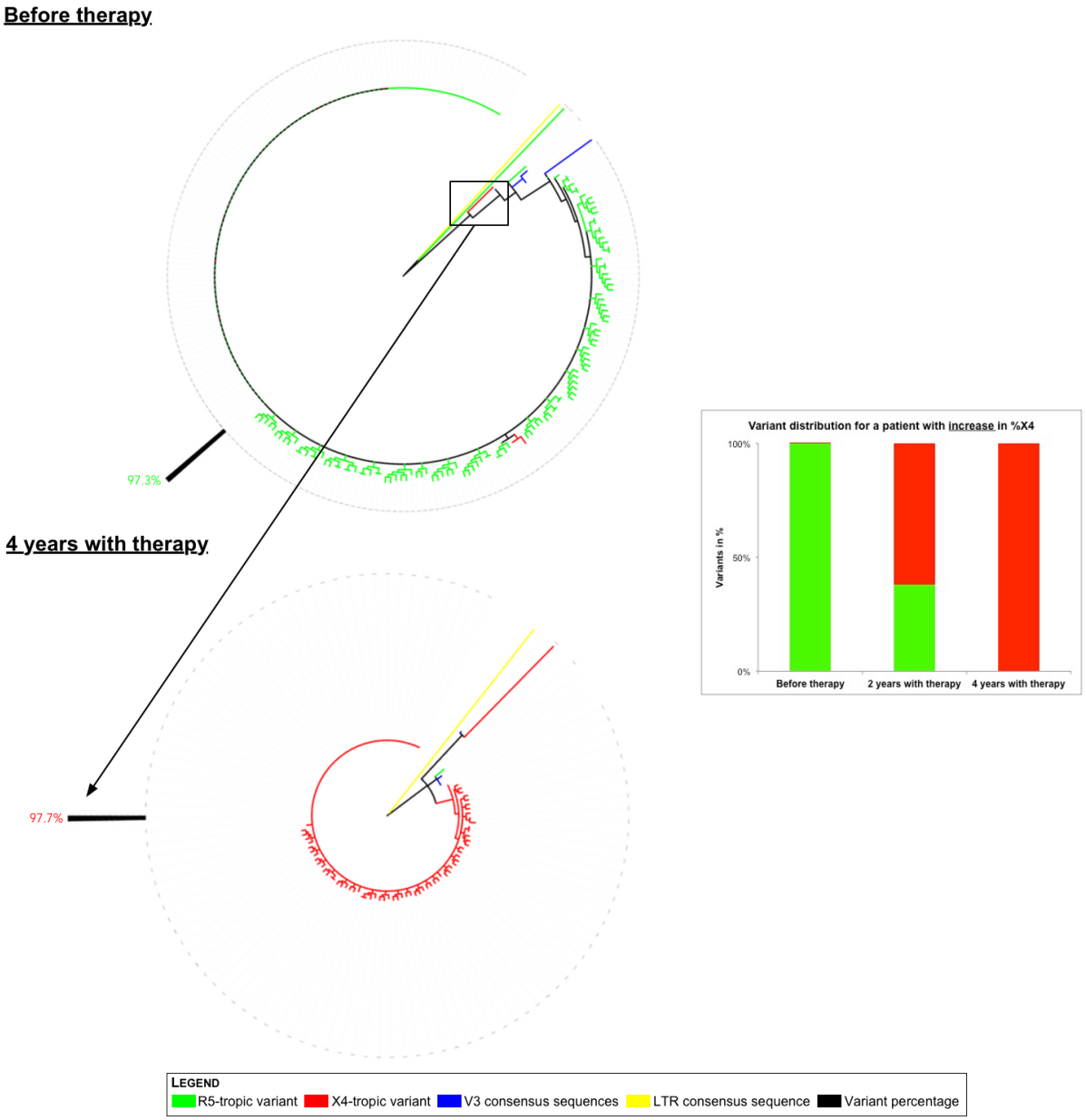

FIGURE 9 Phylogenetic trees with percentage of variant frequency before and with therapy for all present V3 variants for a representative patient with increasing %X4 and decreasing diversity. The green leaves represent R5-tropic HIV-variants and the red leaves X4-tropic variants. Blue leaves represent the consensus V3 sequences of all subtypes, subtype A, B and C. The individual yellow leaf represents the consensus HIV-1 subtype B LTR sequence used for rooting. The height of the black bars indicates the frequency percentage of a variant in the whole sequence pool. Main variants are marked with a percentage number, colored according to their tropism. The variant that is responsible for the outgrowth is marked with a square before therapy. The separate bar chart represents the change in tropism frequencies counting in all variants over time. Abbreviations: V3, variable loop 3; LTR, long terminal repeat.

IV. Discussion

It is well established in the course of untreated disease that X4-tropic HIV-1 associates with disease progression[30,56,57]. The mechanism of how the X4-tropism contributes to disease progression is still not well understood, and in the context of antiretroviral therapy the validity of this link has not yet been demonstrated. The paper by Kaufmann et al. has described an impaired recovery of CD4 T-cells despite the successful suppression of HIV by cART[61]. Especially as X4-tropic viruses are known to associate with faster CD4 T-cell decline[35] it was addressed whether a tropism determination prior to the initiation of cART would enable the prediction of a poorer immune recovery. Sixty percent of all patients with X4-tropism at therapy initiation experienced only an impaired recovery of CD4 T-cells (delta CD4 T-cell count below 400 cells/µL). Although the majority of patients with X4-tropic viruses tended to be in the incomplete responder group the p-value did not reach the significance level. As a possible confounder the lower baseline CD4 T-cell count in the incomplete responder group had to be considered. However, in a sub analysis both groups were significantly overlapping: some patients had a baseline CD4 T-cell count below 150 cells/µL but were still classified into the responder group, whereas some of the patients with a baseline CD4 T-cell count above 150 cells/µL did not reach a delta CD4 T-cell count of more than 400 cells/µL.

Although X4-tropic viruses have been associated with faster CD4 T-cell loss and disease progression[35,36], this could not be significantly associated in this patient population in a treated situation. This observation is in line with the data from Waters et al. who found that the tropism did not influence the success of therapy, although X4-tropism was associated with progressing CD4 T-cell loss before initiation of treatment[35]. Already in the early days of HIV tropism research, where combination therapy was not available, it was recognized that although X4-tropism was a predictor of CD4 T-cell decline it was not an indicator for a higher death risk compared to patients with R5-tropism[65]. It is still not clear how X4-tropic variants trigger a faster CD4 T-cell depletion, but all these findings suggest that this is only happening in a treatment free situation. Speculating of possible reasons, one indication might be the reported elevated levels of T-cell activation markers (HLA-DR, CD38) in treatment-naïve patients with X4-tropic viruses[66].

The paper by Doitsh et al.[67] has shown what can drive CD4 T-cell depletion. After infection of an activated cell viral transcripts are released into the cytosol. The intracellular mediator of the innate immune system, caspase-1, can sense these

transcripts and induces cell death upon pyroptosis. A mechanism characterized by inflammation due to the release of pro-inflammatory cytokines and other cellular components upon cell death. These inflammatory signals attract other CD4 T-cells on site that in turn can get infected.[67] Therefore, higher activation levels reported in patients with X4-tropic viruses[66] might favor a faster depletion of CD4 T-cells. In contrast to treatment-naïve patients, long-term infected patients on successful therapy show similar levels of inflammation markers such as interleukin-6 or D-dimers, irrespective of the viral tropism[68].

A generally faster depletion of a cell infected by X4-tropic viruses may also be caused by the distinct characteristics, like cell turn-over rate, of this cell population. Investigation of this aspect showed that T-cells infected either via R5- or X4-receptor have similar turn-over rates and show also no compartmentalization[69]. Also is the mentioned caspase-1 dependent mechanism of CD4 T-cell death (pyroptosis) independent of the viral tropism and the used receptor for infection[67]. As cells infected via X4-tropic viruses show similar characteristics to cells infected via R5-receptor, this aspect might be excluded as possible reason for faster CD4 T-cell depletion associated with X4-tropism.

The subsequent comparison of the tropism before and after five years of treatment revealed that for the majority of patients, the tropism changed from X4-tropic to R5-tropic during therapy. This was in contrast to viruses with R5-tropism at baseline, which in most cases retained their R5-tropism over time.

The observation that the majority of patients with X4-tropic viruses at baseline changed to R5-tropic viruses during successful therapy was surprising, especially as it is known that X4-tropic variants appear later in disease and associate with rapid disease progression[35,36]. This finding argues against persisting X4-tropic viruses during therapy. On the contrary, it provides evidence that R5-tropic variants might have higher proviral stability during suppressive therapy and are therefore found more often after years of treatment. As the treatment effect was steady over the years, proven by the full suppression of the free plasma virus, other mechanisms have to influence this change in tropism over time. One of these managing agents might be the recovering immune system that acts selectively against X4-tropic viruses and/or infected cells. Support for this hypothesis comes from earlier reports that have demonstrated that the recognition of the envelope glycoprotein by neutralizing antibodies is centrally involved in the

elimination of a viral variant[54]. X4-tropic viruses are often less glycosylated than R5-tropic viruses and might therefore be more readily recognized and eliminated by the host defense[33,52,53,70]. In turn, it has been suggested that R5-tropic viruses could have a significant selection advantage during chronic infection based on their evasion mechanism (through specific glycosylation sites) from the immune system[55]. Moreover and of critical importance, the non-pathogenic SIV in the natural host, the African green monkey is known to be solely R5-tropic, and these viruses are associated with an infection that is non-destructive for CD4 T-cells with no damage to the GALT (gut associated lymphoid tissue)[71-73].

Analysis on glycosylation sites revealed that in half of the cases changes to X4-tropism coincided with the loss of a potential N-linked glycosylation site. This is well in line with the finding that a progressive loss of glycosylation sites correlates with the emergence of X4-tropism[33,53,70]. Further, it was noted that in cases where the tropism changed from R5 to X4 only few nucleotide changes in V3 were sufficient to alter the amino acid sequence and obtain an X4-tropism. This can be explained by one of the used G2P algorithm rules that assign an X4-tropism if a charged amino acid is found at positions 11, 24 or 25. In contrast, a tropism change from X4 to R5 required many changes in the V3 sequence. This observation hints that tropism changes to R5 reflect the emergence of an already pre-existing viral minority, as that many changes in a sequence are unlikely to occur as a consequence of spontaneous mutation. On the other hand, the changes from R5 to X4-tropism were mostly characterized by G to A transitions at positions 11, 24, or 25 of the V3 loop. Such G to A transitions are known to be induced by APOBEC3G/F (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 3G/F) which are part of the innate immunity of the host and act as antiviral factors[74-76]. It has also been suggested already that APOBEC3G/F might be directly responsible for or involved in tropism changes and the occurrence of X4-tropic variants[77,78].

The identified shift to R5-tropism in a majority of patients under treatment from a pre-treatment X4-tropism, analyzed with bulk sequencing (only majority viral variant is detected), was subsequently confirmed when the frequencies of X4-tropic variants during treatment were assessed with next generation sequencing. In that part of the study, in the great majority of patients the percentage of X4-tropic variants decreased during therapy intervention, accompanied by a good CD4 T-cell response. Even applying non-standard, more stringent FPR cut-offs (5% and 10%) did not change the

primary outcome of the study. This indicates the solidity of the analysis. These findings are supported by several other studies that utilized theoretical modeling to predict that the initiation of cART would lead to a preferential suppression of X4-tropic variants[79-81]. Additional support comes from the MOTIVATE (Maraviroc versus Optimized Therapy in Viremic Antiretroviral Treatment-Experienced Patients) studies in which the R5-receptor antagonist maraviroc was administered to patients in salvage therapy situations. These patients were long-term infected, treatment experienced and the acquired resistant viruses allowed only for limited additional therapy options. Interestingly, the majority of these pre-treated patients had an R5-tropic virus at baseline, despite the fact that they have been infected and treated for long periods before[60]. After a long history of infection it might be expected that up to 50% of the patients will have X4-tropic viruses[30,56,57], however, this was not the case. This has also been confirmed by another study, where patients under treatment and virological failure presented in the majority R5-tropic viruses[59].

It is therefore even more tempting to speculate that during successful treatment the recovering immune system might be able to preferentially target such "more visible" X4-tropic HIV variants or cells infected with these viruses and expressing their glycoproteins, which could explain the "asymmetric" reduction of X4-tropic provirus observed in this thesis.

Along with the overwhelming trend of an X4 decline this study identified in a minority of patients the increase in X4-tropic variants. In all these cases this was characterized by the outgrowth of a single genetic virus variant, originating from a small minority, which was confirmed by NGS to have been present already prior to therapy initiation. Similar observations were reported by two earlier studies: Westby et al.[82] had shown that solely X4-tropic variants emerged during application of the R5-receptor antagonist maraviroc. This stemmed from a pre-treatment reservoir with an ancestral background distinct from the main R5-tropic variants in the circulation. A case study by Verheyen et al. had identified an emerging X4 variant after stem cell transplantation, which again had emerged from a pre-existing minority variant before treatment[83,84]. The observation in all these studies that the emerging variant originated from an archived virus minority and that this increase was not principally associated with a greater virus diversity over time hints a clonal expansion of individual, infected cells in the population of provirus-carrying CD4-positive cells. In fact, for an individual patient such

a mechanism of clonal virus expansion has been demonstrated to represent the origin of an emerging virus[85].

The detailed evolutionary analysis of the variants in R5- and X4-tropic samples revealed for proviruses with assigned X4-tropism that overall the viral population had a much greater sequence variation. Furthermore, in over 70% of the patients molecular evidence suggests that a most prevalent X4-tropic variant at therapy start changed to an R5-tropic variant during therapy. This type of viral transformation was not observed where pre-treatment samples contained predominant R5-variants: In these patients 60% of the proviral HIV sequences were preserved over time. The higher dynamics in X4-tropic samples with a greater number of variants overall, and a lower sequence conservation in the proviral pool throughout time might lend further support for the hypothesis that a better immunologic "visibility" and a higher immune pressure on X4-tropic variants is critical for such "asymmetric viral dynamics and stability".

None of the patients in all study settings received R5-receptor antagonists. This is important as its administration favors the emergence of X4-tropic viruses[82]. Furthermore, the naturally occurring mutation in the CCR5 gene could also lead to a favorable usage of X4-tropic viruses[84]. The identified frequency of heterozygosis in all the patients did not greatly differ from the prevalence reported for the Caucasian population[13]. For the first part of the study the heterozygous patients were equally distributed among the responders and incomplete responders group. Overall, the majorities of patients in all study settings with a heterozygous mutation in the R5 gene had R5-tropic viruses and were not associated with an increase in the percentage of X4-tropic variants under therapy. The observation that also in patients with a heterozygous delta32 genotype %X4 declined may indicate that even the assumed lower surface expression of R5-receptors did not favor the X4-receptor use by the virus in these infections. It provides additional evidence that in the immune competent host the X4-tropism does not appear to be a favorable phenotype of HIV-1. This speculation is in full agreement with the clinical finding for patients who receive an R5-receptor antagonist[82]. For all who developed failure, the drug-driven emergence of X4-tropic variants immediately reverted after suspension of the R5-receptor antagonist[82].

The observed reduction of X4-tropic variants in this study is in line with some reports that support the decrease of X4-tropic variants after initiation of antiviral therapy[79-81]. In contrast, another study supports the emergence of X4-tropic variants in the cell compartment during therapy[86]. Even so, the findings here cannot support the over-

representation of X4-tropic variants in the proviral reservoir under treatment. Furthermore, another study identified also a higher proportion of R5-tropic viruses in the cellular compartment[87]. However, the difference was that the authors concluded that based on the under-representation of X4-tropic viruses the proviral tropism testing system is insufficient for use[87]. Yet the observations of that study are in line with the findings here, as they also showed more R5-tropic viruses in the cellular compartment.

The findings of this thesis have revealed that under therapy the X4-tropic HIV variants experience a strong downward trend in cells, even during complete viral suppression. This development was not expected and may even be misinterpreted in clinical studies[87]. The lower representation of X4-tropic variants is indeed real and could be explained best by the loss of X4-tropic infected cells during antiretroviral therapy due to the recovering immune system.

The observations in this thesis strongly support the clinical concept of early treatment initiation and favor the early use of R5-receptor antagonists. X4-tropic variants are known to be rare after infection and mostly accumulate later in disease[30,47,48,56,57]. Along with this goes the observation that during failure of first line therapy most viruses continue to be R5-tropic[59,60]. Therefore, the preservation of potent immune function itself may contribute to suppressing or even eliminating X4-tropic virus variants. Hence, with initiation of early therapy the emergence of X4-tropic variants may be suppressed maintaining the full therapeutic potency of a regimen containing inhibitors such as maraviroc. Most recently the concept of early therapy initiation has received massive support from data of the START (Strategic Timing of Antiretroviral Therapy) trial, which proved that early administration of cART has great net benefits for the patient[88].

Further studies on the role of the HIV tropism during infection are needed, but obtained data strongly support the "Treatment as Prevention" strategy to help improving patient quality of life and to limit the further spread of the disease.

V. Conclusion and outlook

V.I. CONCLUSION

Over the years of HIV research X4-tropic viral variants have become known as the ones associated with disease progression and CD4 T-cell loss in therapy free situations[30,56,57]. How the viral tropism behaves in a treated situation was not clear. This work now elucidates new ways of understanding how the tropism contributes to the disease development. The main findings of this work indicate that…

…THE PRESENCE OF X4-TROPIC VIRUSES BEFORE THERAPY DOES NOT NECESSARILY LEAD TO AN IMPAIRED CD4 T-CELL RECOVERY UNDER TREATMENT.

…PROVIRAL TROPISM CHANGES TO A PREDOMINANCE OF R5-TROPIC VIRAL VARIANTS UNDER THERAPY.

…THE AMOUNT OF X4-TROPIC VIRAL VARIANTS GENERALLY DECREASES OR REMAINS STABLE UNDER THERAPY.

…EMERGENCE OF X4-TROPIC VIRAL VARIANTS UNDER THERAPY CORRELATES WITH THE OUTGROWTH OF A SINGLE VARIANT ALREADY PRESENT AS MINORITY BEFORE THERAPY.

Furthermore, X4-tropic viruses…

…MIGHT BE RECOGNIZED BETTER BY THE IMMUNE SYSTEM AND COULD THEREFORE BE SELECTIVELY ELIMINATED BY THE IMMUNE COMPETENT HOST.

…MIGHT CONTRIBUTE TO CD4 T-CELL LOSS DUE TO A HIGHER VISIBILITY, INDUCED INFLAMMATION, AND SELECTIVE CELL ACTIVATION.

V.II. OUTLOOK FOR FUTURE RESEARCH TOPICS

Further studies with larger sample sizes will be needed to clinically verify the results obtained in this thesis. Furthermore, the results presented here may pave the way for interesting additional research topics. This thesis identified that an X4-tropic variant increase was characterized by the outgrowth of an individual minority variant already present before treatment initiation. It therefore seems likely that such an outgrowth occurs as clonal expansion of a single cell. This could be elucidated by integration site analysis; if truly proliferation were responsible all proviral genomes would be characterized by integrating in the same position in the host genome. Furthermore, this analysis could reveal the genes in which HIV has integrated. Although integration is random, recently presented work elucidated evidence that HIV might favor integration

into genes involved in cell proliferation[85]. Another interesting question would concern the infected cell type responsible for an increase in X4-tropic variants. It has been shown that chemokine receptors are differently expressed on various cell types and during different stages of the disease[89], also cancer might play a certain role here[90]. Maybe X4-tropic proviruses in proliferating cells can be linked to cancer and metastasis or to a special subset of infected T-cells, like naïve T-cells[90,91].

V.III. OUTLOOK FOR THE CLINICS

The net benefit of early antiretroviral therapy has been shown in the START trial earlier this year[88]. Initiating therapy at a CD4 T-cell count higher than 500 CD4 T-cells/µL showed a lower risk to develop AIDS related events compared to patients that initiated therapy when CD4 T-cell counts were below 350 CD4 T-cells/µL[88]. The findings of this thesis support early therapy initiation as well. The obtained results favor a decrease of X4-tropic viral variants during the recovery of the immune system under therapy. Therefore, the emergence of X4-tropic variants in a treatment free situation might be the consequence of the accelerated damage of the immune system. Data from the clinics already support this idea, as the majority of patients in a treated and failing regimen present R5-tropic viruses[59,60]. Early therapy initiation would maintain the potency of the immune system, thereby enabling it to control X4-tropic variants. Therefore, the other therapeutic target would be the R5-tropic virus that seems not to be readily controlled by the immune system. However, these variants have been shown to be efficiently reduced by the administration of the R5-receptor antagonist maraviroc that prohibits the infection via the R5-receptor[92,93]. An additional way of controlling the viral infection would hence include strategies to hit on both viral variants. This might be achieved by the use of early antiretroviral therapy with the combination of an R5-receptor antagonist.

VI. Materials and methods

VI.I. MATERIALS

VI.I.I. CHEMICALS

Name	Supplier
RNA isolation	
Prepito NA Body Fluid Kit	Chemagen
DNA isolation and purification	
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel
RT-PCR	
Affinity Script One-Step RT-PCR Kit	Stratagene
Standard PCR	
PfuUltra II Fusion HS DNA polymerase	Agilent Technologies
iProof™ High-Fidelity DNA polymerase	Bio-Rad
dNTPs (dATP, dCTP, dGTP, dTTP), 10mM	Sigma
illustra ExoStar 1-Step	GE Healthcare
Gel electrophoresis	
Agarose	Invitrogen
TBE buffer, 10x	Amresco
Ethidiumbromide solution (10 mg/mL)	Sigma
RedSafe™ Nucleic Acid Staining Solution	Lucernachem
Gel loading dye, blue	New England Biolabs
100 bp DNA ladder (500 µg/mL)	New England Biolabs
1 kb DNA ladder (500 µg/mL)	New England Biolabs
Glycerol anhydrous	Fluka
Sanger sequencing	
BigDye® Terminator v3.1	Applied Biosystems
BigDye® v1.1/3.1 Sequencing Buffer (5x)	Applied Biosystems

Next generation sequencing	
Agencourt AMPure XP	Beckman Coulter
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen
Nextera XT DNA Library Preparation Kit	illumina
XTrack double strand separation	
Dynabeads M-280 Streptavidin (10mg/µL)	Invitrogen
Tris	Sigma
EDTA	Sigma
NaCl	Fluka
NaOH	Fluka
KCl	Fluka
Capillary electrophoresis	
Polymer - POP conformational analysis polymer	Applied Biosystems
Buffer 10x with EDTA	Applied Biosystems
Glycerol (87%)	Fluka
TBE buffer, 10x	Amresco
General chemicals	
Ethanol (100%)	Fluka
MilliQ H_2O	In house

VI.I.II. PRIMERS AND PROBES

Nomenclature: F forward primer, R; reverse primer. The number in general (excluded "others" section) indicates the 5' end on the HIV genome reference (pNL-4-3) and allows the calculation of the amplified PCR fragment.

Name	Sequence 5' – 3'
F_6943	CAC AGT ACA ATG YAC ACA TGG AAT
R_7365	AGT AGA AAA ATT CYC CTC YAC AAT TAA A
F_7092	GAA TCT GTA GAA ATT AAT TGT ACA AGA C
R_7232	TGC TCT ACT AAT GTT ACA ATG TGC TTG TCT TAT
F_6553	ATG GGA TCA AAG CCT AAA GCC ATG TG
R_7801	AGT GCT TCC TGC TGC TCC CAA GAA CCC AAG
F_6848	CCA ATT CCC ATA CAT TAT TGT GCC CCG GCT GG
R_7371	TTA CAG TAG AAA AAT TCC CCT CCA CAA TTA AA
FAM_V3_7092_F	FAM-GAA TCT GTA GAA ATT AAT TGT ACA AGA C
Biotin_V3_7232_R	Biot-TGC TCT ACT AAT GTT ACA ATG TGC TTG TCT TAT
F-522	GCC TCA ATA AAG CTT GCC TTG A
R-643	GGG CGC CAC TGC TAG AGA
R-584 (LTR-Probe)	FAM-CCA GAG TCA CAC AAC AGA CGG GCA CAG GTC TCAG-BHQ
Others	
2818_bcrabl	TAMRA-CCT CAG GGT CTG AGT GAA GC
2886_revbcra	AGT TAT GCT TAG AGT GTT ATC TCC A
D32_forward	GAT AGG TAC CTG GCT GTC GTC CAT
D32_reverse	ACC AGC CCC AAG ATG ACT ATC T
F-GAPDH	GAA GGT GAA GGT CGG AGT C
R-GAPDH	GAA GAT GGT GAT GGG ATT TC
GAPDH-probe	VIC-CAA GCT TCC CGT TCT CAG CC-BQH

VI.II. METHODS

VI.II.I. EXTRACTION

RNA and DNA extraction was performed with chemagen Prepito (Perkin Elmer, Baesweiler, Germany) using the NA Body Fluid Kit according to protocol.

VI.II.II. PCR PREPARATION FOR BULK SEQUENCING AND XTRACK

One-step reverse transcriptase PCR

Reverse transcriptase was performed with Affinity Script One-Step RT-PCR Kit (Agilent Technologies), enabling reverse transcription and PCR in one tube. The total reaction volume was 50µL, containing 45µL of master mix and 5µL of patient RNA. The master mix included 17µL of MilliQ H_2O, 25µL of Herculase II RT-PCR 2x Master Mix, 1µL of 10µM F-6943 primer, 1µL of 10µM R-7365 primer and 1µL Affinity Script RT/RNAse Block. PCR reaction started with an initial step of five minutes at 45°C for reverse transcription and a second step at 92°C for one minute to denature the reverse transcriptase. Then 40 cycles of denaturing at 92°C for 20 seconds, annealing at 51°C for 20 seconds and elongation for 30 seconds at 68°C followed. There was a final elongation step for three minutes at 68°C.

Nested PCR

Reaction was performed with PFU Ultra II HF (Agilent Technologies) in a volume of 20µL. 15µL of master mix and 5µL of patient DNA. The master mix contained 10µL of MilliQ H_2O, 2µL of PfuUltra II Reaction Buffer, 0.5µL of dNTP, 1µL of 10µM F-7092 primer, 1µL of 10µM R-7232 primer and 1µL PfuUltra II polymerase. PCR reaction started with an initial step of one minute at 92°C. Then 30 cycles of denaturing at 92°C for 20 seconds, annealing at 51°C for 20 seconds and elongation for 15 seconds at 72°C followed. There was a final elongation step for three minutes at 72°C.
For proviral DNA the first PCR was carried out with PfuUltra II with primers F-6943 and F-6977 under the same conditions as for the second nested PCR.

VI.II.III. PCR PREPARATION FOR NEXT GENERATION SEQUENCING

A nested PCR approach was used; both reactions were performed with PFU Ultra II HF (Agilent Technologies) in a volume of 20µL. 15µL of master mix and 5µL of patient DNA. The master mix contained 10µL of MilliQ H_2O, 2µL of PfuUltra II Reaction Buffer,

0.5µL of dNTP, 1µL of 10µM F-6848 (1st PCR) or F-6553 (2nd PCR), 1µL of 10µM R-7371 (1st PCR) or R-7801 (2nd PCR) and 1µL PfuUltra II polymerase. First PCR reaction started with an initial step of one minute at 92°C. Then one cycle of denaturing at 92°C for 20 seconds, annealing at 65°C for 20 seconds and elongation for 30 seconds at 72°C followed. Afterwards one cycle of denaturing at 92°C for 20 seconds, annealing at 60°C for 20 seconds and elongation for 30 seconds at 72°C followed by 45 cycles of denaturing at 92°C for 20 seconds, annealing at 55°C for 20 seconds and elongation for 30 seconds at 72°C followed. A final elongation step was carried out at 72°C for three minutes. Condition for second nested PCR were exactly the same except for the annealing temperatures that were 65°C for the first cycle, 63°C for the second cycle and 60°C for the following 45 cycles.

VI.II.IV. DNA CLEANING FOR DOWN STREAM APPLICATION

Gel electrophoresis

To make the PCR product visible and to validate it the DNA was loaded onto a 2% agarose gel containing 2g of agarose in 100µL of 1x TBE. After agarose was dissolved by heat and cooled down, 5µL of ethidium bromide was added. The 20µL PCR product was mixed with 3µL of 6x blue loading dye and loaded onto the gel. The gel was run at 210V for 45 minutes.

Gel extraction

Gel extraction was performed according to the protocol of the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). DNA was eluted in 35µL of elution buffer.

VI.II.V. SANGER SEQUENCING

Sequencing reaction mix contained 1µL Big Dye Terminator v3.1, 3µL Big dye v1.1/v1.3 Sequencing Buffer, 3µL of 1µM forward or reverse primer and 7µL of cleaned DNA filled up with MilliQ H_2O to a final volume of 20µL. Sequencing reaction included 40 cycles of denaturing at 96°C for 20 seconds, annealing at 50°C for 20 seconds and elongation for four minutes at 60°C.

The reaction was cleaned through a Sephadex™ G-50 superfine column. The final cleaned PCR product was sequenced with the ABI 3130 Genetic Analyzer with a five seconds injection protocol.

VI.II.VI. NEXT GENERATION SEQUENCING

Agencourt AMPour XP

After PCR the DNA product was cleaned with Agencourt AMPour XP beads (Beckmann Coulter) according to protocol.

DNA quantification

For quantification the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) was used according to protocol.

Library preparation

DNA concentration was adjusted to 0.2ng/µL and the Nextera XT DNA Library Preparation Kit (Illumina) was used to prepare the library according to Kit instructions.

Sequencing

Sequencing was performed with a Illumina MiSeq Benchtop sequencer with 2x250bp reads.

VI.II.VII. XTRACK

Generation of single stranded fluorescent labeled V3 probes

The fluorescent-labeled probe was generated by labeling PCR with a fluorescent labeled forward primer and a biotin tagged reverse primer. The PCR was performed with iProof™ High-Fidelity DNA polymerase. The total reaction was performed in a volume of 50µL. 45µL of master mix and 5µL of 10ng/µL DNA. The master mix contained 31.5µL MilliQ H_2O, 10µL iProof HF Buffer, 1µL of dNTP, 1µL of 10µM FAM_V3_7092_F primer, 1µL of 10µM Biotin_V3_7232_R primer and 0.5µL iProof™ polymerase. PCR was performed under the following conditions: 98°C for two minutes, 35 cycles at 98°C for ten seconds, 48°C for 15 seconds and 72°C for ten seconds, followed by ten minutes at 72°C.

The PCR product was mixed with 6x glycerol loading dye, containing 675µL anhydrous glycerol and 1325µL MilliQ H_2O. Then it was loaded on a 2% agarose gel in 100µL 1x TBE and gel extracted with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) according to protocol. DNA was eluted in 50µL elution buffer. All the steps were performed as much in the dark as possible.

For the separation of the double strand the following procedure was performed. 40µL of Streptavidine magnetic beads were washed three times with binding buffer containing 20mM Tris-HCl pH 7.5, 10mM EDTA and 2M NaCl. To the eluted DNA 50µL of binding buffer was added and the mixture was incubated 15 minutes with the washed magnetic beads in the dark. Afterwards the mixture was washed three times with wash buffer containing 20mM Tris-Cl pH 7.5, 10mM EDTA and 1M NaCl. The magnetic beads were mixed with 20µL of fresh 0.2N NaOH to separate the single strand. The beads were washed with 10µL of MilliQ H_2O and put together with the 20µL. 20µL of 1M Tris-HCl was added which gave a final volume of 50µL.

Generation of molecular weight marker (MWM)

The MWM was generated by labeling PCR with iProof™ High-Fidelity DNA polymerase. The total reaction was performed in a volume of 50µL. 45µL of master mix and 5µL of 10ng/µL DNA. The master mix contained 31.5µL MilliQ H_2O, 10µL iProof™ HF Buffer, 1µL of dNTP, 1µL of 10pM/µL 2818_bcrabl primer, 1µL of 10pM/µL 2886_revbcra primer and 0.5µL iProof™ polymerase. PCR reaction was performed under following conditions: 98°C for two minutes, 40 cycles at 98°C for ten seconds, 53°C for 15 seconds and 72°C for five seconds, followed by five minutes at 72°C.
The PCR product was mixed with 6x glycerol loading dye, containing 675µL anhydrous glycerol and 1325µL MilliQ H_2O. Then it was loaded on a 2% agarose gel in 100µL 1x TBE and gel extracted with the NucleoSpin® Gel and PCR Clean-up Kit according to protocol. DNA was eluted in 25µL elution buffer.

Generating the heteroduplex

1µL of 2ng fluorescent labeled single strand probe and 7µL of patient DNA in 1x TKE buffer in a total volume of 10µL was denatured for five minutes at 95°C and held for ten minutes at 4°C. The 10µL were mixed with 5ng of molecular weight marker in 10µL MilliQ H_2O, which gave a final volume of 20µL, which was loaded onto the ABI PRISM® 310 Genetic Analyzer.

VI.II.VIII. PROVIRAL LOAD TESTING

Proviral loads were assessed using an in house multiplex real time PCR approach with a VIC labeled GAPDH probe and a FAM labeled LTR probe. 5µL of extracted patient DNA was mixed with 1µL of 10µM forward primer (GAPDH), 1µL of 10µM reverse

primer (GAPDH), 1µL of 10µM forward primer (LTR), 1µL of 10µM reverse primer (LTR), 0.5µL of 10µM GAPDH probe, 0.5µL of 10µM LTR probe and 10µL of 2x TaqMan® Fast Universal PCR Master Mix (Life Technologies) in a final volume of 20µL. The PCR reaction was: two minutes at 50°C, ten minutes at 95°C, 40 cycles of (15 seconds at 95°C, one minute at 60°C).

VI.II.IX. DELTA32 GENOTYPE TESTING

The total reaction volume was 50µL, containing 45µL of master mix and 5µL of patient DNA. The master mix included 35.8µL of MilliQ H_2O, 5µL of Taq ThermoPol Buffer (Bioconcept), 2µL of 10mM dNTP, 1µL of 10µM D32-forward primer, 1µL of 10µM D32-reverse primer and 0.2µL Taq polymerase (Bioconcept). PCR reaction started with an initial step of five minutes at 95°C followed by 35 cycles of (95°C for five seconds, 55°C for ten seconds, 72°C for one minute) and a final elongation step at 72°C for two minutes[94].

VI.II.X. COMPUTATIONAL ANALYSIS

Geno2Pheno

Geno2Pheno was used for tropism determination based on sequence characteristics. For the analysis in chapters III.I. and III.II. bulk sequencing was performed and therefore $Geno2Pheno_{coreceptor}$ with a FPR cut-off of 5% was used. In chapter III.III. next generation sequencing was performed and the tropism determination was done with $Geno2Pheno_{454}$ with a FPR cut-off value of 3.5%. Using next generation sequencing, an R5-tropism was assigned if the relative amount of X4-variants in the patient pool was below 2%. If the frequency of X4-variants changed by less than 1% between time points it was designated as "stable".

Evolutionary analysis and phylogenetic trees

All calculations were performed with MEGA 6.0. The distance relatedness was calculated between the prevalent variant and all remaining variants for a sample. iTOL 2.0 was used for phylogenetic tree visualization[95,96].

Statistics

Statistical analysis were performed with R 0.97.312 and Stata 12.1. Categorical data were compared by means of Chi-square test, whereas continuous data were compared by Mann-Whitney-Wilcoxon test.

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IX. FIGURE AND TABLE INDEX

IX.I. FIGURE INDEX

IX.II. TABLE INDEX

X. PUBLICATIONS

X.I. CORRELATING HIV TROPISM WITH IMMUNOLOGICAL RESPONSE UNDER CART

Bader J, Schöni-Affolter F, Böni J, Gorgievski-Hrisoho M, Martinetti G, Battegay M, Klimkait T, and the Swiss HIV Cohort Study. HIV Medicine. Copyright © 2015 John Wiley & Sons, Inc., Wiley Online Libary (accepted for publication).

Correlating HIV Tropism With Immunological Response Under cART

Running Head: Coreceptor Use and Disease Progression

Joëlle Bader[1]*, Dr.med. Franziska Schöni-Affolter[2], PD Dr. Jürg Böni[3], Dr. med. Meri Gorgievski-Hrisoho[4], Sig.ra Dr. FAMH Gladys Martinetti[5], Prof. Dr. med. Manuel Battegay[6], Prof. Dr.rer.nat. Thomas Klimkait[1], and the Swiss HIV Cohort Study

[1]Molecular Virology, Department Biomedicine - Petersplatz, University of Basel, Basel, Switzerland;

[2]Swiss HIV Cohort Study (SHCS) Data Center, University Hospital Lausanne, Lausanne, Switzerland

[3]Institute of Medical Virology, National Center for Retroviruses, University of Zürich, Zürich, Switzerland

[4]Institute for Infectious Diseases, University of Berne, Berne, Switzerland

[5]Department of Microbiology, Ente Ospedaliero Cantonale, Bellinzona, Switzerland

[6]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Basel, Switzerland

* Corresponding author

Joelle Bader, University of Basel, Department Biomedicine, Molecular Virology, Petersplatz 10, 4009 Basel, Switzerland, joelle.bader@unibas.ch, 0041 61 267 09 81

Keywords: HIV, tropism, cART, immune response

Abstract

Objectives: A significant percentage of patients infected with HIV-1 experience only suboptimal CD4 cell recovery while treated with combination therapy (cART). It is still unclear, whether viral properties such as cell tropism play a major role for such incomplete immune response. This study therefore intended to follow the tropism evolution of the HIV-1 envelope during periods of suppressive cART.

Methods: Virus from two distinct patient groups, one with good and another one with poor CD4-recovery after five years of suppressive cART, were genotypically analyzed for viral tropism at baseline and the end of the study period.

Results: Patients with CCR5-tropic viruses at baseline tended to maintain this tropism to the study end. Patients who had a CXCR4-tropic virus at baseline were overrepresented in the poor CD4 recovery group. Overall, however, the majority of patients presented with CCR5-tropic viruses at follow-up.

Conclusions: Our data lend support to the hypothesis that tropism determination can be used as parameter for disease progression even if analyzed long before the establishment of a poorer immune response. Moreover, the lasting predominating CCR5-tropism during periods of full virus control suggests the involvement of cellular mechanisms that preferentially reduce CXCR4-tropic viruses during cART.

[195 words]

X.II. OUTCOMES OF CHILDREN ON ANTI-RETROVIRAL THERAPY IN NURSE-LED CLINICS IN RURAL LESOTHO.

Puga D, Cerutti B, Molisana C, **Bader J**, Faturiyele O, Ringera I, Lejone T, Pfeiffer K, Klimkai T, Labhardt ND. The Pediatric Infectious Disease Journal. Copyright © 2015 Wolters Kluwer Health, Inc., (ePub).

BRIEF REPORT

Title: Outcomes of children on anti-retroviral therapy in nurse-led clinics in rural Lesotho

Abbreviated Title: Virologic Outcomes of Children on cART in Lesotho.

Running Head: Virologic Outcomes of Children on cART

Authors:

Daniel PUGA[1] (MD)¶, Bernard CERUTTI[2] (PhD), Cheleboi MOLISANA (MLT)[3], Joëlle BADER (M.Sc)[4], Olatunbosun FATURIYELE[1] (MD), Isaac Ringera[1] (RN), Thabo LEJONE[1] (RN), Karolin PFEIFFER[5] (MD, MPH), Thomas KLIMKAIT[4] (Prof. rer. nat.), Niklaus D. LABHARDT[6,7] *¶(MD, MIH)

Authors' affiliations

[1] SolidarMed, Swiss Organization for Health in Africa, Maseru, Lesotho

[2] Faculty of Medicine, University of Geneva, Geneva, Switzerland

[3] Laboratory Services, Seboche Hospital, Butha-Buthe, Lesotho

[4] Molecular Virology, Department of Biomedicine – Petersplatz, University of Basel, Basel, Switzerland

[5] SolidarMed, Swiss Organization for Health in Africa, Lucerne, Switzerland

[6] Clinical Research Unit, Swiss Tropical and Public Health Institute, Basel, Switzerland

[7] University of Basel, Basel, Switzerland

¶ Equally contributed to this manuscript

***correspondence to:** Niklaus Daniel Labhardt, Clinical Research Unit, Medical Services and Diagnostic, Swiss Tropical and Public Health Institute, Socinstrasse 57, 4051 Basel, Switzerland; n.labhardt@unibas.ch

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Key-words

- Viral load
- Virologic Outcomes
- pediatric
- Children
- Africa
- HIV
- Anti-retroviral therapy

Abstract

This survey assessed virologic outcomes of children on antiretroviral therapy and potential predictors in 10 nurse-led clinics in Lesotho. Success was achieved in 72% of the 191 children. No predictors for virologic outcome were found, underlining the need for routine viral load-testing in resource-limited settings in order to achieve 90-90-90.

Tropical Medicine and International Health doi:10.1111/tmi.12509

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Is zidovudine first-line therapy virologically comparable to tenofovir in resource-limited settings?

Niklaus D. Labhardt[1,2], Joëlle Bader[3], Thabo Ismael Lejone[4], Isaac Ringera[4], Daniel Puga[4], Tracy R. Glass[2,5] and Thomas Klimkait[3]

1 *Medical Services and Diagnostics, Clinical Research Unit, Swiss Tropical and Public Health Institute, Basel, Switzerland*
2 *University of Basel, Basel, Switzerland*
3 *Molecular Virology, Department Biomedicine – Petersplatz, University of Basel, Basel, Switzerland*
4 *SolidarMed, Swiss Organization for Health in Africa, Maseru, Lesotho*
5 *Biostatistics Department, Epidemiology and Public Health Unit, Swiss Tropical and Public Health Institute, Basel, Switzerland*

Abstract

OBJECTIVE To compare virologic success between adult patients on tenofovir (TDF) and zidovudine (AZT)-containing first-line antiretroviral (ART) regimens in 10 rural clinics in Lesotho, Southern Africa.

METHODS Multicentre cross-sectional study, patients ≥16 years, on first-line ART ≥6 months, receiving AZT/lamivudine (3TC) or TDF/3TC combined with efavirenz (EFV) or nevirapine (NVP). Patient characteristics and clinical/therapeutic history were collected on the day of blood draw for viral load (VL). Analysis was stratified for non-nucleoside reverse transcriptase inhibitor (EFV or NVP). A logistic regression model weighted for patients' baseline characteristics was used to assess the likelihood of virologic success (<80 copies/ml) in patients with TDF- as compared to AZT-backbones.

RESULTS In total 1539 patients were included in the analysis. Most were clinically and immunologically stable (clinical failure: 2.7% (AZT) and 2.8% (TDF); immunological failure: 4.6% (AZT) and 4.8% (TDF)). In EFV-based regimens (n = 1162), TDF was significantly associated with higher rates of virologic suppression than AZT (93.8% *vs.* 88.1%; weighted odds ratio: 2.15 (95% CI: 1.29–3.58; P = 0.003)). In NVP-based regimens, a similar trend was observed, but not significant (89.4% *vs.* 86.7%; 1.99 (0.83–4.75, P = 0.121)).

CONCLUSION These findings support the WHO recommendation to use TDF/3TC/EFV as first-line regimen. They do, however, not support the recommendation that patients who are clinically stable on AZT should continue on this first-line regimen.

keywords nucleoside reverse transcriptase inhibitor, tenofovir, zidovudine, viral load, HIV, Africa, anti-retroviral therapy

Introduction

The 2013 consolidated WHO guidelines on the use of antiretroviral drugs for treating and preventing HIV infection recommend the combination of tenofovir (TDF), lamivudine (3TC) and efavirenz (EFV) as the preferred first-line antiretroviral therapy (ART) regimen in treatment naïve adults. The guidelines favour as nucleoside reverse transcriptase inhibitor (NRTIs) TDF over zidovudine (AZT) due to its superior side effects profile. However, the guidelines state that the two NRTIs have comparable therapeutic efficacy and that patients who are clinically stable on AZT should continue on this regimen [1]. Due to lower cost and comparable efficacy to TDF, nearly 40% of ART patients in sub-Saharan Africa still receive an AZT-containing first-line regimen, and forecasts expect that about 30% will still be in 2016 [2]. While several studies from sub-Saharan Africa report lower rates of substitution, lower attrition from care and better immunological response for patients on TDF than AZT [3–6], virologic information comparing the two NRTIs in resource-poor settings is very limited. A recent cohort analysis from South Africa comparing TDF to stavudine (d4T), a thymidine analogue closely related to AZT, found no significant differences in mortality, loss to follow-up or virologic success [7].

We report on rates of virologic suppression in patients taking a TDF-containing *vs.* an AZT-containing first-line regimen in a multicentre cross-sectional study in rural Lesotho, Southern Africa.

Methods

Data were derived from a registered observational study on virologic suppression and chronic comorbidities among patients on ART, conducted in two district hospitals and eight health centres in Lesotho, Southern Africa (www.clinicaltrials.gov; NCT02126696). SolidarMed, a Swiss non-profit organisation, supports the clinics through training, mentoring and supervision in the provision of ART. The specific setting of hospitals and affiliated health centres has been described before [8, 9]. The study received ethical clearance by the National Research and Ethics Committee of the Ministry of Health of Lesotho (ID 2014-01). Here, we present an analysis of potential predictors for virologic success among study participants aged 16 years and older, who have been on AZT- or TDF-containing first-line regimens for 6 months or longer.

Data on patient characteristics and clinical and therapeutic history were collected on the day of blood draw for viral load (VL) analysis. VL samples were centrifuged and plasma frozen on place and subsequently transported on dry ice to a reference laboratory in Switzerland for analysis. Viral RNA was prepared using an automated extractor (NucliSENS® easyMAG®, Biomerieux, Switzerland) and quantified using a quantitative, validated protocol as published [10].

Statistical analyses were stratified by the type of non-nucleoside reverse transcriptase inhibitor (NNRTI). Virologic success was defined as VL < 80 copies/ml. Characteristics of patients on different regimens are listed in Table 1. For assessing differences between TDF and AZT in the likelihood to achieve virologic success, a weighted logistic regression model was used. The model was weighted for all variables assumed to influence the likelihood of a patient receiving either TDF or AZT. Variables in the weighted model were as follows: age, sex, clinical WHO stage at baseline, time on ART, history of single-drug substitution, history of anti-retroviral drug exposure for prevention of mother-to-child transmission (PMTCT) prior to starting ART, type of facility (hospital *vs.* health centres), baseline haemoglobin, baseline CD4-count and creatinine clearance.

As a sensitivity analysis, a non-weighted multivariate logistic regression model was estimated including all variables potentially associated with virologic success. Covariates included age, sex, time on ART, baseline haemoglobin, baseline CD4-count, clinical WHO stage at baseline, history of treatment interruption >1 day, history of PMTCT prior to starting ART, history of single-drug substitution, adherence (measured by pill-count), travel time to the facility, alcohol consumption and educational level. Additional sensitivity analyses considered the definition of virologic success according to WHO and Lesotho guidelines, VL < 1000 copies/ml.

Results

Of 1598 patients enrolled, 35 were excluded due to missing data and 24 were excluded because they were on a first-line regimen that did not contain AZT or TDF. All remaining 1539 patients were included in analysis. Rates of clinical and immunological failure by WHO definition were comparable for patients on AZT or TDF (clinical failure: 2.7% and 2.8%; immunological failure: 4.6% and 4.8%). Rates of virologic success were highest for TDF/3TC/EFV (93.8%), followed by TDF/3TC/NVP (89.4%), AZT/3TC/EFV (88.1%) and AZT/3TC/NVP (86.7%) (Table 1). The unadjusted odds ratio (OR) for virologic success with TDF as compared to AZT was 2.03 (95% CI:1.29–3.19, P = 0.002) for EFV-based and 1.29 (0.64–2.59, P = 0.471) for NVP-based regimens. The weighted ORs were 2.15 (95% CI:1.29–3.58; P = 0.003) and 1.99 (0.83–4.75, P = 0.121) for EFV- and NVP-based regimens, respectively. The multivariate logistic regression model allowing for all covariates expected to be associated with virologic success resulted in an adjusted OR of 1.98 (1.15–3.42; P = 0.014) and 1.22 (0.49–3.08; P = 0.670) for EFV- and NVP-based regimens, respectively.

Sensitivity analyses using the less stringent VL cut-off of 1000 copies/ml found similar results: weighted ORs for suppression under TDF compared to AZT were 1.96 (1.11–3.44, P = 0.020) and 2.42 (0.86–6.84, P = 0.095) for EFV- and NVP-based regimens respectively.

Discussion

This is one of the first studies comparing virologic outcomes between patients on TDF- or AZT- containing first-line regimens in remote, rural, nurse-led clinics in Africa. The majority of patients enrolled in the study had no clinical or immunological failure, according to the World Health Organization [1] definition. We found that among patients on EFV-based first-line ART, a TDF backbone was associated with higher rates of virologic success compared to AZT. For NVP-based regimens, we observed a similar trend but the confidence interval was wide due to limited sample size. These results imply that, contrarily to what is stated in the guidelines, even in clinically stable patients AZT may be virologically inferior to

Table 1 Virologic success and characteristics of patients on different AZT- or TDF-containing first-line regimens

Patient characteristics	EFV-based (n = 1162)		NVP-based (n = 377)	
	TDF/3TC/EFV (n = 884)	AZT/3TC/EFV (n = 278)	TDF/3TC/NVP (n = 113)	AZT/3TC/NVP (n = 264)
Virologic success (VL < 80 copies/ml) (%)	829 (93.8; 95% CI: 91.9–95.3)	245 (88.1; 95% CI: 83.7–91.7)	101 (89.4; 95% CI: 82.2–94.4)	228 (86.7; 95% CI: 81.9–90.6)
Median age (IQR)	41.3 (33.8–51.3)	47.3 (37.5–56.3)	43.7 (34.3–50.4)	43.6 (34.8–54.4)
Female gender (%)	573 (64.8)	184 (66.2)	86 (76.1)	214 (81.1)
Followed at hospital (*vs.* health centre) (%)	349 (39.5)	95 (34.2)	60 (53.1)	80 (30.3)
Years on ART (IQR)	3 (1.3–4.8)	4.2 (2.3–5.5)	5.7 (3.5–6.5)	5.2 (3.8–6.3)
History of single-drug substitution (%)	171 (19.3)	46 (16.6)	62 (54.9)	89 (33.7)
PMTCT prior to ART (%)	16 (1.8)	2 (0.7)	1 (0.9)	1 (0.4)
Estimated creatinine-clearence <50ml/min (%)	168 (19.0)	79 (28.4)	23 (20.4)	52 (19.7)
Clinical WHO stage at baseline (%)				
Stage 3 or 4	258 (29.2)	106 (38.1)	34 (30.1)	64 (24.2)
Baseline CD4-count (%)				
<100 cells/ml	148 (16.7)	66 (23.7)	22 (19.5)	39 (14.8)
100–199 cells/ml	220 (24.9)	66 (23.7)	32 (28.3)	69 (26.1)
200–349 cells/ml	343 (38.8)	120 (43.2)	50 (44.3)	135 (51.1)
≥350	140 (15.8)	20 (7.2)	8 (7.1)	19 (7.2)
Missing	33 (3.7)	6 (2.2)	1 (0.9)	2 (0.8)
Baseline haemoglobin (g/dl) (%)				
<10 g/dl	55 (6.2)	13 (4.7)	12 (10.6)	11 (4.2)
10–11.9 g/dl	130 (14.7)	55 (19.8)	23 (20.4)	33 (12.5)
≥12 g/dl	294 (33.3)	128 (46.0)	41 (36.3)	113 (42.8)
Missing	405 (45.8)	82 (29.5)	37 (32.7)	107 (40.5)
Adherence				
Pill-count (IQR)	100 (97–100)	99 (96–100)	100 (96–100)	99 (96–100)
History of treatment interruption > 1 day (%)	197 (22.3)	71 (25.5%)	18 (15.9)	56 (21.2)
Regular alcohol consumption (%)	115 (13.0)	42 (15.1)	12 (10.6)	24 (9.1)
Travel time to facility (h) (IQR)	1 (0.7–2)	1.5 (0.8–2)	1 (0.5–2.1)	1 (0.5–2)
Social situation				
Education (%)				
No school	416 (47.1)	147 (52.9)	52 (46.0)	122 (46.2)
Primary	238 (26.9)	82 (29.5)	31 (27.4)	69 (26.1)
Secondary	77 (8.7)	19 (6.8)	10 (8.9)	26 (9.9)
Tertiary	142 (16.1)	29 (10.4)	19 (16.8)	43 (16.3)
Employment status (%)				
Employed	129 (14.6)	42 (15.1)	23 (20.4)	38 (14.4)
Self-employed	220 (24.9)	62 (22.3)	23 (20.4)	53 (20.1)
No work	510 (57.7)	167 (60.1)	62 (54.9)	169 (64.0)
Missing	25 (2.8)	7 (2.5)	5 (4.4)	4 (1.5)
Disclosed HIV-status to sexual partner (%)				
Yes	647 (74.7)	190 (69.6)	81 (76.4)	184 (71.3)
No	40 (4.6%)	13 (4.8)	4 (3.8)	13 (5.0)
NA	179 (20.7)	70 (25.6)	21 (19.8)	61 (23.6)
Persons knowing HIV-status (IQR)	5 (3–7)	5 (3–8)	6 (4–15)	5 (3–8)

EFV, efavirenz; NVP, nevirapine; TDF, tenofovir; AZT, zidovudine; 3TC, lamivudine; IQR, interquartile range; 95% CI, 95% confidence interval; PMTCT, prevention of mother-to-child transmission; NA, not applicable.

TDF. This raises the question whether clinically stable patients on AZT should be changed to TDF.

Interpretation of our data is limited to adult patients who are retained in care for ≥6 months. Given its cross-sectional design, no conclusion can be drawn in terms of mortality and retention in care. Moreover, although two models were run to account for potential confounders, the possibility of confounders that were not accounted for remains. Two randomized trials compared TDF backbone and AZT backbone in EFV-based regimens: The GS-01-934 trial, conducted in Europe and USA, demonstrated higher rates of patients reaching a VL < 400 copies/ml in the TDF arm at 96 weeks as well as 144 weeks follow-up [11, 12]. However, this result has been questioned by the PEARLS trial conducted in high-, middle- and low-income countries, including three African countries (Zimbabwe, Malawi and South Africa) [13]. In the PEARLS trial, AZT/3TC/EFV and TDF/3TC/EFV were comparable in achieving VLs of <400 copies/ml. The discrepancy of the two trial results could probably be explained by a different definition of endpoints as the GS-01-934 trial used a composite endpoint consisting of virologic failure and drug substitution within the regimen, whereas the PEARLS trial looked at virologic failure alone [14].

Although randomized trials are the gold standard for determining efficacy of drug regimens, observational studies have the advantage of providing a picture of the 'real-life' effectiveness of ART-regimens. In routine care in rural African settings, where patients receive less support and follow-up than during trials, the challenges of adherence to a twice-daily *vs.* a once-daily regimen might in the long run result in lower virologic success rates in patients on AZT [15]. Currently, there are few published observational data from low-resource settings on virologic success comparing TDF and AZT backbones. Velen *et al.* [16] reported higher rates of drug substitution, loss from care or death but no significant difference in virologic success in patients on AZT than TDF in an urban South African cohort. In our rural cohort, we now observed considerably lower rates of virologic success under AZT.

Overall, published data are coherently showing clinical inferiority of AZT in real-life settings with important questions remaining: Should patients, who are clinically stable on AZT/3TC/EFV be routinely changed to TDF/3TC/EFV or should they continue on AZT to conserve TDF as a NRTI option in second line? Given the current state of evidence, single-drug substitution from AZT to TDF in patients who are clinically stable should only be performed if viral suppression is documented prior to the change. However, for a recommendation in settings where virologic treatment success cannot be monitored on a regular basis, studies from larger cohorts comparing virologic outcomes under TDF and AZT are needed.

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Corresponding Author Niklaus Daniel Labhardt, Medical Services and Diagnostic, Clinical Research Unit, Swiss Tropical and Public Health Institute, Socinstrasse 57, 4051 Basel, Switzerland. E-mail: n.labhardt@unibas.ch

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A Diagnostic HIV-1 Tropism System Based on Sequence Relatedness

Suzanne Edwards,[a] Heinz Stucki,[a]* Joëlle Bader,[a] Vincent Vidal,[b] Rolf Kaiser,[c] Manuel Battegay,[d] Thomas Klimkait,[a] the Swiss HIV Cohort Study

Molecular Virology, Department of Biomedicine, University of Basel, Basel, Switzerland[a]; InPheno AG, Basel, Switzerland[b]; Institute of Virology, University of Cologne, Cologne, Germany[c]; Division of Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Basel, Switzerland[d]

Key clinical studies for HIV coreceptor antagonists have used the phenotyping-based Trofile test. Meanwhile various simpler-to-do genotypic tests have become available that are compatible with standard laboratory equipment and Web-based interpretation tools. However, these systems typically analyze only the most prominent virus sequence in a specimen. We present a new diagnostic HIV tropism test not needing DNA sequencing. The system, XTrack, uses physical properties of DNA duplexes after hybridization of single-stranded HIV-1 *env* V3 loop probes to the clinical specimen. Resulting "heteroduplexes" possess unique properties driven by sequence relatedness to the reference and resulting in a discrete electrophoretic mobility. A detailed optimization process identified diagnostic probe candidates relating best to a large number of HIV-1 sequences with known tropism. From over 500 V3 sequences representing all main HIV-1 subtypes (Los Alamos database), we obtained a small set of probes to determine the tropism in clinical samples. We found a high concordance with the commercial TrofileES test (84.9%) and the Web-based tool Geno2Pheno (83.0%). Moreover, the new system reveals mixed virus populations, and it was successful on specimens with low virus loads or on provirus from leukocytes. A replicative phenotyping system was used for validation. Our data show that the XTrack test is favorably suitable for routine diagnostics. It detects and dissects mixed virus populations and viral minorities; samples with viral loads (VL) of < 200 copies/ml are successfully analyzed. We further expect that the principles of the platform can be adapted also to other sequence-divergent pathogens, such as hepatitis B and C viruses.

The predominant virus variant in early stages of the clinical manifestation of disease, CCR5-tropic HIV, is found in approximately 80% of treatment-naive patients (1, 2). Although this number can vary for the different virus subtypes, the percentage of CXCR4-tropic HIV isolates is generally low and tends to rise with disease progression (3–6). Nevertheless, the fraction of CCR5-tropic viruses in clinical specimens continues to stay at >50% throughout the course of infection (7, 8). As such, the molecular interactions between the viral envelope and the cellular chemokine receptor CCR5 were recognized as potentially attractive targets for drug development and have yielded compounds and drugs able to specifically block CCR5-tropic HIV (9–11). It is this selectivity of the inhibition of one (CCR5) and not the other (CXCR4) viral coreceptor that necessitates tropism testing prior to prescribing drugs of this particular class. Although the chemokine receptor binding site in the HIV envelope is constituted mainly by the V3 loop, the V1/V2 regions, and the C4 conserved region in the HIV protein gp120, coreceptor tropism is dictated predominantly by amino acid sequences of the V3 region (12, 13). But also sequences of other variable *env* regions can contribute as secondary sites to the viral tropism (14–17).

Initially, all tropism determinations in the key clinical studies during development of CCR5 antagonists, e.g., maraviroc (Celsentri/Selsentry), used several sensitivity versions of the Trofile test, a phenotype-based system developed by Monogram Biosciences. And along with the approval of maraviroc as the first drug in class, the HIV-1 authorities required a mandatory tropism determination prior to any prescription (18). The phenotypic Trofile test, particularly its enhanced-sensitivity version (TrofileES), had proven to represent an excellent tool for determining the tropism of HIV in patients, particularly when the question was to detect with highest sensitivity CXCR4-tropic viruses. At that time, it was crucial for the salvage studies to exclude affected patients from studies in order to minimize therapy failure. However, such highly sophisticated mostly centralized phenotypic testing turned out to be problematic for the everyday settings when introducing this drug class into clinical practice (19). Particularly for requests from Europe, the need for international sample shipment, a limited test sensitivity (>1,000 copies/ml), and long turnaround times were recognized as inacceptable obstacles. As a consequence, various simpler diagnostic tools have been developed and validated (20–23). And this development of genotypic tests created a need for a further refinement of the tools to be able to characterize mixed virus populations, detect CXCR4 tropism in viral minorities, and define predictors of disease progression.

Various methods were developed and evaluated as possible options for simplifying the diagnostic procedures. We, as others, have used amplified HIV-1 *env* sequences for tropism analysis based on a heteroduplex tracking assay (HTA) (4, 24), Pathway Diagnostics (SensiTrop)-developed commercial HTA-based tests, and later Quest Diagnostics-developed commercial HTA-based tests.

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Address correspondence to Thomas Klimkait, thomas.klimkait@unibas.ch.
S.E. and H.S. contributed equally to this work.
* Present address: Heinz Stucki, Roche Diagnostics AG, Rotkreuz, Switzerland.

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The apparent complexity of the subject is exemplified in a recent publication by Cabral et al., who compared genotypic methods with Trofile as the phenotypic standard and concluded that "composite algorithms may be needed" for predictively assessing the viral tropism when only V3 sequences are analyzed (25). Comparative studies of the commercial genotypic test with the validated Trofile assay found the SensiTrop test to be inferior in identifying CXCR4 tropism in clinical specimens. Hence, the use of the SensiTrop test was suspended and has in the meantime been replaced with sequence-based methods, including sensitive next-generation sequencing (Reflex; Quest Diagnostics). One key element not revealed for most published HTA-based tropism tests is the strategy by which the hybridization oligonucleotide of the test was identified or the methods by which the relative tropism specificity of such oligonucleotides was validated for the commercial test.

This study presents a new approach attempting to improve and simplify genotypic tropism testing. The method presented here is based on the principles of duplex tracking as initially described by Delwart et al. (26); only for a limited number of critical and ambiguous samples (<10%) did it require complementation by sequence information or phenotyping. As is principally typical for homo- and heteroduplex tracking, our system utilizes the analysis of double-stranded hybrids between a patient-derived HIV-1 sequence(s) and a small set of defined synthetic V3 sequences in a standardized capillary assay format. A key element of this development was the primer optimization based on numerous, characterized sequence pairs.

MATERIALS AND METHODS

Clinical specimens were from routine testing in the frame of the Swiss HIV Cohort Study (SHCS). As no preselection of patients or selection during sampling was performed, the genotypic properties were similar to those observed in routine at the Basel center: over 90% of all samples belong to subtype B, with the next most frequent subtype being C (<5%).

Preparation of labeled probe. Single-stranded (ss) 6-carboxyfluorescein (FAM)-labeled V3 probes were obtained by PCR using commercial FAM-labeled, high-performance liquid chromatography (HPLC)-purified oligonucleotides (FAM-GA ATC TGT AGA AAT TAA TTG TAC AAG AC) in combination with a biotin-tagged oligonucleotide (biotin-TGC TCT ACT AAT GTT ACA ATG TGC TTG TCT TAT) for the opposite strand (Microsynth, Balgach, Switzerland) covering the HIV-1 V3 region. Ten microliters of 5× iProof HF buffer, 1 μl 10 mM deoxynucleoside triphosphate (dNTP) mix, 1 μl of each primer corresponding to 10 pmol, 0.5 μl iProof DNA polymerase (Bio-Rad, Reinach, Switzerland), and 30 ng of DNA template were mixed on ice and water added to 50 μl. After 2 min at 98°C, a standard cycling protocol with 35 cycles (10 s at 98°C, 15 s at 48°C, 10 s at 72°C) was performed with a final extension for 10 min at 72°C.

Single-stranded DNA separation. PCR products were fixed via a biotin tag onto streptavidin. Forty microliters of Dynabeads M-280 streptavidin suspension (Life Technologies, Zug, Switzerland) was washed three times in 200 μl binding buffer (20 mM Tris-HCl [pH 7.5], 10 mM EDTA, 2 M NaCl) in a 1.5-ml reaction tube (Eppendorf, Hamburg, Germany) by using magnetic separation. Then 50 μl of purified PCR product and beads were mixed in 50 μl binding buffer and incubated for 30 min in the dark. Beads were then separated with the magnet for 2 min, supernatant was removed, and the Dynabead-DNA complex was washed 3 times in 200 μl wash buffer (20 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1 M NaCl). For strand separation, the complex was resuspended in 20 μl 0.2 N NaOH, incubated for 10 min in the dark, and magnetically separated for 2 min. The supernatant containing the single-stranded, labeled DNA was collected in a reaction tube, and 20 μl of 1 M Tris-HCl (pH 7.5) was added. Prior to use, yields were quantified using a NanoDrop ND-1000 photometer (Fisher Scientific, Wohlen, Switzerland). A tetramethylrhodamine (TAMRA)-labeled 80-bp fragment of unrelated *bcr-abl* DNA served as the molecular weight marker (MWM) for each electrophoretic run. Sample preparation and PCR for XTrack was as follows: for RNA extraction from clinical specimens, the lysis protocol of the Prepito NA body fluid kit was followed (PerkinElmer, Baesweiler, Germany), and a one-step reverse transcription-PCR (RT-PCR) was then performed.

For one-step reverse transcription, primers F-6943 (CAC AGT ACA ATG YAC ACA TGG AAT) and R-7365 (AGT AGA AAA ATT CYC CTC YAC AAT TAA A), each 10 pmol, were mixed with 5 μl RNA template, 25 μl of Herculase II RT-PCR 2× master mix, and 1 μl Affinity Script RT/RNase block (Affinity Script One-Step RT-PCR kit; Agilent Technologies, Basel, Switzerland) according to the manufacturer's instructions in a final volume of 50 μl. Incubation was for 5 min at 45°C, 1 min at 92°C, 40 cycles of 20 s at 92°C, 20 s at 51°C, and 30 s at 68°C, and then 3 min at 68°C.

After the one-step RT-PCR and Illustra Exostar 1-step treatment (GE Healthcare, Opfikon, Switzerland) for 15 min at 37°C and 15 min at 80°C, the 2nd nested PCR step yielded products of ca. 140 bp in length using the following conditions: 5 μl of the one-step RT-PCR product was added to 10 pmol of primer F-7092 (GAA TCT GTA GAA ATT AAT TGT ACA AGA C) and 10 pmol of primer R-7232 (TGC TCT ACT AAT GTT ACA ATG TGC TTG TCT TAT), dNTPs, and 1 μl of PfuUltra II enzyme (Agilent Technologies) in PfuUltra II buffer in a reaction volume of 20 μl. PCR was carried out for 1 min at 92°C, and then 30 cycles of 20 s at 92°C, 20 s at 51°C, and 15 s at 72°C, and then 3 min at 72°C. The product was purified from a 2% agarose gel by excision and recovery of the V3 fragment and quantified. Approximately 20 ng V3 DNA was then mixed with 2 ng single-stranded probe, 2 μl 10× TKE buffer (10 mM Tris-Cl [pH 7.5], 10 mM KCl, 1 mM EDTA), and H_2O to a final volume of 10 μl. The mix was denatured at 95°C for 5 min and rapidly cooled on wet ice in the dark for 10 min, after which 10 ng of the *bcr-abl* molecular weight marker was added. Samples were loaded onto an ABI 310 genetic analyzer fitted with a 47-cm capillary.

Fragment analysis. The structure-based "genosorting" fragment analysis system uses POP conformational analysis polymer (CAP; ABI-Life Technologies catalog no. 4340379) at 5%, 1× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA), and 10% glycerol. Electrophoresis was carried out in 1× TBE and 10% glycerol; samples were injected at 15 kV for 10 s and run for 30 min at 10 kV at a temperature of 30°C.

The resulting electropherograms were used to calculate for each probe the relative run length of a patient-derived HIV-1 sample, compared to the run of the perfect match (equal to a value of 1) and to the prototypic clonal CCR5-tropic reference virus HIV_{BaL}. Based on the validation with phenotypically characterized isolates, this resulted in the reliable rule that values for the sample hybrid/perfect match ratio of below 0.7 were found for X4-tropic viruses, those above 0.79 were found for R5-tropic viruses, and values between 0.7 and 0.79 were called "indeterminate."

Dideoxy sequencing. Sequencing was carried out using BigDye Terminator kit v3.1 according to the manufacturer's instructions (Life Technologies, Zug, Switzerland). Reaction products were processed on an ABI3100 sequence analyzer.

rPhenotyping. Principles and details of the replicative phenotyping protocol have been described elsewhere (27, 28) with the following adaptations for tropism analysis. Amplification of the HIV-1 Env gene isolated from clinical samples was successfully performed when virus loads were greater than 500 copies/ml (29). The HIV-1 envelope was amplified using the AffinityScript One-Step RT-PCR kit (Agilent Technologies, Basel, Switzerland) on a Biometra T3000 cycler (Biometra, Goettingen, Germany); primers matched nucleotide positions as indicated by their numbers: F_5700, GAA ACT TAT GGG GAT ACT TGG; R_8494, AGC TGA AGA GGC ACA GGC TCC. After primer annealing at 65°C for 5 min, 42°C for 2 min, and 25°C for 10 min, reverse transcription was conducted at 45°C for 30 min, 92°C for 1 min, followed by PCR at 40 cycles of 92°C for 20 s, 55°C for 20 s, and 68°C for 3 min, and then extension was at 68°C

for 5 min. For the 2nd, nested, PCR for envelope and for introducing the cloning sites MluI and NgoMIV, the *Pfu*-ultraII Fusion HS DNA polymerase was used (Agilent Technologies, Basel, Switzerland). Primers were F_6435M (CYA CCA ACG CGT GTG TAC CCA C) and R_8319N (TGA RTA TCC CTG CCG GCC TCT ATT YAY TAT AGA AA); cycling conditions were 95°C for 2 min, 35 cycles of 95°C for 20 s, 50°C for 20 s, and 72°C for 1 min and 20 s, followed by an extension at 72°C for 3 min.

Products were cut with MluI and NgoMIV (New England Biolabs; Bioconcept, Allschwil, Switzerland) and purified over a 0.8% agarose gel. Then the respective fragments of 1.9 kbp were ligated into an NL4-3 backbone to reconstitute fully functional proviruses. After transformation of Top10 bacteria (Life Technologies), 4 ml of standard LB broth-Amp was directly inoculated without plating in order to retain viral diversity. Transformed bacteria were grown at 37°C overnight. Plasmid DNA was purified (DNA MiniPrep kit; Macherey-Nagel AG, Oensingen, Switzerland) and directly used for cell transfection. The drug susceptibility assay was performed as described previously (28) by using serial dilutions of the CCR5 antagonist TAK-779 (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, MD, USA) (30) or the CXCR4 inhibitor AMD3100 (Sigma-Aldrich, St. Louis, MO, USA) (31). Briefly, the susceptible human cells were transfected with recombinant HIV-1 plasmids to produce replication-competent virus. Cultures were maintained in the presence of active concentrations of the respective antagonists. The time window of 4 days permitted up to 4 rounds of viral replication.

For phenotypic comparison, samples were sent to Monogram Biosciences (South San Francisco, CA, USA) for analysis using the TrofileES version of the commercial Trofile test.

Ethics statement. The SHCS has been approved by the responsible ethical committees of all participating institutions (Ethikkommission Beider Basel; Kantonale Ethikkommission Bern; Comité d'Éthique du Département de Médicine de Hôpitaux Universitaires de Genève; Commission d'Éthique de la Recherche Clinique, Lausanne; Comitato Etico Cantonale, Bellinzona; Ethikkommission des Kanton St. Gallens; and Ethik-Kommission Zürich [all from Switzerland]).

Written informed consent was obtained from and is on file for all study participants. This study has been approved by the scientific review board of the Swiss HIV Cohort Study.

RESULTS

By targeting a host protein, HIV coreceptor antagonists represent a distinct, unique class of HIV inhibitors. As for cell binding, the virus has the principal option to use one of its two main coreceptors, CCR5 or CXCR4; it appears mandatory to assess this preference of the virus prior to prescribing an inhibitor that is restricted to CCR5-tropic virus variants. A test therefore has to provide reliable means for predicting whether or not the virus in the respective patient will respond to the treatment.

Here, we describe a refined genotypic test which is based on sequence hybridization and which does not require knowledge of the genetic envelope sequence.

For an extensive test validation, representative viruses with known tropism were utilized. The *env* regions of the CXCR4-tropic clonal HIV-1 strain NL4-3 (32), the CCR5-tropic AD87 (33), or the CCR5-tropic BaL (34) were utilized (reviewed in reference 35) and inserted into a viral NL4-3 genome backbone. Virus tropism and replicative fitness of the resulting constructs were assessed by replicative HIV phenotyping. This test, termed PhenXR, permits up to four rounds of virus replication in the presence of inhibitor. Details have been described elsewhere, and the test has been validated for diagnostic drug resistance testing (27). For all above-mentioned *env* variants, a replicative fitness of >70% compared to that of the wild-type NL4-3 was noted. This confirms that an exchange of larger segments of the *env* gene is sufficient for a functional tropism determination; it further shows that the exchange retains envelope functionality.

Properties of the "genosorting tropism test" (XTrack). Although some reports have indicated a tropism-determining role for regions outside the V3 region (36), most tests focus today on V3-derived information (19, 37, 38). The tropism test described here is based on the sequence along with a sequence-implied structure characterized by the high variability of the V3 region of the envelope glycoprotein gp120 of HIV-1. Env sequences of this variable region in clinical samples are hybridized to double strands with an exogenously added sequence probe of known tropism. This step will constitute a more or less perfect double strand. The use of carefully designed, representative probes for diagnostics has successfully been compared against the genotypic method of Geno2Pheno and against a phenotype-based method (39). In the past, different HTA-based approaches have been described by others (40), and also the use of capillary analysis systems has been presented, e.g., by Baumann et al. (41). Principally, a fluorescently labeled single-stranded DNA probe representing the V3 region of known coreceptor tropism is hybridized under nondenaturing conditions to V3 loop sequences which were obtained from the virus in a given clinical sample. The degree of matching between sample and probe will determine a certain electrophoretic mobility that discriminates more perfect duplexes (homoduplex of almost identical strands) from imperfectly matched DNA (heteroduplexes). The principle of the test is to associate the respective mobility areas with a given tropism. This can be accomplished only with the most carefully selected probes that represent a given tropism and cover the various HIV subtypes.

Technical discrimination of R5/X4. A principal limitation of the HTA approach is that the envelope V3 regions of individual HIV-1 isolates and of the various virus subtypes can differ dramatically from each other. In fact, this is the main reason for a certain inherent limitation of any genotypic prediction of an HIV tropism. Nevertheless, clinical comparisons have clearly demonstrated that for the vast majority of cases, genotyping and phenotypic methods are in quite good agreement, particularly for R5-tropic virus (25, 42). In an HTA approach, the degree of matching between patient-derived virus and the known sequence probes defines discrete migration zones. The capillary running characteristics of the duplexes under semi-denaturing conditions form the technical basis for the definition of these zones. The tropism prediction is based on the ratio between the distance from the start of the sample-probe duplex ("duplex") and the single-stranded probe termed "1" divided by the distance of the perfectly matched probe-probe duplex, termed "2" (Fig. 1A). The designation of an "area of migration" forms the basis for defining discrete cutoffs between R5- and X4-tropic isolates. The shorter the distance of a sample-probe duplex from the perfect match (PM), the smaller the difference in character or sequence between probe and sample. Hence, the sample is more likely to share the tropism of the probe.

Of note, in earlier studies, which had recruited mostly patients in the U.S. or Europe, the number of non-B-subtype samples was quite small. Comparisons of older and more recent studies thus have the potential limitation that the more divergent non-B samples affect test performance (amplification success) as well as interpretation in a negative way. This is a principal caveat for comparing tests in different settings and time periods.

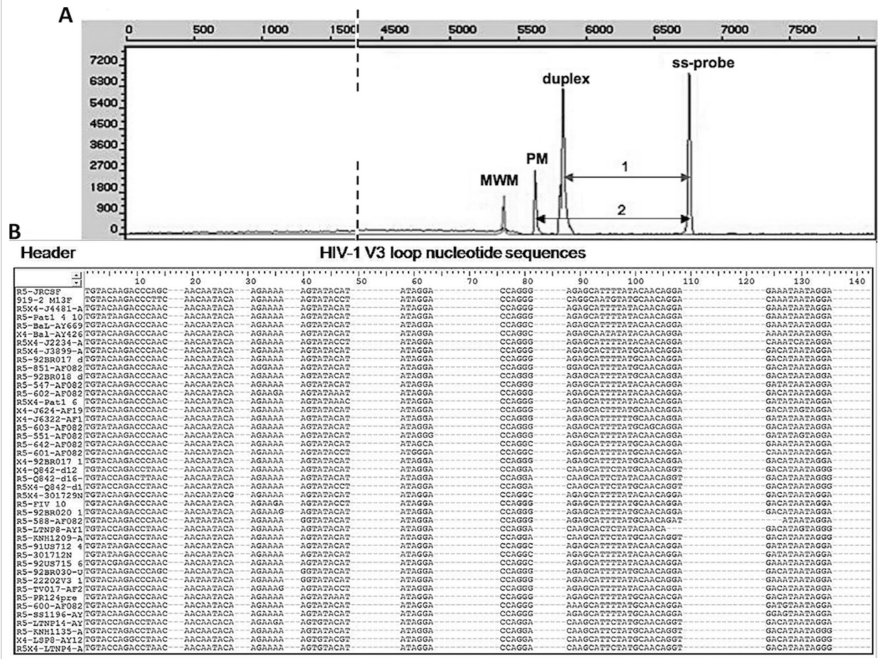

FIG 1 (A) Typical electropherogram depicting the test principle. Four types of mass peaks are resolved: 80-bp molecular weight marker (MWM); single-stranded V3 probe (ss-probe); hybrid of patient-derived V3 sequence and probe (duplex). Residual double-stranded probe material serves as the ds-control. PM, perfect match; 1 and 2 indicate relative peak distances for tropism calculation. (B) Representative section of the list of V3 loop nucleotide sequences after grouping according to their relatedness to the probe candidate in the top line: HIV-$1_{JR\text{-}CSF}$.

A major task lies in the broad divergence of HIV-1 sequences, particularly for the variable regions of *env*. From the literature, only a small amount of information is available for guiding the sequence optimization for a given diagnostic test. Yet it is quite likely that oligonucleotides chosen for amplification or hybridization are responsible for sequence-biased amplification. This will affect the results of the test. In order to systematically approach the design of suitable diagnostic probes, we tried several algorithms and utilized sequences available through the Los Alamos database (43). Its >1,000 *env* entries formed the basis for designing a larger number of probes. The best-fitting ones were sequentially tested on the same set of specimens.

Probe design strategy 1: direct sequence alignment. This approach was based on a large sequence alignment with the aim to identify X4- and/or R5-tropic consensus sequences that could serve as representative probes.

For primary amplification of the V3 *env* target of HIV-1, the primer pair V3_7092F/V3_7232R was used yielding a 140-bp fragment; the primer positions were chosen based on published information (44, 45). Env sequences from 635 patient-derived viruses were obtained from the Los Alamos database (43) and from an in-house set with available subtype and tropism information. All sequences were computed to yield a generic HIV-1 consensus. Using ClustalW (EMBL-EBI, Cambridge, United Kingdom), sequences were aligned, compared to one another, and grouped according to relatedness and homology. Figure 1B shows a representative section of this alignment. Stretches of dots in the sequences indicate polymorphisms and reflect regions, where individual sequences had insertions of the respective length (not shown). The SplitsTree4 software (University of Tubingen, Tubingen, Germany) was then applied to yield a graphical dendrogram (Fig. 2) with discrete branches, largely reflecting the different subtypes of HIV-1. About 67% of the available sequences belonged to R5-tropic isolates (blue) mostly clustering tightly. In contrast, X4-tropic sequences (red) were distributed more toward the periphery. Of note, for subtype D, the available data set contained more X4-tropic sequences, and branches tend to have longer distances than other subtypes from a virtual "best probe" de-

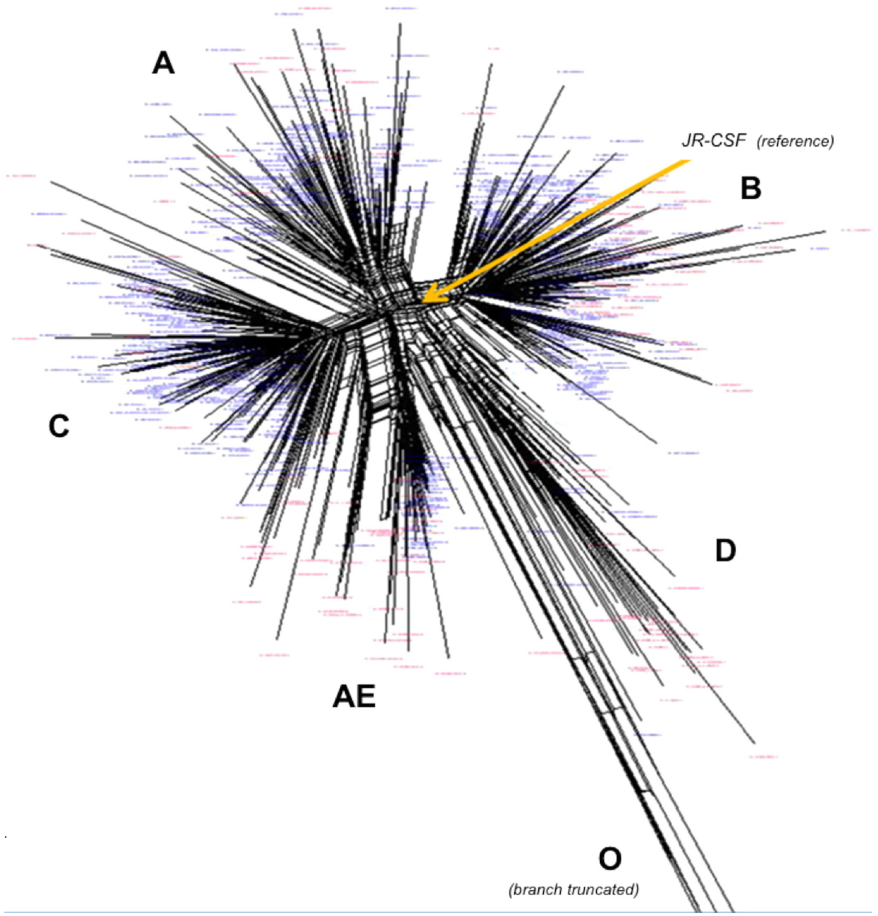

FIG 2 Dendrogram of 655 HIV-1 sequences depicting the maximal relative relationship to one another (Los Alamos database and data from the Swiss HIV Cohort Study). Blue letters, R5-tropic viruses; red letters, X4-tropic isolates. Lobes of related sequences correspond with subtypes/groups (capital letters); the group O branch with 4 isolates is truncated. Yellow arrow indicates arbitrary reference sequence HIV-$1_{JR\text{-}CSF}$

fining the center of the dendrogram. As an interesting correlate with published observations, subtype D associated with the highest rates of dual/mixed (D/M) tropism (2) in a phenotypic test. A distinct set of exclusively R5-tropic sequences (Fig. 2, bottom right) belonged to group O isolates that, in agreement with its phylogeny, possessed the longest distance to our B subtype reference JR-CSF near the center (branch length truncated). Moreover, recent investigations suggest that for CRF01_AE, algorithms for the genotypic tropism prediction have a much higher failure rate than for other subtypes (46). It is therefore interesting to note that the Clustal analysis in Fig. 2 produced a dendrogram that very discretely separates subtype A from CRF01_AE.

TABLE 1 Binding enthalpies of three R5-specific probes to a selection of R5 or X4 sequences[a]

Probe	Identifier	Enthalpy (kcal/mol)
NH45_B	NH45_B	−189.04
	BaL	−181.94
	92BR018	−178.64
	PR107	−176.04
	TV017	−175.53
	XJ624	−175.05
	AY426	−173.57
	AC10	−173.27
	851	−169.49
	91US712	−166.72
	1DRPT	−166.58
	QH0692	−166.21
	LDLOCE	−163.77
	IN_1	−163.08
	92BR021	−162.63
	LDLPMON	−157.84
	C113	−155.01
	FIV_10	−153.01
	92US727	−150.03
	R5-92HT596	−149.97
	92BR004	−149.20
KNH_C	KNH_C	−199.23
	C113	−171.89
	92BR021	−159.42
	92US727	−158.11
	PR117	−156.83
	J624	−155.85
	BaL	−155.55
	851	−155.03
	QH0692	−154.35
	TV017	−154.02
	AY426	−150.44
	LDRPT	−148.66
	92BR0041	−147.23
	FIV_10	−146.55
	LDPMON	−146.12
	LDLOCE	−144.17
	AC10	−143.92
	91US712	−143.76
	IN_1	−141.42
	92BR021	−137.85
	92HT596	−127.65
TAZ_A	TAZ_A	−201.33
	92BR018	−146.15
	C113	−145.12
	PR107	−143.56
	FIV_10	−143.17
	J624	−142.60
	QH0692	−142.03
	851	−141.80
	BaL	−141.72
	LDRPT	−141.72
	92BR004	−141.01
	TV017	−139.01
	AY426	−138.79
	AC10	−136.52
	LDLOCE	−136.18
	92US727	−135.50
	LDLPMON	−134.79
	91US712	−133.17
	IN_1	−127.96
	92BR021	−126.00
	92HT596	−116.14

[a] Unshaded, R5 sequences; shaded, X4 sequences. The top value for each probe corresponds to the perfect match of the probe. Values are sorted by decreasing ΔH values.

For a best representation of the different clades, we chose several R5- or X4-tropic V3 sequences as single-stranded candidate DNA probes and tested them on a representative subset of 20 HIV-1 samples with known coreceptor tropism to verify a correct assignment. FAM-919 produced for 17 samples an interpretable result and yielded the highest number of distinct results (R5, X4, or mix) with 88% concordance with the TrofileES test. In comparison, the reference FAM-JRCSF yielded 19/20 (95%) interpretable results but tended to be too high on the R5 side (85%), which led to 10 false predictions; only 9 samples (47%) were in agreement with TrofileES. And FAM-JRCSF failed to dissect mixed virus populations.

Based on its favorable properties, FAM-919 was kept as one promising probe for the XTrack test system. In order to further minimize ambiguous results, we set out to define a second probe, which was, however, not readily identified with this strategy.

Probe design strategy 2: binding enthalpies linked to phylogenetic relatedness. This approach considered physical properties of base pairing stability and sequence relatedness. The algorithm, kindly provided by Alex Thielen (Max Planck Institute, Kaiserslautern, Germany), compared each individual candidate R5 probe to every other sequence in the entire sample set of 655 R5 and X4 sequences. Each sample sequence would be sorted in relation to the one chosen to serve as the reference. The degree of relatedness between the two assigns a value to this pair. A value of 0 stands for an identical sequence, and a value of 1 stands for the most distant one in the set. Hence, a closer relatedness of any given sequence to the chosen R5 reference is reflected in a smaller resulting value. The aim of this process was to identify a set of sequences with very low scores that would allow a best separation of R5-tropic from X4-tropic viral sequences. Ten R5-tropic sequences representing the main lobes of the dendrogram of Fig. 2 were chosen, and they were aligned one by one to the entire sequence set from the database. A representative section of the results is shown in Table 1.

From the sum of distance values for each sample, a total XR score was calculated for every probe candidate. Table 2 summarizes, e.g., the result for probe candidate 01PB27ZA. This XR algorithm was run against all available isolates, and the obtained values are listed in the "Distance" column. The table is sorted by increasing distance values, with a score of 0 indicating 100% relatedness. In the "1/0 R5" column, a value of 1 is then entered for known R5 tropism according to the Los Alamos database, and a value of 0 for X4-tropic sequences. The "Index" column counts numerically down from 1 for the top sequence (for reference sequence 01PB27ZA) to index 655. A smaller index number thus indicates a closer relatedness to 01PB27ZA. A multiplication of columns "1/0 R5" and "Index" yields the next column, named "Value R5." It generates a positive value solely for R5-tropic se-

TABLE 2 Relative enthalpy-based sequence distance from each V3 sequence to every other one in the list of published isolates[a]

Tropism	Isolate (GenBank accession no.)	Sequence	Distance	1/0 R5	Index R5	Value R5	1/0 X4	Index X4	Value X4
R5	R5-S123 (AF153176)	TGTACAAGAC	0.021858	1	3	3	0	3	0
R5	R5-S194 (AF153164)	TGTATAAGAC	0.021858	1	4	4	0	4	0
R5	R5-S083 (AF153189)	TGTACAAGAC	0.027322	1	5	5	0	5	0
R5	R5-98ZABLM84 (DQ235618)	TGTATAAGAA	0.027322	1	6	6	0	6	0
X4	X4-CHN19	TGTACAAGAC	0.027322	0	7	0	2.3	7	16.1
R5	R5-A125 (AY253304)	TGTGTAAGAC	0.027322	1	8	8	0	8	0
R5	R5-C054 (AF153155)	TGTACAAGAC	0.027322	1	9	9	0	9	0
R5	R5-93MW_965 (AY713413)	TGTACAAGAC	0.027322	1	10	10	0	10	0
R5	R5-S071 (AF153162)	TGTACAAGAC	0.027322	1	11	11	0	11	0
R5	R5-TV002 (AF254767)	TGTACAAGAC	0.027322	1	12	12	0	12	0
X4	X4-CTSC2 (AY043176)	TGTACAAGAC	0.027322	0	13	0	2.3	13	29.9
R5	R5-TV019 (AF254783)	TGTACAAGAC	0.027322	1	14	14	0	15	0
R5	R5-C022 (AF153186)	TGTACAAGAC	0.027322	1	15	15	0	15	0
R5	R5-TV014A (AF391247)	TGCACAAGAC	0.027322	1	16	16	0	16	0
R5	R5-99ZASW5 (AY170658)	TGTACAAGAC	0.027322	1	17	17	0	17	0
R5	R5-99ZASW38 (AY170667)	TGTACAAGGC	0.027322	1	18	18	0	18	0
R5	R5-99ZASW38 (AY505002)	TGTACAAGGC	0.027332	1	19	19	0	19	0
R5	R5-99ZATM19 (DQ235629)	TGTACAAGAC	0.027322	1	20	20	0	20	0
R5	R5-98ZA502 (AY158534)	TGTACAAGAC	0.032787	1	21	21	0	21	0
R5	R5-S147 (AF153169)	TGTACAAGAC	0.032787	1	22	22	0	22	0
R5	R5-S171 (AF153150)	TGCACTAGAC	0.032787	1	23	23	0	23	0
R5	R5-01PB15ZA (AY510062)	TGTACAAGGC	0.032787	1	24	24	0	24	0
X4	X4-C070 (AF153143)	TGTACAAGGC	0.032787	0	25	0	2.3	25	57.5
R5	R5-C109 (AF153142)	TGTACAAGAC	0.032787	1	26	26	0	26	0
R5	R5-97ZAPET100 (DQ235617)	TGTACAAGAC	0.032787	1	27	27	0	27	0
R5	R5-01PB21ZA (AY510065)	TGTACAAGAC	0.032787	1	28	28	0	28	0
R5	R5-S059 (AF153151)	TGTACAAGAC	0.038251	1	29	29	0	29	0
R5	R5-TV005 (AF254770)	TGTACAAGAC	0.038251	1	30	30	0	30	0
R5	R5-TC25 (AY265945)	TGTACAAGAC	0.038251	1	31	31	0	31	0

[a] Highest scores for various HIV-1 isolates to the reference (line 1), with "Distance" value = 0 and sorted by increasing values; columns as detailed in the text.

quences. When the same procedure is now reiterated for every one of the 655 sequences, each reference sequence itself will receive an overall qualifier through the total sum of all "Value R5" scores. This procedure allowed us to define an overall relatedness for all R5 sequences to one another. Simultaneously, it reveals the greatest distance to CXCR4-tropic HIV-1. Subsequently, the same procedure was run with X4-tropic sequences ("1/0 X4" column for X4 viruses). As X4 isolates are underrepresented in the Los Alamos database (only 43% of all entries), we compensated for this by applying the multiplier of 2.3, and cumulative value R5 was divided by cumulative value X4 to yield a final XR score for the winning reference. The top scoring two sequences were used for further experiments. As an example, sequence 01PB27ZA had a final score of 0.93 (shaded in Table 3). The lowest overall score (greatest separation of R5 and X4) within the set of 655 samples was 0.46 and the maximal score was 4.9. As the score for a random distribution was 1.23, meaningful sequences have to possess a score below this. Interestingly, the candidate sequences in the top section of the list were contributed mainly by virus subtypes A and C (Table 3).

In summary, sequence 01PB27ZA scored favorably with 0.93 and was therefore tested in the XTrack assay.

Probe design strategy 3: free enthalpy (Δ*H*). As V3 sequences in clinical specimens typically differ in sequence, a "best match" between sample and probe is expected to yield the most stable hybrid. In this approach, the theoretically best probe candidates were hybridized *in silico* with each one of the available 655 sequences using the online tool RNAfold (Institute for Theoretical Chemistry, University of Vienna, Vienna, Austria). However, the three probe candidates representing the HIV-1 subtypes B, C, and A (NH45_B, KNH_C, TAZ_A) yielded no consistent ranking. And although X4 sequences principally tended to be closer to the bottom of the rank, there was no clear separation from R5-tropic sequences. We therefore concluded that free enthalpy alone was not suitable for predicting a tropism-specific clustering (data not shown).

In summary, of the three strategies, the highest predictive power was reached with probes targeting subtypes A and C:

TABLE 3 XR scores of relatedness[a]

Sequence identifier	Subtype	XR score
01PB27	C	0.93
02CM	A	0.99
UGSE81	C	1.06
KH024	A	1.10
93BR	A	1.10
SF-162	C	1.13
LSP3	A	1.15
US01	A	1.17
92US	C	1.19
93TH	C	1.19
Random distribution		1.23

[a] Calculated for the top 10 R5 sequences of HIV-1 subtypes A or C. Random distribution, reference score for a random distribution of R5 and X4 sequences. Shading indicates the top scoring two sequences, which were used for further experiments.

TABLE 4 XTrack results[a]

Sample no.	FAM probe result 919	CM_A	PB_C	TrofileES result	Size (bp)	Subtype
1	X4	R5	R5	R5	105	A/AG
2	Mix	Mix	Mix	D/M	105	B
3	R5	R5	R5	R5	105	B
4	Mix	?	?	D/M	105	B
5	Mix	Mix	Mix	D/M	105	A/AG
6	Mix	R5	R5	D/M	105	B
7	R5	R5	R5	R5	105	A/AG
8	R5	X4	X4	R5	102	B
9	Mix	Mix	Mix	D/M	108	B
10	R5	R5	R5	R5	105	B
11	R5	R5	?	R5	105	B
12	R5	X4	X4	R5	102	B
13	?	R5	R5	D/M	105	A/AG
14	?	R5	R5	R5	105	B
15	Mix	Mix	R5/?	D/M	105	B
16	R5	R5	R5	R5	105	B
17	R5	R5	R5	D/M	105	B
18	?	?	?	D/M	105	B
19	R5	R5	R5	R5	105	B
20	R5	?	R5/?	R5	105	B

[a] Using probes FAM-919, FAM-CM_A, or FAM-PB_C in columns 2, 3, and 4, respectively, and in comparison to Trofile (column 5). Column 6 lists the V3 loop length and column 7 the subtype for each sample. Samples with shorter V3 loops (no. 8 and 12) are shaded. R5, CCR5-tropic virus; X4, CXCR4-tropic virus; Mix, mixed virus population with both tropisms; D/M, dualtropic or mixed virus; ?, noninterpretable result.

01PB27ZA (termed FAM-PB_C) and 02CM (termed FAM-CM_A). By sequence alignment, differences were found at 10 positions, distributed throughout the V3 region (not shown). We added a subtype B candidate, FAM-919. Then the 140-bp DNA segment of each of these V3-probe candidates was generated synthetically by tiling PCR and the gel-purified products cloned into the vector pCR-blunt II-TOPO (Invitrogen).

These probes for XTrack analysis were tried on 20 patient-derived V3 sequences. In parallel, the same patients were analyzed with the TrofileES test. Previous studies have confirmed the good agreement between the phenotypic format of TrofileES and the genotyping tool Geno2Pheno on a retrospective comparison of a large study population in clinical studies (47, 48). As further confirmation, we also performed a parallel analysis with TrofileES on a limited set of 20 clinical samples. Results are summarized in Table 4. For this trial set, the tropism results with FAM-CM_A and FAM-PB_C were identical and concordant with the TrofileES in 13 out of 20 cases. Shorter or longer length than the canonical 105 bp of the V3 loop is likely to affect the hybridization behavior. Among the discordant samples, two had shorter V3 loops (102 bp in length). Related difficulties have also been reported for the genotypic interpretation by G2P (48) and require further attention. Overall, we found for FAM-919 an 88% agreement with the TrofileES results, 71% for FAM-CM_A, and 67% for FAM-PB_C. For test validation and for establishing interpretation rules, we focused on the combination of FAM-CM_A plus FAM-919 due to the superior agreement with the TrofileES test. We chose to accept the results of the clinically validated TrofileES test by default as true. It should be remembered, however, that in clinical studies, TrofileES results were handled quite restrictively in the way that any sign of "non-R5 tropism" or any "uninterpretable result" led to patient exclusion from the respective clinical studies (49). Such policy potentially restricted the number of valid R5 participants by eliminating all less clear cases. A clinical proof for the validity of this exclusion is not available, and tropism changes between screening and baseline in the clinical studies may reflect the test variability or a certain instability of the viral tropism (50–52). Recent deep-sequencing analyses have demonstrated that low proportions of X4-tropic viruses can be found in almost any clinical sample, yet the clinical relevance, e.g., of X4-tropic virus minorities below 2% in a given virus population found in clinical specimens remains unclear (53).

Capillary analysis of duplex species. A PCR product of about 140 bp containing V3 sequences from HIV-1 in clinical specimens is hybridized to the fluorescently labeled, single-stranded V3 DNA probe of known sequence and tropism. A double-stranded molecular weight marker is mixed with the sample prior to electrophoresis, serving as the migration standard. The relative migration of the products in relation to the marker and to the remaining single-stranded probe allows to determine their relatedness to the homoduplex probe.

In order to assess which of the probes was suitable for analyzing the HIV-1 tropism of clinical samples, we adapted the heteroduplex tracking to a capillary electrophoretic format. A typical electropherogram is depicted in Fig. 3.

The tropism of sample peaks is calculated based on percent migration, (position of single strand − position of duplex of patient sample and probe)/(ss-probe position − ds-probe [PM] position), as described in Fig. 1 (ds is "double-stranded"). With the help of samples with known tropism in a large validation set, two distinct migration zones corresponding to R5-tropic (80 to 100% migration) and X4-tropic (0 to 69% migration) were defined. Signals mapping to the region between these defined zones (70 to 79%) indicate unassignable samples.

For specimens simultaneously containing several distinct HIV variants, several duplexes will form with different, separable electrophoretic migration, therefore "genosorting." We have shown that our system will identify, e.g., X4-tropic viral subpopulations that may represent as little as 1% of the total quasispecies (4).

As a potential limitation, the XTrack analysis is restricted to the V3 region of envelope and will therefore not consider other tropism-relevant regions of the HIV genome.

In our study setup with 145 available samples, 139 samples yielded an interpretable result with at least one of the systems. Validation was performed as a side-by-side comparison of XTrack, Geno2Pheno (10% false-positive rate), and TrofileES, and for 57 clinical samples results were obtained with all three systems. For 137/139, such comparison was available between two systems. All analyses were blinded to the interpreting expert so that the link between results from the other systems was not available to the operator. In order to avoid interpretation bias, a second independent operator performed linkage of the results summarized in Table 5. The high number of missing results for the TrofileES system is explained mainly by two factors. As all clinical samples stemmed from the routine laboratory for tropism analysis, either not enough material was available for including the external TrofileES test or the samples were not suitable for Trofile testing due to viral loads being below 1,000 copies/ml (not accepted by the provider). Although the small number is acknowledged as a significant potential limitation of our study, results

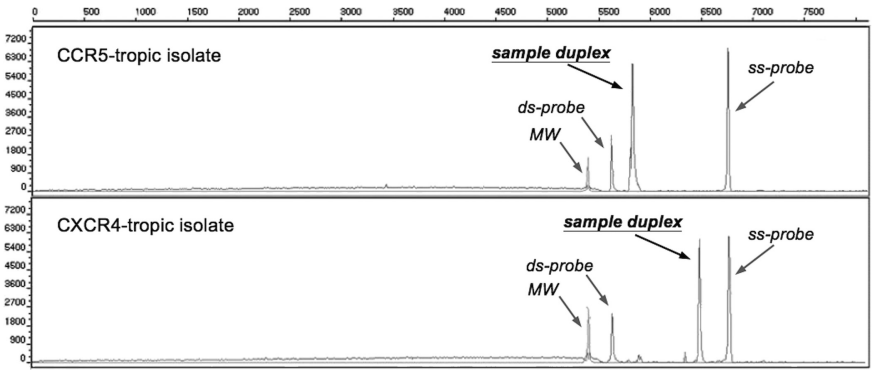

FIG 3 XTrack analysis of one prototypic R5-tropic and one X4-tropic virus at position "sample duplex." MW, double-stranded molecular weight marker; ss-probe, labeled single-stranded probe; ds-probe, double-stranded probe.

from earlier work had clearly demonstrated that genotypic techniques correlated very well with the TrofileES results (25). This agreement hence supports the comparative analysis of this study.

In our analysis, all three systems found R5-tropic virus in 27 samples and X4-tropic HIV or mixes in 15 samples. For 20 samples, TrofileES called D/M, and XTrack identified nine mixed virus populations. All of these correlated with D/M in TrofileES; in contrast, for six of the TrofileES D/M calls, XTrack and Geno2Pheno agreed on X4, and three were called R5. For two samples, XTrack identified an R5 virus and Geno2Pheno an X4 variant. Overall disagreement of at least one system was found in 15 cases (26%).

When assessing the overall performance in six cases, XTrack did not yield a result; in five, no sequence for Geno2Pheno was obtained; and in 56 cases, no TrofileES result was obtained or no such test was performed due to insufficient sample volume or low viral load. Three results were uninterpretable by XTrack due to unusual duplex migration, and for Geno2Pheno four sequences were double sequences that could not be analyzed.

V3 sequence sizes and tentative virus subtypes were determined in order to provide additional information for interpreting discordances. Whereas among the compared samples no link between discordance and subtype was found (not shown), a noncanonical length of the V3 loop (non-105 bp) was more frequently associated with discordances between the tests. With the simultaneous use of probes FAM-CM_A and FAM-919, concordance to TrofileES of over 70% was reached. Less than 5% of XTrack results were noninterpretable.

A statistical analysis produced performance parameters (i) for XTrack versus TrofileES and (ii) for Geno2Pheno versus TrofileES. The sensitivity of determining *R* as the no. of R5-concordant samples/(no. of R5-concordant samples + no. of X4- and mix-discordant samples) was similar or slightly superior for XTrack in the groups with 89.7% or 81.6%, respectively. The test specificity values for X4 tropism as no. of X4- and mix-concordant samples/(no. of X4- and mix-concordant samples + no. of R5-discordant samples) were 73% and 79%, and the negative predictive values toward X4 tropism as no. of X4- and mix-concordant samples/(no. of X4- and mix-concordant samples + no. of X4- and mix-discordant samples) were 80% and 73%. It has been described that tropism changes can be achieved by exchange of single amino acids in the V3 loop (54), and we set out to investigate whether, as reported earlier in a study for subtype A and D viruses (2, 55), the D/M viruses were dualtropic or alternatively mixed virus populations. In the analysis shown in Table 5, for more than half of the TrofileES D/M results (11/20), the XTrack system did not identify a mixed virus population, and in two samples the V3-based genotypes disagreed. As suggested by Huang et al. (2), a likely explanation is that additional determinants outside the V3 region contributed to the viral tropism. The data of Table 5 are depicted in the histogram of Fig. 4, highlighting the good agreement of 83.0 to 84.9% between the systems, with identical results for all three in 76% of the specimens. The proportions of X4-tropic samples in our patient population were determined to be 38.7% by XTrack, 40.3% by G2P (10% false-positive rate), and 37.7% by TrofileES.

By including data from ongoing genotypic testing at the Basel center, a total of 256 samples could be analyzed. Full agreement was found in 79.3% of samples, and the proportion of X4-tropic/mixed viruses represented 25% (54) of all analyzed specimens.

In order to address this possibility further, we employed a replicative phenotyping system (deCIPhR), which uses patient-derived full-envelope sequences for recombinantly reconstituting a fully infectious virus, similar to the format described earlier (27). This test allows for the expansion of virus during four replication cycles in the presence of inhibitors, and the virus carries envelopes from clinical specimens. Residual virus after *in vitro* treatment with, e.g., a CCR5 antagonist can be used for subsequent infections in the presence of a second inhibitor class, e.g., CXCR4 inhibitors. For 23 specimens with available sufficient sample volume, a comparison was conducted for TrofileES and our replicative phenotyping system (PhenXR). All 14 TrofileES analyses with assigned R5 tropism and the only X4 sample were confirmed by PhenXR. TrofileES called eight virus samples D/M, four of which were congruent between both systems and were in our tests exclusively inhibited by the X4 antagonist AMD3100. The remaining four D/M samples by TrofileES were not confirmed by the replicative system PhenXR to contain both tropisms or dualtropic virus. All samples were exclusively inhibited by the CCR5 antagonist in the PhenXR system, and no inhibition or plateau was found with AMD3100; they were thus classified as R5. The four discordant samples were all of subtype B.

TABLE 5 Side-by-side comparison of XTrack, Geno2Pheno, and Trofile

No.	Result[a] XTrack	G2P	TrofileES
1	R5	R5	R5
2	**X4**	**X4**	**D/M**
3	R5	R5	R5
4	**Mix**	**X4**	**D/M**
5	**Mix**	**X4**	**D/M**
6	**Mix**	?	–
7	**Mix**	**X4**	**D/M**
8	R5	R5	R5
9	R5	R5	R5
10	**Mix**	**X4**	**D/M**
11	R5	R5	–
12	R5	R5	R5
13	R5	R5	R5
14	R5	**X4**	R5
15	R5	R5	**D/M**
16	R5	R5	R5
17	**Mix**	R5	**D/M**
18	**X4**	**X4**	–
19	R5	R5	R5
20	R5	R5	**D/M**
21	?	**X4**	**D/M**
22	R5	R5	R5
23	R5	**X4**	R5
24	?	**X4**	R5
25	R5	R5	R5
26	R5	R5	–
27	R5	**X4**	**D/M**
28	R5	**X4**	**D/M**
29	R5	R5	–
30	?	**X4**	R5
31	**Mix**	**X4**	**D/M**
32	R5	R5	R5
33	R5	?	R5
34	**X4**	R5	R5
35	R5	**X4**	–
36	R5	R5	R5
37	R5	R5	R5
38	R5	R5	R5
39	R5	R5	R5
40	R5	R5	–
41	R5	R5	–
42	?	**X4**	**D/M**
43	?	**X4**	–
44	R5	R5	–
45	R5	R5	–
46	**X4**	**X4**	**D/M**
47	**Mix**	**X4**	**D/M**
48	R5	R5	R5
49	R5	**X4**	–
50	R5	**X4**	–

TABLE 5 (Continued)

No.	Result[a] XTrack	G2P	TrofileES
51	**X4**	R5	–
52	R5	R5	–
53	**X4**	**X4**	**D/M**
54	**Mix**	**X4**	**D/M**
55	R5	R5	–
56	**X4**	R5	R5
57	?	R5	R5
58	R5	R5	R5
59	**Mix**	**X4**	**D/M**
60	R5	R5	R5
61	?	R5	**D/M**
62	R5	R5	–
63	?	R5	–
64	**X4**	R5	–
65	**X4**	**X4**	**D/M**
66	**X4**	R5	–
67	R5	–	R5
68	R5	R5	–
69	?	–	**D/M**
70	?	R5	–
71	R5	–	–
72	**Mix**	**X4**	–
73	R5	R5	R5
74	R5	**X4**	R5
75	R5	R5	R5
76	R5	R5	–
77	?	R5	–
78	R5	?	–
79	?	R5	–
80	**X4**	**X4**	–
81	R5	R5	R5
82	?	R5	–
83	R5	R5	R5
84	**X4**	**X4**	**D/M**
85	R5	R5	R5
86	**X4**	R5	–
87	**X4**	R5	–
88	R5	R5	–
89	R5	?	R5
90	R5	R5	R5
91	R5	R5	–
92	R5	R5	–
93	R5	?	**D/M**
94	R5	R5	–
95	**X4**	R5	–
96	**X4**	**X4**	–
97	R5	R5	–
98	**X4**	**X4**	R5
99	?	?	R5
100	**X4**	R5	R5

(Continued on following page)

TABLE 5 (Continued)

No.	Result[a]		
	XTrack	G2P	TrofileES
101	R5	R5	**D/M**
102	**X4**	**X4**	–
103	**X4**	**X4**	–
104	R5	R5	–
105	R5	R5	–
106	R5	R5	–
107	R5	R5	R5
108	R5	R5	–
109	?	**X4**	–
110	**X4**	**X4**	–
111	R5	R5	–
112	**X4**	**X4**	–
113	**X4**	**X4**	–
114	**X4**	**X4**	–
115	**X4**	**X4**	–
116	**X4**	**X4**	–
117	**X4**	**X4**	–
118	**X4**	**X4**	–
119	**X4**	**X4**	–
120	**X4**	**X4**	–
121	?	**X4**	–
122	R5	R5	–
123	R5	R5	–
124	R5	R5	–
125	R5	R5	–
126	**Mix**	**X4**	–
127	**X4**	**X4**	–
128	R5	R5	–
129	R5	R5	–
130	R5	R5	R5
131	R5	?	R5
132	**X4**	**X4**	**X4**
133	**X4**	**X4**	**D/M**
134	R5	R5	R5
135	R5	R5	–
136	R5	**X4**	R5
137	?	**X4**	–
138	**X4**	**X4**	–
139	?	R5	–

[a] G2P, Geno2Pheno; D/M, dual/mixed tropism; Mix, two viral species with R5 and X4 tropism in the same specimen; ?, no tropism assignable by the respective method (uninterpretable results); –, missing value. X4 viruses, mixes, and D/M are indicated with bold letters, R5 viruses are shaded.

The well-characterized CCR5 antagonist TAK-779 was utilized for all studies, as earlier reports had demonstrated its excellent agreement in specificity and potency with maraviroc in cellular systems (56).

Although the PhenXR results were confirmed in repeat experiments (not shown), it cannot be excluded that amplification bias during PCR could have contributed to this discordance between the systems.

Although this report describes the properties of a hybridization-based genotyping principle only for HIV samples, the same physical principles will apply for any genetically divergent pathogen. Hence, an application and similar optimization approaches will be useful for designing suitable test systems also for pathogens such as other highly variable viruses, such as hepatitis B (HBV) and C (HCV) viruses. The same methodology might help to identify optimal probes there, too. This has been suggested already in 1998 by Calvo et al. for genotype 2 of HCV (57).

DISCUSSION

We describe a strategy for complementing and improving the diagnostic genotype-based tropism determination of HIV for clinical use. Sequence-based tropism testing allows a more rapid turnaround time than phenotyping; here, we validated a hybridization-based "genosorting" method, which can further simplify the analysis process by omitting the need for sequencing. In addition, the short PCR fragment required for analysis (<150 bp) allows for the successful application of this system to clinical samples with viral loads below 200 copies/ml, assessed by routine VL testing (Ampliprep-TaqMan; Roche, USA). This poses a major challenge to phenotypic methods that depend on cloning large segments of DNA. Our validation data suggest that by optimization based on enthalpy and sequence relatedness, only 2 or 3 probes are sufficient to predict tropism with precision similar to that of phenotyping or Geno2Pheno (10% false-positive rate). Moreover, genotyping would benefit from additional properties, such as the resolution of mixed virus populations. The simple diagnostic format of XTrack renders the system suitable for diagnostic purposes in routine settings with standard equipment.

The XTrack system confirmed the mixed nature of viral isolates in about 50% of unselected cases of this study, called "dual or mix" by TrofileES. As the other half was assigned to a single tropism, it is likely that this part of the samples is either difficult to judge, truly made up of dualtropic viruses, or misclassified. A functional verification was not available for these samples.

Certain principal shortcomings of genotype-based methods have been identified, particularly for noncanonical V3 loop lengths shorter or longer than 105 bp. The current limitation for those variants is that the respective interpretation rules are still lacking and need to be established. In relation, the suitability for the genetically distant group O variants of HIV-1 could not yet be assessed. Another principal limitation is the fact that the genotyping assays described here restrict the analysis to the Env V3 region. Thereby, this study did not take into consideration the possible contributions to a viral tropism by regions outside this peculiar peptide structure.

It should also be noted that the true value of the term "correct tropism" was not clinically verified for any of the tests. Until today, it remains unclear how meaningful minority viruses of the opposite tropism in clinical specimens are. It also is to be determined whether viruses with unclear tropism assignment (e.g., medium false-positive rate in the Geno2Pheno system or intermediate migration in XTrack) have a higher chance of switching their tropism. For standardization purposes, this study rated TrofileES results as "correct" and set them as the default. Subsequent investigations will have to demonstrate the validity of this relationship using larger panels of molecularly and clinically defined virus isolates.

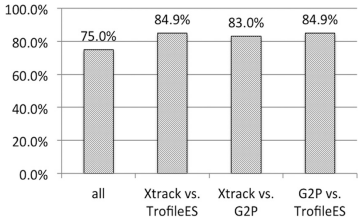

FIG 4 Degree of agreement between the three systems, based on the data in Table 5. Bars indicate either the overall agreement between the three systems ("all") or between XTrack and TrofileES, or XTrack and Geno2Pheno, or Geno2Pheno and TrofileES, as indicated. Percent agreement is given above each bar.

However, additional validation came from a European ring trial (First European Collaborative Study on HIV Tropism), where various genotypic and phenotypic tropism systems were assessed on blinded identical sets of 12 virus samples by 36 participating laboratories. Results, as reported by Guertler et al. (39), confirmed the very good overall performance of the XTrack and PhenXR systems.

Future work will be needed for connecting XTrack and Geno2Pheno with results from rPhenotyping in order to further improve test performances. By adaptation of the choice of hybridization primers and through primer shortening utilizing minor-groove-binding modifications, this system will also be adaptable to putative emerging new HIV variants.

It is technically likely that the same test principle may also be applicable toward new pathogens, such as hepatitis viruses and other genetically highly variable entities.

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XI. POSTERS

XI.I. 11TH EUROPEAN MEETING ON HIV & HEPATITIS, ROME, ITALY (MARCH 2013)

HIV Tropism as a suitable tool to predict immune response?

P_41

Joëlle Bader[1], Franziska Schöni-Affolter[2], Manuel Battegay[3], Thomas Klimkait[1], and the Swiss HIV Cohort Study

[1]Department Biomedicine - Petersplatz, University of Basel, Switzerland
[2]Swiss HIV Cohort Study (SHCS) Data Center, University Hospital Lausanne, Switzerland
[3]Division of Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Switzerland

Abstract

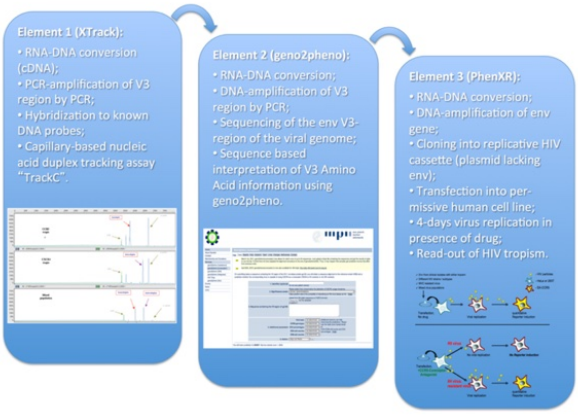
CD4 cell recovery in patients infected with HIV-1 under ART is often not sufficient. We searched for a possible correlation of incomplete immune response with CXCR4 tropism, which is known to correlate with faster disease progression. Ninety-five patients of the Swiss HIV Cohort Study (SHCS), 48 with CD4 T cell counts >500 cells/µL (responder) and 47 with <500 cells/µL (plateau) after three years on ART were assessed for their tropism at baseline. HIV-1 RNA load was below 1000 copies/mL during treatment. Tropism was determined by genotyping through XTrack and geno2pheno (G2P), discordant results were verified by the replicative phenotyping assay PhenXR. The plateau group could be associated with more X4-tropic and mixed tropic viruses than the responder group. We suggest therefore the use of early tropism testing as a predictor of disease progression.

Background

HIV infection via the chemokine receptor CXCR4 (X4) correlates with faster disease progression, a more rapid decline in CD4 cells, and therefore earlier signs of AIDS related illnesses. We hypothesize that impaired CD4 cell response directly correlates with the viral tropism considering that X4 tropism seems to associate with a poorer outcome of the disease and that in a substantial number of HIV infected individuals even under suppressive ART CD4 cell recovery is often not adequate. Kaufmann et al. had shown in a previous SHCS-study that a subset of virologically suppressed patients did not experience a sufficient CD4 cell recovery: One group had a continuous CD4 cell increase during ART and a second group an impaired CD4 cell response with a persisting plateau below 500 cells/µL. The study indicated that age, duration of infection, or baseline CD4 count qualified as determinants of an incomplete immune response. The aim of this study was to substantiate a link between viral characteristics and immunological outcome.

Methods

This retrospective study included only patients from the SHCS who were under ART for > 3 years, with complete virus suppression, no virological failure and previously analyzed for CD4 characteristics by Kaufmann et al. Patients were grouped according to their CD4 counts after 3 years on ART: >500 cells/µL (responder) or <500 cells/µL (plateau). Plasma samples were analyzed for viral tropism at the time of initiation of ART using a three-elements-based system. Genotyping was performed with XTrack (InPheno, Basel) and geno2pheno$_{[coreceptor]}$ (MPI, Germany) simultaneously. In addition discordant cases were analyzed with the replicative phenotyping assay PhenXR (InPheno, Basel).



Results

Ninety-five patients, 48 responders and 47 plateaus, were included in the study. For the respective CD4 range in this population, literature reports about 82% of patients to carry R5 tropic viruses (Brumme et al, 2005). Our data with 81.3% R5 (74/91) correlate well with this expectation (geno2pheno data). Baseline CD4 counts and viral loads were significantly different in the plateau group with 96 cells/µL vs. 264 cells/µL [p=<.0001] and 5.57 $\log_{10}$ copies/mL vs. 5.19 $\log_{10}$ copies/mL [p=0.006]. Average CD4 cell increase in responder group was 508 cells/µL vs. 225 cells/µL in plateau group [p=<.0001]. As a surprising fact viral kinetics and level of suppression were indistinguishable for both groups.

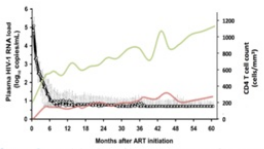


Figure 1: Viral load profile against time after ART initiation for all patients enrolled in the Kaufmann et al. SHCS study. Coloured lines indicate a typical CD4 profile of a patient in the responder group (green) and a patient in the plateau group (red).

	XTrack						geno2pheno (FPR 5%)			
	R5	X4	Mix	% X4	% Mix	p value	R5	X4	% X4	p value
Responder (n=48)	34	5	9	10%	19%	0.011	43	3	7%	0.0061
Plateau (n=47)	19	9	19	19%	40%		31	14	31%	

Table 1: XTrack and geno2pheno tropism results grouped according to responder and plateau patients. Four samples were indeterminate by G2P.

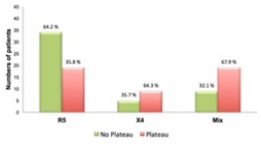


Figure 2: Tropism analysis according to XTrack split into patients experiencing a CD4 plateau or not.

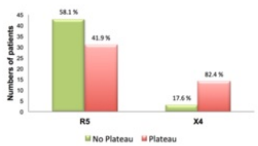


Figure 3: Tropism analysis with G2P at an FPR threshold of 5%.

Overall we found in the study population that 64.3% of all X4 and 67.9% of all mixed viruses by XTrack and 82.4% of all X4 viruses by geno2pheno could be assigned to the plateau group.
We investigated nine discordant cases with our replicative phenotyping system PhenXR which did not detect one of the genotyping systems as the more sophisticated. Furthermore, the observation that more patients in the plateau group could be assigned to either X4 or mixed tropic viruses was still correct after inclusion of the phenotypic results.

Proviral tropism analysis on the same samples five years after ART initiation are currently ongoing trying to answer the following questions:

- Is a tropism analysis predictive over 5 years?
- Are there differences between plasma and proviral samples?
- Is X4 or R5 the dominant tropism under immunological failure?

Conclusion

Unexpectedly up to 80% of patients with a X4 tropic virus at initiation of ART ended up with an impaired immune response, characterized by a plateauing CD4 recovery below 500 cells/µL. Since tropism determination was performed on specimens years before the development of a CD4 plateau, our data support the benefit of early tropism testing.

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joelle.bader@stud.unibas.ch , thomas.klimkait@unibas.ch

XI.II. 12TH EUROPEAN MEETING ON HIV & HEPATITIS, BARCELONA, SPAIN (MARCH 2014)

Will eradication of CXCR4-tropic HIV-1 under cART be possible?

3

Joëlle Bader[1], Franziska Schöni-Affolter[2], Jürg Böni[3], Meri Gorgievski[4], Thomas Klimkait[1], and the Swiss HIV Cohort Study

[1]Molecular Virology, Department Biomedicine - Petersplatz, University of Basel, Switzerland
[2]Swiss HIV Cohort Study (SHCS) Data Center, University Hospital Lausanne, Switzerland
[3]Institute of Medical Virology, Swiss National Center for Retroviruses, University of Zürich, Switzerland
[4]Institute of Infectious Diseases, University of Berne, Switzerland

Abstract

Detectable CXCR4-tropic (X4-) HIV is known to correlate with accelerated CD4 cell deterioration but little is known about the role and dynamics of the tropism of cell-associated proviruses. Here we present evidence that prevalent X4-tropic HIV at cART initiation correlates with a later impaired CD4 response. Aim of this study was to follow the evolution of the proviral tropism under suppressive cART. Paired PBMC samples from 44 patients (and 57 pairs of plasma/PBMC) in the Swiss HIV Cohort Study were analyzed at cART initiation and after 5 years of complete virologic suppression using geno2pheno (FPR 5%). The majority of patients presented with R5-tropic provirus (89%; 39/44) at baseline (BL) – the same was true for plasma samples in 86% of cases (49/57 R5-tropic viruses at BL). After 5 years of successful therapy 85% of the provirus and 86% of the plasma samples with initial R5 tropism continued to carry a R5-tropic provirus. Our analysis revealed that surprisingly 10 of our 13 patients with BL X4-tropic HIV in plasma or provirus presented with R5-tropic provirus five years later. The study suggests that under successful cART the deposition of R5-tropic HIV variants appear to be strongly favored. It further provides evidence for a successful and possibly lasting functional suppression of X4-tropic HIV that might lead a way to eliminating certain long-lived, provirus-carrying cells.

Background

Early in HIV-history it was observed that infection through the chemokine receptor CXCR4 (X4) correlates with a more rapid disease progression, accelerated decline of CD4 cells, and therefore earlier signs of AIDS-related illnesses. This was mostly observed in patients experiencing uncontrolled virus infection in the absence of treatment. Several studies in the past provided evidence that X4-tropic viruses may in deed be better eliminated under therapy than CCR5- (R5) tropic ones. Among the reasons altered glycosylation pattern of the viral envelope were discussed. R5-tropic viruses tend to be more extensively glycosylated than X4-tropic ones and might therefore hide more effectively from antibody recognition and elimination through the immune system. Earlier studies have also established that under cART R5-tropic HIV becomes the dominant one. As PI and RT drugs have no tropism-selectivity we hypothesize that the improving immune functions could act more efficiently on X4-tropic HIV strains. This study had the aim to follow over time the evolution of the viral tropism under successful cART.

Methods

Paired PBMC samples from 44 patients (and 57 pairs of plasma/PBMC) in the Swiss HIV Cohort Study were analyzed at ART initiation and after 5 years of complete virologic suppression. All patients were virologically suppressed at <50c/mL (with no CCR5 antagonist administered). HIV tropism was assessed with geno2pheno (restrictive setting of FPR 5%) and XTrack (InPheno AG, Basel)

Conclusion

- We provide first evidence that under successful cART the deposition of CCR5-tropic rather than CXCR4-tropic HIV is favored.
- An early therapy start and the maintenance of a potent immune function might be key for sustained virus control and eventually towards an elimination of CXCR4-tropic virus variants (confirmation in larger study pending).
- This study also suggests that proviral testing does not underestimate CXCR4 tropic viruses in the cellular compartment.

Molecular Evidence for Virus Evolution

Population-based sequencing in our evaluation revealed a marked difference for the direction of switching:

- In all examined cases the tropism switch from
- X4 → R5 required several nucleotide changes to facilitate the change. Sequences harbored also insertions and/or deletions.
- R5 → X4 required only single critical amino acid changes responsible for switching the tropism. They affected a charged amino acid in the V3-sequence.

- Often a G to A nucleotide transition at positions 11, 24 or 25 of the V3 loop was responsible for the change in charge.
- In other cases an N-linked glycosylation site was lost.

Further Information: joelle.bader@unibas.ch , thomas.klimkait@unibas.ch

Results

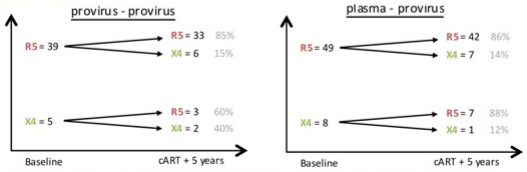

Figure 1: Comparison of BL tropism and tropism after five years on treatment for different compartments with geno2pheno (FPR 5%).

- The majority of patients had CCR5-tropic proviruses (39/44) at BL. Thirty-three patients (85%) continued to have a CCR5-tropic provirus five years later; in the remaining six patients HIV tropism had changed to CXCR4.
- We then analyzed how the tropism of *free* virus at BL correlated with the tropism of *pro*viral DNA in later samples using a larger data set of 57 paired samples (plasma/provirus). CCR5-tropic virus at BL was identified in 49 of 57 patient samples, and 42 (86%) were CCR5-tropic also five years later, quite similar to the proportion in the provirus/provirus comparison. This similarity allowed us to pool plasma- and provirus results for patients with CXCR4-tropic viruses at BL in order to reach higher numbers in total.

In contrast the **majority of patients with BL CXCR4-tropic HIV (plasma or provirus) (10/13) presented with CCR5-tropic strains** five years later (provirus). Plasma and proviral samples of a given baseline time point were concordant in 93% of all cases.

When analyzing the same samples with the Xtrack system similar results were obtained; in addition the it allowed to discriminate mixed viruses.

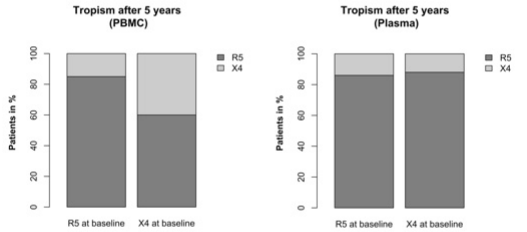

Figure 2: Geno2Pheno (FPR 5%) analysis showing the tropism comparison for proviral compartments ("PBMC") or for free virus versus provirus ("Plasma") after five years on therapy for all patients with R5 or X4 tropic HIV at baseline. Abbreviations: PBMC: peripheral blood mononuclear cell; R5: CCR5 tropic HIV; X4: CXCR4 tropic HIV.

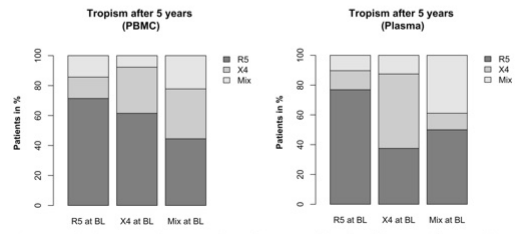

Figure 3: XTrack analysis comparing the tropism in the proviral compartments ("PBMC") or of the tropism of free virus with provirus ("Plasma") after five years on suppressive therapy for all patients with R5-, X4- or mixed tropic HIV at baseline. Abbreviations: PBMC: peripheral blood mononuclear cell; BL: baseline; R5: CCR5 tropic HIV; X4: CXCR4 tropic HIV.

Preferential suppression of CXCR4-tropic HIV-1 under cART?

Bader J[1], Schöni-Affolter F[2], Böni J[3], Gorgievski M[4], Klimkait T[1], and the Swiss HIV Cohort Study

[1]Department Biomedicine - Petersplatz, University of Basel, Switzerland
[2]Swiss HIV Cohort Study (SHCS) Data Center, University Hospital Lausanne, Switzerland
[3]Institute of Medical Virology, Swiss National Center for Retroviruses, University of Zürich, Switzerland
[4]Institute of Infectious Diseases, University of Berne, Switzerland

Introduction

Early in HIV-history it was observed that infection through the chemokine receptor CXCR4 (X4) correlates with a more rapid disease progression, accelerated decline of CD4 cells, and therefore earlier signs of AIDS- related illnesses. This was mostly observed in patients experiencing uncontrolled virus infection in the absence of treatment. Several studies in the past provided evidence that X4-tropic viruses may indeed be better eliminated under therapy than CCR5- (R5) tropic ones. Among the reasons altered glycosylation pattern of the viral envelope were discussed. R5-tropic viruses tend to be more extensively glycosylated than X4-tropic ones and might therefore hide more effectively from antibody recognition and elimination through the immune system. Earlier studies have also established that under cART R5-tropic HIV becomes the dominant one. As PI and RT drugs have no tropism-selectivity we hypothesize that the improving immune functions could act more efficiently on X4-tropic HIV strains. We report here results from a study with the aim to follow over time the evolution of the viral tropism under successful cART.

Materials and methods

Pilot study

Paired PBMC samples of 45 patients (and 59 pairs of plasma/PBMC) in the Swiss HIV Cohort Study (SHCS) from the time of ART initiation and after 5 years of complete virologic suppression were analyzed. All patients were virologically suppressed at <500c/mL (with no CCR5 antagonist administered). HIV tropism was assessed with geno2pheno (restrictive setting of FPR 5%) and XTrack (InPheno AG, Basel)

Trend study

Nineteen patients from the SHCS were selected with available PBMC samples in yearly intervals from three years before cART initiation until three years of follow-up. All patients were virologically suppressed (<100c/mL) with good immune recovery (>500 CD4 cells/mm³). We assessed proviral loads and HIV tropism with geno2pheno (restrictive setting of FPR 5%).

Results

Pilot study

- The majority of patients had CCR5-tropic proviruses (39/45) at BL. Thirty-three patients (85%) continued to have a CCR5-tropic provirus five years later; in the remaining six patients HIV tropism had changed to CXCR4 use.

Other studies have suggested that X4 tropism in proviral DNA could not reliably be assessed and is thus underrepresented.

- We analyzed if and how the tropism of free virus at BL correlated with the tropism of proviral DNA in later samples using a larger data set of 59 paired samples (plasma/provirus). CCR5-tropic virus at BL was identified in 50 of 59 patient samples, and 43 (86%) of these patients harbored CCR5-tropic HIV also five years later, quite similar to the proportion in the provirus/provirus comparison. Based on this good correlation we pooled plasma- and provirus results for patients with CXCR4-tropic viruses at BL.
- In contrast to the high stability of R5 tropism over time the majority of patients with BL CXCR4-tropic HIV (plasma or provirus) (11/15) presented with CCR5-tropic strains five years later (provirus). Plasma and proviral samples of a given baseline time point were concordant in 88% of available cases.

When analyzing the same samples with the duplex-tracking system Xtrack similar results were obtained; the system allowed to also discriminate mixed virus populations.

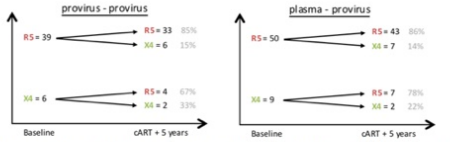

Figure 1: Comparison of BL tropism and tropism after five years on treatment for different compartments with geno2pheno (FPR 5%).

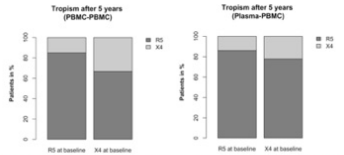

Figure 2. Geno2Pheno (FPR 5%) results showing the tropism comparison between proviral compartments (PBMC) or from free virus (plasma) at baseline with PBMC after five years on therapy for all patients with R5 or X4 tropic HIV at baseline. Abbreviations: PBMC, peripheral blood mononuclear cell; R5, CCR5 tropic HIV; X4, CXCR4 tropic HIV.

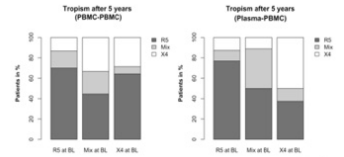

Figure 3. XTrackC results showing the tropism comparison between proviral compartments (PBMC) or from free virus (plasma) with PBMC after five years on therapy for all patients with R5-, X4- or mixed tropic HIV at baseline. Abbreviations: PBMC, peripheral blood mononuclear cell; BL, baseline; R5, CCR5 tropic HIV; X4, CXCR4 tropic HIV.

Trend study

We analyzed proviral loads at different time points in the course of the disease and compared these data with CD4 cell counts and viral loads.

- CD4 cells as well as VL show typical profiles with increasing CD4 cell counts and rapidly dropping VLs after cART start. Some patients experienced low viral blips (<100c/mL).
- Analysis of proviral loads revealed in most cases an increase until cART initiation and a drop thereafter. However, the drop in proviral load occurs often in a big delay of more than one year after cART has been commenced. In some cases proivral HIV rises even after cART start.
- Nonetheless proviral loads were in no case stable over time with in- and decreasing numbers even under cART. This might be explained by episodes of immune activation during generalized infections, allergies etc.
- Tropism analysis with geno2pheno was performed on the time point three years after cART initiation to check the observed overrepresentation of R5 tropic virus in the pilot study. Despite highly variable profiles for the proviral loads, the analysis of up to here 6 patients samples yielded only R5 tropic virus strains by population sequencing.

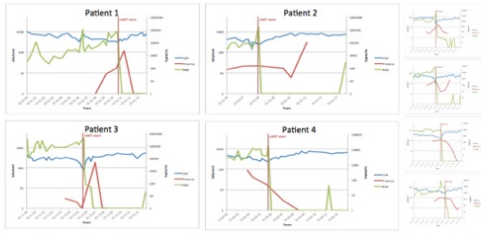

Figure 4. Logarithmic presentation of CD4 cell counts (blue), RNA viral load (green) and proviral load (red) of representative patients in the study group. Time point of cART start is indicated by a red line.

Conclusions

We provide first evidence that under successful cART the deposition of CCR5-tropic rather than CXCR4-tropic HIV is strongly favored leading to a reduction in X4-tropic viral loads. Early therapy start and maintenance of a potent immune function might be key for sustained virus control and eventually towards an elimination of CXCR4-tropic virus variants (confirmation in larger study pending). This study suggests that proviral testing does not underestimate CXCR4 tropic viruses in the cellular compartment.

Outlook

As population sequencing cannot dissect assumed mixed viral populations and will not reveal minorities or their fluctuations over time, we will employ next generation sequencing to precisely determine the proportion and possible role of X4-tropic viruses over time.

joelle.bader@unibas.ch

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cART Driven Recovery of Immune Function Preferentially Targeting CXCR4-Tropic HIV-1

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Joëlle Bader[1], Martin Däumer[2], Alexander Thielen[2], Jürg Böni[3], Meri Gorgievski-Hrisoho[4], Thomas Klimkait[1], and the Swiss HIV Cohort Study

[1]Molecular Virology, Department Biomedicine - Petersplatz, University of Basel, Switzerland
[2]Institute for Immunogenetics, Kaiserslautern, Germany
[3]Institute of Medical Virology, Swiss National Center for Retroviruses, University of Zürich, Switzerland
[4]Institute of Infectious Diseases, University of Berne, Switzerland

Abstract

It has been shown that CXCR4-tropic HIV is better neutralized by the immune system than CCR5-tropic variants. Reasons therefore might be the less glycosylated envelope on CXCR4-tropic viruses and the antibody binding due to less steric hindrance. We hypothesize that CXCR4-tropic variants should be better eliminated by a competent immune system generated through efficient cART. Therefore we monitored the frequency of CXCR4-tropic HIV by NGS on a set of 17 patients through six consecutive time points before and after cART initiation. We could identify a decrease in the frequency of CXCR4-tropic viruses in the majority of patients with time on therapy. Patients which on the contrary showed an increase of CXCR4-tropic variants under therapy had all increasing proviral loads. In the control patients we could show that without therapy and weakening of the immune system CXCR4-tropic variants start to increase. We suggest that CXCR4-tropic variants can be better eliminated by the recovering immune system under cART and similarly in early stages of the infection, when immune surveillance is still largely intact. On contrast, weakening of the immune system leads to an increase in CXCR4-tropic viruses. Therefore early therapy initiation and maintaining of an effective immune state might help to better control CXCR4-tropic HIV variants.

Background

In early HIV infection over 80% of patients carry HIV strains with CCR5 tropism. Only late in the disease, along with a deterioration of the immune situation, the proportion of CXCR4-tropic virus isolates in the circulation rises to >40%. It is not known, however, whether X4-tropic HIV appearing late in the infection is cause or consequence of the immune failure. The observation that X4-tropic virus is less frequently detected early during infection has at least two plausible explanations: Either R5-tropic HIV is more infectious or more readily transmitted, or X4 viruses are less well retained or more easily eliminated. This last view could be explained by the envelope glycosylation patterns of CXCR4-tropic viruses, as they tend to have less glycosylation sites than CCR5-tropic viruses and might be more readily neutralized by the immune system. Based on these observations we hypothesize that CXCR4-tropic variants do emerge without therapy and with a weakening of the immune system and in contrast should disappear with time under therapy. Aim of this study was to monitor the frequency of CXCR4-tropic viruses throughout phases of immune recovery by cART.

Methods

Seventeen patients in the Swiss HIV Cohort Study were followed annually after virologic suppression by tropism testing. In all patients HIV-1 was fully suppressed throughout study time with good CD4 T cell restoration to >250 cell/mm^3 in 3 years after cART initiation. For eight we included also three consecutive time points prior to cART. Frequency of CXCR4-tropic variants was analyzed by Illumina Miseq sequencing (FPR 3.5%, R5 < 2% X4).

Results

- Of the total 17 patients, ten patients (59%) had exclusively CCR5-tropic viruses after cART initiation, which stayed CCR5-tropic during follow-up. Of the seven remaining patients with CXCR4-tropic HIV at start, four (57%) showed decreasing frequencies of CXCR4-tropic variants during suppressive therapy (Figure 1.).
- For eight patients consecutive time points before cART initiation were included. Of six patients with an R5-tropic virus at start 4 remained R5-tropic; two of them presented with an X4-tropic virus after cART initiation and over time. Two patients with X4 virus pre cART kept the virus tropism throughout. In all cases X4-tropic viruses showed increasing frequencies of CXCR4-tropic viruses over time and without therapy. (Figure 2):

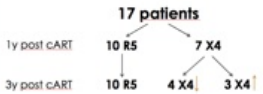

Figure 1. Tropism determination on all patients one and three years after cART initiation.

Figure 2. Tropism determination of patients with available samples before and after therapy initiation.

- For 15 of the 17 patients proviral load testing was successful at all time points. For four (27%) we observed an initial increase of proviral loads under therapy. Interestingly, this increase in patients correlated with an increase in CXCR4-tropic virus frequencies by NGS.
- In patients with declining proviral loads after therapy this decrease occurred only with a delay of up to one year after therapy initiation.

Graphical representation of descriptive parameters for two patients

- Patient 1 with good therapy response experienced a slowly decreasing frequency of CXCR4-tropic variants (light blue) and declining proviral load (red).
- Patient 2 had a good therapy response, however with repeated blips (green) after the study period (< 100 c/mL). Increasing frequencies of CXCR4-tropic variants (light blue) and rising proviral load (red) under therapy are appreciated.

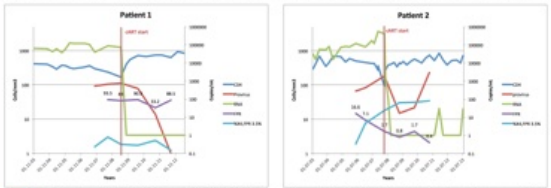

Figure 3. Logarithmic presentation of CD4 cell counts (blue), RNA viral load (green), proviral load (red), Geno2Pheno FPR (violet), and frequency of CXCR4-tropic variants (light blue) of two representative patients from the study group. Time point of cART initiation is indicated by a vertical red line.

Conclusions

- We observed a declining frequency of CXCR4-tropic HIV variants during successful cART in a majority of our patients in the study.
- An increase in X4-frequency under therapy correlated with rising proviral loads.
- This might suggest a selectively superior elimination of CXCR4-tropic HIV variants by an intact immune surveillance.
- Clinical studies will have to verify if early therapy initiation and maintaining of an effective immune state might help controlling CXCR4-tropic HIV variants.

Further information: joelle.bader@unibas.ch , thomas.klimkait@unibas.ch

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TUPEA053

Evolution of the HIV-1 Envelope gene during suppressive cART

Joëlle Bader[1], Martin Däumer[2], Alexander Thielen[2], Jürg Böni[3], Meri Gorgievski-Hrisoho[4], Thomas Klimkait[1], and the Swiss HIV Cohort Study

[1]Molecular Virology, Department Biomedicine - Petersplatz, University of Basel, Switzerland
[2]Institute for Immunogenetics, Kaiserslautern, Germany
[3]Institute of Medical Virology, Swiss National Center for Retroviruses, University of Zürich, Switzerland
[4]Institute of Infectious Diseases, University of Berne, Switzerland

Abstract

The genetic diversity of HIV-1 presents currently a major obstacle for controlling and eventually curing infection. It has been claimed that sufficiently potent combination therapy (cART) blocks viral replication, thus not allowing a molecular evolution of the targeted viral genes over years of therapy. In this context the role of the viral cell tropism of HIV-1, affecting critical infection events, has only rarely been addressed. Aim of this study was to monitor the sequence evolution of the V3 loop during cART, particularly of cell-associated virus in the peripheral blood. We used NGS analysis of the viral genomes. Distance relatedness calculations between the dominant variant found at baseline (time of therapy initiation) and all variants at follow-up time points revealed evidence for sequence-based provirus evolution in five of eight cases in our study group (62.5%) during the pre-treatment period. During cART, of the total 17 patients seven (41.2%) continued to show evidence for evolution in their proviruses. In five (29.4%) patients the virus developed a greater diversity over time and therapy. CXCR4-tropic variants were in general not increasing under therapy and therefore unlikely to be responsible for the diversity increase. In cases, where a CXCR4-variant became the dominant variant under therapy the viral population was always characterized by the proliferation of one variant, which had already been present as a minority before therapy.

We conclude that genetic envelope gene evolution and diversification under suppressive therapy is possible, suggesting some ongoing replication in sanctuary sites and that, at least in selected cases ongoing infection might be driven by proliferation of infected cells. The wider clinical implication could become to initiate cART as early as possible for limiting the proviral reservoir. The decrease of CXCR4-tropic variants during treatment may suggest that they are not the main cause of ongoing infection/replication.

Background

The envelope gene is one of the most variable regions in HIV and its genetic diversity is a main barrier for virus neutralization. During infection the general diversity of the viral population is continu-ously increasing due to the missing proof reading activity of the reverse transcriptase. However, it has been reported that during successful therapy there be no evidence for molecular evolution correlating with a stable proviral reservoir once cART is initiated. Most of these results were based on sequencing data of reverse transcriptase and protease, which are the main targets by cART. To our knowledge the genetic diversity of envelope and its viral tropism under therapy has only rarely been addressed so far. We therefore aimed at investigating the sequence-based evolution of the V3 loop under cART, focusing on cell-associated virus.

Methods

The Illumina Miseq platform (NGS) was used to obtain deep sequencing results on provirus from 17 chronically HIV-1 infected patients in the Swiss HIV Cohort Study prior to (= 8 control cases) and during periods of virologic suppression. Virus was permanently fully suppressed throughout the study time, and all patients had experienced a good CD4 T cell recovery. Calculations were performed with MEGA 6.0.

Results

Evolution and Diversity

- In five (62.5%) of eight cases HIV-1 showed an envelope evolution by sequence, and in six (75%) cases an increased diversity during their pre-treatment period.
- During cART, seven (41.2%) of the total 17 patients continued to show evidence for evolution in their proviruses.
- In five (29.4%) patients the virus developed a greater diversity over time and therapy.

Tropism association

- Overall the frequency of CXCR4-tropic variants in the overall HIV-1 population pool decreased under therapy.
- In samples with increasing viral diversity this associated with a stable or declining frequency of CXCR4-tropic variants.
- In a minority of patients CXCR4-tropic virus variants increased under therapy; this was, however, in no case associated with a higher diversity but the increase was always characterized by the emergence of one dominant HIV-1 variant, which had been present as a minority already before therapy.

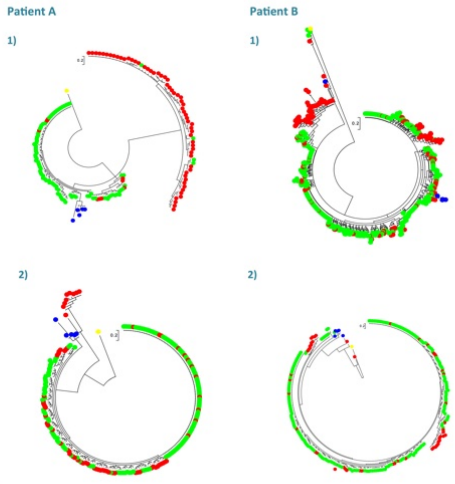

Figure 1. Phylogenetic trees before (1) and after treatment initiation (2) for a patient A with decreasing HIV diversity and a patient B with increasing virus diversity. The green dots represent CCR5-tropic HIV-variants, and the red dots CXCR4-tropic variants.
Blue dots reflect the consensus V3 sequences of subtypes A, B, and C, and the individual yellow dot represents the consensus HIV-1 subtype B LTR sequence used for rooting.

Conclusions

- ***We confirm genetic HIV env evolution prior to treatment.***
- ***HIV persistence might be driven directly by proliferation of infected cells.***
- ***CXCR4-tropic variants are unlikely to be responsible for an increased diversity as they decrease under therapy.***
- ***Replication in sanctuary sites might be the driver of ongoing evolution and diversification.***
- ***Ongoing cell-driven processes that permit viral genetic modification and diversification may support the idea of early therapy initiation in the clinics.***

Further Information: joelle.bader@unibas.ch , thomas.klimkait@unibas.ch