# Novel insights into the effect of CCR5 inhibition on HIV treatment, pathogenesis and cure

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# Novel insights into the effect of CCR5 inhibition on HIV treatment, pathogenesis and cure

## Nieuwe inzichten in het effect van CCR5 inhibitie op HIV behandeling, pathogenese en genezing

(met een samenvatting in het Nederlands)

**Proefschrift**

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## Contents

**Chapter 1**

**General introduction**

Partly derived from:

**HIV population genotypic tropism testing and its clinical significance**

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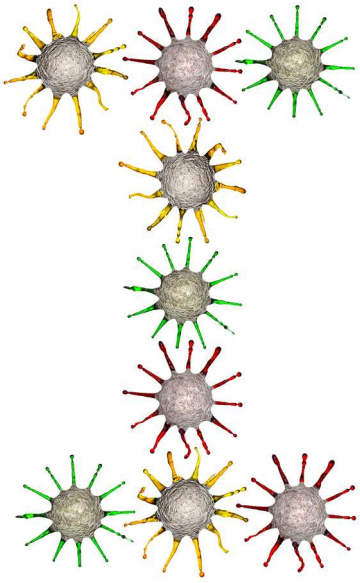

The human immunodeficiency viruses HIV-1 and HIV-2 are enveloped retroviruses of the lentiviridae genus and are the causative agents of acquired immunodeficiency syndrome (AIDS)[1,2]. Since the discovery of HIV-1 in 1983[1,2] over 35 million people have died due to AIDS related diseases. In 2012 about 2.3 million people were newly infected with HIV and 1.6 million people died due to AIDS[3]. Currently over 35.3 million people live with HIV worldwide[3]. In absence of treatment, HIV infection generally leads to the progressive depletion of $CD4^+$ T-lymphocytes[4-6], resulting in the loss of cellular immunity and the development of AIDS defining events[7].

Worldwide sexual contact is the major route of HIV transmission (horizontal)[8]. The rate of transmission is influenced by several behavioural and biological factors. Behavioural factors include type of sexual contact (anal, vaginal, oro-genital) number of partners and serosorting[9]. Other routes of horizontal transmission are transfusion of blood or blood products, intravenous drug use or needle stick injuries[10]. HIV can also be vertically transmitted from an HIV-infected mother to the fetus in utero, through intrapartum inoculation from mother to infant or during breast-feeding[11]. Biological factors that influence transmission rate include HIV RNA level, co-infections, inflammation and host genetics of the recipient[12].

Within a host HIV infects cells that carry the CD4 antigen. The envelope glycoprotein 120 (gp120) of HIV binds to receptors on the target cell[4,5,13]. Following receptor binding the viral particle fuses with the host cell membrane and the viral core is deposited in the cytoplasm. Uncoating of the core releases in addition to others two copies of positive single stranded viral-RNA and the viral replication enzymes: reverse transcriptase and integrase[14]. The error prone reverse transcriptase transcribes the viral-RNA into double stranded DNA[15]. The HIV dsDNA together with viral integrase is translocated to the nucleus where integrase facilitates integration of the viral dsDNA into the host genome[16]. Activation of the cell induces transcription of the proviral-DNA into viral messenger RNA (mRNA) which is translated into polyproteins by cellular ribosomes. Following this translation two newly transcribed copies of viral genomic RNA, the replication enzymes and the structural proteins assemble at the cellular membrane[17]. Subsequently, an immature capsid is formed and the viral particle buds from the cell acquiring a new envelope with viral and host proteins. Finally, cleavage of the viral polyproteins by HIV protease results in the generation of mature infectious virus particles (Figure 1)[18-20].

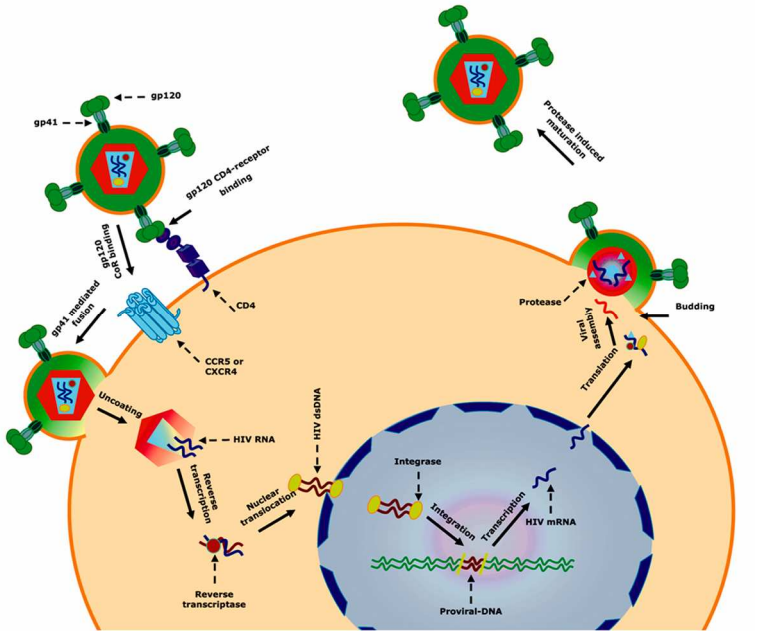


**Figure 1. HIV life cycle.** Schematic overview of the HIV live cycle.

## HIV entry into the target cell and HIV co-receptor tropism

The first essential step of the HIV life cycle is viral entry. This is a multistep process which is critically dependent on the chronological interaction with two host receptors, the CD4 receptor and a co-receptor. Binding of the envelope glycoprotein gp120 to the CD4 receptor induces a conformational change resulting in structural rigidity of gp120 and protrusion of the co-receptor binding site (Figure 2a, part 1)[20]. This co-receptor binding site consists of the V1V2 loop, the bridging sheet and the hypervariable loop V3 (Figure 2b). The latter will bind to the extracellular loop 2 of the co-receptor[21-24] (Figure 2a, part 2). The main co-receptors used by HIV are the seven transmembrane C-C chemokine receptor 5 (CCR5) and the seven-transmembrane C-X-C chemokine receptor type 4 (CXCR4)[25,26]. Binding to the co-receptor evokes another conformational change in gp120 resulting in exposure of the hydrophobic fusion domain of gp41 which facilitates gp41 to penetrate the cellular membrane mediating virus-cell fusion (Figure 2a, part 3)[27]. Viral co-receptor tropism is determined by its co-receptor usage; viruses that enter $CD4^+$ cells by the CCR5 receptor are commonly named R5-tropic viruses and viruses that enter via the CXCR4 receptor X4-tropic viruses[28]. Dual-tropic viruses are capable of using both co-receptors whereas mixed tropism refers to a population containing both R5- and X4- tropic viruses[29,30]. Although CCR5 and CXCR4 are the major co-receptors, alternative co-receptors have been identified for viral entry *in vitro*, these include CCR1, CCR2b, CCR3,

CCR8, CX3CR1, CXCR6, FPRL1, GPR1, GPR15, APJ, STRL33 and D6[31]. The relative contribution of these alternative co-receptors to viral entry in the human host is unclear.

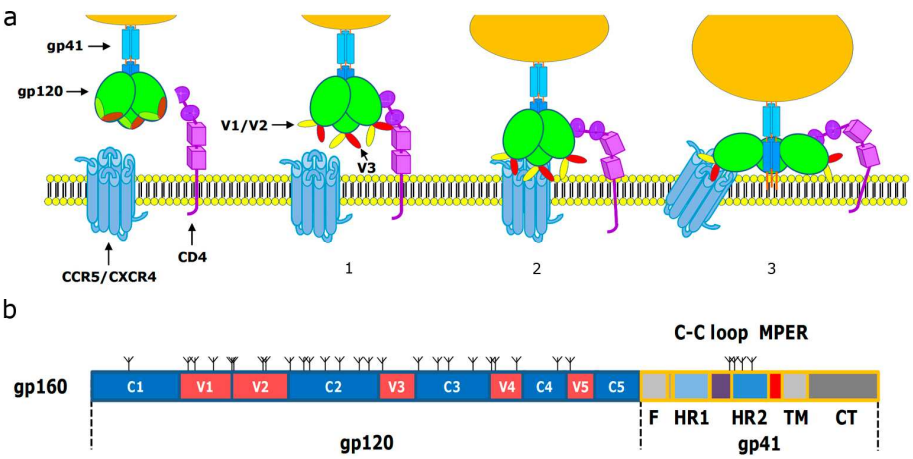


**Figure 2. a) HIV entry in the target cell.** The envelope protein gp120 binds to CD4 receptor, evoking a conformational change (1) resulting in binding of the co-receptor binding region of gp120 to the co-receptor(2). This facilitates another conformational change which enables gp41 to penetrate the cellular membrane (3).

**Figure 2. b) Composition of the HIV envelope glycoprotein160.** C1-C5 are the conserved regions and V1-V5 the variable regions of gp120. The F, fusion peptide; HR1, heptad repeat 1; C-C loop, the immunodominant loop with a conserved disulfide bond; HR2, heptad repeat 2; TM, transmembrane anchor; CT, cytoplasmic tail of gp41 is given. Glycans are represented by tree-like symbols (adapted from G. Fray, J Chen, S Rits-Volloch, *et al*. Distinct conformational states of gp41 are recognized by neutralizing and non-neutralizing antibodies. *Nature Struc. & Mol. Biol.* 2010 **17**:1486-1491)[32].

## Genetic determinants of co-receptor tropism

The V3-loop is usually comprised of 35-amino acid residues and bookended by cysteine residues resulting in the loop structure[33]. In HIV-1 subtype B, the most consistent amino acid changes influencing viral co-receptor tropism are those leading to a positive charge at positions 11 and or 25[34,35] (Figure 3). These positive amino acid residues can more easily interact with the negatively charged extracellular loop 2 of CXCR4. Loss of the N-glycosylation site at the base of the V3 loop is also associated with CXCR4-usage[36,37]. The N-glycosylation sequon N-X-T is lost whenever the asparagine or threonine mutates to another amino acid or when the X is mutated into a proline or asparatic acid[38].

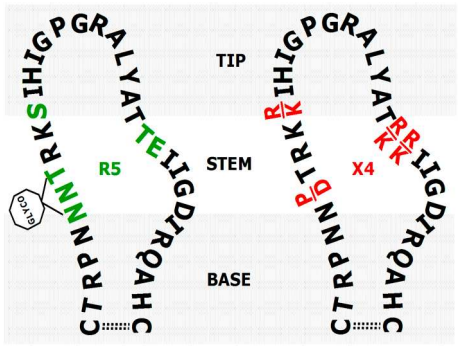


**Figure 3. V3-loop secondary structure.** The left V3-loop is the consensus V3-loop amino acid sequence of the R5-tropic HIV-1 reference strain BaL. In green are amino acid positions associated with CCR5 usage. The right V3-loop represents the amino acid mutations in the HIV-1 reference strain BaL associated with CXCR4 usage given in red.

Besides the V3-loop which is the main determinant in co-receptor usage[39,40] there are other positions which can influence binding to the co-receptor, these positions are observed in both gp120 and in the fusion peptide gp41, which form the envelope protein gp160 (Figure 2b). An insertion of a hydrophobic residue after the third amino acid in gp41 is associated with CXCR4 usage as are the mutations L43M and A303T[41]. Furthermore, mutations in variable loop 1 (V1) (S141N) and variable loop 2 (V2) (S159A, N185K) are associated with viral tropism[42,43]. However, these mutations may be compensatory mutations for the loss of replication capacity due to mutations in the V3-loop[44].

**Co-receptor tropism during HIV disease progression**

The clinical progression of HIV disease has been well documented and consists of three phases; the acute phase, the clinically latency phase, and AIDS. The first phase is characterized by a steep increase in viral load and a rapid decline in $CD4^+$ T-cells. Regardless of the route of infection it has been shown that R5-tropic HIV-1 is preferentially transmitted over X4-tropic virus[45-47]. Several factors may be involved in the preferential transmission of R5-tropic virus. Dendritic cells (DC) in mucosal tissues bind HIV by interaction of the C-type lectin DC-SIGN and the high-manose on gp120[48,49]. It has been demonstrated that R5-tropic virus is preferentially transmitted at the DC cell–T cell infectious synapse[50,51]. Furthermore, it was suggested that the epithelial cells, important in vertical transmission and oral horizontal transmission, mainly express CCR5 and not CXCR4 and selectively transfer R5-tropic virus to CCR5 positive cells in the mucosa[52]. In addition, the preferred R5-tropic virus transmission is underlined by the fact that individuals homozygous for a deletion of 32 nucleotides in the extracellular loop 2 of CCR5 (CCR5-Δ32) are largely protected against HIV-1 infection[53]. This natural polymorphism occurs in ~1% of the Caucasian population. The deletion induces a stop codon by

frame shift mutation resulting in retention of the truncated CCR5 protein in the endoplasmic reticulum. As a consequence cell surface expression of CCR5 in homozygous genotypes is absent, with no apparent clinical consequences[54,55].

A recent study has shown that the overall presence of X4-tropic virus early in infection is around 15% suggesting a less stringent bottleneck for X4-tropic transmission than previously thought[56]. In some cases that both R5 and X4-tropic viruses are transmitted it has been shown that CXCR4 using viral strains were selectively cleared from the circulating viral population[57,58]. The strong negative selection of X4-tropic HIV-1 during the early course of disease may be caused by mechanisms that hamper replication of X4-tropic variants. For instance, in the mucosal-associated lymphoid tissue GALT, the major site of HIV-1 replication during primary infection, the T-lymphocytes mainly express CCR5 and provide an optimal environment for R5-tropic amplification[59,60]. Furthermore, in lymphoid tissues there is high expression of the CXCR4 ligand, CXCL12, by dendritic cells and lymphoid epithelial cells specifically inhibiting X4-virus replication[51,61].

In the second phase, characterised by clinical latency, there is an initial (partial) recovery of the number of $CD4^+$ T-cells and stabilisation of the viral load to a certain setpoint. Thereafter, $CD4^+$ T-cells gradually decrease in most patients weakening the host immune system[62-64]. At the same time continuous viral replication is associated with persistent high levels of immune activation and destruction of the lymphoid architecture[65,66]. Over the course of disease in about 50% of subtype B infected individuals a switch in co-receptor use from CCR5 to CXCR4 is observed in the viral population[67,68]. The cause of this co-receptor switch remains elusive. Although it is assumed that the continuous attrition of the immune system during the course of infection facilitates co-receptor switch. The switch is associated with accelerated $CD4^+$ T-cell decline[69,70] and rapid disease progression. Ultimately these events result in the third and final phase AIDS (Figure 4)[71]. In this phase the $CD4^+$ T-cells have declined below the threshold needed for protection against opportunistic infections.

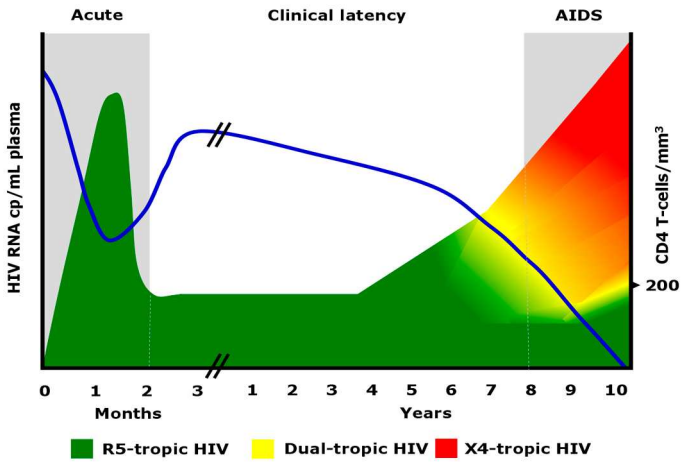


**Figure 4. A generalized schematic reprensitation of HIV disease progression in patients that switch co-receptor tropism.** The CD4+ T-cell count is given in blue. The viral load depicted in HIV RNA cp/mL is given in green representing the R5-tropic viral load, in yellow dual-tropic viral load and in red representing the X4-tropic viral load. Adapted from Schuitemaker H, van 't Wout AB, Lusso P. Clinical significance of HIV-1 coreceptor usage. J Transl Med. 2010; 9(suppl 1) S5[72].

## Phenotypic co-receptor tropism testing

The discovery of the two major co-receptors CCR5 and CXCR4 provided the molecular explanation for differences observed in HIV phenotypes for over a decade. HIV isolates were characterized based on their ability to induce syncytia in CD4+ T-cell lines that only express CXCR4 as their co-receptor[73,74]. This classical phenotypic tropism test is the MT-2 assay in which patient derived peripheral blood mononuclear cells or established viral isolates are co-cultured with MT-2 cells without PCR amplification. These MT-2 cells only express CXCR4 as a co-receptor and when infected form syncytia. The viral population will consequently be classified as syncytium inducing (SI). Co-cultures negative for syncytia formation will be classified as non-syncytia inducing (NSI)[74]. The appearance of syncytia inducing (SI) variants was predictive for an increased CD4+ T-cell decline and therefore a binary marker for progression to AIDS[73-75].

Currently several other phenotypic tropism tests are available. Most phenotypic tests require a PCR amplification step. In these assays full length or subunits of *gp160* of the viral population are generated and cloned to obtain replication competent or pseudoviruses representing the viral population of a patient[76]. The tropism of the viral population is subsequently assessed in cell-cultures. Several commercial and non commercial assays are available. The Trofile assay of Monogram inc. is the most frequently used phenotypic assay. The Original Trofile Assay (OTA) is a single-cycle recombinant assay in which the entire *gp160* gene of the viral population is amplified and pseudoviruses are generated. Co-receptor usage is subsequently assessed in U87-MAGI cells

which express either CCR5 or CXCR4. OTA has a 100% sensitivity for viral population with >10% X4-tropic virus[76]. OTA was replaced by the Enhanced Sensitivity Trofile Assay (ESTA) which could detect a X4-tropic minority population comprising 0.3% of the total viral population with 100% sensitivity when clonal mixtures were used[76].

**Genotypic co-receptor tropism prediction**

Genotypic tropism testing as performed in diagnostic settings is based on the amplification and sequencing of the V3 region of the viral envelope[76-78]. Two different sequencing approaches are available for tropism testing: population-based sequencing and deep sequencing. Although population or bulk sequencing methods are currently most often applied for diagnostic purposes, next generation sequencing methods allow for the analysis of minority variants. However, there are limitations to be considered with next generation sequencing, especially the limitation in accurately sequencing highly repetitive regions[79]. Furthermore, the methods and facilities are not commonly available for diagnostic purposes. The sequences generated by both sequencing methods are used to predict the co-receptor tropism *in silico*. The most frequently used bioinformatics tools for co-receptor use are the position-specific scoring matrix (PSSMX4-R5, WebPSSM)[80] and geno2pheno$_{[co\text{-}receptor]}$ which uses support vector machine technology[81]. A common principle of all the tools is training of the prediction models by analysing paired data of genotypes and phenotypes. Datasets used for these analyses are derived from the Los Alamos HIV Sequence Database[82] or from special clinical cohorts like the HAART Observational Medical Evaluation and Research (HOMER) cohort[83]. Furthermore, the geno2pheno$_{[co\text{-}receptor]}$ prediction model has been trained with clinical outcome data from several cohort studies and retrospective analyses of clinical trials[81]. PSSM uses amino acid sequence as input and provides a sum of the scores for all positions in which sequences with a higher total sum have a higher likelihood of being derived from an X4-tropic virus[80]. Nucleotide sequences are used as input in geno2pheno$_{[coreceptor]}$ therefore amino acid mixtures are taken into consideration. The result is given as a false-positive rate (FPR), indicating the chance of falsely predicting a virus as an X4-tropic variant[81]. Low cut-offs increase the specificity but reduce the sensitivity of X4-tropic prediction.

**Inhibition of HIV entry by blockage of the CCR5 co-receptor**

The introduction of combination anti-retroviral therapy (cART) targeting different steps of the viral life cycle has significantly reduced HIV disease progression[84,85].

HIV entry inhibitors form together with reverse transcriptase inhibitors, protease inhibitors and integrase inhibitors the current armamentarium of antiretroviral compounds. Inhibition of viral entry by blocking CCR5 is a relatively new and valuable approach. CCR5 blockers specifically bind to transmembrane CCR5 co-receptor cavity and thereby inhibit

entry of R5-tropic viruses by an allosteric non-competitive mechanism[86,87].Of interest, CCR5 blockers are the first antiretroviral drugs that target a host receptor. As targeting host proteins interfere with cellular function this anti-retroviral strategy might be hazardous. However, individuals who lack a functional CCR5 receptor due to a genetic defect in chromosome 3 (CCR5-Δ32) appear to be healthy[88,89]. Furthermore, HIV-infected individuals harbouring a heterozygous CCR5-Δ32 gene defect often display delayed disease progression[90,91], indicating that blocking of CCR5 can be a successful antiretroviral strategy. Several CCR5 inhibitors such as vicriviroc (VVC), TAK-220 and cenicriviroc a CCR2/CCR5 antagonist, are or have been studied in clinical trials[92,93]. So far maraviroc (MVC) is the only clinically available and approved CCR5 antagonist. MVC is a small molecule that specifically and selectively binds to the transmembrane helices of CCR5. This interaction stabilizes the conformation of CCR5 and thereby inhibits binding of R5-tropic virus to the co-receptor in an allosteric manner[94]. MVC has potent activity against R5-tropic HIV, with a low geometric mean 90% inhibitory concentration of 2.0 nM[95] and is well tolerated[96].

The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have approved MVC for use in treatment-experienced adults. The FDA has also approved MVC for use in treatment-naive individuals[97,98].For both indications it is explicitly stated that viral tropism should be assessed before clinical use and that the inhibitor should only be used in patients in whom R5-tropic virus is exclusively detected[97,98]. At present the European guidelines on the clinical management of HIV-1 tropism testing prefer for practical reasons population genotype testing, whereas the US Department of Health & Human Services guidelines specifically advise to obtain a phenotype[76,99].

**MVC treatment in R5-tropic and in dual- or mixed tropic infected patients**

In two clinical trials, MOTIVATE 1 and MOTIVATE 2, 1049 individuals infected with R5-tropic HIV-1, as assessed with OTA, randomly received placebo or MVC, once or twice daily. The mean decrease in HIV-1 RNA in plasma was greater in the MVC arm. Moreover, the increase in $CD4^+$ T-cell count was greater in the MVC arm as compared to placebo. Frequencies of adverse events were similar among the groups[100]. Follow-up of the MOTIVATE 1 and 2 trials demonstrated maintained durable virological responses over 96 weeks with a safety profile similar to placebo[101].

In a different study, the A4001029 study, MVC or placebo both with optimized backbone therapy was administered once or twice daily to 167 patients infected with dual- or mixed tropic HIV-1 as assessed with OTA. The decrease in HIV-RNA levels was similar between groups. The largest increase in $CD4^+$ T-cells was observed in the MVC arms, however, not significantly different from the placebo arm. The incidence of adverse events was similar between all groups[102].

Interestingly, in these clinical trials the largest increase of $CD4^+$ T-cells was observed in the MVC arms. Furthermore, a meta-regression analysis of clinical trials with CCR5 antagonists in antiretroviral treatment-experienced patients demonstrated a significant additional increase in $CD4^+$ T-cells compared to when CCR5 antagonists were not administered[103].The clinical trials with MVC demonstrated that MVC based therapy was effective in R5-tropic infected individuals but confers little to no virologic benefit for patients with dual- or mixed-tropic HIV-1 infection. However, MVC induced reduction of lymphocyte trafficking to inflamed sites might reduce immune activation and microbial translocation[104] and as such might be beneficial for HIV infected patients.

**CCR5 inhibitor therapy failure**

HIV-1 has a high mutation rate of approximately $3.4 \times 10^{-5}$ mutations per base pair per replication cycle[105]. This error rate leads to the generation of viral variants which differ in viral fitness within the host. This characteristic of HIV ensures that the viral population quickly adapts to the host environment and may escape immune pressure and or antiretroviral therapy. There are two mechanisms for HIV to escape the effect of CCR5 inhibition. One apparent route of CCR5 antagonist escape is a switch in tropism from CCR5 to CXCR4 usage. In a clinical cohort study, in which patients received MVC monotherapy for 10 weeks, therapy failure was observed due to the emergence of X4-tropic variants. Of note, several studies demonstrated that therapy failure resulted from viral expansion of a pretreatment CXCR4-using reservoir[106,107]. These CXCR4-using viruses were not detected before the start of MVC therapy by the OTA [106,107].

MVC therapy failure can also occur without a shift in co-receptor usage. In these cases, viral variants escape the CCR5 inhibitor because they can use inhibitor bound CCR5 for viral entry. These resistant variants have mutations in the V3-loop which shift the binding ability from the extracellular loop 2 of CCR5 to the N-terminus region of CCR5 [109-111]. Berro *et al.* demonstrated that resistance mutations can be selected at 7 positions located in the V3-loop tip and stem regions[109]. MVC escape was most often observed in relation to the substitution I26V. This mutated residue disrupts a β-sheet in the stem of the V3-loop and occupies less space, therefore reducing the interface areas of V3-loop and the N-terminal region of CCR5[112]. VVC-CCR5 bound resistance mutations were more scattered along the V3-loop and involved at least 15 positions in the tip, stem and base of the V3-loop[109]. The most frequently observed VVC escape mutations was K10R accompanied with a G21E/A substitution[109]. The mutations observed were at positions in the V3-loop that are known to be polymorphic. Thus it was postulated that the selection pressure of VVC and MVC may favour expansion of natural resistance related minority variants[113,114]. Also mutations in the conserved region 1, V2-loop and gp41 can contribute to resistance to CCR5 antagonists[115]. In addition, mutations at the start of gp41 allow entry despite presence of the vicriviroc-

CCR5 complex. Furthermore, the combination of G516V, M518V and F519I also confers resistance to VVC[116] but the mechanism is not yet understood. Of note, since most CCR5 antagonists in clinical practice or under development bind to approximately the same region of CCR5 cross resistance may become an issue if more compounds would become available

## Scope of this thesis

The aim of this thesis was to investigate the effect of inhibition of the CCR5 co-receptor on HIV treatment, pathogenesis and HIV cure. Our research questions and the chapters in which they have been investigated are listed below:

1) Is there a difference between phenotypic and genotypic tropism assays in predicting clinical outcome of MVC containing regimens and does genotypic tropism testing in triplicate increase X4-tropic minority detection? Chapter **2** and **3**.

2) What is the inhibitory effect and the selective pressure of MVC in patients infected with X4-tropic, and dual-tropic viruses? Chapter **4** and **5**.

3) Does MVC intensification have an effect on virological parameters in immunologic non-responders? Chapter **6**.

4) Why is there no rebound observed of predicted X4-tropic virus after stem cell transplantation with CCR5 dysfunctional cells which resulted in the first case of HIV cure? Chapter **7**.

The results of these studies and implications for future therapies and curative strategies are discussed in chapter **8**.

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## Chapter 2

# Clinical outcome of maraviroc containing therapy in HIV-1 infected patients

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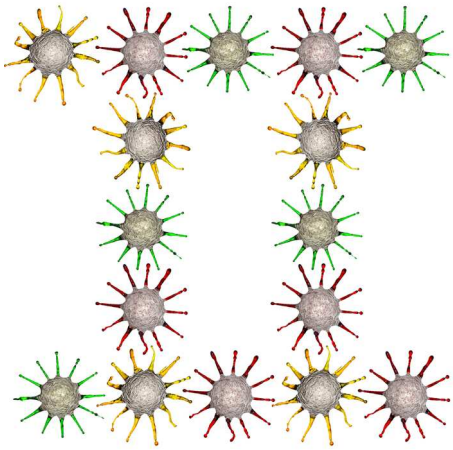

**Abstract**

**Objectives:** The available data on the use of maraviroc (MVC) in clinical settings is limited. We analysed the clinical outcome of patients treated with MVC and compared the predictive value of different tropism assays.
**Methods:** HIV-1 infected patients treated with MVC were included in this cohort study. Baseline viral tropism was assessed and compared by phenotypic (Trofile, MT-2) and genotypic assays, and a Genotypic Sensitivity Score (GSS) of the backbone was calculated. The virological and immunological response was evaluated.
**Results:** 62 predominantly extensive pre-treated patients started MVC (median GSS was 2.0 (2.0-2.5)). Tropism assays were performed on baseline samples of 54 patients (87.1%). Thirty-two (80.0%) viral samples were classified as R5 by Trofile, 26 (89.7%) by genotypic tropism test (GTT) and 18 (81.8%) by MT-2. On samples of 22 patients > 1 type of tropism assay was performed, with an observed concordance of 86.7-94.1%. Plasma HIV-RNA was undetectable (<50 copies/mL) in 82.1, 85.0 and 68.8%, of patients after 12, 24 and 36 months; the median CD4 cell increase was 194 (IQR 108-283), 291 (IQR 187-413) and 234 (IQR 106-444). Three patients stopped MVC treatment because of suspected side-effects. Five patients died during follow-up.

**Conclusions:** In this heavily pre-treated cohort, treatment with MVC was tolerated well and resulted in a good immunological and virological response. The results generated by the different tropism assays correlated well with each other and clinical outcome.

**Introduction**

Maraviroc (Celsentri®, Selzentry®) is the first drug of the class of entry inhibitors to be registered for treatment of HIV-1 infected antiretroviral therapy naive (USA only) and experienced patients, based on the 'Maraviroc plus Optimized Background Therapy in Viremic, ART-Experienced Patients (MOTIVATE) 1 and 2 studies[1,2]. Maraviroc (MVC) is an antagonist for the chemokine (C-C motif) receptor 5 (CCR5 co-receptor), which is, next to the chemokine (C-X-C motif) receptor 4 (CXCR4), a co-receptor for HIV-entry[3,4]. Binding of MVC to CCR5 prevents interaction with the V3-loop of the viral envelope protein gp120, and thereby HIV entry and fusion. 'Co-receptor tropism' refers to the ability of HIV-1 to enter $CD4^+$ cells using the CCR5 co-receptor ('R5-tropic virus'), the CXCR4 co-receptor ('X4-tropic virus'), or both co-receptors ('dual-tropic virus')[5]. Some patients are infected with a population of viruses with different co-receptor tropisms ('mixed-tropism virus population'). Since MVC is registered for treatment of patients infected with R5-tropic HIV-1, viral co-receptor tropism has to be assessed before start of treatment with MVC[6]. Genotypic tropism tests (GTT) predict viral co-receptor usage based on the sequence coding for the gp120-V3 loop by means of interpretation algorithms (R5 or X4 prediction)[5].

Although MVC has been registered since 2008 (Europe), data from clinical practice regarding efficacy, tolerability and the predictive value of different tropism assays is limited. The aim of this study is to analyse the clinical outcome of treatment of a heterogeneous group of HIV-infected patients with a MVC containing regimen in clinical practice, and to compare the use of different tropism assays for clinical purposes.

**Patients and methods**

The University Medical Centre Utrecht (UMCU) is one of the Dutch HIV treatment centres. All patients participate in the AIDS Therapy Evaluation in the Netherlands (ATHENA) observational cohort, which has been approved by local and national institutional review boards. As of June 2013, 21,990 patients were registered in this database.

*Study population*

We conducted a retrospective cohort study in the UMCU, and included all patients with an HIV-1 diagnosis, aged $\geq$18 years and who had taken at least one dose of MVC. Patients were followed until discontinuation of MVC, death or May 1, 2012.

*Data collection*

Collected data included information about demographics, date of HIV diagnosis, antiretroviral medication use, clinical diagnoses, laboratory data, and patient outcomes. The most recent $CD4^+$ T (CD4) cell counts and plasma HIV-RNA measurements before the start of MVC were recorded as baseline values. The indications for MVC use were recorded; these were categorized as failure of previous ART, intolerance to other antiretroviral drugs, MVC intensification therapy (either in attempt to increase CD4 cell count in patients with suppressed plasma HIV-RNA or to suppress plasma HIV-RNA in patients with low level viremia) or other indications. The optimized backbone regimen (OBR) that was used in combination with MVC was recorded, as were changes in OBR during MVC therapy. In case MVC was stopped, the date and indication for stopping were captured.

Immunological and virological response was assessed at time points with a three monthly interval from baseline. CD4 cell count and plasma HIV-RNA measurements within the range of one month before and one month after these time points were included.

Virologic response was evaluated as the proportion of patients with an HIV-RNA plasma level below a threshold of 50 copies/mL and 400 copies/mL, whereas the immunologic outcome was determined as the median change in CD4 cell count (cells/μL) compared to baseline.

Virological failure was defined as a viral load >50 copies/mL in two consecutive HIV-RNA measurements during ART (preceded by virological suppression <50 copies/mL) and/or as selection of resistance related mutations based on the IAS mutation list[7].

For evaluating tolerability of a MVC containing regimen, laboratory abnormalities were graded according to the Division of Microbiology and Infectious Diseases (DMID)-defined grades of toxicity (DMID Adult Toxicity Table May 2001, National Institute of Allergy and Infectious Diseases, National Institutes of Health). If ALT values were 1.25-2.5 times above upper limit of normal reference (ULN) it was graded as 'mild' toxicity, a value of >2.5-5.0 times ULN was graded as 'moderate', >5.0-10.0 times ULN as 'severe' and a value of more than 10.0 times ULN was graded as 'very severe' toxicity. For creatinine these cut-off levels were >1.0-1.5, >1.5-3.0, >3.0-6.0 and >6.0 times ULN.

*Co-receptor tropism testing*

Baseline plasma samples or the last sample with detectable plasma HIV-RNA were used for assessment of HIV-1 co-receptor tropism.

*Phenotypic co-receptor tropism testing*

Samples were sent to Monogram Biosciences (San Francisco, USA) for phenotypic tropism testing. Initially, the Origial Trofile™ (OTA) assay was used for tropism assessment. Results of the OTA are reported as R5 (CCR5 tropism), X4 (CXCR4 tropism) or D/M (dual or mixed tropism). When, due to technical reasons, no tropism results can be generated this will be reported as 'non-reportable'. In 2008, the OTA has been replaced by an improved version, the 'enhanced sensitivity Trofile™ assay' (ESTA). It has been reported that the ESTA is able to detect X4 virus clones with 100% sensitivity when they comprise at least 0.3% of the virus population[8]. OTA was performed on samples with at least 1000 HIV-RNA copies/mL, whereas ESTA was performed on samples with a minimum of 500 copies/mL.

The MT-2 assay is a phenotypic in-house tropism assay using an MT-2 cell line expressing the CXCR4 co-receptor. MT-2 cells were maintained in culture medium [CM; RPMI1640 with L-glutamine (BioWhittaker), 10% fetal bovine serum (FBS; Biochrom AG) and 10 mg/L gentamicin (Gibco)]. Patient-derived PBMCs ($1\times10^6$) prepared by Ficoll–Paque density gradient centrifugation were co-cultured in triplicate with $1\times10^6$ $CXCR4^+CCR5^-$ MT-2 cells in CM[9]. Viral cultures were maintained for 3 weeks and monitored for syncytium formation. A viral population is reported to be 'syncytium-inducing' (X4-, dual-, or mixed-tropic), or 'non-syncytium-inducing' (R5-tropic).

*Genotypic co-receptor tropism prediction*

For genotypic prediction of HIV-1 co-receptor tropism (Genotypic tropism test (GTT)), viral RNA was isolated from 200–1000 mL of plasma/serum as described previously[10] or viral DNA was isolated from $5\times10^6$ PBMCs. GTT using viral RNA was performed on either baseline samples with a detectable plasma HIV-RNA or (in case of undetectable plasma HIV-RNA at baseline) on the last available sample with detectable plasma HIV-RNA.

Alternatively, when no samples with detectable plasma HIV-RNA were available, viral DNA was isolated from PBMCs for tropism prediction. The V3 region of the viral envelope was amplified as previously reported[11]. In brief; V3 region was amplified with primers 6206V3F 5'-AGAGCAGAAGACAGTGGCAATGAGAGTGA-3', 7785R 5'-AGTGCTTCCTGCTGCTCCYAAGAACCC-'3 (Titan One Tube RT-PCR kit, Roche). The nested-PCR was performed with primers 6658F 5'-TGGGATCAAAGCCTAAAGCCATGTG-'3, 7371R 5'-GAAAATTCCCCTCCACAATT-'3 (Expand High-Fidelity PCR-System, Roche). Sequenced using primers 6957F 5'-GTACAATGTACACATGGAAT-'3 and 7371R or V3-4 5'-ACAGTACAATGTACACATGGAATTA-3' and V3-3 5'-AATTCCCCTCCACAATTAAAASTGTG-3' (Big dye Terminator Cycle seq kit v3,1, Applied Biosystems). Viral co-receptor tropism was predicted using 'geno2pheno$_{[co\text{-}receptor]}$ algorithm'. (http://www.geno2pheno.org/) [R5 prediction, >10%; and X4, ≤10% false positive rate (FPR)][5]. When patients experienced virological failure on MVC containing regimen co-receptor tropism of the viral population at rebound was assessed phenotypically with an MT-2 assay and predicted by genotypic tropism testing.

*Cumulative Genotypic Sensitivity Score of optimized backbone regimen*

The Cumulative Genotypic Sensitivity Score (GSS) of the virus population to antiretroviral drugs was calculated for each patient using all HIV *pol* sequences obtained before start of the MVC containing regimen. The sequences were uploaded in the Genotypic Resistance Interpretation Algorithm of the Stanford University HIV drug resistance database (http://hivdb.stanford.edu/pages/algs/HIVdb.html). Antiretroviral drugs were scored as 1 when the virus was 'susceptible' or 'potential resistant', 0.5 when the virus was 'low' or 'intermediate' resistant and zero in case of 'high resistance'.

*Statistical analysis*

Continuous variables were compared using Student's t-test or the Mann-Whitney test, for categorical variables Chi-square or Fisher's exact test were used. Statistical significance was determined at $p < 0.05$. Missing CD4 and HIV-RNA values were imputated according to 'last observation carried forward' method. All analyses were performed with SAS software (version 9.2, SAS institute Inc.).

## Results

*Baseline characteristics*

Sixty-two patients were included, their baseline characteristics are depicted in table 1. In 40 patients the plasma HIV-RNA was detectable (≥50 copies/mL) at the start of MVC, whereas in 22 patients MVC was started when plasma HIV-RNA was undetectable. Thirty-four (54.8%) patients started MVC because of virological failure of their previous regimen, whereas 15 (24.2%)

patients switched to a MVC containing regimen because of toxicity problems. In 12 (19.4%) patients cART was intensified with MVC with the aim to increase CD4 count or to suppress low level viremia (<500 copies/mL).

The mean age of the included patients was 48.8 years (95% confidence interval 46.0-51.5), 75.8% were male, and the majority acquired HIV through homosexual contact (54.8%) and originated from Western Europe (79.0%). Although in general the included patients had long-standing HIV-infection and were heavily pre-treated (median 14.2 (interquartile range (IQR) 9.3-16.2) years since HIV diagnosis, median 11.5 (IQR 8.5-12.7) years since start cART), an optimized backbone regimen with a median cumulative GSS of 2.0 (2.0-2.5) could nevertheless be constructed. Only five patients had a cumulative GSS ≤1.

**Table 1** Baseline characteristics

| | Total population (*n* = 62) | Patients with VL ≥50 copies/ml (*n* = *40*) | Patients with VL <50 copies/ml (*n* = *22* ) | *p*-value |
|---|---|---|---|---|
| Age (years)[a] | 48.8 (46.0-51.5) | 46.2 (42.9-49.5) | 53.5 (49.1-57.8) | 0.01 |
| Male sex | 47 (75.8%) | 26 (65.0%) | 21 (95.5%) | 0.01 |
| Time since HIV diagnosis (years)[1] | 14.2 (9.3-16.2) | 14.2 (8.9-16.0) | 14.3 (8.9-16.2) | 0.79 |
| Duration of ART (years)[2] | 12.9 (8.5-14.6) | 12.3 (5.4-14.6) | 13.2 (9.3-14.9) | 0.30 |
| Duration of cART (years)[3] | 11.5 (8.5-12.7) | 11.5 (8.5-11.8) | 12.5 (8.5-13.9) | 0.10 |
| Previous CDC-C diagnosis | 34 (54.8%) | 22 (55.0%) | 12 (54.6%) | 0.97 |
| GSS[4] | 2.0 (2.0-2.5) | 2.0(1.5-2.0) | 2.0 (2.0-3.0) | 0.04 |
| CD4 nadir[5] | 52 (19-158) | 38 (12-158) | 65 (37-190) | 0.21 |
| $CD4^+$ cell count (cells/$mm^3$) | 282 (96-485) | 165 (49-395) | 363.5 (271.0-760.0) | 0.002 |
| $CD4^+$ cell count < 200 cells/mm | 23 (37.1%) | 21 (52.5%) | 2 (9.1%) | 0.0007 |
| HIV-RNA ($log_{10}$ copies/mL) | 3.0 (1.7-4.9) | 4.7 (3.2-5.3) | <1.7 | <0.000 |
| ALT (U/L)[6] | 25 (19.-40) | 24 (19-39) | 26 (18-43) | 0.96 |
| Creatinine (µmol/l)[7] | 89 (74-103) | 71 (71-96) | 99.5 (79-138) | 0.06 |
| **Region of origin:** | | | | |
| Western Europe | 49 (79.0%) | 28 (70.0%) | 21 (95.5%) | 0.03 |
| Sub-Saharan Africa | 8 (12.9%) | 7 (17.5%) | 1 (4.6%) | 0.24 |
| North/Latin America, Caribbean | 4 (6.5%) | 4 (10.0%) | 0 (-) | 0.29 |
| **Transmission route:** | | | | |
| Homosexual | 34 (54.8%) | 18 (45.0%) | 16 (72.7%) | 0.04 |
| Heterosexual | 14 (22.6%) | 11 (27.5%) | 3 (13.6%) | 0.34 |
| IV drug use | 2 (3.2%) | 0 (-) | 2 (9.1%) | 0.11 |
| **Indication for MVC use:** | | | | |
| Virological failure | 34 (54.8%) | 34 (85.0%) | 0 (-) | <0.000 |
| Intolerance to previous ARV(s) | 15 (24.2%) | 2 (5.0%) | 13 (59.1%) | <0.000 |
| Intensification | 12 (19.4%) | 3 (7.5%) | 9 (40.9%) | 0.001 |
| Other | 1 (1.6%) | 1 (1.6%) | 0 (-) | - |

Values expressed as median (interquartile range) or number of cases (%), unless otherwise indicated. [a]Mean (95% confidence interval). Abbreviations: n = number of patients, VL = viral load, ART = antiretroviral therapy, cART= combination antiretroviral therapy, ARV = antiretroviral drug, ALT = alanine aminotransferase, IV = intravenous. [1]Data available for 59 patients, [2]for 61 patients, [3]for 59 patients and [4]for 59 patients, [5]for 61 patients, [6]for 61 patients and [7]for 61 patients.

*Co-receptor tropism*

In 54 patients (87.1%) a tropism assay was performed (Figure 1A and Table 2). An OTA/ESTA was performed in 43 patients (69.4%). In three out of 43 OTA/ESTA assays no result was generated ('not reportable', NR) (7.0%). The majority of samples tested had a plasma viral load >1000 HIV RNA cp/mL plasma (90.7%). Of four samples the plasma viral load at time of testing was >50 and <1000 HIV RNA cp/mL of which two were classified as D/M and two as

R5-tropic. In total OTA/ESTA resulted in an R5 tropism classification in 32 (80%) patient samples.

The phenotypic MT-2 assay was performed for 21 patient samples. The viral load at time of testing all exceeded 1000 HIV RNA cp/mL plasma. For 17 (81%) samples non-syncytia inducing (NSI) viral population was reported. For the remaining four the viral population was reported as syncytia inducing (SI). These viral populations harboured variants able to utilize the CXCR4 co-receptor (Figure 1B).

Genotypic tropism prediction was based on V3-sequences. The GTT was performed in 29 patients (46.8%), resulting in a mean FPR of 50.2 (95% CI 39.3-61.1). If we stratify according to HIV RNA cp/mL plasma at time of tropism testing, all eight samples with a viral load <50 cp/mL were assessed with GTT on proviral DNA, of which seven were predicted R5-tropic and one was predicted X4-tropic. The remaining 21 samples (72.4%) all had a viral load >1000 HIV RNA cp/mL plasma and GTT was performed on plasma HIV RNA. Of these 19 were predicted to be R5-tropic. In total, three patients (10.3%) harboured a viral population predicted to be X4-tropic .

In 8 (12.9%) patients no tropism assay was performed. Of these patients (with plasma HIV-RNA <50 cp/mL), seven patients started MVC as intensification therapy and one because of toxicity of the previous regimen.

In 22 (35.5%) patients at least two different tropism assays were performed (Figure 1c). Comparison of two different tropism assays resulted in a concordance of 86.7% (13/15) for OTA/ESTA compared to GTT, 94.1% (16/17) for MT-2 assay compared to OTA/ESTA and 88.9% (16/18) for MT-2 compared to the GTT assay

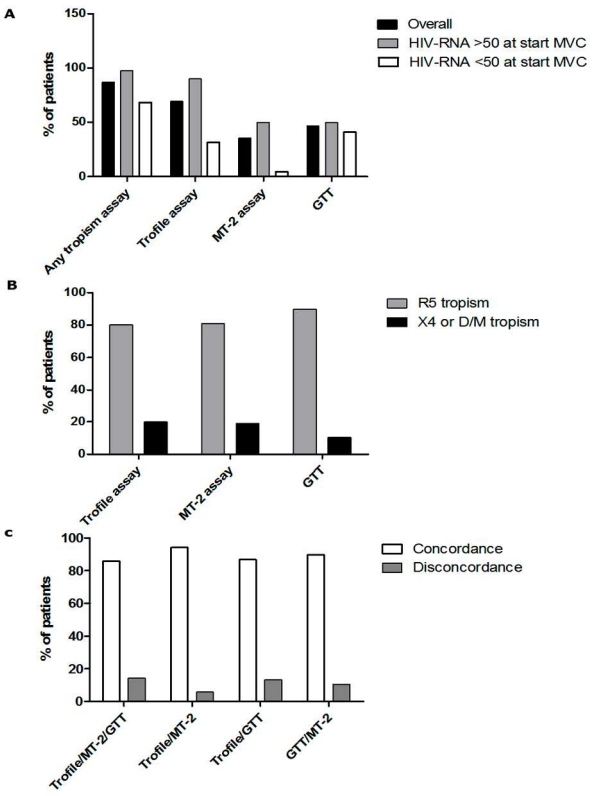


**Figure 1. Overview and results of the three performed tropism assays (Trofile assay, MT-2 assay and genotypic assay).** Overview of the percentage of patients in which the Trofile assay [OTA ( original Trofile assay) or ESTA (Enhanced Sensitivity Trofile Assay)], MT-2 assay or genotypic tropism test (GTT) is performed in the total study population, patients with detectable and undetectable (<50 copies/mL) baseline plasma HIV-RNA (A). Observed concordance between Trofile, MT-2 and GTT; between Trofile and MT-2 assay; between Trofile and GTT; and, between GTT and MT-2 assay (B). Results of the Trofile assay, MT-2 assay and GTT (C).

*Tolerability*

In general, MVC containing regimens were well tolerated. Twelve patients (19.3%) discontinued MVC therapy for various reasons. Of these patients, three restarted MVC treatment after an average of 6 months. Three patients discontinued MVC because of side effects possibly due to MVC. In the remaining patients, MVC was stopped for various reasons. Although four patients developed increased ALT levels that could be classified as severe or very severe toxicity (grade 3-4), there seemed to be no relation to MVC therapy. In 5 patients severe or very severe (grade 3-4) increased plasma creatinine levels were found. However all five patients were known with renal insufficiency before the start of MVC, and no relation to MVC use was found. MVC was therefore continued.

Five patients (8%) deceased during follow up. Causes of death were pneumonia (two times), B-cell lymphoma, squamous cell carcinoma, cardiomyopathy in combination with pulmonary hypertension. All patients had extensive co-morbidity, present before the start of MVC. There seemed to be no direct relation between MVC therapy and any of these deaths.

**Table 2. HIV-subtypes, plasma HIV-RNA and results of the various tropism assays.** Abbreviations: MVC = maraviroc; OTA = original Trofile assay; ESTA = Enhanced Sensitivity Trofile Assay; GTT=genotypic tropism assay; R5 = CCR5-tropic; DM = dual/mixed-tropic; X4 = CXCR4-tropic; NSI = non-syncytium inducing; SI = syncytium inducing; FPR = false-positive rate; plasma RNA = plasma HIV-RNA of the sample on which the tropism assay is performed; subtype = HIV-subtype.

| patient | subtype | Trofile | | | MT-2 | | GTT | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | type | plasma RNA | result | plasma RNA | result | plasma RNA | FPR | V3-sequence | result |
| 1 | B | ESTA | 3,6 | R5 | | | | | | |
| 2 | B | . | | | | | 6,3 | 90,9 | RNA | R5 |
| 3 | B | OTA | 3,7 | R5 | | | | | | |
| 4 | C | ESTA | 6,5 | R5 | | | | | | |
| 5 | B | ESTA | 5,5 | DM | 5,6 | NSI | 5,6 | 41,4 | RNA | R5 |
| 6 | unknown | . | | | | | | | | |
| 7 | A1 | . | | | | | 1,7 | 74 | DNA | R5 |
| 8 | B | . | | | | | 1,7 | 42,5 | DNA | R5 |
| 9 | B | OTA | 5,7 | R5 | 5,7 | NSI | 5,7 | 58,6 | RNA | R5 |
| 10 | B | . | | | | | 5,7 | 22 | RNA | R5 |
| 11 | J | ESTA | 4,7 | R5 | 4,7 | NSI | 4,7 | 82,3 | RNA | R5 |
| 12 | B | . | | | 4,0 | NSI | 4,0 | 9 | RNA | X4 |
| 13 | B | . | | | | | | | | |
| 14 | B | ESTA | 5,0 | DM | 5,0 | SI | | | | |
| 15 | B | . | | | | | | | | |
| 16 | unknown | . | | | | | | | | |
| 17 | B | OTA | 5,6 | R5 | 5,7 | NSI | 5,6 | 41,6 | RNA | R5 |
| 18 | B | ESTA | 4,7 | R5 | 4,7 | NSI | 4,7 | 79,5 | RNA | R5 |
| 19 | B | ESTA | 6,1 | NR | 6,1 | NSI | 4,1 | 38 | RNA | R5 |
| 20 | D | ESTA | 5,0 | DM | | | | | | |
| 21 | CRF02_AG | ESTA | 4,5 | NR | 3,6 | NSI | 5,8 | 69,1 | DNA | R5 |
| 22 | unknown | ESTA | 2,5 | R5 | | | | | | |
| 23 | B | ESTA | 4,9 | R5 | | | | | | |
| 24 | unknown | OTA | 4,9 | R5 | | | | | | |
| 25 | CRF02_AG | OTA | 5,3 | R5 | 5,3 | NSI | 5,3 | 58,6 | RNA | R5 |
| 26 | B | ESTA | 3,6 | R5 | | | | | | |
| 27 | B | ESTA | 2,9 | R5 | | | | | | |
| 28 | A1 | OTA | 5,7 | R5 | 5,7 | NSI | 5,7 | 96,5 | RNA | R5 |
| 29 | B | ESTA | 5,3 | R5 | 5,3 | NSI | 5,3 | 28,8 | RNA | R5 |
| 30 | B | ESTA | 3,7 | R5 | | | | | | |
| 31 | unknown | . | | | | | | | | |
| 32 | B | . | | | | | 1,7 | 1,7 | DNA | X4 |
| 33 | B | . | | | | | | | | |
| 34 | B | ESTA | 2,9 | DM | | | | | | |
| 35 | CRF03_AB | . | | | | | | | | |
| 36 | B | OTA | 5,0 | DM | 4,7 | SI | 4,7 | 10,5 | RNA | R5 |
| 37 | B | OTA | 5,8 | R5 | | | | | | |
| 38 | B | ESTA | 5,1 | R5 | 5,1 | NSI | 5,1 | 73,3 | RNA | R5 |
| 39 | C | . | | | | | 1,7 | 74,4 | DNA | R5 |
| 40 | B | OTA | 3,7 | R5 | | | | | | |
| 41 | unknown | . | | | | | 1,7 | 71,7 | DNA | R5 |
| 42 | B | OTA | 3,6 | R5 | | | | | | |
| 43 | B | ESTA | 5,9 | R5 | | | | | | |
| 44 | B | ESTA | 5,9 | R5 | 5,9 | NSI | 4,5 | 36,5 | RNA | R5 |
| 45 | B | OTA | 5,3 | R5 | 5,3 | NSI | 5,3 | 42,5 | RNA | R5 |
| 46 | B | OTA | 4,3 | DM | 4,3 | SI | | | | |
| 47 | B | ESTA | 4,7 | R5 | | | | | | |
| 48 | B | ESTA | 3,9 | R5 | | | | | | |
| 49 | B | . | | | | | 1,7 | 33,9 | DNA | R5 |
| 50 | B | OTA | 4,3 | R5 | | | | | | |
| 51 | B | OTA | 3,0 | DM | | | | | | |
| 52 | B | OTA | 3,7 | R5 | 3,7 | NSI | 3,7 | 27,2 | RNA | R5 |
| 53 | B | ESTA | 4,1 | R5 | 4,1 | NSI | | | | |
| 54 | unknown | . | | | | | | | | |
| 55 | B | ESTA | 3,0 | R5 | | | 3,0 | 87,8 | RNA | R5 |
| 56 | CRF03_AB | . | | | | | 1,7 | 23,6 | DNA | R5 |
| 57 | C | OTA | 5,9 | R5 | | | | | | |
| 58 | B | ESTA | 4,1 | DM | 4,9 | SI | 4,1 | 4,8 | RNA | X4 |
| 59 | C | . | | | | | 1,7 | 38,8 | DNA | R5 |
| 60 | B | ESTA | 3,3 | R5 | | | | | | |
| 61 | C | ESTA | 4,9 | NR | 3,9 | NSI | 4,9 | 96,2 | RNA | R5 |
| 62 | B | OTA | 6,5 | R5 | | | | | | |

*Immunological and virological response*

The cumulative follow up period was 166.0 years Forty patients with a detectable plasma HIV-RNA (median 4.7 (IQR 3.2-5.3) $log_{10}$ copies/mL) started a MVC containing regimen (median GSS 2.0). The plasma HIV-RNA of the majority of patients became suppressed ( < 50 copies/mL) after 3 months (59.0%), whereas after 12, 24 and 36 months, plasma HIV-RNA was suppressed in 82.1, 85.0 and 68.8%, respectively (Figure 2a).

Of these 40 patients an incomplete virologic response was observed in four patients (10.0%). All of these patients were infected with a multi-drug resistant HIV virus population. Baseline viral tropism was classified as DM in two patients, and maraviroc was included in salvage cART for a possible additive virological effect. In three patients drug compliance issues were recorded whereas in the fourth patient (with DM tropism at baseline) only a regimen with a backbone GSS of 1.5 could be constructed due to extensive drug-resistance. In this patient with persistently detectable plasma HIV-RNA despite maraviroc-containing cART, the viral population was phenotypically assessed to be SI and had an extreme X4-prediction (FPR 1.1-1.8).

Virologic rebound was observed in another four patients (10%). All of these patients were infected with an extensive drug-resistant virus population, and as a result only cART regimens with backbone GSS of 1.0-1.5 could be constructed. Although in one patient the virus population was assessed to be DM tropic at baseline (GSS of backbone cART 1.5), maraviroc was included in a salvage cART regimen. In the other three patients tropism was classified as R5 ( GSS 1.0, 1.5 and 1.5). In two of these patients compliance issues were noted. Switching to a new antiretroviral regimen in these three patients resulted in full virological suppression. In the group of patients with an undetectable plasma HIV-RNA at baseline (n = 22), virologic rebound was not observed.

During follow-up a sustained immunological response was observed for patients with a detectable plasma HIV-RNA at baseline (Figure 2b). In the group with baseline plasma HIV-RNA > 50 copies/mL at the start of MVC, the median CD4 cell increase was 199 (IQR 108-283), 291 (IQR 187-413) and 234 (IQR 106-444) after 12, 24 and 36 months, respectively ($p < 0.0001$, $= 0.001$ and $= 0.0002$). However, In patients who switched to a MVC containing regimen (either intensification therapy or switch to a complete new regimen) while having a plasma HIV-RNA of <50 copies/mL, no significant increase in CD4 cell count was observed. The twelve patients who started MVC as intensification therapy (nine patients with plasma HIV-RNA <50 copies/ml, three with low level viremia), had a median CD4 cell count increase at 12 months of 145 (IQR 57-196) cells/μL which was not significantly different from baseline ($p = 0.06$).

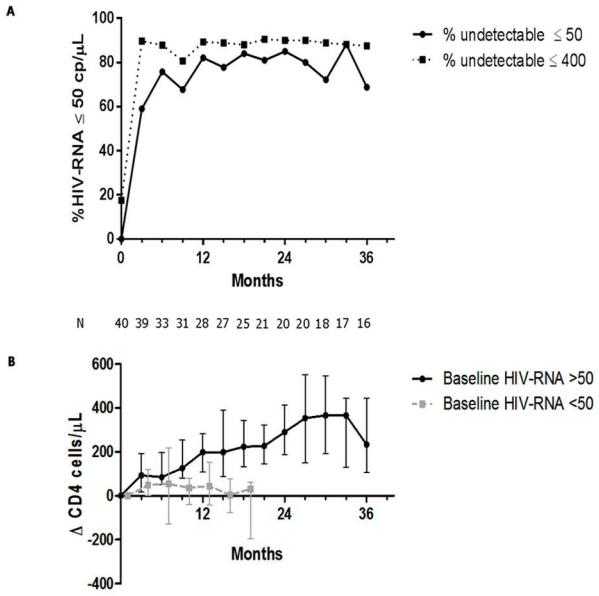


**Figure 2. Virological and immunological response on a maraviroc containing regimen.**
Virological (percentage of patients <50 copies/mL) response on a maraviroc containing regimen of the patients with a detectable plasma HIV-RNA at baseline (A; N = 40). Immunological response (Change in CD4 cell count (delta)) is given for those patients with detectable and undetectable baseline plasma HIV-RNA (B). N = number of remaining patients during follow up

*Predictive value of the different tropism assays*

We analysed the predictive value of the different tropism assays with respect to virological and immunological outcome. When the virus was classified as R5 according to Trofile, 92.3% of patients (with detectable viral load at baseline) had undetectable (<50 copies-mL) plasma HIV-RNA month 24, compared to 88.9 and 100.0% according to respectively GTT and MT-2 (Figure 3a). The median CD4 cell count increase after 24 months in this group of patients was 291 (IQR 187-389) for R5 classification according to Trofile, compared to 339 (IQR 274-552) and 339 (IQR 274-552) according to respectively GTT and MT-2 (Figure 3b).

Seven patients started an MVC containing regimen while harbouring a virus population classified as DM by Trofile at baseline (median GSS 2.0 (IQR 1.5-3.0). At 24 weeks in 60% (three out of five) of patients plasma HIV-RNA was suppressed. Out of the four patients with a virus population classified as SI by MT-2 assay (median GSS 2.3 (IQR 1.5-3.0), two patients had suppressed plasma HIV-RNA at 24 months follow up. Only two patients with detectable baseline plasma HIV-RNA harboured a virus population predicted to be X4-tropic by GTT, and both had a very limited follow up period (three and six months).

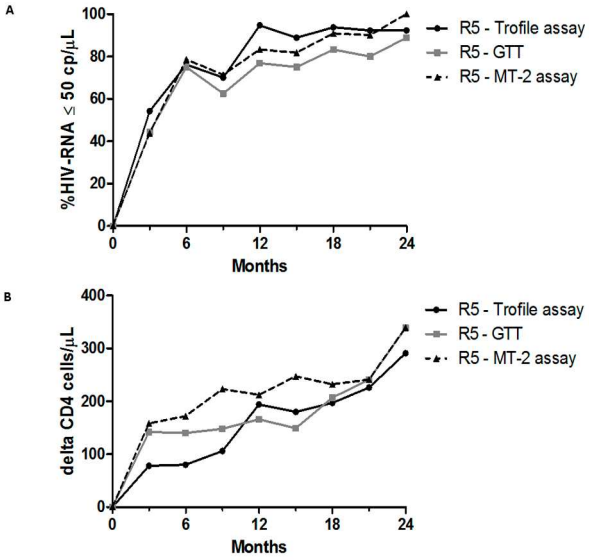


**Figure 3. Virological and immunological outcome according to different tropism assays.**
Virological outcome (percentage of patients <50 copies/mL) of those patients classified as R5-tropic by Trofile, genotypic tropism test (GTT) or MT-2 assay (A). Immunological outcome (Change (delta) in CD4 cell count) of those patients classified as R5-tropic by Trofile, GTT or MT-2 assay (B).

*Discussion*

The present study discusses the experiences and outcome of MVC containing therapy in clinical practice in a heterogeneous, heavily pre-treated cohort of 62 HIV-1 infected patients. Treatment with MVC was tolerated well and resulted in a good immunological and virological response. The results generated by the different tropism assays correlated well with each other and clinical outcome.

Despite the heterogeneous and heavily antiretroviral treatment experienced cohort, 85.0% of patients who initiated MVC with a detectable viral load at baseline had undetectable plasma HIV-RNA and a median increase in CD4 cell count of 249 (IQR 164-412.5) cells/µL after 24 months. In general, MVC therapy was tolerated well. None of the liver and kidney toxicity, nor the deaths that were recorded were classified by the treating physicians as related to MVC therapy. These toxicity characteristics merely seem to be a reflection of the extensive co morbidity of the described cohort.

Although the quantity of reported efficacy and tolerability data of MVC containing regimens from routine clinical practice are very limited, our findings are in line with the few smaller studies that have been published. In a Spanish cohort 96.3% of patients (N = 46) had undetectable plasma HIV-RNA at week 48, with a mean CD4 cell count increase of 151 cells/µL[12]. In a German cohort (N = 44) 78% of patients had undetectable plasma HIV-RNA at month 6,

whereas a median CD4 cell count increase of 124 cells/μL was observed. The major indication for MVC containing therapy in this heavily pre-treated cohort (median duration of cART 10.1 years; median number of previous drug combinations nine) was virological failure (56%)[13]. The efficacy and safety of the combination MVC, raltegravir and etravirine was evaluated in 28 antiretroviral therapy experienced patients in a no randomised prospective study[14]. All patients participated in the expanded access programs for these drugs. (At week 48, 93% of patients had a plasma HIV-RNA <50 copies/mL, with a median CD4 cell count increase of 267 cells/μL.

The reasons for initiating MVC in the present study were most commonly virological failure (54.8%), toxicity of the previous regimen (24.2%) and drug intensification strategy (19.4%). None of the patients that switched to a MVC containing regimen because of previous toxicity experienced virological failure during follow up, whereas the CD4 cell count of these patients remained stable. These data underline the fact that switching to a MVC containing regimen for toxicity reasons can be a good alternative. In twelve patients MVC was started as intensification of cART. Although a trend for an increase in CD4 cell count after 12 months was observed this was not statistically significant. This is in agreement with other intensification studies, that report minor or no changes in CD4 cell count after intensification of cART with MVC[15-18].

Since MVC is registered for treatment of patients infected with R5-tropic HIV-1, viral co-receptor tropism has to be assessed before start of treatment with MVC[6]. To our knowledge, this is the first published study in which three different HIV co-receptor tropism assays have been used, namely two phenotypic (OTA/ESTA and MT-2 assay) and one genotypic assay. The concordance between the various tropism assays was very high ranging from 85.7 to 94.1%. This is comparable or better than other comparisons between different tropism assays that have been reported so far[13,19]. In addition, the predictive value of the different tropism assays with respect to virological and immunological outcome was excellent and correlated well with each other. MVC selected for X4-predicted viral variants with an extremely low FPR in two patients who experienced a viral rebound or a slow virological response during MVC containing therapy. In three out of the four patients with viral rebound, a subsequent switch to a new antiretroviral regimen resulted in adequate suppression of plasma HIV-RNA. The European guidelines for HIV-1 tropism recommend to use the population-based genotypic tropism test, mostly because of practical and costs issues. These guidelines advise to use a cut-off FPR of 10% in most situations for both viral RNA and pro-viral DNA. This may be a rather conservative approach since at virological failure only viral variants with an extremely low FPR were observed[20]. Studies to prospectively explore the optimal cutt-off (i.e. FPR) should be performed. Nevertheless, the data of the present study underline that a genotypic tropism test is a solid alternative for predicting HIV co-receptor tropism in clinical practice.

One of the strengths of this study is the heterogeneous group of patients described in this cohort, which makes the results more applicable to

clinical practice. Other strengths are the larger sample size as compared to previously published studies and the relatively long period of follow-up. Besides the strengths, this study has some limitations. First, this study was of a retrospective nature. This could have resulted in uncontrolled confounders, because it was predetermined which patients received MVC. In this study MVC was in particular prescribed to heavily pre-treated patients what could have led to an underestimation of the virological and immunological outcome and a higher rate of the adverse events. Second, this study was conducted at a single institution and therefore limits the generalizability of our results. Third, studies with a larger study population might generate more statistical power to detect differences in virological and immunological response.

In conclusion, MVC containing therapy in a heterogeneous, mostly heavily pre-treated cohort was tolerated well and resulted in good virological and immunological response during a substantial follow-up period. Moreover, the tropism data of this study show that prediction of HIV co-receptor tropism by a genotypic tropism assay is a reliable option in clinical practice.

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## Chapter 3

# Impact of triplicate testing on HIV genotypic tropism prediction in routine clinical practice

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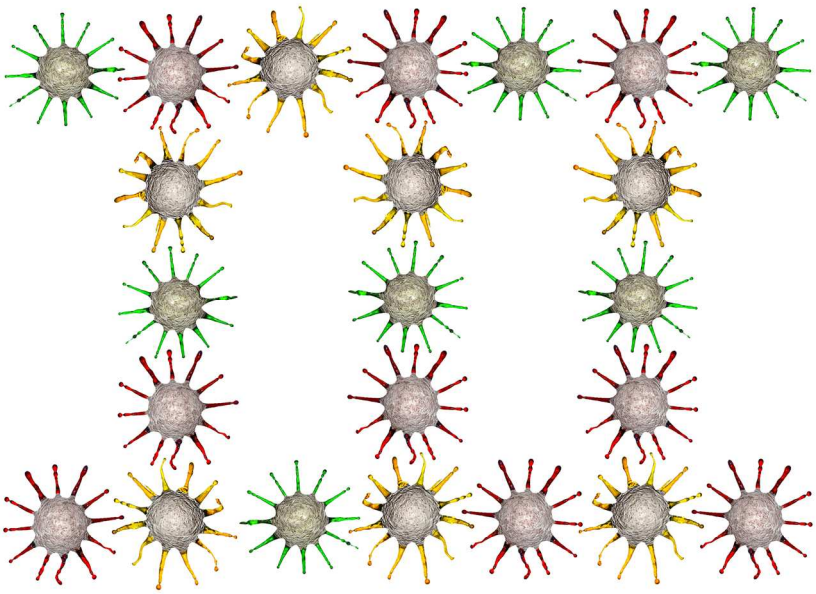

**Abstract**

Guidelines state that the CCR5-inhibitor Maraviroc should be prescribed to patients infected with R5-tropic HIV-1 only. Therefore, viral tropism needs to be assessed phenotypically or genotypically. Preliminary clinical trial data suggest that genotypic analysis in triplicate is associated with improved prediction of virological response by increasing the detection of X4-tropic variants. Our objective was to evaluate the impact of triplicate genotypic analysis on prediction of co-receptor usage in routine clinical practice. Samples from therapy naive and therapy-experienced patients were collected for routine tropism testing at three European clinical centres. Viral RNA was isolated from plasma and proviral DNA from peripheral blood mononuclear cells. Gp120-V3 was amplified in a triplicate nested RT-PCR procedure and sequenced. Co-receptor usage was predicted using the Geno2Pheno$_{[coreceptor]}$ algorithm and analysed with a false positive rate (FPR) of 5.75%, 10%, or an FPR of 20% and according to the current European guidelines on the clinical management of HIV-1 tropism testing. A total of 266 sequences were obtained from 101 patient samples. Discordance in tropism prediction for the triplicates was observed in ten samples using an FPR of 10%. Triplicate testing resulted in a 16.7% increase in X4-predicted samples and to reclassification from R5 to X4 tropism for four cases rendering these patients ineligible for Maraviroc treatment. In conclusion, triplicate genotypic tropism testing increases X4 tropism detection in individual cases, which may prove to be pivotal when CCR5-inhibitor therapy is applied.

**Introduction**

Maraviroc (MVC) is the first available antiretroviral drug targeting a human receptor. It binds to the CCR5 co-receptor thereby inhibiting replication of CCR5 using (R5-tropic) HIV-1[1,2]. MVC has been approved for HIV-1-infected patients that exclusively harbour R5-tropic viruses and is licensed in Europe for therapy-experienced patients and in the USA for both therapy-experienced and therapy-naive patients. As MVC has no antiretroviral effect on strains using the CXCR4 co-receptor (X4-tropic), determination of co-receptor usage (viral tropism testing) is needed to exclude the presence of X4-tropic HIV-1 strains. For determination of viral tropism several phenotypic and genotypic assays have been developed. Among phenotypic tropism tests, the 'enhanced sensitivity Trofile assay' (ESTA; Monogram Biosciences, San Francisco, CA) is most often used[3,4]. However, for clinical centres, ESTA has several limitations: testing is only performed in California (USA), resulting in logistical problems, long turnaround time and high costs. Furthermore, the assay is only available in Europe for samples with HIV RNA >1000 copies/mL. For these reasons tropism testing is increasingly performed using genotypic assays. Genotypic tropism tests analyse the sequence of the HIV-1 envelope gp120 variable 3 (V3) loop, the main determinant for co-receptor usage.

To predict viral tropism the generated V3 sequences are interpreted using publicly available algorithms, such as Geno2Pheno$_{[coreceptor]}$ (G2P) and

position specific scoring matrices ($PSSM_{X4-R5}$)[5,6]. Genotypic tropism testing can be applied on population sequences obtained from either HIV RNA or HIV proviral DNA. The latter is recommended if HIV RNA levels are below the level of reliable amplification[7]. Population sequencing, the most frequently used method of genotypic tropism testing, is hampered by limited sensitivity for detecting minority X4-tropic strains in the quasi-species. As such, minority X4-tropic variants may remain undetected when they represent <10–25% of the total population[8–10]. Despite limitations in sensitivity compared with ESTA, population genotypic tropism testing demonstrated equal predictive value for virological outcome of MVC-containing therapy in antiretroviral naive individuals[11]. In this particular retrospective analysis a genotypic testing procedure was performed in triplicate to increase detection of minority X4 populations. The rationale for performing genotypic tropism testing in triplicate, instead of using a single procedure as usually performed for resistance testing on pol, is based on differences in selective pressure on the viral envelope protein compared with pol, which are reflected by the nine-fold higher nucleotide substitutions/site/year in env[12]. The relatively high levels of variation in env may be better captured in a triplicate procedure.

In therapy-experienced patients, re-analysis of three clinical trials demonstrated that triplicate genotypic tropism testing increased the number of X4-predicted samples[13]. Preliminary data suggest that testing in triplicate has a beneficial effect on predicting clinical outcome of MVC-containing regimens[13]. However, in clinical cohort studies triplicate genotypic tropism testing is not performed routinely. Still a good correlation between genotypic tropism testing and ESTA in predicting virological outcome to MCV-containing therapy has been observed[14–17]. As such, the added value of triplicate testing in routine care is still under debate. In the absence of a direct comparison of single and triplicate test procedures in clinical practice, the recently formulated European guidelines advise triplicate testing with a false-positive rate (FPR) of 10%. If single testing is performed then a more conservative FPR of 20% for RNA samples witha viral load <1000 copies/mL and for proviral DNA samples is recommended[7].

We investigated the influence of triplicate testing on tropism prediction during routine clinical practice in three European clinical centres.

## Materials and Methods

Patient samples on which routine tropism testing was performed in clinical practice were randomly selected from three European centres. HIV-1 plasma RNA levels and counts of CD4+ cells/mm3 at nadir and at time of sampling were collected, HIV proviral DNA was not measured. HIV-1 pol subtyping was based on IDNS (Smartgene, Lausanne, Switzerland) or the Rega HIV-1 subtyping tool[18].

*Viral RNA, DNA isolation*

Viral RNA was isolated from 200–500 lL EDTA-plasma with the Viroseq HIV-1 sample preparation module (Abbott, Hoofdorp, the Netherlands) or a high pure viral RNA kit (Roche, Vilvoorde, Belgium). If no plasma was available or the HIV RNA level was below the level of amplification, proviral DNA was extracted from $1.0E^7$ peripheral blood mononuclear cells with QIAamp DNA Blood Mini Kit (QIAGEN, Madrid, Spain).For each sample, one isolation was performed. Subsequent processing of the samples, amplification and sequencing, were performed in triplicate. In each isolation and amplification round two or three negative controls were included, depending on the number of isolations and amplifications.

*Viral RNA amplification*

For amplification of the V3-loop, two in-house protocols were used. Protocol one; 10 µL of RNA, with primers 6206V3F 5'-AGAGCAGAAGACAGTGGCAATGAGAGTGA-3', 7785R 5'-AGTGCTTCCTGCTGCTCCYAAGAACCC-3' (Titan One Tube RT-PCR kit, Roche, Woerden, the Netherlands) for RT-PCR. Subsequently a nested-PCR was performed using primers 6658F 5'-TGGGATCAAAGCCTAAAGCCATGTG-3', 7371R 5'-GAAAATTCCCCTCCACAATT-'3 (Expand High-Fidelity PCR-System, Roche, Woerden, the Netherlands). Sequencing was performed with primers 6957F 5'-GTACAATGTACACATGGAAT-3' and 7371R or V3-4 5'-ACAGTACAATGTACACATGGAATTA-3' and V3-3 5'-AATTCCCCTCCACAATTAAAASTGTG-3' (Big dye Terminator Cycle seq kit v3,1, Applied Biosystems, Nieuwekerk ad IJsel, the Netherlands). Protocol two; for the RT-PCR 10 µL RNA and a mixture of the primers sense ENV_11 5'-GGATATAATCAGYYTATGGGA-3', antisense ENV_22 5'-GGTGGGTGCTAYTCCYAITG-3', sense-ENV_1 5'-GAGGATATAATCAGTTTATGG-3' and antisense-7294 5'-GGTGGGTGCTATTCCTAATGG-3' (Titan One Tube RTPCR kit, Roche, Vilvoorde, Belgium) were used. These primer mixtures cover a broader range of HIV variants The nested-PCR was performed using primers sense-ENV_33 5'-GATCAAAGCCTAAARCCATGT-3', antisense-ENV_44 5'-CTCCAATTGTCCYTCATHTYTCC-3', sense-ENV2 5'-GATCAAAGCCTAAAGCCATG-3' and antisense-7238 5'-ACTTCTCCAATTGTCCCTCATAT-3' with AmpliTaq DNA polymerase (Applied Biosystems, Halle, Belgium). Amplified product was sequenced with primers sense-6951 5'-AGYRCAGTACAATGYACACATGG-3', sense-6690 5'-TCAACHCAAYTRCTGTTAAATGG-3' and antisense-7336 5'-ATTTCTRGRTCYCCICCYG-3' (Big dye Terminator Cycle seq kit v3,1, Applied Biosystems). Proviral DNA amplification For amplification 3 lL DNA was used to amplify full-length envelope with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Barcelona, Spain) using primers 5677U24 5'-ATG GCTTAGGGCAACATATCTATG-3' and 9687L24 5'-CTGAGGGATCTCTAGTTACCAGAG-3' or primers 5954U29 5'-CACCTAGGCATCTCCTATGGCAGGAAGAAG-3' and 8904L22 5'-GTCTCGAGATACTGCTCCCACCC-3'. Nested-PCR using primers 5954U29 5'-CACCTAGGCATCTCCTATGGCAGGAAGAAG-3' and 8904L22 5'-

GTCTCGAGATACTGCTCCCACCC-3' or 6373U22 5'-CCACTCATTTTGTGCATCAGA-3' and 7855L25 5'-AAYTGTCTGCCTGTACCGTCAGCG-3' (Platinum Taq DNA Polymerase High Fidelity, Invitrogen). Sequenced using primers 7002U20 5'-CTGTTAAATGGCAGTCTAGC-3' and 7374L25 5'-AGAAAAATTCYCCTCYACAATTAAA-3', or 6959U25 5'-ACAATGYACACATGGAATTARGCCA-3' and 7365L21 5'-CCCCTCCACAATTAAAACTGT-3' (Big dye Terminator Cycle seq kit v3.1, Applied Biosystems, Madrid, Spain).

*Tropism prediction*

Nucleotide sequence tropism prediction was performed in silico using G2P and an FPR of 5.75%, 10%, 20% and according to the current European guidelines[7]. For web-PSSMX4-R5 the amino acid sequence was used. In the case of mixtures all possible amino acid sequences were analysed and the highest value was reported (R5 prediction: ≤6.69, X4: ≥2.88, the 11/25 rule was applied at intermediate values)[19]. If an isolate was predicted to be X4-tropic in at least one of the three tests the viral population was reported to be X4-tropic.

*Statistical analysis*

Pearson chi-squared test was used to compare the ratio X4-predicted sequences between low (<350) and high (≥350) $CD4^+$ cells/$mm^3$, to compare the ratio of X4-predicted sequences of G2P versus PSSMX4-R5, and to evaluate the ratio and number of X4-predicted sequences, and samples between single, duplicate or triplicate testing. Mann–Witney U-test was used to compare viral RNA load between samples with or without amplification failure. Furthermore, data were randomized with randperm in MATLAB 2010b. Values below 0.05 were regarded statistically significant.

**Results**

The majority of 101 patients (70) were infected with subtype B, followed by: C (6), CRF02_AG (6), CRF01_AE (5), A (4), A1 (2), G (1), H (1), J (1), CRF15_01B (1), CRFAB (1), CRF30 (1), CRF18_cpx (1) and one unclassified strain. The median viral load was 8.35 $E^3$ copies/mL HIV RNA (interquartile range (IQR) 7.26 $E^4$). The median $CD4^+$ T-cell count at time of sampling was 422 $CD4^+$ (IQR 439) and the median nadir $CD4^+$ T-cell count was 310 (IQR 261) cells/$mm^3$. Neither low $CD4^+$ cell-count at time of sampling (p 0.636) nor low nadir $CD4^+$ count (p 0.462) was associated with either X4 or R5 prediction.

Tropism was predicted using two interpretation algorithms; G2P and $PSSM_{X4\text{-}R5}$. Using an FPR of 10% the number of X4-predicted sequences did not significantly differ between G2P and $PSSM_{X4\text{-}R5}$ (p 0.186), 28 sequences were predicted to be X4-tropic in G2P and R5-tropic in $PSSM_{X4\text{-}R5}$. Conversely, 15 sequences were predicted to be R5-tropic in G2P and X4-tropic in $PSSM_{X4\text{-}R5}$. As G2P is the most commonly used interpretation algorithm in clinical practice in Europe further analysis was performed with G2P only. Using a triplicate

procedure a total of 266 (87.8%) sequences were generated (156 from 58 viral RNA samples and 110 from 43 proviral DNA samples) (Figure 1).

Amplification failures were observed in 14% of proviral DNA samples and in 10% of viral RNA samples. Amplification failures were not specifically associated with low viral RNA load (p 0.249). Median viral RNA load of samples with an amplification failure was 7.9 $E^3$ (IQR 1.221 $E^5$) compared with 8.7 $E^3$ (IQR 5.479 $E^4$) for samples with no amplification failure. The majority of sequences had an FPR above 10% (n = 202, 75.9%). After dividing the data into 10% FPR increments, these sequences are distributed throughout the different categories. The remaining 24.1% (n = 64) fall into the 0–10% FPR increment, which therefore was the largest category (Figure 2). Using single genotypic tropism testing successful tropism results were generated for 92.1% of the samples. The success rate increased into 100% tropism results when tested in duplicate and triplicate. Pooled analysis of triplicatesequence data from individual patient samples resulted in an X4 prediction in 25.9% of the viral RNA samples and 30.2% of the viral DNA samples (FPR 10%; p 0.628) (Figure 1a).

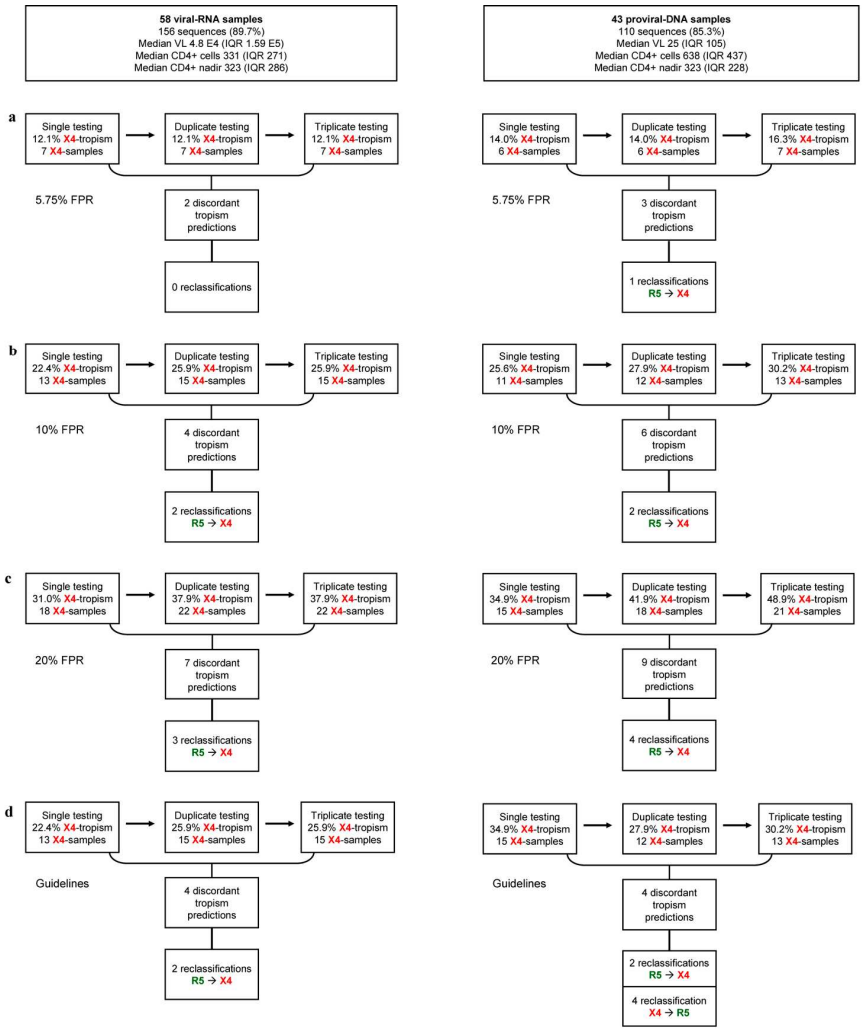


**Figure 1**. A total of 101 patient samples tested in a single versus triplicate genotypic tropism procedure (58 viral RNA samples and 43 proviral DNA samples). VL = HIV-1 RNA level copies/mL, number of successfully analysed sequences are listed. Tropism was predicted using a false-positive rate (FPR) of 5.75% (a), 10% (b), 20% (c) or according to the current European guidelines (d)[7]. Percentage X4-predicted samples is depicted.

Samples for which one of the sequences resulted in an R5 prediction while at least one of the other sequences yielded X4 results, were considered discordant.

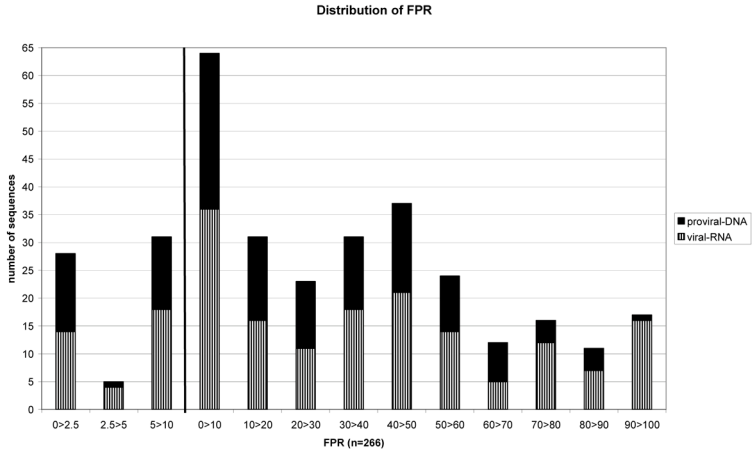


**Figure 2**. Distribution of Geno2Pheno[coreceptor] results of 266 sequences obtained from 101 patient samples. Black bars represent proviral DNA sequences, striped bars represent viral RNA sequences. Distribution of 0>10 false-positive rate (FPR) is also subdivided in categories 0>2.5, 2.5>5 and 5>10.

**Table1**. Overview of reclassified samples using a triplicate tropism procedure. X4 prediction is given in red and R5 prediction in green, – indicates amplification failure and NA = not assessed. Guidelines: European guidelines on clinical management of HIV-1 tropism testing[7].

| | | | | Single | Triplicate procedure | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FPR | Sample id | Sample | Subtype | FPR | FPR 1st | FPR 2nd | FPR 3rd | Viral load | CD4 count | CD4 nadir | Reclassification |
| 5.75% | A | *DNA* | B | 8.2 | 8.2 | - | 4.1 | 20 | 669 | NA | R5 → X4 |
| 10% | B | RNA | CRF01_AE | 33.0 | 33.0 | 7.4 | - | 48100 | 5 | 5 | R5 → X4 |
| | C | RNA | B | 16.9 | 16.9 | 6.6 | - | 74651 | 467 | 467 | R5 → X4 |
| | D | *DNA* | B | 53.5 | 53.5 | 17.9 | 7.8 | 25 | 1085 | 635 | R5 → X4 |
| | E | *DNA* | B | 55.8 | 55.8 | 8.7 | 17.0 | 130 | 658 | 378 | R5 → X4 |
| 20% | B | RNA | CRF01_AE | 33.0 | 33.0 | 7.4 | - | 48100 | 5 | 5 | R5 → X4 |
| | F | RNA | AB | 20.1 | 20.1 | 18.3 | - | 30000 | NA | NA | R5 → X4 |
| | G | RNA | B | 33.7 | 33.7 | 17.0 | 33.7 | 648 | 318 | 281 | R5 → X4 |
| | D | *DNA* | B | 53.5 | 53.5 | 17.9 | 7.8 | 25 | 1085 | 635 | R5 → X4 |
| | E | *DNA* | B | 55.8 | 55.8 | 8.7 | 17.0 | 130 | 658 | 378 | R5 → X4 |
| | H | *DNA* | B | 34.8 | 34.8 | 32.6 | 16.9 | 25 | 621 | 283 | R5 → X4 |
| | I | *DNA* | B | 49.9 | 49.9 | 24.7 | 13.7 | 25 | 761 | 278 | R5 → X4 |
| Guidelines* | B | RNA | CRF01_AE | 33.0 | 33.0 | 7.4 | - | 48100 | 5 | 5 | R5 → X4 |
| | C | RNA | B | 16.9 | 16.9 | 6.6 | - | 74651 | 467 | 467 | R5 → X4 |
| | D | *DNA* | B | 53.5 | 53.5 | 17.9 | 7.8 | 25 | 1085 | 635 | R5 → X4 |
| | E | *DNA* | B | 55.8 | 55.8 | 8.7 | 17.0 | 130 | 658 | 378 | R5 → X4 |
| | J | *DNA* | B | 16.9 | 16.9 | 49.7 | 19.6 | 600 | 458 | 378 | X4 → R5 |
| | K | *DNA* | B | 14.7 | 14.7 | 20.8 | - | 130 | 245 | NA | X4 → R5 |
| | L | *DNA* | B | 17.0 | 17.0 | - | 15.7 | 25 | 750 | 413 | X4 → R5 |
| | M | *DNA* | B | 13.7 | 13.7 | 15.6 | 12.8 | 25 | 890 | 4 | X4 → R5 |

* The European guidelines advice an FPR of 10% for triplicate procedures. For a single procedure an FPR of 20% for RNA-samples with a viral load <1000 cp/mL and for pro-viral DNA samples is advised.

Analysis of the data with an FPR of 10% resulted in ten discordant tropism results, four viral RNA and six proviral DNA samples (9.9%) had discordant tropism results (Figure 1). Discordance does not always result in reclassification of the tropism report. If a population is predicted to be X4-tropic in the first replicate, finding an R5-tropic virus in the second or third

replicate will not change the tropism prediction. Therefore we analysed for each individual patient sample the influence of triplicate testing on the reported tropism result. If only the first replicate was taken into account, 24 samples (both RNA and DNA samples) were predicted to be X4-tropic (23.8%). Adding the second replicate increased the number of X4-predicted samples to 27 (26.7%) and addition of the third replicate resulted in 28 samples with X4 tropism results (27.7%) (Fig. 1). Hence, triplicate testing using an FPR of 10% resulted in a 16.7% increase of X4-predicted samples. This corresponds to a 4% increase in X4 prediction for the total study population (p 0.730) and reclassification from R5 to X4 tropism in four patients (RNA n = 2; DNA n = 2) (Table 1).

Additionally we analysed the data with three random sets of first replicates using an FPR of 10%. These three data sets resulted in ten discordant samples with four, five or eight reclassifications from R5 to X4 tropism, respectively (p ≥0.278). Randomizing the order of replicates did not influence our results. Triplicate analyses using a more conservative 20% FPR increased the total number of discordant samples to 16 (15.8%) (RNA n = 7; DNA n = 9) (Figure 1) and the number of reclassifications from R5 to X4 tropism to seven (6.9%, p 0.353) (Table 1). Analysis of the sequence replicates in a different order did not significantly change the level of reclassifications (all p values ≥0.310). The number of discordant samples did not significantly differ in proviral DNA samples (low viral load) or RNA samples (high viral load) (p ≥0.228 depending on FPR).

We also analysed the samples according to the European guidelines for clinical management of HIV-1 tropism testing[7]. In this analysis the first replicate resulted in 28 (27.7%) samples predicted to be X4-tropic. Triplicate genotypic tropism testing did not change the overall number of X4-predicted samples (Figure 1). However, this analysis resulted in eight discordant samples (RNA n = 4, DNA n = 4). Furthermore, two viral RNA samples and two proviral DNA samples were reclassified from R5-tropic to X4-tropic and four proviral DNA samples were reclassified from X4-tropic to R5-tropic (Table 1). In literature, a low FPR of 5.75% was found to be a good predictor for response to MVC treatment in phenotypically pre-screened patients[20]. In general, application of a lower FPR results in less frequent prediction of X4 virus and therefore a decreased level of discordance. In our data set triplicate testing with an FPR of 5.75% yielded five discordant samples (RNA n = 2, DNA n = 3) resulting in reclassification from R5 to X4 tropism in only one patient (p 0.995) (Figure 1).

**Discussion**

We evaluated the added value of triplicate versus single testing on genotypic tropism prediction in routine clinical practice. Co-receptor usage of virus isolates from 101 patient
samples was predicted after V3 sequencing and applying publicly available and commonly used interpretation algorithms. In this study, patient samples with a

broad range of HIV RNA plasma levels as well as proviral DNA samples were analysed with G2P with an FPR of 5.75%, 10%, 20% and according to current European guidelines on tropism testing. A considerable number of samples with discordant triplicate results was observed. In one out of every ten samples X4-predicted as well as R5-predicted sequences were detected. However, in only half of these cases did the discordance result in a reclassification of the final tropism call from R5 to X4.

One could argue that in samples with a low viral input the observed discordance in the triplicate analyses results from stochastic errors in sampling and amplification. However, the number of discordant results did not significantly differ between samples with low or high viral load. Therefore it seems more likely that the overall high levels of variation in env is the reason for the observed discordance in the triplicate analysis of viral tropism. Detection of nucleotide mixtures in a sequence complicates the tropism prediction. In the G2P algorithm nucleotide sequences are used as input and amino acid mixtures are therefore considered. In $PSSM_{X4\text{-}R5}$ amino acid sequences are used as input and all possible amino acid combinations have to be considered manually. Taking mixtures into account may overcall X4 prediction because not every combination may actually be present in the viral population. Next generation, ultra-deep pyrosequencing may partly solve this issue because every strain is separately sequenced using this technique. Our results could not be compared with ESTA because almost half of our samples had a viral load below the minimum requirement of 1000 copies/mL for European samples.

Our study is the first that compares single with triplicate genotypic tropism testing in clinical practice. A recent study investigated the added value of tropism determination using duplicate PCR amplification and pooled sequencing. All possible amino acid sequences of the V3 loop were interpreted with G2P using an FPR of 10%[21]. The number of X4 tropism results in this particular duplicate approach increased from 25 to 30 (3.3%), which is in line with our observations.

On the individual patient level, our triplicate procedure increased the detection of X4 variants, thereby decreasing the number of patients eligible for MVC treatment. Unfortunately we cannot present clinical outcome data comparing single with triplicate testing because our study was designed as a prospective analysis and triplicate testing results were part of the clinical decision-making. Triplicate testing may have implications for the efficacy of MVC-containing therapy. Failure to detect an X4 virus in a single genotypic procedure may lead to selection of X4 virus, MVC therapy failure and loss of backbone activity[22]. In conclusion, independent of the applied FPR, triplicate testing increased X4 prediction in individual cases.

Our results illustrate that comparison of single with triplicate amplification procedures in relation to clinical outcome data is urgently needed. Pending these data, we prefer to be conservative and increase the

sensitivity of genotypic tropism testing by performing a triplicate procedure in routine clinical practice.

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**Chapter 4**

## Maraviroc is able to inhibit dual-R5 viruses in a dual/mixed HIV-1-infected patient

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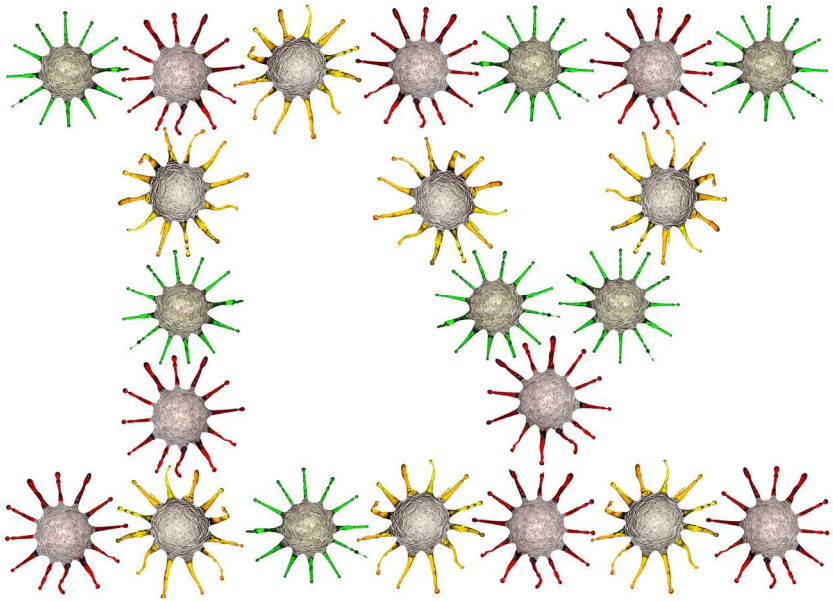

**Abstract**

**Objectives:** Maraviroc is the first licensed chemokine co-receptor 5 (CCR5) co-receptor antagonist in clinical practice. It is currently being used in patients harbouring exclusively CCR5-tropic virus. The objective of the study was to investigate the impact of maraviroc on viruses with different co-receptor preferences in a patient with a dual/mixed (D/M) infection.

**Methods:** We present a case report of an HIV-1 patient infected with a D/M virus population. Co-receptor tropism was determined by phenotypic and genotypic tests. Biological clones from pre- and post-maraviroc therapy were generated. Tropism of these infectious clones was investigated in U373-MAGI cells expressing $CD4^+CCR5^+$ or $CD4^+CXCR4^+$. Maraviroc susceptibility and viral replication were determined using donor peripheral blood mononuclear cells (PBMCs).

**Results:** In-depth clonal genotypic analysis revealed the presence of both R5-tropic variants and X4-tropic viruses before the start of maraviroc. During maraviroc therapy all R5-predicted viruses were suppressed. Phenotypic analyses revealed that all biological clones before maraviroc therapy could infect both CCR5- and CXCR4-bearing U373-MAGI cells, demonstrating dual tropism. The baseline biological clones preferentially infected the CCR5 cell line and were fully susceptible to maraviroc in PBMCs (dual-R5). In contrast, during maraviroc therapy the dual-R5-tropic viruses were replaced by more X4-tropic viruses (dual-X4), which could not be inhibited by maraviroc.

**Conclusions:** This case report demonstrates that dual-tropic viruses, capable of using both co-receptors in phenotypic assays, can be inhibited by maraviroc if they have a CCR5 co-receptor preference in vivo.

**Introduction**

HIV-1 entry into host cells requires binding of the viral envelope protein to the CD4 receptor and subsequently to a chemokine co-receptor (CCR5, CXCR4)[1,2]. Viral populations in an infected patient can be categorized by phenotypic tests as R5-tropic, X4-tropic or dual/mixed [D/M; use of both co-receptors by one virus (dual) and/or a mixture of CCR5-using (R5) and CXCR4-using (X4) viruses (mixed)][3]. Genotypic tests predict viral co-receptor tropism based on the sequence of the viral envelope by means of interpretation algorithms (R5 or X4 prediction). Inhibition of co-receptor usage is a new antiretroviral strategy and multiple compounds are now being studied. Maraviroc is the first licensed CCR5 antagonist and in clinical trials demonstrated potent activity in patients in whom only CCR5-tropic viruses were detected[4,5]. In general, no added value of maraviroc with respect to viral efficacy was observed in patients harbouring D/M-tropic viral populations, which are capable of using the CXCR4 co-receptor[6]. Nevertheless, detailed genotypic analysis demonstrated that virological response could be achieved in a

subset of these patients with ,10% of X4-predicted viruses in their viral population[7].

Here we present a report of a patient with a D/M viral population where maraviroc inhibited not only R5-tropic viruses but also a range of dual-tropic viruses.

## Methods

The patient participates in the AIDS Therapy Evaluation in the Netherlands (ATHENA) observational cohort, which has been approved by local and national institutional review boards.

*Genotypic analysis*

Viral RNA was isolated from 200–1000 mL of plasma/serum as described previously[8]. The V3 region of the envelope was amplified using primers V3-1 (5′-TATCCTTTGARCCAATTCCCAT-3′) and V3-2 (5′-CAGTAGAAAAATTCCCCTCCACAA-3′) (Superscript-III One-Step Platinum Taq, Invitrogen). Nested PCR using primers V3-3 (5′-AATTCCCCTCCACAATTAAAASTGTG-3′) and V3-4 (5′-ACAGTACAATGTACACATGGAATTA-3′) was performed (Expand High Fidelity PCR System, Roche). PCR-amplified products were ligated (pGEM-T Easy Vector; Promega) and sequenced using nested primers. Viral co-receptor tropism was predicted using Geno2Pheno(co-receptor) [R5 prediction, .10%; and X4, ≤10% false positive rate (FPR)] and Web PSSM (where PSSM stands for position-specific scoring matrices) (R5 prediction, ≤26.69; and X4, ≥22.88; the 11/25 rule was applied at intermediate values)[9]. Genotypic sensitivity scores (GSSs) were calculated using the Stanford HIVdb algorithm[10].

*Phenotypic analysis*

*Cells*

MT-2 cells were maintained in culture medium [CM; RPMI1640 with L-glutamine (BioWhittaker), 10% fetal bovine serum (FBS; Biochrom AG) and 10 mg/L gentamicin (Gibco)]. U373-MAGI cell lines were maintained as recommended by the NIH AIDS Research and Reference Reagent Program. Donor peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll–Paque density gradient centrifugation of heparinized blood from five HIV-seronegative donors (CCR5-homozygous wild-type for Δ32). The mix was stimulated for 2–3 days with phytohaemagglutinin (2 mg/L) in CM. Cells were incubated at 37°C and 5% $CO_2$. MT-2 cell culture and generation of biological clones. Patient-derived PBMCs ($1\times10^6$) prepared by Ficoll–Paque density gradient centrifugation were co-cultured in triplicate with $1\times10^6$ $CXCR4^+CCR5^-$ MT-2 cells in CM[11]. Viral cultures were maintained for 3 weeks and monitored for syncytium formation. Positive viral cultures were used for the generation of biological clones by infecting $4\times10^4$ MT-2 cells/well in a 96-well plate in a 5-fold dilution series. In cases where less than one-third of viral cultures were positive, virus was harvested. These biological clones were expanded by infecting $1\times10^6$ MT-2 cells. Supernatant was harvested, and p24 was measured

and sequenced. No nucleotide differences were observed after expansion and the biological clones corresponded to the dominant viral population at the specific timepoints. The 50% tissue culture infective dose ($TCID_{50}$) was determined on donor PBMCs. These biological clones were subsequently used in phenotypic analysis.

*Co-receptor usage and inhibition in U373 cells*

At day 0, 1×104 cells/well of U373-MAGI-CCR5E or U373-MAGICXCR4$_{CEM}$, expressing $CD4^+CCR5^+CXCR4^-$ and $CD4^+CCR5^-CXCR4^+$, respectively, were plated into a 96-well plate in 100 mL of Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) with 10% FBS and 10 mg/mL gentamicin. Subsequently, at day 1, medium was discarded and replaced by 150 mL DMEM with 10% FBS and 10 mg/mL gentamicin, with a final concentration of 10 mM maraviroc, 1 mM AMD-3100 (a CXCR4 inhibitor) or no inhibitor. This was incubated for 1 h at 37°C. Subsequently, 1 ng of p24 of the biological clones, BaL or HXB2 suspended in 50 mL of CM was added and incubation was continued for 2 days at 37°C. Subsequently, luminescence was measured using the Galacto-Star™ b-Galactosidase Reporter Gene Assay System for Mammalian Cells (Applied Biosystems) according to the manufacturer's protocol using 20 mL of lysis buffer and 60 mL of reaction buffer. Background activity (cells without virus in the absence or presence of inhibitor) was subtracted from the activity of the wells containing virus.

*Analysis of maraviroc and AMD-3100 susceptibility in PBMCs*

Donor PBMCs were infected with a biological clone using a multiplicity of infection (MOI) of 0.001 (PBMC titration) in CM with 5 U/mL IL-2 and incubated for 2h at 37°C, after which cells were washed twice. Subsequently, $0.2\times10^6$ cells/well were plated into a 96-well plate with 5 U/mL IL-2 in CM containing increasing concentrations of maraviroc or AMD-3100. p24 was analysed on days 0 and 7.

*Analysis of viral replication capacity in PBMCs*

Donor PBMCs ($5\times10^6$) were infected with a biological clone (50 ng of p24) in 1 mL of CM, incubated for 2 h at 378C and washed twice. Cells were cultured in 10 mL of CM with 5 U/mL IL-2. Cells were incubated for 14 days and p24 was analysed daily. Viral replication of all biological clones was comparable.

**Results**

A 51-year-old man was diagnosed with HIV-1 subtype B infection in 1992. Zidovudine monotherapy was initiated and he was subsequently treated with multiple antiretroviral regimens, including integrase and fusion inhibitors. Initial antiretroviral monotherapy and add-on therapy together with intolerability to enfuvirtide, darunavir and other drugs resulted in frequent

virological failure and selection of multidrug-resistant HIV (cumulative resistance profile: RT, 41L-67S-69del-74I/V-98G-103N-118I-Y181C-184V-190A-210W-215Y-219E; PR, 10I-20I-36I-43T-46I-54V-62V-63P-71V-73S-82C-84V-90M; and IN, Q95K-V151I-N155H). Due to a lack of alternative treatment options, sustained virological suppression was never achieved and immunological deterioration was observed ($CD4^+$ cell count ,50 cells/$mm^3$).

In 2008, the patient received tenofovir, raltegravir and tipranavir/ritonavir and presented with a $CD4^+$ cell count of 4 cells/$mm^3$ and plasma HIV-RNA of $5.6\times10^4$ copies/mL. Efforts to design an effective antiretroviral regimen included determination of viral co-receptor tropism using phenotypic assays, namely Trofile (original Trofile assay and enhanced sensitivity Trofile assay); Monogram Biosciences] and MT-2[11]. These assays demonstrated that the viral population was capable of using the CXCR4 co-receptor (D/M and syncytium-inducing, respectively, Figure 1). However, genotypic analysis using two interpretation algorithms predicted CCR5 usage (Geno2Phenoco-receptor, 10.5% FPR; and Web PSSM, -8.61). Despite the ability of the viral population to use the X4 co-receptor in phenotypic tests, the potential value of maraviroc as an addition to the ongoing therapy was investigated, while raltegravir was discontinued to preserve future options.

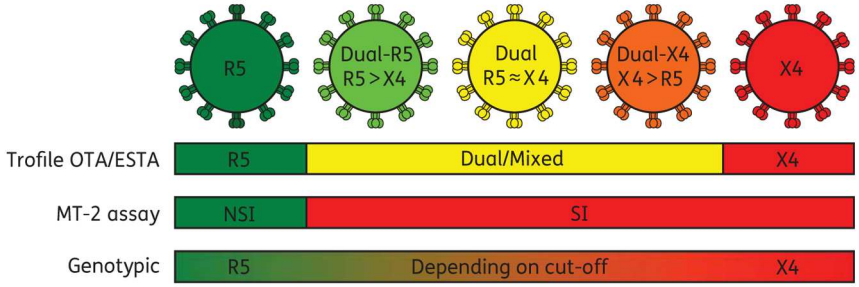


**Figure 1. Schematic representation of HIV-1 tropism and corresponding phenotypic and genotypic test results**. OTA, original Trofile assay; ESTA, enhanced sensitivity Trofile assay; NSI, non-syncytium-inducing (correlates to CCR5 usage); SI, syncytium-inducing (correlates to CXCR4 usage).

During 3 weeks of treatment intensification no clear effect on HIV-RNA concentration or $CD4^+$ cells was observed (Figure 2a). Nevertheless, the patient reported substantial improvement in his clinical condition and was motivated to continue with maraviroc as part of a mega-HAART (highly active antiretroviral therapy) regimen consisting of tenofovir/emtricitabine, etravirine, raltegravir and fosamprenavir/ritonavir. Despite the fact that the GSS of this therapy was only 1 based on the most recent genotype, or 0 according to the cumulative genotype, a rapid viral and immunological response was observed (Figure 2a).

We set out to investigate the impact of maraviroc in this D/M-infected patient by

performing in-depth in vivo and in vitro analysis. In-depth clonal genotypic analysis at baseline (t0) revealed the presence of X4-predicted viruses in plasma, in line with phenotypic tropism data (Figure 2b). However, these viruses constituted only a minority of the analysed clones, with most predicted to be R5-tropic. Furthermore, all viable viral variants generated from infected cells (biological clones) had V3 sequences identical to the dominant plasma population and were predicted to be R5-tropic. Within 1 week of maraviroc intensification, the R5-predicted viral population was replaced by X4-predicted viruses (Figure 2b, t1). Follow-up during mega-HAART revealed no additional changes in the viral V3 envelope and corresponding co-receptor tropism (Figure 2b, t3). In addition, no new resistance mutations were observed in reverse transcriptase and protease.

Co-receptor usage of the biological clones at baseline (t0-I and t0-II) and at t2 (t2-I, t2-II and t2-III) was assessed in X4 and R5 cell lines. The biological clones at t0 (t0-I and t0-II) demonstrated a higher entry efficacy in the CCR5 cell line as compared with the CXCR4 cell line. In contrast, the biological clones obtained at t2 (t2-I, t2-II and t2-III) showed a higher entry efficacy in the CXCR4 cell line as compared with the CCR5
cell line (Figure 3a).

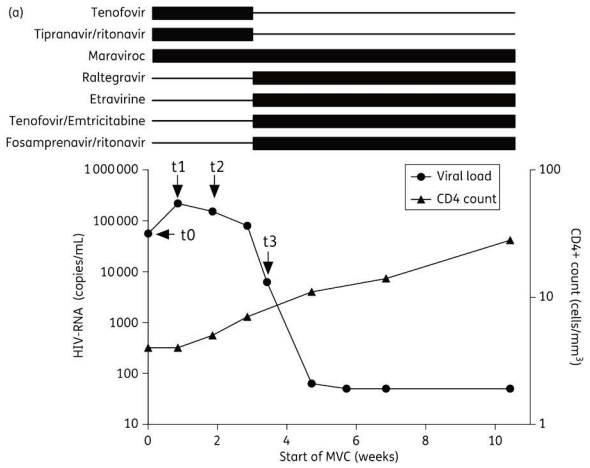


**Figure 2**. (a) Plasma HIV-RNA levels and CD4+ cells/mm3 during maraviroc (MVC) therapy. (b) V3 loop sequence analysis at start of MVC (t0) and subsequent timepoints (t1–t3) during MVC therapy. Amino acid (AA) positions associated with HIV tropism are boxed, and amino acid changes as selected in the patient associated with X4 prediction are depicted in red italics. Genotypic interpretation of co-receptor tropism by Geno2Phenoco-receptor using an FPR of 10% and Web PSSM is indicated (R5 prediction is given in green and X4 prediction is given in red italics).

Since all biological clones were able to use both co-receptors, we asked why the dominant viral population in the patient shifted from R5- to X4-

predicted viruses after administration of maraviroc in vivo. To gain more insight into co-receptor preference, we investigated maraviroc susceptibility in the CCR5 and CXCR4 cell lines and in the natural target cells (PBMCs). Viral entry in the CCR5 cell line was inhibited by maraviroc, whereas addition of AMD-3100 did not affect viral entry (Figure 3b). Vice versa, viral entry in the CXCR4 cell line could be inhibited by AMD-3100, whereas maraviroc did not inhibit viral entry in this cell line (Figure 3b). Interestingly, in PBMCs in which both co-receptors were expressed, all baseline biological clones could be fully inhibited by maraviroc, indicating their CCR5 co-receptor preference in natural host cells, whereas the
biological clones obtained during subsequent maraviroc exposure (t2) were only partly inhibited by maraviroc (Figure 3c). These maraviroc-resistant biological clones (t2) mainly used the CXCR4 co-receptor and not maraviroc-bound CCR5 co-receptor, as indicated by full inhibition by a CXCR4 co-receptor antagonist.

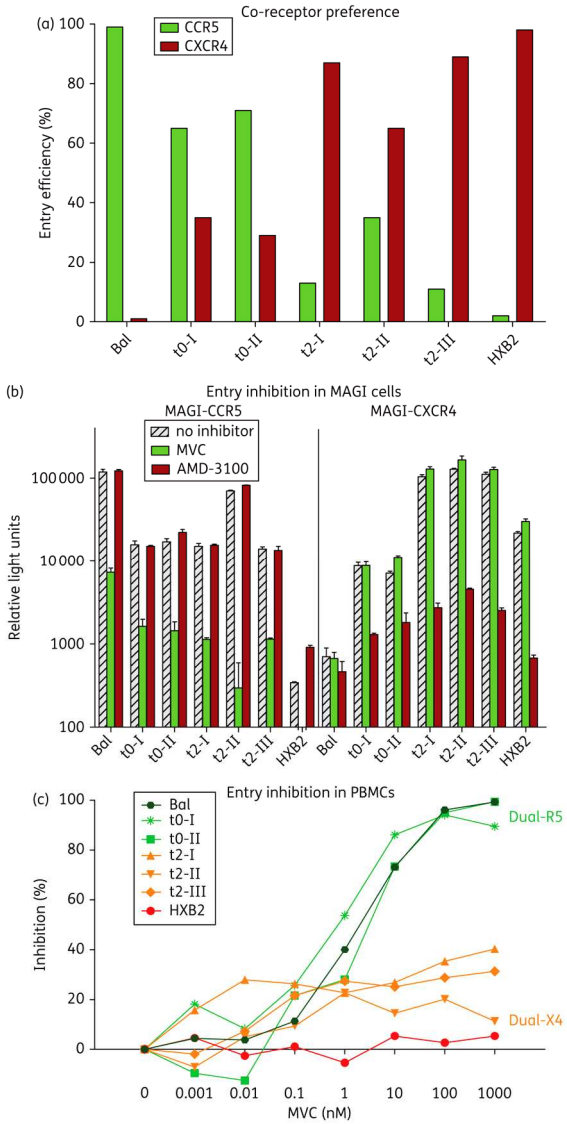


**Figure 3.** (a) Co-receptor preference in the CCR5 and CXCR4 cell lines (n¼3). (b) Entry inhibition in the CCR5 and CXCR4 cell lines with no inhibitor, maraviroc (MVC) or AMD-3100. Standard error of the mean is depicted (n¼3). (c) Representative MVC susceptibility assay in donor-derived PBMCs. Biological clones pre-MVC therapy (t0-I and t0-II), biological clones obtained during MVC therapy (t2-I, t2-II and t2-III) and the reference strains
HXB2 and Bal were used.

## Discussion

In this case report we describe the ability of maraviroc to inhibit a dual-tropic virus population in an HIV-1-infected patient. It has been reported earlier that certain dual-tropic viruses prefer usage of the CCR5 co-receptor and are typed as R5>X4 or dual-R5-tropic, whereas others use the CXCR4 co-receptor more efficiently (X4>R5 and dual-X4) in PBMCs[12,13]. In our case, the patient harboured a viral population that was reported to be D/M in the Trofile assays and syncytium-inducing in the MT-2 assay, but R5-tropic in two genotypic prediction algorithms. In vitro experiments demonstrated that this dominant baseline viral population could be considered R5>X4 or dual-R5 since it was capable of using the CXCR4 co-receptor, but preferentially used the CCR5 co-receptor in cell lines and natural target cells (PBMCs). Furthermore, entry of these dual-R5 viruses in the CCR5 cell line and in PBMCs could be inhibited by maraviroc. Also, in vivo these dual-R5 variants were suppressed by maraviroc, demonstrating their preferential usage of the CCR5 co-receptor.

Unfortunately, in our patient no viral efficacy of maraviroc was observed, which could be explained by the presence of a dual-X4 minority at baseline that was rapidly selected in the absence of an active backbone regimen.

These results indicate that viruses capable of using both co-receptors in vitro may be inhibited by maraviroc. Further research is warranted to establish whether maraviroc in combination with an active backbone might be of added value in patients harbouring dual-tropic virus.

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## Chapter 5

# Maraviroc treatment in non-R5-HIV-1-infected patients results in the selection of extreme CXCR4-using variants with limited effect on the total viral setpoint

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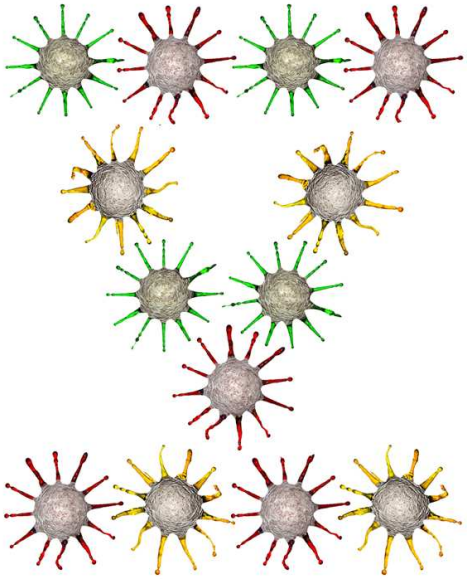

**Abstract**

**Objectives**: Using deep sequencing methods, we intensively investigated the selective pressure of maraviroc on the viral population in four patients with dual/mixed HIV-1 experiencing treatment failure.

**Methods**: Patients received maraviroc add-on therapy (n¼4). Tropism was determined by Monogram's Trofile assay and/or 'deep' sequencing. Longitudinal 'deep' sequence analysis used triplicate HIV V3 RT–PCR on plasma samples. Sequences were interpreted using the geno2phenocoreceptor algorithm with a 3.5% false positive rate (FPR) cut-off.

**Results**: Patients had a median viral load of 4.7 log10 HIV RNA copies/mL with a median of 24% chemokine (C-X-C motif) receptor 4 (CXCR4)-using virus at baseline. Following maraviroc exposure, the chemokine (C-C motif) receptor 5 (CCR5)-using virus (R5) plasma viral load decreased by at least 1 log10, and only non-R5 variants with extremely low FPR values predominated after 21 days. Virus with an FPR ≤1.8% accounted for more than 90% of the circulating virus, having expanded to occupy the 'space' left by the suppression of R5 variants. Population genetic estimates of viral fitness in the presence of maraviroc showed a steep rise around an FPR value of 2%.

**Conclusions**: Longitudinal analysis of independent R5 and non-R5 HIV populations shows that maraviroc selects viruses with an extremely low FPR, implying that the antiviral activity of maraviroc may extend to a broader range of HIV variants than previously suspected.

## Introduction

HIV-1 infection is dependent on viral entry into a susceptible host cell. Entry is mediated by the binding of virus to the host CD4 receptor and one of two chemokine co-receptors present on the cell surface—either chemokine (C-C motif) receptor 5 (CCR5) or chemokine (C-X-C motif) receptor 4 (CXCR4)[1,3]. The majority of new infections appear to be due to CCR5-using 'R5' virus, which continues to predominate during the course of the infection[4,5]. As time progresses however, ~50% of patients with HIV subtype B have a shift in their viral population to include a growing amount of CXCR4-using 'X4' virus, which is strongly correlated with rapid disease progression[5-7]. Many patients with X4 virus have a viral population that contains both X4 and R5 virus (M; mixed infection). In some cases, the virus may also be able to use both co-receptors (D; dual infection)[8,9]. For the purposes of this paper, both X4 and dual/mixed (D/M) populations will be referred to as 'non-R5'.

The co-receptor that HIV uses to enter the host cell ('tropism') is largely but not entirely dependent on the characteristics of the V3-loop region within the gp120 protein encoded by the env gene[10,11]. The V3-loop region is usually 35 amino acid residues in size and bookended by cysteine residues between which a disulphide bridge forms, resulting in the loop structure[12]. Particular mutations have been associated with CXCR4 co-receptor use, including the substitution of basic residues at V3-loop positions 11 and/or 25[13,14]. This observation formed the basis of the simpler rule-based co-

receptor prediction methods[13]. Multiple interpretive algorithms have since been designed to predict co-receptor use based on the sequence of relevant regions of the viral genome, in particular the envelope's V3-loop region. The use of machine-learning techniques has allowed prediction methods to become more complex, resulting in the improved sensitivity of genotypic methods[15,16].

Maraviroc is the first clinically available CCR5 antagonist[17]. Patients screened as having R5 virus in the clinical trials of maraviroc had a significantly greater virological response to maraviroc than those with non-R5 virus populations, demonstrating the need to identify HIV tropism prior to therapeutic initiation of maraviroc[18-20]. A number of both phenotypic and genotypic screening methods have been developed in order to determine HIV-1 tropism in patient samples. Initially, the most widely used method of identifying tropism was the phenotypic Original Trofile Assay (Monogram Biosciences)[21]. Since its introduction, adjustments have been made to increase its sensitivity and it is now referred to as the Enhanced Sensitivity Trofile Assay (ESTA)[22]. More recently, a population-based genotypic tropism assay has been developed in which the V3 loop is sequenced and interpreted using a bioinformatic algorithm such as geno2pheno$_{[coreceptor]}$ (g2p)[16,23,24]. The same concept can be achieved with enhanced sensitivity using next-generation sequencing (NGS) technology[25,26]. NGS can be used to quantify viral variants accounting for ,2%–3% of the viral population, in comparison with the approximate 20% limit of standard sequencing technologies[25-27]. This allows a more thorough evaluation of the non-R5 and R5 variants present within a sample.

Using deep sequencing methods to identify tropism, we longitudinally investigated and quantified the selective pressure of maraviroc on the dynamics and composition of the viral population in four patients with non-R5 virus who had experienced maraviroc treatment failure.

## Methods

*Study population*

The four patients were participants in the AIDS Therapy Evaluation in the Netherlands (ATHENA) observational cohort, which has been approved by local and national institutional review boards. Three patients harboured non-R5 HIV populations by ESTA. The remaining patient harboured R5 virus, but was predicted to harbour non-R5 virus by 'deep' sequencing. Despite the presence of non-R5 virus, the potential value of maraviroc as an addition to an ongoing failing therapy regimen was investigated. No other drugs were added, to preserve future treatment options in these heavily pre-treated individuals.

Patient A started treatment in 1995 and was treated sequentially with two duo nucleoside reverse transcriptase inhibitor (NRTI) regimens, followed by four protease inhibitor (PI)-based regimens and one nonnucleoside reverse transcriptase inhibitor (NNRTI)-based regimen. Patient A discontinued all highly active antiretroviral therapy components (Combivir, tenofovir and ritonavir-boosted atazanavir) on his own initiative prior to starting maraviroc.

Patient B started treatment in 1995 with mono and duo NRTI-based and PI-based regimens followed by one PI+NNRTI-based regimen. At the time of starting maraviroc, Patient B was being treated with Combivir, tenofovir and ritonavirboosted tipranavir. Patient C had started triple drug therapy in 1997 with a PI-based regimen followed by two NNRTI-based regimens. Patient C was being treated with tenofovir, emtricitabine and nevirapine when maraviroc was introduced; however, during the course of maraviroc treatment, nevirapine was substituted with ritonavir-boosted lopinavir as part of the background regimen. Patient D started therapy in 1995 and was treated with mono and duo NRTI regimens, three PI-based regimens and one NNRTI-based regimen. Patient D was being treated with tenofovir and ritonavir-boosted tipranavir when maraviroc was added.

Four longitudinal samples were collected from each patient over a median of 53 days (IQR 27 days). The median length of maraviroc add-on therapy was 3 weeks (range 13–26 days). The period between the start of maraviroc and the first sample analysed ranged from 6 to 26 days (median 11 days). Three of the four patients had two postmaraviroc samples sequenced.

*Laboratory methods*

RNA was extracted from frozen plasma and the V3 loop of the gp120 protein encoded by the HIV-1 env gene was amplified in triplicate using nested RT–PCR[25,28]. Second-round PCR was performed with primers incorporating unique sample-specific tags, 'barcodes', for sample identification. In preparation for clonal amplification using emulsion PCR, the resulting triplicate PCR amplicons were quantified, combined in equal proportions and purified[25,28]. Amplicon libraries were diluted to $1\times10^7$ molecules/mL, such that five molecules of DNA were present for every DNA capture bead. Approximately 790000 beads were loaded into each of the four regions of the 454 pyrosequencing picotitre plate. Samples were sequenced in both the forward and reverse directions using 454 GS-FLX (Roche, 454 Life Sciences).

*Sequence processing and tropism interpretation*

A median of 14000 sequence reads per sample was obtained. After processing for sequence quality, a median of 12500 sequence reads per sample was included in the analyses. Viral tropism was interpreted using the g2p algorithm with a false-positive rate (FPR) cut-off of 3.5% for each variant sequence. As previously reported for 'deep' sequence analyses, sequences below this FPR value were inferred as being non-R5[25,26]. The percentage of non-R5 variants within each viral population was determined from the total useable read counts for each sample. Having calculated this, the viral load of non-R5 and R5 populations could be determined separately. The non-R5 plasma viral load (pVL) was calculated by simply multiplying the percentage of non-R5 by the total pVL, with the R5 pVL as the remainder.

*Fitness analysis*

Following g2p scoring, sequences were stratified into five bins based on their g2p FPR (FPR ,2%, 2% ≤ FPR,3.5%, 3.5% ≤ FPR,10%, 10% ≤ FPR,20%, 20% ≤ FPR). The observed frequency of the sequences within each bin was calculated and plotted over time for each timepoint and patient (allele frequencies). Each bin represents a different 'level' of tropism and level of response to maraviroc.

Relative viral fitness in the presence of maraviroc was calculated according to Wright's recursive formula for allele frequency evolution under haploid selection, with the generation time assumed to be 1 day[29]. We fitted a four-parameter fitness curve based on the flexible gamma cumulative distribution function to the longitudinal deep sequence data, given this model. The expected allele frequency of evolution was calculated from the initial observed allele frequencies at the earliest timepoint given the four model parameters of the fitness function. Here, a relative fitness of 1.0 is equal to the most-fit allele in the patient viral population.

**Results**

All four patients were confirmed as having non-R5 virus at baseline by 454 deep sequencing, the baseline being defined as the most recent sampling timepoint prior to the start of maraviroc treatment. The viral populations were composed of between, 1% and 58% non-R5 virus at baseline (median 24%, IQR 6%–44%).

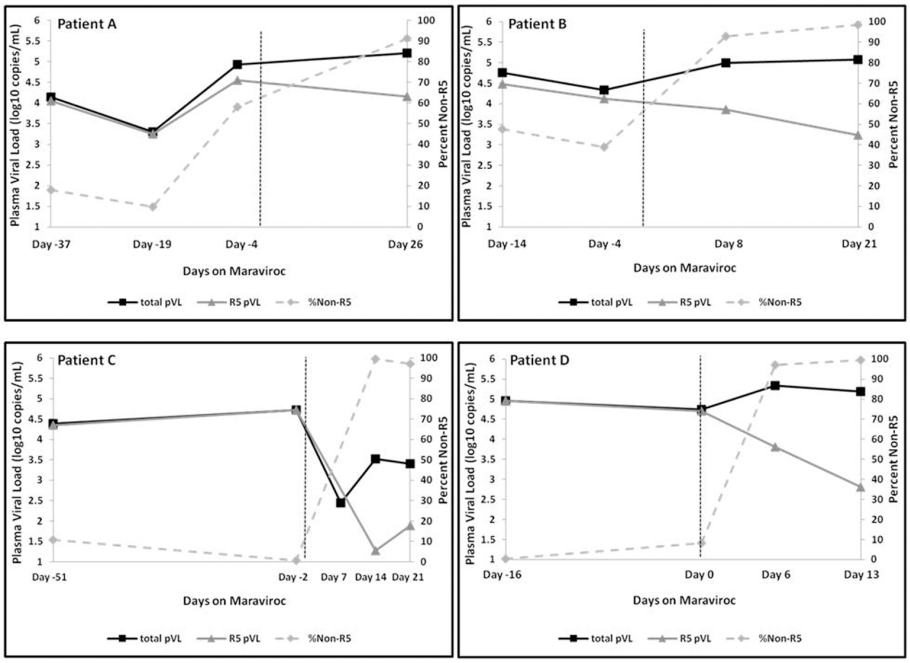


**Figure 1**. Change in pVL ($log^{10}$ copies/mL) and non-R5 percentage following exposure to maraviroc in four patients experiencing treatment failure and screened as having D/M HIV-1. Longitudinal samples were collected over a median of 53 days; patients were exposed to maraviroc for a median of 21 days. Viral tropism was determined from the V3 sequences generated using deep sequencing (454 GS-FLX titanium) and interpreted using the g2p algorithm with a 3.5% FPR cut-off point. The vertical broken line indicates the date at which the patient started maraviroc as an additive therapy. When the patient was exposed to maraviroc the overall pVL remained relatively constant, despite the decrease of at least 1 $log^{10}$ in the R5 population, due to the expansion of the non-R5 population.

Even at the first timepoint following the start of maraviroc treatment, it was apparent that non-R5 virus was not inhibited by maraviroc as the percentage of non-R5 variants increased in all patients (Figure 1). In fact, the proportion of non-R5 populations
increased by a median of 71% (IQR 49%–91%) after a median of 11 days of maraviroc exposure (Figure 1). We note an increase in the percentage of non-R5 virus in Patient A several days before the documented start of maraviroc; we could not find an explanation for this observation. When tested using ESTA, Patient C was determined to have R5 virus only at baseline (<1% non-R5 virus using deep sequencing). The result from the previous timepoint, 49 days prior to baseline, was determined as 11% non-R5 virus using deep sequencing. This non-R5 population subsequently increased to 99% after 21 days of maraviroc exposure. In all four patients, the predominant non-R5 variant contained a basic residue (Lys or Arg) at V3-loop positions 11 or 25 (Figure 2).

| Patient ID | Days on MVC | V3 loop amino acid sequence | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | G2P FPR | Pre-therapy Prevalence (%) | Post-therapy Prevalence (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | | | |
| A** | -37 | C | T | R | P | N | N | N | T | R | K | G | I | H | I | G | P | G | G | A | L | Y | A | T | G | Q | I | I | G | N | I | R | E | A | H | C | 90.7 | 51.2 | 2.5 |
| | 26 | - | - | - | - | - | - | - | - | - | - | - | Y | - | - | - | - | R | - | V | - | T | - | - | R | - | - | - | D | - | - | K | - | - | - | - | 1.3 | 14.7 | 77.5 |
| B | -14 | C | T | R | P | N | N | N | T | R | K | G | I | H | I | G | P | G | R | A | F | Y | T | T | E | R | I | I | G | D | I | R | K | A | H | C | 1.7 | 22.5 | 0.0 |
| | 21 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | V | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1.7 | 12.9 | 61.5 |
| C** | -2 | C | T | R | P | N | N | N | T | R | K | S | I | H | M | G | P | G | G | A | L | Y | A | T | G | E | I | V | G | D | I | R | Q | A | H | C | 80 | 72.2 | 2.3 |
| | 21 | - | - | - | - | - | - | - | - | - | - | G | - | R | V | - | - | - | R | T | - | - | - | - | E | K | - | I | - | - | - | - | - | - | - | - | 1.8 | 0.36 | 80.1 |
| D | -16 | C | T | R | P | N | N | N | T | R | K | S | I | T | L | G | P | G | R | V | F | Y | T | T | G | D | I | I | G | D | I | R | Q | A | H | C | 10.2 | 84.1 | 0.19 |
| | 24 | - | - | - | - | - | - | - | - | - | - | R | - | - | - | - | - | - | - | - | - | - | - | - | E | - | V | - | - | - | - | - | - | - | - | - | 1.1 | 0.02 | 88.7 |

**Figure 2.** Summary of the change in prevalence and FPR for the most common HIV-1 nucleotide sequence translated into amino acids from each of the four patients with D/M HIV-1, before and after exposure to maraviroc (MVC). Amino acid substitutions between the most common sequence at the first and last timepoints are noted; dashes represent no change at the position. Sequence prevalence, as a percentage of the number of useable reads obtained from the sample, was calculated for each sequence as found at the pre-therapy and post-therapy timepoints. A variant with a g2p FPR of <3.5% was considered to be X4. Amino acid positions marked by a square are associated with mutations characteristic of CXCR4-using virus. **Unusually, the majority of sequences in the forward direction from the first two timepoints for Patients A and C contain a frameshift mutation and were omitted from the analyses.

The results here demonstrate a rapid replacement of the R5 population by non-R5 variants following the start of maraviroc treatment (Figure 3). The overall pVL increased, with a median of 0.36 $\log^{10}$ copies/mL by the final timepoint (Figure 1). Viral loads in patients A, B and D increased (0.27, 0.73 and 0.45 $\log^{10}$ copies/mL, respectively) after 26, 21 and 13 days of exposure, respectively. Patient C showed a decrease in viral load, with 1.32 $\log^{10}$ copies/mL after 21 days of maraviroc. When separated into R5 and non-R5 viral loads, it is evident that changes in the overall pVL are due to a large decrease in the R5 populations (median 21.64 $\log^{10}$ copies/mL, IQR 21.85 to 21.27) and the outgrowth of the non-R5 populations, which expanded to comprise a median of 98% (IQR 99%–96%) by the last timepoint (Figure 1).

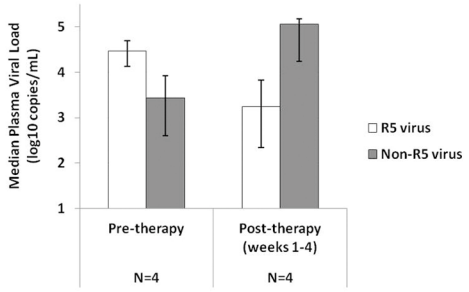


**Figure 3.** Change in the median pVL of non-R5 and R5 variants prior to and following the start of maraviroc treatment in the viral populations of four patients with D/M HIV-1 who experienced treatment failure. Patients were exposed to maraviroc for a median of 21 days. V3 sequences

generated using 454 GS-FLX titanium were interpreted for tropism using the g2p algorithm with an FPR cut-off of 3.5%. The grey bars represent the change in the non-R5 population and the white bars represent the change in the R5 population. Error bars indicate the IQR. Following maraviroc exposure, all four individuals showed decreases in R5 viral load of at least 1 log10; the R5 population was replaced by non-R5 variants (FPR <3.5%).

Variants were divided into five strata (FPR ,2%, 2%≤ FPR, 3.5%, 3.5%≤ FPR, 10%, 10%≤ FPR, 20%, 20%≤ FPR) according to their g2p FPR in order to estimate relative viral fitness in vivo in the presence of maraviroc. The frequency of the sequences was plotted for each bin at each timepoint, showing the change in FPR over time. The non-R5 variants with extremely low FPR values (<2%) had the most substantial increase in prevalence. R5 variants (FPR ≥3.5%) were represented by three bins, the higher being considered increasingly characteristic of R5 virus and most likely to respond to maraviroc. There was no corresponding increase in the proportion of virus with an FPR between 3.5% and 20%, thus implying the relative inhibition of these variants (Figure 4).

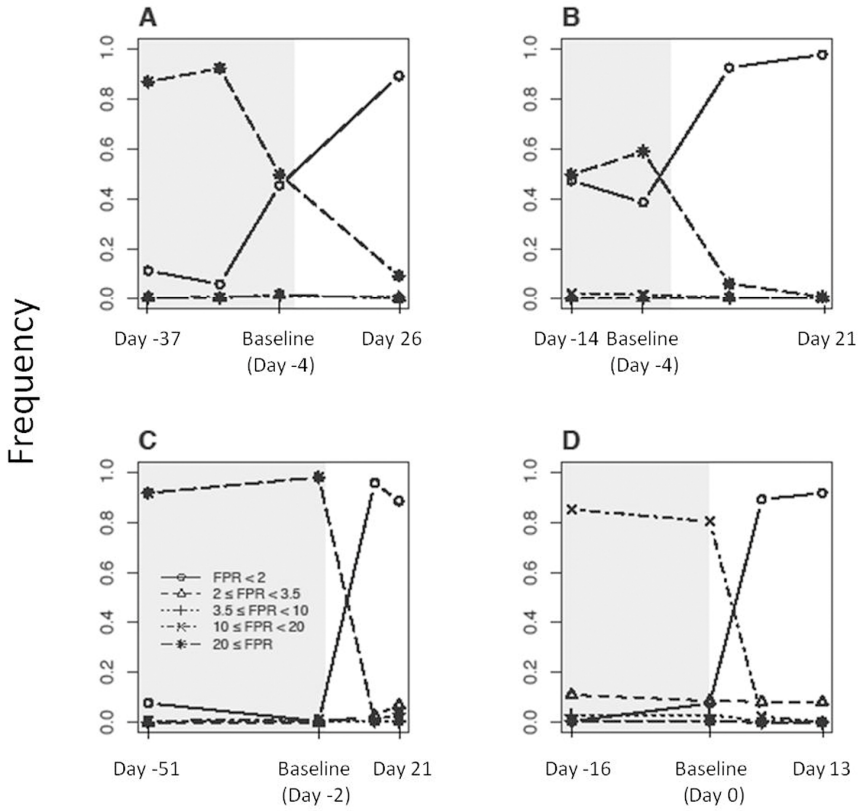


**Figure 4.** Viral sequences were stratified based on FPR; the frequency of sequences within each stratification was then plotted against the time since baseline. Plasma samples were taken from patients experiencing treatment failure who were exposed to maraviroc despite having D/M HIV-1. Sequences were generated using 454 deep sequencing and the FPR was determined using the g2p algorithm. The grey shaded area of each graph represents the time prior to the start of maraviroc treatment and the white shaded area of each graph represents the time after the start of maraviroc. Circles represent non-R5 variants with extremely low FPR values (FPR <2%) and triangles and plus signs represent variants with low FPR values bordering the non-R5 or R5 classification (2% ≤ FPR<3.5% and 3.5% ≤ FPR,10%, respectively). Crosses and asterisks represent R5 variants (10% ≤ FPR,20%), the asterisks indicating variants with the highest FPR values and most likely to respond to maraviroc (20% ≤ FPR). Non-R5 variants with extremely low FPR values predominated after maraviroc—virus with FPR values of 1.3%, 1.7%, 1.8% and 1.1% accounted for >90% of the circulating virus populations in patients A–D, respectively.

Based on the data collected, we employed a population genetic model to measure the elative fitness advantage under haploid selection (with a generation time of 1 day) of predicted X4 viral variants in the presence of maraviroc in vivo. The relative frequencies of these allelic classes over time

were fitted to Wright's haploid selection model to estimate four parameters of a flexible monotonic function relating relative fitness to FPR
(Figure 5). The viral fitness of variants with an FPR >5% has little selective advantage in the presence of maraviroc, remaining relatively constant at a relative fitness of 0.2, where 1.0 is equal to the most-fit allele in the patient viral population. However, in variants with an FPR <5%, the relative fitness began to increase arkedly. At an FPR between 2% and 0%, the relative fitness was shown to increase from ~0.65 to 1.0, representing an increase in fitness of ~0.2 units for every FPR unit (Figure 5).

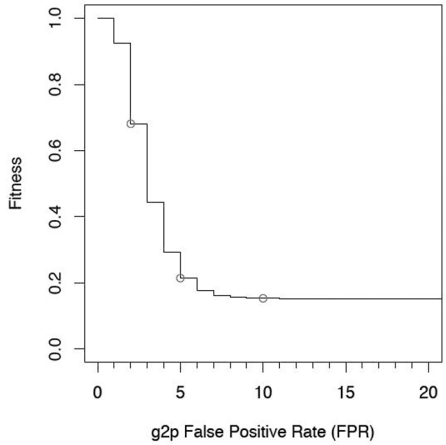


**Figure 5.** Data from four individuals with D/M HIV-1 who had been exposed to maraviroc were pooled to analyse viral fitness as a function of g2p FPR in the presence of maraviroc. Relative fitness in the presence of maraviroc was calculated according to Wright's recursive formula for allele frequency evolution under haploid selection, with the generation time assumed to be 1 day. A four-parameter fitness curve was fitted based on the gamma cumulative distribution function to the longitudinal deep sequence data. Sequences were categorized into bins based on FPR cut-off points (FPR <2%, 2%≤ FPR,3.5%, 3.5%≤ FPR,10%, 10%≤ FPR<20%, 20%≤ FPR) and observed allele frequencies were calculated across bins for each timepoint and patient. Expected allele frequencies were calculated from the initial observed allele frequencies (earliest timepoint) given the model parameters (four parameters of the fitness function). Here, a relative fitness of 1.0 is equal to the most-fit allele in the patient viral population.

The most common sequences from the pre-therapy and post-therapy samples tested were compared for each patient (Figure 2 and Table 1). The prevalence of the most common sequences as a percentage of the total useable sequences obtained for the
sample was calculated. For each patient, the most common sequence was different between the pre-therapy and post-therapy timepoints; however, the most common sequence at the post-therapy timepoint was always present at a pre-therapy timepoint (Figure 2 and Table 1). In the case of Patient C, the most common sequence at the post-therapy timepoint was not found at the first pre-

therapy timepoint but was found at baseline. With the exception of Patient B, the most common pre-therapy sequence was an R5 variant, whereas the post-therapy sequence was a non-R5 variant. The most common sequences for Patient B, pre- and post-therapy, were both non-R5 variants. Notably, the non-R5 variants that became the most common sequence all had extremely low FPR values (between 1.1% and 1.8%). For each patient, the most common sequence at the post-therapy timepoint had a greater prevalence than the most common sequence at the pre-therapy timepoint, showing a decrease in sequence variation and a selective advantage for those sequences with a low FPR in the presence of maraviroc (Figure 2).

**Table 1.** Summary of the three most prominent sequences for each of the four patients with D/M HIV-1 before and after the addition of maraviroc (MVC) to their treatment regimen. Sequences in bold were identified as X4 by deep sequencing in conjunction with the g2p algorithm (FPR cut-off 3.5%). These X4 sequences were observed at pre-maraviroc timepoints, becoming the most prevalent sequence by the final post-maraviroc timepoint. A fourth sequence has been listed for Patient C at the second pre-therapy timepoint, indicating the presence of this sequence before maraviroc. Despite a low prevalence this sequence was later observed as the most prominent sequence at the final post-maraviroc timepoint.

| Patient | Days on MVC | Amino acid sequence | Prevalence (% of sequence reads) | Tropism | g2p FPR |
|---|---|---|---|---|---|
| A | | CTRPNNNTRKGIHLGPGGAFYATGQIIGNIREAHC | 51.2 | R5 | 90.7 |
| | −37 | **CTRPNNNTRKGIYIGPGRAVYTTGRIIGDIRKAHC** | **14.7** | **X4** | **1.3** |
| | | CTRPNNNTRKGIHLGPGGAFYATGQIIGNIREAHC | 7.6 | R5 | 61.8 |
| | | CTRPNNNTRKGIHIGPGGALYATGQIIGNIREAHC | 43.0 | R5 | 90.7 |
| | −19 | CTRPNNNTRKGIHLGPGGAFYATGQIIGNIREAHC | 19.2 | R5 | 61.8 |
| | | **CTRPNNNTRKGIYIGPGRAVYTTGRIIGDIRKAHC** | **7.3** | **X4** | **1.3** |
| | | **CTRPNNNTRKGIYIGPGRAVYTTGRIIGDIRKAHC** | **47.1** | **X4** | **1.3** |
| | −4 | CTRPNNNTRKGIHIGPGGALYATGQIIGNIREAHC | 23.2 | R5 | 90.7 |
| | | **CTRPNNNTRKGIHLGPGGAFYATGQIIGNIREAHC** | 5.4 | R5 | 61.8 |
| | | **CTRPNNNTRKGIYIGPGRAVYTTGRIIGDIRKAHC** | **77.5** | **X4** | **1.3** |
| | 26 | CTRPNNNTRKGIHIGPGGALYATGQIIGNIREAHC | 3.5 | R5 | 90.7 |
| | | CTRPNNNTRKGIHIGPGKAFYATGQIIGNIRQAHC | 2.5 | R5 | 44.1 |
| B | | CTRPNNNTRKGIHIGPGRAFYTTERIIGDIRKAHC | 22.5 | X4 | 1.7 |
| | −14 | **CTRPNNNTRKGIHIGPGRAVYTTERIIGDIRKAHC** | **12.9** | **X4** | **1.7** |
| | | CTRPNNNTRKGIHLGPGSSFYTTGEIIGDIRQAHC | 12.4 | R5 | 26.9 |
| | | CTRPNNNTRKGIHLGPGSSFYTTGEIIGDIRQAHC | 19.7 | R5 | 26.9 |
| | −4 | CTRPNNNTRKGIHIGPGRAFYTTERIIGDIRKAHC | 17.9 | X4 | 1.7 |
| | | CTRPNNNTRKGIHIGPGSAFYTTGEIIGDIRQAHC | 13.4 | R5 | 25.3 |
| | | CTRPNNNTRKGIHIGPGRAFYTTERIIGDIRKAHC | 43.5 | X4 | 1.7 |
| | 8 | **CTRPNNNTRKGIHIGPGRAVYTTERIIGDIRKAHC** | **28.1** | **X4** | **1.7** |
| | | CTRPNNNTRKGIHIGPGRAVYTTERIIGDIRKAHC | 5.1 | X4 | 1.7 |
| | | **CTRPNNNTRKGIHIGPGRAVYTTERIIGDIRKAHC** | **61.5** | **X4** | **1.7** |
| | 21 | CTRPNNNTRKGIHIGPGRAVYTTDRIIGDIRKAHC | 8.7 | X4 | 1.7 |
| | | CTRPNNNTRKGIHIGPGRAVYTTDRIIGDIRKAHC | 3.3 | X4 | 1.7 |
| C | | CTRPNNNTRKSIHMGPGGALYATGEIVGDIRQAHC | 70.7 | R5 | 80 |
| | −51 | CTRPNNNTRKGIRVGPGRTVYAAEKIIGDIRQAHC | 10.3 | X4 | 1.3 |
| | | CTRPNNNTRKSIHMGPRGALYATGEIVGDIRQAHC | 7.3 | R5 | 85.9 |
| | | CTRPNNNTRKSIHMGPGGALYATGEIVGDIRQAHC | 72.2 | R5 | 80 |
| | −2 | CTRPNNNTRKSIHMGPGGALYATGEVVGDIRQAHC | 5.6 | R5 | 72 |
| | | CTRPNNNTRKSIHMGPGGALYATGEVVGDIRQAHC | 3.2 | R5 | 72 |
| | | **CTRPNNNTRKGIRVGPGRTLYATEKIIGDIRQAHC** | **0.4** | **X4** | **1.8** |
| | | **CTRPNNNTRKGIRVGPGRTLYATEKIIGDIRQAHC** | **87.2** | **X4** | **1.8** |
| | 14 | CTRPNNNTRKGIRVGPGRTLYATEKIIGDIRQAHC | 1.4 | X4 | 1.8 |
| | | CTRPNNNTRKGIRVGPGRTLYATERIIGDIRQAHC | 0.5 | X4 | 1.7 |
| | | **CTRPNNNTRKGIRVGPGRTLYATEKIIGDIRQAHC** | **80.1** | **X4** | **1.8** |
| | 21 | CTRPNNNTRKGIRVEPGRTLYATEKIIGDIRQAHC | 3.8 | X4 | 2.5 |
| | | CTRPNNNTRKSIHMGPGGALYATGEIVGDIRQAHC | 2.3 | R5 | 80 |
| D | | CTRPNNNTRKSITLGPGRVFYTTGDIIGDIRQAHC | 84.1 | R5 | 10.2 |
| | −16 | CTRPNNNTRKSITLGPGKVFYTTGDIIGDIRQAHC | 3.2 | R5 | 12.8 |
| | | CTRPNNNTRKSITLGPGRVFYTTGDIIGDIRQAHC | 0.8 | R5 | 10.2 |
| | | CTRPNNNTRKSITLGPGRVFYTTGDIIGDIRQAHC | 77.4 | R5 | 10.2 |
| | 0 | **CTRPNNNTRKRITLGPGRVFYTTGEIVGDIRQAHC** | **6.9** | **X4** | **1.1** |
| | | CTRPNNNTRKSITLGPGKVFYTTGDIIGDIRQAHC | 3.5 | R5 | 12.8 |
| | | **CTRPNNNTRKRITLGPGRVFYTTGEIVGDIRQAHC** | **87.6** | **X4** | **1.1** |
| | 6 | CTRPNNNTRKSITLGPGRVFYTTGDIIGDIRQAHC | 2.3 | R5 | 10.2 |
| | | CTRPNNNTRKRITLGPGRVFYTTGEIVGNIRQAHC | 0.4 | X4 | 1.1 |
| | | **CTRPNNNTRKRITLGPGRVFYTTGEIVGDIRQAHC** | **88.7** | **X4** | **1.1** |
| | 13 | CTRPNNNTRKRITLGPGRVFYTTGEIVGNIRQAHC | 0.7 | X4 | 1.1 |
| | | CTRPNNNTRKRITLGPGRVFYTTGEIVGDIRQAHC | 0.3 | X4 | 1.1 |

**Discussion**

Using 454 deep sequencing, viral tropism was examined in plasma samples from patients with non-R5 HIV-1 who were exposed to maraviroc add-on therapy to attempt to obtain a potential clinical benefit. The R5 viral population in all patients responded to the presence of maraviroc, with a median decrease in R5 pVL of 2 $\log^{10}$ copies/mL within 21 days. However, consistent with the A4001029 study, the total viral population did not have a significant virological response to maraviroc[19]. In fact, alongside the suppression of R5 virus, the non-R5 populations modestly expanded such that the total viral load remained nearly constant. Interestingly, the most successful non-R5 variants were those with an extremely low FPR using g2p, which suggests that maraviroc may inhibit HIV with a lower g2p FPR than previously thought.

It has previously been suggested that the emergence of non-R5 variants is due not to a switch in co-receptor use by variants but to the selective outgrowth of a pre-existing non-R5 reservoir in the presence of maraviroc or vicriviroc[30,31]. This is consistent with the more recent data generated by applying the same deep sequencing screening method to a group of 24 individuals from Spain with non-R5 HIV populations who had been exposed to maraviroc for 8 days[32]. Unlike most previous analyses using individuals participating in clinical trials of maraviroc and vicriviroc, patients here were given maraviroc despite having been screened as having non-R5 HIV by ESTA. In heavily pre-treated patients experiencing drug-related toxicity, such as those studied here, clinicians have to apply individually tailored empirical therapy regimens. Although this is not recommended, all four patients received maraviroc to assess its virological or immunological efficacy. This study was a retrospective analysis of four of these cases.

After a median of 21 days of maraviroc add-on therapy, patients with non-R5 virus at baseline had a dramatic increase in their non-R5 population, such that despite a minimum decrease of 1 $\log^{10}$ copies/mL in the median R5 pVL, the overall pVL remained nearly constant. In each patient from the study presented here, the most prevalent sequence after ~3 weeks of exposure to maraviroc was non-R5, and in agreement with the findings of Westby et al.[30] these sequences had already been observed at a pre-therapy timepoint. Given the short amount of time that elapsed between samplings, it is unlikely that the mutations conferring non-R5 usage that had been observed in the pre-maraviroc samples had disappeared only to re-emerge de novo after the start of maraviroc therapy. The more parsimonious interpretation of our results is that non-R5 variants that had been present before maraviroc therapy continued to persist in the population until they gained a selective advantage at the start of maraviroc therapy.

There is an ongoing debate regarding the appropriate FPR cut-off point to apply in order to effectively predict a patient's viral response to maraviroc. Genotypic tropism testing in British Columbia, Canada, uses a cut-off point of

5.75% in population based sequencing methods and 3.5% in NGS methods, both of which have been retrospectively validated using populations from clinical trials of maraviroc[25,33]. However, the European guidelines recommend a more conservative FPR cut-off point of 10%[24]. Similarly, the German–Austrian guidelines recommend an FPR cut-off range of 5%–15% depending on the treatment options (www.daignet.de). Longitudinal analysis of the independent CCR5- and non-CCR5-using HIV populations shows that maraviroc selects for viruses with an extremely low FPR (between 0% and 2%), suggesting that the FPR cut-off points as high as 10% may exclude individuals who could respond to maraviroc.

This analysis provides another way in which to look at and interpret FPR cut-off points clinically. By composing a fitness map, measuring the shape of a fitness function over the range of co-receptor-tropism predictions from g2p, there is a possibility of identifying results that are overlooked when making inferences based on outcome. The data are more consistent with the lower g2p cut-offs of the newer German–Austrian guidelines (as low as 5%) compared with the values of up to 20% suggested in older guidelines. Based on the results of this study and a retrospective analysis of clinical trials, a cut-off of 5% may be more applicable and should be explored further. However, due to our small sample size, this finding needs to be approached with caution, and oversampling is a concern as patients, with the exception of Patient C, generally had a high pVL following the start of maraviroc treatment. Unfortunately, we were unable to use the primer ID method more recently described by Jabara et al.,[35] and therefore the viral input copy number as well as the effects of PCR and sequencing error are unknown.

In conclusion, when patients with D/M virus were exposed to maraviroc add-on therapy, selection for non-R5 populations caused them to expand to fill the space once occupied by R5 variants. Selection favoured non-R5 variants with an extremely low FPR, which may indicate that the antiretroviral activity of maraviroc may be effective in a broader range of HIV variants than currently suspected.

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## Transparency declarations

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## Chapter 6

# Impact of maraviroc therapy intensification on virological parameters in HIV-1 infected patients with a poor immunological recovery

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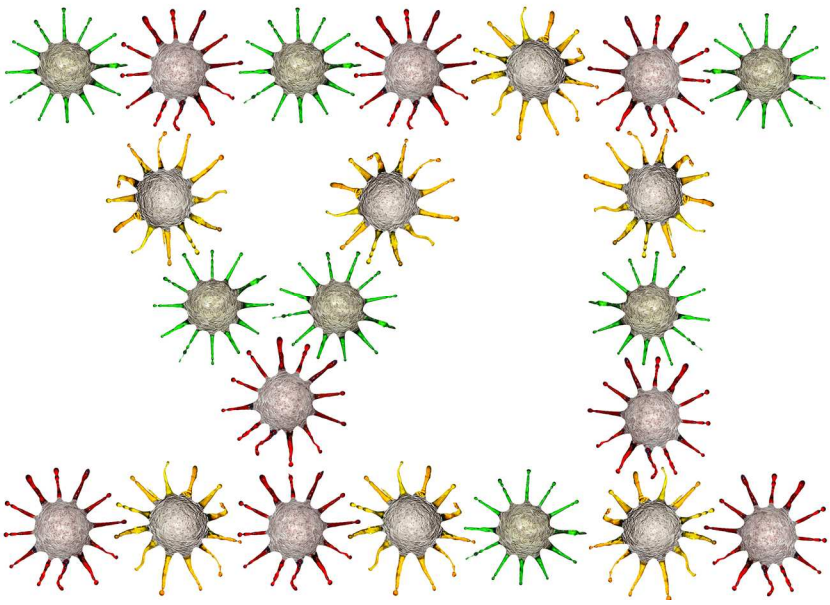

**Abstract**

**Background:** Clinical trials demonstrated a more pronounced increase in $CD4^+$ T cells in patients treated with a CCR5 inhibitor. Several studies showed that chemokine signalling through the CCR5 pathway mediates T-cell trafficking and activation. Inhibition of CCR5 mediated trafficking of T-cells to areas of inflammation could potentially reduce the level of T-cell activation and thereby HIV production. Blocking the co-receptor with the CCR5 antagonist maraviroc (MVC) could therefore be a potential beneficial strategy, especially in immunological non-responders.

The objective of our study was to investigate the impact of MVC intensification on the size of the viral reservoir and the level of viral production in immunologic non-responders. Using novel ultra-sensitive digital PCR we performed a detailed longitudinal virological analysis in 15 patients who participated in a 48-week, double-blind, placebo-controlled trial.

**Methods:** We assessed changes in relative HIV RNA expression and total HIV DNA, per million peripheral blood mononuclear cells by digital droplet PCR. In addition we analysed differences in HIV RNA cp/mL plasma and changes in $CD4^+$ T-cell count during MVC intensification. All these data were related to viral co-receptor tropism and the host CCR5 genotype (CCR5Δ32).

**Results:** Patient characteristics and baseline values did not differ between the MVC and placebo group. We observed no significant effect of MVC during 48 weeks intensification on the virological parameters or on $CD4^+$ T-cell recovery. Although we found a non-significant increase in relative HIV RNA expression in the maraviroc group (10 patients) as compared to a non-significant decrease in the placebo group (five patients) during the first 8 weeks of the study, the difference in relative HIV RNA expression was significant between the groups.

**Conclusions**: In general, there seems to be no effect over 48 weeks of maraviroc intensification of cART in patients with suboptimal immunological recovery on virological parameters. However, the study demonstrates that maraviroc intensification could have an early effect on relative HIV RNA expression. The study warrants further investigation into underlying mechanisms of possible MVC induced HIV expression.

**Introduction:**

The introduction of combination antiretroviral therapy (cART) in 1996 has significantly reduced HIV related morbidity and mortality[1,2]. Treatment with effective cART suppresses viral replication below the limit of detection of clinical assays (<50 HIV RNA/mL plasma) in many patients. Although, most infected individuals on cART show a significant recovery of their $CD4^+$ T-cell count, some patients treated with effective cART have a suboptimal immunological recovery despite virological suppression (<50 HIV RNA/mL plasma)[3,4]. Poor immunological recovery is associated with a higher risk for

overall morbidity and mortality[5]. Factors associated with poor immunological recovery include low nadir CD4+ T-cell count, viral co-infection and persistent immune activation[6,7]. Furthermore, residual viremia (between 0 and 50 copies of HIV RNA/mL plasma) has been more frequently detected in immunologic non-responders compared to immune responders[8]. Of note, it has been demonstrated that a higher frequency of CD4+ T-cells were infected in patients with residual viremia compared to patients with no detectable plasma viral load[9]. These data suggest that the latent viral reservoir may be larger in immunologic non-responders compared to immune responders on effective cART[10]. This larger reservoir, increased residual viremia and persistent high levels of immune activation during effective cART[8] could cause increased HIV antigen–driven CD4+ T-cell loss in immunologic non-responders.

Several studies demonstrate that chemokine signalling through the CC chemokine receptor type 5 (CCR5) pathway mediates cellular trafficking and activation[11,12]. This is underlined by the observation that the CCR5 antagonist maraviroc (MVC) decreases the visceral acute graft-versus-host disease in HIV negative recipients of allogeneic hematopoietic stem-cell transplantation most likely by inhibition of T-cell trafficking[13]. Inhibition of CCR5 mediated cellular trafficking to areas of inflammation could influence the level of T-cell activation, microbial translocation and total immune activation thereby influencing HIV production. Blockage of the CCR5 co-receptor with MVC could therefore be a potential beneficial strategy, especially in immunological non-responders. A meta-regression analysis of clinical trials with CCR5-antagonists in antiretroviral treatment-experienced patients found that CCR5-antagonist based therapy was associated with a significant higher increase in CD4+ T-cell count over 24 weeks of treatment compared to treatment groups not using CCR5 antagonists[14]. These data suggest that MVC reduces immune activation by modulation of CCR5 induced T-cell trafficking and as such may impact viral production.

The objective of our study was to investigate the effects of MVC intensification on virological parameters in immunologic non-responders. Patients participated in a 48 week MVC intensification double-blind, placebo-controlled trial studying the effect of MVC intensification of cART on CD4+ T cell recovery[15]. We performed a detailed virological analysis of a subgroup of these patients (n=15) using novel ultra-sensitive techniques measuring changes in total HIV DNA and HIV RNA expression in peripheral blood mononuclear cells (PBMCs). Since MVC is a CCR5 antagonist and thus only inhibits entry of CCR5 using (R5-tropic) HIV and not of viruses that use the alternative CXCR4 co-receptor (X4-tropic), we predicted co-receptor tropism of the viral populations at baseline and after 48 weeks of treatment with MVC or placebo. Furthermore, host *ccr5* genotype and changes in CD4+ T-cell count were assessed in the same sub-group of patients.

**Methods:**

This study is a detailed virological longitudinal sub-analysis of a 48-week, double-blind, placebo-controlled trial containing 85 patients to determine the effects of treatment intensification with MVC in immunological non-responders[14]. All patients studied provided written informed consent. This study was approved by the Ethical Committee of the University Medical Centre Utrecht, The Netherlands (ClinicalTrials.gov identifier: NCT00875368; EudraCT number 2008-003635-20). The inclusion criteria for the clinical trial were: age ≥ 18 years; either a $CD4^+$ T- cell count <350 cells/μL > two years on cART, or $CD4^+$ T cell count <200 cells/μL> one year on cART. All patients had viral suppression (plasma HIV-RNA <50 copies/ml) for at least the previous 6 months. Exclusion criteria were: previous use of MVC; HIV-2 infection; cART regimen containing a combination of tenofovir and didanosine; active infection treated with antimicrobial therapy; immunosuppressive medication; and, radiotherapy or chemotherapy in the previous 2 years. For the detailed virological sub-analysis, 15 patients from this study were selected based on the predicted co-receptor usage of their viral population.

*Plasma Virological Analyses*

Differences in HIV RNA cp/mL plasma were assessed as qualitative measurements. Since values between 0 and 20 HIV RNA cp/mL plasma are qualitative values all data was transformed to qualitative measurements. We scored all values >0 HIV RNA cp/mL plasma as positive signal and target non detected TND (0 HIV RNA cp/mL plasma) as negative signal. To asses if there was a difference in plasma viral load above the quantitative measurement cut-off of the assay (20 HIV RNA cp/mL plasma) we also scored ≥20 HIV RNA cp/mL plasma as positive values and signals <20 HIV RNA cp/mL plasma as negative signal.

*Immunologic Analyses*

Absolute $CD4^+$ T cell counts were assessed by flow cytometry at each visit. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood via density gradient centrifugation, cryopreserved and stored at -135.

*DNA and RNA isolation*

For the longitudinal analysis of total HIV DNA, total DNA was isolated according to manufactures protocol from frozen samples containing $5x10^6$ viable PBMCs and eluted in 75μL 60°C preheated elution buffer to increase the yield (DNeasy Blood & Tissue Kit, QIAGEN). For the longitudinal analysis of cell associated HIV RNA during MVC intensification, total RNA was isolated from frozen samples containing $5x10^6$ viable PBMCs and eluted in 30μL according to manufactures protocol (RNeasy mini kit, Qiagen). Subsequently, 1,5μg total RNA was converted into cDNA using iScriptcDNA synthesis kit with 30 μL (BioRad).

*Digital droplet PCR*

**usRNA:** The ddPCR mix for the usRNA assay consisted of: 10 µl 2x ddPCR™ super mix for probes (Bio-Rad); 500 nM of primers; 250 nM probe mix and 4 µl of cDNA into a final volume of 20 µl. A qPCR was performed on crude Dnase treated RNA (-RT control) to assess possible incomplete DNA digestion. No signal was obtained demonstrating complete DNA digestion.

**Total HIV DNA:** Total isolated DNA was digested with EcoRI (Promega) for 1h at room temperature at a concentration of maximally 430 ng/µl. Per replicate reaction, the ddPCR mix consisted of 10 µl 2x ddPCR™ super mix for probes (Bio-Rad), 800 nM primers, 300 nM probe and 2 µl of crude restriction digest for the HIV DNA assays or 400 nM primers, 300 nM probe and 1 µl of crude restriction digest for the RPP30 assay and into a final volume of 20 µl.

The HIV DNA and RNA reactions were run in triplicate, the RPP30 reactions in duplicate. Of the total mix dDroplets were formed in the QX100™ droplet generator (Bio-Rad). Droplet in oil suspensions were transferred to an Eppendorf® 96 well plate (Eppendorf, Germany) and placed into the T100™ Thermal Cycler (Bio-Rad). Cycling conditions were as follows: 5' 95°C, followed by 40 cycles of 15" 95°C and 60" 58°C. The droplets were subsequently read automatically by the QX100™ droplet reader (Bio-Rad) and the data was analyzed with the QuantaSoft™ analysis software 1.3.2.0 (Bio-Rad).

*Normalization of the ddPCR data for usRNA samples*

Absolute HIV DNA quantities as obtained by ddPCR were divided by the amount of cells per reactions as assessed by the RPP30 assay.

Normalization of the HIV RNA was performed was performed by validating multiple selected reference genes from a set of pre-validated reference genes since single or non-validated reference genes may induce experimental bias in the data[16,17]. The most stable and optimal number of reference genes were validated from a panel of seven reference genes by qPCR. Expression levels were assessed on an LC480 qPCR cycler (Roche Diagnostics) using LightCycler® 1536 DNA Probes Master mix (Roche) with 1x ResoLight Dye (Roche), 10 µM of each primer (Table 1), and 0.5 µl cDNA. Cycling conditions were 5' 95°C, followed by 45 cycles of 15" 95°C, 30" 60°C and 15" 72°C. The geometric mean of the most stable reference genes (HMBS, HPRT, TBP) was used to calculate the normalization factor of each sample using the Genorm method in qBase Plus (Biogazelle, Ghent)[18]. Subsequently raw ddPCR values were divided by the normalization factors to obtain normalized usRNA values for all samples.

**Table 1: Panel of seven reference genes.**

| Gene name | Accession number | 5'-3' forward primer sequence | 5'-3' reverse primer sequence |
|---|---|---|---|
| B2M | NM_004048 | AGATGAGTATGCCTGCCGTGTGAA | TGCTGCTTACATGTCTCGATCCCA |
| PLOD1 | NM_000302.3 | CAACAACAAGGACAACCGCATCCA | GAATTTGTGCCACTCCCGCTCAAA |
| RPL13A | NM_012423 | CCTGGAGGAGAAGAGGAAAGAGA | TTGAGGACCTCTGTGTATTTGTCAA |
| HMBS | NM_000190 | CCCACGCGAATCACTCTCAT | TGTCTGGTAACGGCAATGCG |
| HPRT1 | NM_000194 | TGACACTGGCAAAACAATGCA | GGTCCTTTTCACCAGCAAGCT |
| TBP | NM_003194 | CAAGCGGTTTGCTGCGGTAATCAT | TGCCAGTCTGGACTGTTCTTCACT |
| GAPDH | NM_002046 | AGCCTCAAGATCATCAGCAATGCC | TGTGGTCATGAGTCCTTCCACGAT |

*Co-receptor tropism prediction*

The V3 region of the viral envelope was amplified from isolated PBMC DNA using primers envF1.1 (forward) 5'-GGATATAATCAGYYTATGGGA-3', envF1.2 (forward) 5'-GAGGATATAATCAGTTTATGG-3', envR1.1 (reverse) 5'-GGTGGGTGCTAYTCCYAITG-3' and envR1.2 (reverse) 5'-GGTGGGTGCTATTCCTAATGG-3' (Titan One Tube RT-PCR kit, Roche). Nested PCR using primers envF2.1 (forward) 5'-GATCAAAGCCTAAARCCATGT-3', envF2.2 (forward) 5'-GATCAAAGCCTAAAGCCATG-3', envR2.1 (reverse) 5'-CTCCAATTGTCCYTCATHTYTCC-3' and envR2.2 (reverse) 5'-ACTTCTCCAATTGTCCCTCATAT-3' (Expand High Fidelity PCR System, Roche). Co-receptor tropism was predicted using Geno2Pheno$_{[co\text{-}receptor]}$ according to the European guidelines on the clinical management of HIV-1 tropism testing. The viral population was scored as X4-tropic if X4-tropism was predicted in at least one of the three tests. If the viral population was predicted to be X4-tropic at week 48, amplification and sequencing of samples obtained at week 0 was performed to determine whether the virus switched from R5- to X4-tropism during MVC intensification. All samples with an X4-tropic prediction at week 48 also were predicted to be X4-tropic at baseline.

*CCR5 genotyping*

All patients were screened for CCR5Δ32 genotype. The CCR5 gene was amplified with the primers flanking the CCR5Δ32 deletion; CCR5f (forward) 5'-GATAGGTACCTGGCTGTCGTCCAT-3' and CCR5d (reverse) 5'-CCTGTGCCTCTTCTTCTCATTTCG-3' (Expand High-Fidelity PCR-System, Roche). Amplification products of 324 bp (wildtype CCR5) and 292 bp (CCR5Δ32) were visualized by gel electrophoresis. Heterozygosis was confirmed by cloning the PCR products into a pGEM-T Easy Vector (Promega) and subsequent sequencing (Big dye Terminator Cycle seq kit v3.1, Applied Biosystems) with primer M13F (forward) 5'-TGTAAAACGACGGCCAGT-3'.

*Statistical analysis*

Differences between treatment groups and co-receptor tropism in the MVC group were assessed with linear regression models. Continuous variables were compared using Mann Whitney U-test. Differences in HIV RNA cp/mL plasma were assessed as qualitative measurements. Signals >0 were taken as positive signal and target non detected TND (0 HIV RNA cp/mL plasma) as

negative signal. Significance was calculated with Chi square test. Differences were considered statistically significant at $p < 0.05$.

**Results:**

*Patient population*

We analysed the effect of MVC intensification on virological parameters in a sub-set of patients (n=15) who participated in a 48 week MVC intensification study. We included 10 patients who received MVC intensification and five patients who received placebo to their current cART. Of the 10 patients who received MVC, five were infected with a viral population predicted to be R5-tropic (FPR range 12-91) and the other five patients harboured a population predicted to be X4-tropic (FPR range 1.1-1.7). The placebo group harboured three patients with an R5-predicted viral population (FPR range 32-94) and two with an X4-predicted viral population (FPR range 0.2-1.7). In addition, the host CCR5 genotype was assessed. Of the 15 patients, 11 had a wild-type *ccr5* gene in both chromosomes (CCR5WT/WT). The other four patients were heterozygous for the *ccr5Δ32* deletion (CCR5WT/Δ32) and all four patients were infected with a viral population predicted to be X4-tropic (2 in placebo and 2 in MVC group). In addition, we analysed the change in $CD4^+$ T-cells in relation to MVC or placebo intensification and predicted co-receptor usage during the initial 8 weeks of intensification or the full observation period of 48 weeks. In this subset of patients, no differences were observed in $CD4^+$ T-cell recovery within the groups or between the groups, data not shown.

*Baseline characteristics*

The median age of the 15 patients selected was 49 years (interquartile range (IQR):43-61), and the median overall baseline $CD4^+$ T-cell count was 174 (IQR: 146-185) cells/μL. The median HIV RNA cp/mL plasma at baseline was 0 (target not detected) and only two patients had a positive signal (between 0 and 20 HIV RNA cp/mL plasma). The median duration of cART was 5.8 (IQR: 3.5-8.9) years and median time to an undetectable viral load was 4.5 (IQR: 3.4-8.0] years. Of note, in the MVC group, patients that were infected with a predicted R5-tropic viral population were significantly shorter on cART compared to patients predicted to be infected with an X4-tropic viral population (p=0.008) (Table 2).

**Table 2. Baseline characteristics of 15 patients included.** All values are given as median and interquartile range between brackets, except number of patients N, Sex and CCR5 WT/Δ32. TND is target not detected. Significance was calculated with Mann Whitney U test*.

| Groups | Selected patients | Placebo | MVC | X4 MVC | R5 MVC |
|---|---|---|---|---|---|
| N | 15 | 5 | 10 | 5 | 5 |
| Age | 49 [43-61] | 55 [42-66] | 49 [42-60] | 58 [49-61] | 45 [41-48] |
| Sex | 100% male | 100% male | 100% male | 100% male | 100% male |
| CD4 count baseline | 174 [146-185] | 175 [142-190] | 160 [152-179] | 174 [150-180] | 160 [160-180] |
| Nadir CD4 | 56 [17-89] | 18 [16-22] | 61 [27-108] | 64 [60-122] | 56 [17-88] |
| HIV RNA cp/mL plasma | 0 [0-0] TND | 0 [0-0] TND | 0 [0-0] TND | 0 [0-0] TND | 0 [0-0] TND |
| Time on cART (years) | 5.8 [3.5-8.9] | 5.8 [3.6-6,2] | 6.6 [3.8-9.8] | 9.9 [9.4-12.8] | 3.6 [3.4-4.4]* |
| Time undetectable | 4.5 [3.4-8.0] | 4.6 [3.4-6,3] | 6.6 [3.4-8.2] | 8.2 [2.0-9.8] | 4.1 [3.6-4.6] |
| CCR5 WT/Δ32 | 4 | 2 | 2 | 2 | 0 |

*Difference in total HIV DNA between co-receptor tropism and ccr5 genotype*

We assessed the size of the viral reservoir (corrected for CD4⁺ T-cell counts) at baseline in respect to cART treatment years. Although no significant difference in viral reservoir in respect to treatment years was observed (p=0.13) patients with an R5-predicted viral population were significantly shorter on cART compared to patients with an X4-predicted viral population (p=0.006) (Figure 1a). In our study we included eight patients who were infected with a predicted R5-tropic viral population and 7 who were infected with a predicted X4-tropic viral population. In addition, we analysed the *ccr5* genotype and four patients were heterozygous for the *ccr5Δ32* genotype, all CCR5WT/Δ32 patients were infected with a predicted X4-tropic viral population. We observed that the total HIV DNA load tended to be lower in the X4-tropic baseline samples as compared to the R5-tropic samples (Figure 1b left). Furthermore, in the heterozygous *ccr5Δ32* group (CCR5WT/Δ32) a significant lower number of total HIV DNA was observed per million PBMCs (Figure 1b right).

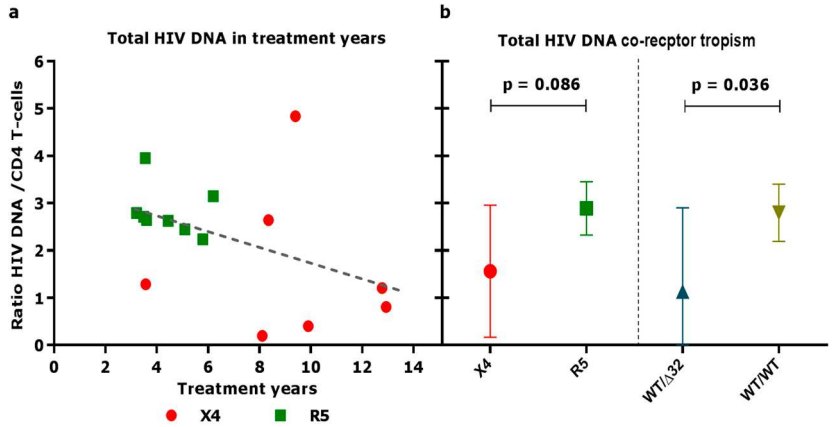


**Figure 1. Differences in total HIV DNA.** Differences in total HIV DNA were assessed. a) Total HIV DNA cp/million PBMCS corrected for CD4⁺ T-cell count with treatment years of cART. With ● X4-tropic and ■ R5-tropic viral population b) Left, copies of total HIV DNA/million PBMCs at baseline of patients infected with a predicted ● X4-tropic and ■ R5-tropic viral population. b) Right, copies of total HIV DNA/million PBMCs at baseline of patients heterozygous for CCR5Δ32 (▲

CCR5WT/Δ32) and homozygous for CCR5 wild type (▼ CCR5WT/WT). Average with standard deviation is given with p values from Mann Whitney U test.

*Effect of MVC intensification on total HIV-DNA*

For all included patients the mean total HIV DNA copies per million cells at baseline was 368 (95% confidence interval [95% CI], 276-460). There was no significant difference in total HIV DNA between the MVC group and placebo group at baseline (p=0.95). Over 48 weeks of MVC intensification no significant difference was observed within the placebo or MVC groups (p=0.75, p=0.87 respectively) or between the placebo and the MVC group (p=0.96) (Figure 2a). When we stratified total HIV DNA according to co-receptor tropism in the MVC group no change in total HIV DNA was observed within the groups (p values >0.74), whereas between the groups no significant difference was detected as well (p values >0.78) (Figure 2b).

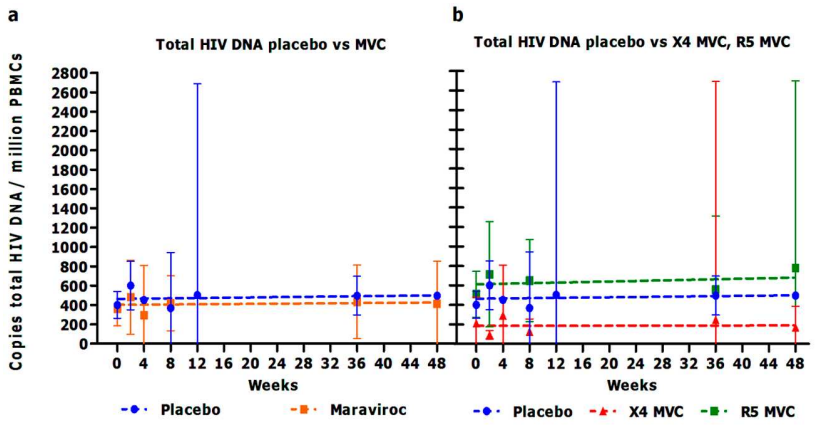


**Figure 2. Total copies of HIV DNA per million PBMCs.** Changes in HIV DNA levels were assessed by linear regression models. a) The estimated mean levels of HIV DNA copies/million PBMCs at each time point are plotted with the 95% confidence interval. In ● blue the placebo group and in ■ orange the MVC group are depicted. b) The estimated mean levels of HIV DNA copies/million PBMCs at each time point are plotted with the 95% confidence interval. In ● blue the placebo group, in ▲ red the predicted X4-tropic group in the MVC group and in ■ green the predicted R5-tropic group in the MVC group are depicted.

Also during the initial period of cART intensification (8 weeks), we did not observe a difference in copies total HIV DNA/million PBMCs between the placebo group and the MVC group (p=0.72) (Figure 3a). We also assessed the difference in total HIV DNA cp/million PBMCs between baseline and week eight of placebo, the MVC group and the MVC group stratified in predicted co-receptor tropism. No significant difference was observed within the groups (p values >0.15). Similar results were found between the groups (Figure 3b).

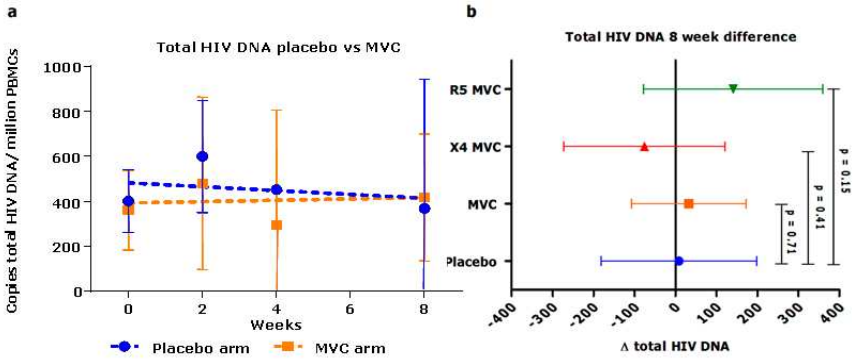


**Figure 3. Total copies of HIV DNA per million PBMCs during the initial eight weeks of MVC intensification.** Changes in HIV DNA levels. a) The estimated mean levels of HIV DNA copies/million PBMCs at each time point are plotted with the 95% confidence interval. In ● blue the placebo group and in ■ orange the MVC group are depicted. Changes were assessed by linear regression models. b) Estimated mean differences in HIV DNA copies/million PBMCs after eight weeks of MVC intensification with the 95% confidence interval. In ● blue the placebo group, in ■ orange the MVC group, in ▲ red the predicted X4-tropic group in the MVC group and in ▼green the predicted R5-tropic group in the MVC group are depicted. p values calculated withMann Whitney U-test

*Changes in relative HIV RNA expression during MVC intensification*

We investigated the effect of MVC intensification on cell-associated HIV RNA. At baseline the mean relative level of cell-associated HIV RNA expression for all patients was 3.1 (95% CI:2.5-3.7). During the entire study length (weeks 0-48) we observed no significant difference in relative HIV RNA expression levels within the placebo and MVC groups nor was there a difference observed between the groups (p values >0.38) (Figure 4a). When the data was grouped according to predicted co-receptor usage in the MVC group we observed no significant difference in 48 weeks of MVC intensification compared to placebo (p values >0.40) (Figure 4b).

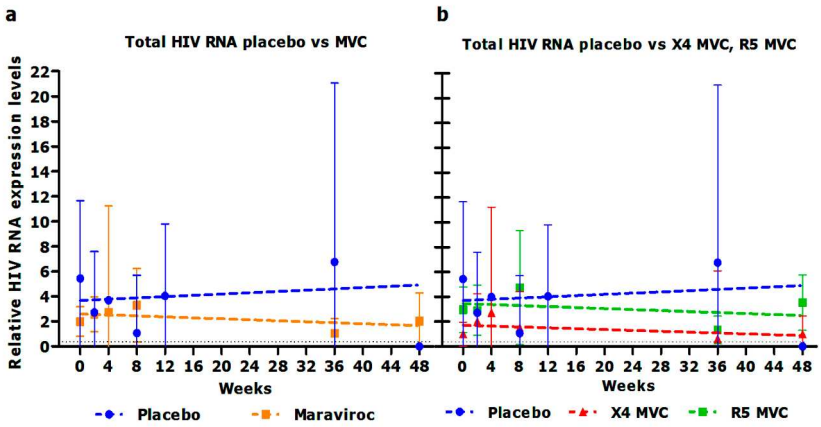


**Figure 4. Relative expression of HIV RNA.** Relative changes in HIV RNA levels were assessed by linear regression models. a) The estimated mean relative levels of HIV RNA expression at each time point are plotted with the 95% confidence interval. In ● blue the placebo group and in ■ orange the MVC group are depicted. b) The estimated mean levels of HIV RNA expression at each time point are plotted with the the 95% confidence interval. In ● blue the placebo group, in ▲ red the predicted X4-tropic group in the MVC group and in ■ green the predicted R5-tropic group in the MVC group are depicted. Values below the dotted black lines represent potential false positive values.

Interestingly, during the initial first eight weeks of MVC intensification the relative levels of HIV RNA expression differed significantly between the placebo and MVC group (p=0.04) (Figure 5a). We observed a decrease in RNA expression in the placebo group and an increase in the MVC group. However, neither the decrease nor the increase in the group was significant (placebo group p=0.17, MVC group p=0.27). Remarkably, a significant difference between the decrease in HIV RNA expression in the placebo group as compared to the increase in the X4-tropic predicted group was observed but not between placebo and the R5-tropic predicted group (Figure 5b).

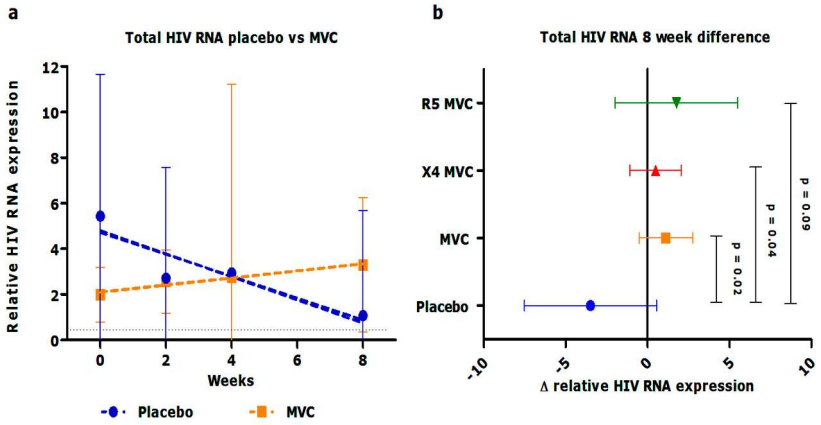


**Figure 5. Relative expression of HIV RNA during initial eight week period.** Relative changes in HIV RNA levels were assessed by linear regression models. a) The estimated mean relative levels of HIV RNA expression at each time point are plotted with the 95% confidence interval. In ● blue the placebo group and in ■ orange the MVC group are depicted. Values below the dotted black line represent potential false positive value. b) Differences in relative HIV RNA expression after eight weeks of MVC intensification. In ● blue the placebo group, in ■ orange the MVC group, in ▲ red the predicted X4-tropic group in the MVC group and in ▼ green the predicted R5-tropic group in the MVC group are depicted. p values calculated with Mann Whitney U-test.

*Effect of MVC intensification on HIV plasma viral load*

We assessed the difference in HIV RNA cp/mL plasma in 13 of the 15 patients (five placebo group, eight MVC group). When we assessed changes in positive signal (every signal >0 HIV RNA cp/mL is a positive signal) no differences were observed between the placebo and MVC group during the study length (p=0.97). We also assessed the differences the between placebo and MVC group in positive signal ≥20 HIV RNA cp/mL which was also not significantly different (p=0.43) (Figure 6a). Similar results were observed when we stratified the MVC group according to predicted co-receptor tropism (p values >0.59) (Figure 6b).

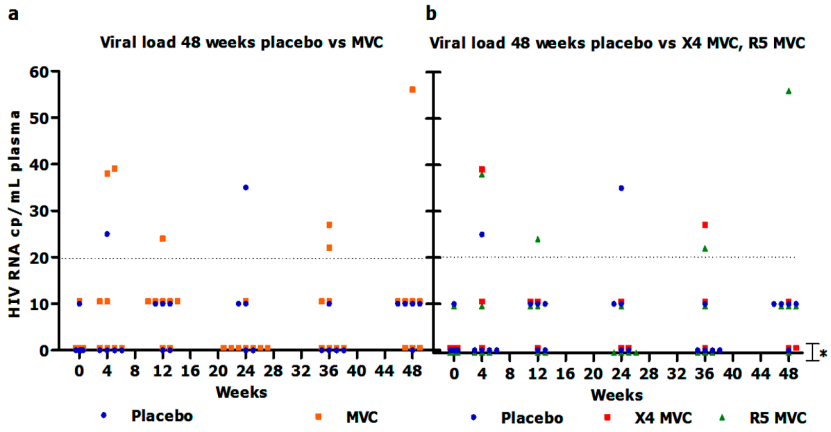


**Figure 6. Changes in HIV RNA plasma viral load.** Differences in positive signal of HIV RNA cp/mL plasma were assessed Chi square test. a) All values of each time point are plotted. Values between 0 and 20 were set at 10 HIV RNA cp/mL. In ● blue the placebo group and in ■ orange the MVC group are depicted. b) All values of each time point are plotted. Values between 0 and 20 were set at 10 HIV RNA cp/mL. In ● blue the placebo group, in ■ red the predicted X4-tropic group in the MVC group and in ▲ green the predicted R5-tropic group in the MVC group are depicted. Dotted lines represent quantitative measurement cut-off. * Target not detected.

## Discussion

Our study is a detailed virological longitudinal sub-analysis of a 48-week, double-blind, placebo-controlled trial containing 85 patients to determine the effects of treatment intensification with MVC in immunological non-responders[15]. We assessed residual viremia (<50 HIV RNA cp/mL plasma) and differences in $CD4^+$ T-cells in a subset of 15 patients. On top of that, we investigated total HIV-DNA and relative HIV RNA expression in PBMCs using cutting-edge ultra-sensitive digital droplet PCR (ddPCR) techniques. This novel technique provides absolute quantification of target sequences without relying on the use of standard curves, like the widely used real-time quantitative PCR (qPCR). Therefore, ddPCR is notably less variable between experiments and it has been shown that ddPCR is substantially more precise detecting HIV template present at very low frequencies compared to qPCR[19,20].

It was hypothesised that MVC intensification could increase $CD4^+$ T-cell recovery[15,21]. A slight increase in median $CD4^+$ T-cell count during 48 weeks of MVC intensification was observed in the 15 immunological non-responders included in this study. However, no significant difference was detected between the placebo group and MVC group. This observation is in line with the outcome as observed for the total group of 85 patients included in this trial[15]. Furthermore, also Hunt *et al.*, could not demonstrate a significant difference in $CD4^+$ T-cell recovery between the placebo and MVC arm in 42 immunological non-responders[21].

Given that the CCR5Δ32 heterozygous genotype is associated with lower expression of CCR5 and is frequently found in long-term non-progressors

who often have a low viral reservoir[22,23], we assessed the CCR5 genotype and reservoir size at baseline. The four patients who were CCR5Δ32 heterozygous show lower HIV DNA levels which could indicate a smaller viral reservoir. Patients infected with a predicted X4-tropic viral population also tended to have a lower reservoir size, in contrast to previous observations that patients infected with an X4-tropic viral population contained a larger viral reservoir[24]. It is difficult to draw definite conclusions based on these data because the patients infected with an X4-tropic population were significantly longer treated with cART and the four CCR5Δ32 heterozygous patients in our study were all infected with an X4-tropic virus which could also affect reservoir size. Unfortunately, a multivariate analysis was not possible due to the relative small number of subjects included in this analysis, which is a limitation of the study.

Using the ultra-sensitive ddPCR technique no effect of MVC intensification on total HIV DNA levels could be demonstrated during 48 weeks of MVC intensification. So far, in none of the cART intensification studies an impact on total HIV DNA levels could be demonstrated[10,25]. This could relate to the fact that the period on cART intensification is relatively short and the size of the viral reservoir is quite stable during long-term cART[26,27].

Several uncontrolled studies investigated the immunological effect of MVC intensification in immunologic non-responders[28,29]. MVC intensification tended to reduce T-cell activation in these studies. Such a reduction in T-cell activation would most likely result in a reduction in HIV RNA transcription[27]. After 48 weeks of MVC intensification no change in T-cell activation was observed in the study from which our subset of patients was derived[15]. Conversely, Hunt *et al.* demonstrated that 24 weeks of MVC intensification caused a modest increase in peripheral blood T-cell activation and a two-fold increase in rectal T-cell activation[21]. In the latter study the CCR5 ligand MIP-1β increased significantly in the MVC arm compared to the placebo arm[22]. Since CCR5 ligands also bind and signal through CCR4 an increase of these ligands could lead to augmented CCR4 mediated activation resulting in production of NF-κB and possibly increase of HIV RNA expression[30]. Remarkably, our results demonstrate a significant difference between the placebo and MVC group in relative cellular HIV RNA expression in response to MVC intensification during the first eight weeks of the study. Within the MVC group cellular HIV RNA expression increased and within the placebo group it decreased, although both not significant. Increase in relative HIV RNA expression without T-cell activation seems contradictory. However, it has been demonstrated that the anti-latency drug disulfiram can induce high levels of HIV transcription without global T-cell activation[31]. In the same study it was observed that the expression of the early activation marker CD69 increased whereas expression of other activation markers was not upregulated[31]. Interestingly, *in vitro* observations demonstrated an increase in expression of CD69 and a down regulation of other activation markers (HLA-DR and CD38) in response to MVC[32]. This slight increase in early activation and augmented CCR4 signalling could possibly result in an increase in HIV RNA expression. Similarly, Gutiérrez

et al. demonstrated an increase in HIV production in a non-placebo controlled MVC intensification study. A significant increase in residual viremia was observed after 12 weeks of MVC intensification compared to baseline[33]. In our subset analysis we observed no difference between the placebo and MVC groups in the effect of 48 weeks MVC intensification on viral production as measured in plasma. Of note, a study investigating the effects of the anti-latency drug vorinostat demonstrated an significant increase in intracellular HIV expression but observed no effect in HIV RNA in plasma[34].

In our study no significant differences in virological parameters were observed between the placebo and MVC groups after 48 weeks of MVC intensification. However, the first eight weeks of the study demonstrates that MVC intensification could have a temporarily effect on relative levels of HIV RNA expression. The interesting observations of differences in relative HIV RNA expression warrants the analysis of a larger number of patients and investigation into underlying mechanisms of possible MVC induced HIV expression.

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**Chapter 7**

## Dependence on the CCR5 co-receptor for viral replication explains the lack of rebound of CXCR4-predicted HIV-variants in the Berlin patient

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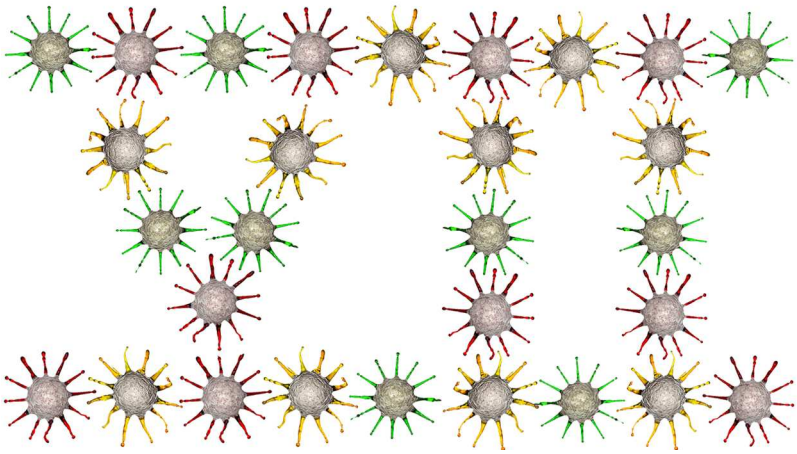
*Submitted*



**Abstract**

The "Berlin Patient" is the first patient cured of HIV-1 infection after allogeneic transplantation with non-functional CCR5 co-receptor stem cells. We demonstrate that CXCR4-predicted minority viruses present prior to transplantation were unable to rebound after transplantation due to their dependence on CCR5 for replication and high genetic barrier towards CXCR4-usage.

**Introduction**

The Berlin Patient is the first individual in whom HIV-1 infection has been cured following allogeneic transplantation to treat acute myeloid leukemia[1,2]. The stem cells used were homozygous for the Δ32 frameshift mutation (CCR5Δ32) resulting in absence of the CCR5 receptor at the cellular surface[3]. Heterozygosis for CCR5Δ32 is associated with slower disease progression and individuals homozygous for CCR5Δ32 are naturally resistant to CCR5-tropic HIV[3].Binding of the viral envelope glycoprotein gp120 to a co-receptor (CCR5 or CXCR4) is essential for HIV entry into $CD4^+$ host cells. HIV co-receptor tropism is mainly determined by the third hypervariable loop of the viral envelope (gp120-V3)[4].

The Berlin Patient had a viral load of $6.9x10^6$ cp/mL during an episode of treatment interruption prior to stem cell transplantation (SCT). The viral population was predicted to be CCR5-tropic based on standard genotypic tropism testing ($geno2pheno_{[coreceptor]}$)[1]. Detailed analysis using ultradeep sequencing detected a 2.9% minority viral population predicted to be CXCR4-tropic (False Positive Rate (FPR) ranging 2.7 to 9.3%). Two months after SCT successful reconstitution of $CD4^+$ T-cells with the homozygous CCR5Δ32 phenotype was observed[1]. These donor derived T-cells displayed normal levels of CXCR4 co-receptor surface expression. Given the detection of CXCR4-predicted viral variants prior to SCT, one would expect these variants to appear after the SCT especially since antiretroviral therapy was discontinued during the transplant procedure. However, no viral rebound was observed and the patient remained free of HIV infection for more than five years post SCT[5].

Previously, we demonstrated that some viruses capable of using CXCR4 have a clear CCR5 co-receptor preference *in vivo*[6]. Based on these findings we postulate that a rebound of the CXCR4-predicted variants in the Berlin Patient did not occur due to dependence on the CCR5 co-receptor for viral replication.

## Methods

*Tropism prediction*

Viral co-receptor tropism was predicted using Geno2Pheno[coreceptor] with a 10% false positive rate (FPR) cut-off[7], position-specific scoring matrices Web $PSSM_{X4\text{-}R5}$ (CCR5-prediction, ≤6.69; and CXCR4, ≥2.88; the 11/25 rule was applied at intermediate values[8]). Net charge was determined by using an online-peptidepropertycalculator (Innovagen; http://www.innovagen.se).

*Generation of recombinant viral constructs*

Recombinant viruses were constructed to investigate replication capacity and co-receptor dependence. An HIV-1 deletion clone lacking the gp120-V3 loop sequence (pHXB2 Δgp120-V3) was generated by introducing a unique BmgBI restriction site at position 7091 and subsequent digestion of the vector with BmgBI and NheI (7260) and insertion of a linker sequence with the unique restriction site AspI. A PCR was performed to generate gp120-V3 amplicons representing the CXCR4-predicted viruses and the most dominant CCR5-predicted strain of the patient using primers BmgBI-V3-f and NheI-V3-r (Table 1) (Expand High-Fidelity PCR-System, Roche, Woerden, the Netherlands). As control viruses we generated constructs of CXCR4-tropic HXB2, and CCR5-tropic BaL. As template for the generation of the amplicons, two nucleotide sequences were used representing the specific gp120-V3 nucleotide sequences (Table 1). The generated PCR products were digested with BmgBI and NheI and ligated into pHXB2 Δgp120-V3 digested with these same restriction enzymes. These ligation products were digested with Asp1 and transformed into Escherichia coli JM109 High Efficiency Competent Cells (Promega), by means of heat shock at 42ºC, and spread on Luria-Bertani agar plates containing 40 mg/mL ampicillin. Colonies were inoculated into 100 mL of Luria-Bertani medium with 40 mg/mL ampicillin. The plasmids were isolated using the Plasmid Midi Kit (Qiagen).

**Table 1.** Nucleotide sequences for synthesis of gp120-V3 constructs, with primers BmgBI-V3f and NheI-V3r and nucleotide sequences used as template with f as forward template and r as reverse template are given.

| | |
|---|---|
| BgmBI-V3f | 5'-GCTGAACACGTCTGTAGAAA-3' |
| NheI1-V3r | 5'-AATTTGCTAGCTATCTGTTTT-3' |
| Rbp-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACAACACAAGAAAAGGTATACATATAGGTCCAGGGAGAGCATTTTAT-3' |
| Rbp-V3r | 5'-AATTTGCTAGCTATCTGTTTTAAAGTGTTATTCCATTTTGCTCTACTAATGTTACAATGTGCTTGTCTTATATCTCCCATTATTTCTCCTGTTGTATAAAATGCTCTCCCTGGAC-3' |
| X1bp-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTGCAAGACCCAACAACAACACAAGAAAAGGTATACATATAGGACCAGGGAGAGCATTTTAC-3' |
| X1bp-V3r | 5'-AATTTGCTAGCTATCTGTTTTAAAGTGTTATTCCATTTTGCTCTACTAATGTTACAATGTGCTTGTCTTATATCTCCCATTATTTCTCCTGTTGTGTAAAATGCTCTCCCTGGTC |
| X2bp-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACAAGACAAGAAAAGGTATACATATAGGACCAGGGAGAGCATTTTAT-3' |
| X2+5+6+7bp-V3r | 5'-AATTTGCTAGCTATCTGTTTTAAAGTGTTATTCCATTTTGCTCTACTAATGTTACAATGTGCTTGTCTTATATCTCCCATTATTTCTCCTGTTGTATAAAATGCTCTCCCTGGTC-3' |
| X3+4bp-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACAACACAAGAAAAGGTATACATATAGGACCAGGGAGAGCATTTTAT-3' |
| X3bp-V3r | 5'-AATTTGCTAGCTATCTGTTTTAAAGTGTTATTCCATTTTGCTCTACTAATGTTACAATGTGCTTGTCTTATATCTCCCATTATTTTTCCTGTTGTATAAAATGCTCTCCCTGGTC-3' |
| X4bp-V3r | 5'-AATTTGCTAGCTATCTGTTTTAAAGTGTTATTCCATTTTGCTCTACTAATGTTACAATGTGCTCGTCTTATATCTCCCATTATTTCTCCTGTTGTATAAAATGCTCTCCCTGGTC-3' |
| X5bp-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACAGCACAAGAAAAGGTATACATATAGGACCAGGGAGAGCATTTTAT-3' |
| X6bp-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACGACACAAGAAAAGGTATACATATAGGACCAGGGAGAGCATTTTAT-3' |
| X7bp-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACAACACAAGAAAAGGTACACATATAGGACCAGGGAGAGCATTTTAT-3' |
| cHXB2-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACAATACAAGAAAAAGAATCCGTATCCAGAGAGGACCAGGGAGAGCAT-3' |
| cHXB2-V3r | 5'-AATTTGCTAGCTATCTGTTTTAAAGTGTTATTCCATTTTGCTCTACTAATGTTACAATGTGCTTGTCTCATATTTCCTATTTTTCCTATTGTAACAAATGCTCTCCCTGGTCCTCTC-3' |
| cBaL-V3f | 5'-GCTGAACACGTCTGTAGAAATTAATTGTACAAGACCCAACAACAATACAAGAAAAAGTATACATATAGGACCAGGCAGAGCATTGTAT-3' |
| cBaL-V3r | 5'-AATTTGCTAGCTATCTGTTTTAAAGTGTTATTCCATTTTGCTCTACTAATGTTACAATGTGCTTGTCTTATATCTCCTATTATTTCTCCTGTTGTATACAATGCTCTGCCTGGTCC-3' |

*Cells*

U373-MAGI-CCR5E and U373-MAGICXCR4$_{CEM}$ cell lines[9] were maintained as recommended by the NIH AIDS Research and Reference Reagent Program. Peripheral blood mononuclear cells (PBMCs) from five healthy donors were prepared by Ficoll–Paque density gradient centrifugation of heparinized blood from five HIV-seronegative donors (homozygous for CCR5WT). The mix was stimulated for 3 days with phytohaemagglutinin (2 mg/L) in culture medium [CM; RPMI1640 with L-glutamine (BioWhittaker), 10% fetal bovine serum (FBS; Biochrom AG) and 10 mg/L gentamicin (Gibco)]. 293T cells were maintained in Dublecco's modified Eagle medium (BioWhittaker) supplemented with 10% fetal bovine serum (Biochrom AG) and 10 mg/mL gentamicin. MT-2 cells were maintained in CM.

*Generation of recombinant viruses*

To obtain the recombinant viruses, 10 μg of the recombinant plasmids was used to transfect 293T cells at 90%–95% confluence. For transfection, lipofectamine 2000 reagent (Invitrogen) was used in accordance with the manufacturer's protocol. After 48 h, recombinant viruses were harvested, and viral supernatant was obtained for p24 analysis and sequence analysis of the gp120-V3 loop. The 50% tissue culture infective dose ($TCID_{50}$) was determined on PBMCs from healthy donors.

*Co-receptor usage in U373-MAGI cells*

At day 0, $2\times10^4$ cells/well of U373-MAGI-CCR5E or U373-MAGICXCR4$_{CEM}$, expressing $CD4^+CCR5^+CXCR4^-$ and $CD4^+CCR5^-CXCR4^+$, respectively, were plated into a 96-well plate in 200 μL of Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) with 10% FBS and 10 mg/mL gentamicin. Subsequently, at day 1, medium was discarded and replaced by 200 μL DMEM with 10% FBS and 10 mg/mL gentamicin containing 2 ng of p24 of the CXCR4-predicted variants, the CCR5-predicted virus or the control viruses and incubation was continued for 2 days at 37ºC. Subsequently, luminescence was measured using the Galacto-StarTM b-Galactosidase Reporter Gene Assay System for Mammalian Cells (Applied Biosystems) according to the manufacturer's protocol using 20 μL of lysis buffer and 60 μL of reaction buffer. Background activity (cells without virus) was subtracted from the activity of the wells containing virus.

*Replication capacity in PBMCs from healthy donors*

PBMCs from healthy donors were incubated for 1 hour at 37ºC in culture medium with 5 U/mL IL-2 with 1 μM MVC, 10 μM AMD-3100 or without HIV inhibitor. The donor PBMCs were infected with a recombinant virus using a multiplicity of infection (MOI) of 0.001 and incubated for 2 hours at 37ºC, after which cells were washed twice. Subsequently, $0.2\times10^6$ cells/well were plated

into a 96-well plate with 5 U/mL IL-2 in culture medium containing 1μM MVC, 10 μM AMD-3100 or no inhibitor. p24 was analyzed on days 0 and 7. Background activity (p24 at day 0) was subtracted from p24 measurements at day 7.

*Replication capacity in post-SCT patient derived PBMCs*

PBMCs derived from the patient after SCT, homozygous for CCR5Δ32 were handled as donor PBMCs with the exception that MVC was not used. Furthermore, patient derived PBMCs were cultured without virus or inhibitor and analyzed for p24 production. No p24 was measured in cells without virus (data not shown).

*Evolution experiments*

At day 0, $3\times10^5$ cells/well U373-MAGI-CCR5E were plated in a 12 well plate in 1 mL (DMEM; BioWhittaker) with 10% FBS and 10 mg/mL gentamicin. Subsequently, at day 1, medium was discarded and replaced by 500 μL DMEM with 10% FBS and 10 mg/mL gentamicin containing 50 ng of p24 of the CXCR4-predicted variants (X2bp and X3bp), the R5 predicted virus Rbp and the cBaL control virus. Incubation was continued for 6 hours at 37ºC. Wells were washed and 2 mL fresh medium was added containing 0.2 mg/ml G418; 0.1 mg/ml hygromycin B, and 1.0 μg/ml puromycin, incubation was continued for 1 week. Supernatant was measured for p24 production and at least 20 ng p24 was passaged on new cells containing 70% U373-MAGI-CCR5E and 30% U373-MAGI-$CXCR4_{CEM}$ and treated as above. Subsequent passages were performed with increasing amounts of U373-MAGI-$CXCR4_{CEM}$ up to 90%, each passage was cultured for 1 week. p24 positive supernatant was tested in a MT-2 assay. MT-2 cells only express CXCR4 as a co-receptor and if infected have the ability to form syncytia. MT-2 culture was continued for three weeks and scored twice weekly on syncytia formation.

**RESULTS**

The 2.9% CXCR4-predicted minority of the viral population observed prior to SCT consisted of seven unique variants with an FPR ranging from 2.7 to 9.3% (X1bp-X7bp). In addition to $Geno2Pheno_{[coreceptor]}$[7] we used three other prediction algorithms (position-specific scoring matrix ($PSSM_{X4-R5}$), net charge rule and 11/25 rule)[8] to assess co-receptor tropism (Table 1). Of the seven variants predicted to be CXCR4-tropic by $geno2pheno_{[coreceptor]}$ only one strain (X3bp) was predicted to use CXCR4 in all other prediction tools. This viral variant harboured the positively charged amino acid lysine at position 25 of the gp120-V3 loop which is strongly associated with CXCR4 usage[10].

**Table 1. Co-receptor tropism prediction of patient derived gp120-V3 loop sequences obtained prior to SCT.** Schematic representation of gp120-V3 loop amino acid sequences of the patient-derived dominant CCR5-predicted variant (Rbp) and CXCR4-predicted minority variants,

X1bp-X7bp, the control viruses cBaL, cHXB2. Genotypic tropism prediction by geno2pheno$_{[coreceptor]}$ (10% FPR)[7], PSSM$_{X4\text{-}R5}$, net charge rule and 11/25 is given. CCR5-prediction is indicated in green, CXCR4-prediction in red.

| | gp120 V3-loop sequences | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | prediction tools | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| viral construct | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 14;1 | 14;2 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | g2p | PSSM | CHARGE | 11/25 |
| Rbp | C | T | R | P | N | N | N | T | R | K | G | I | H | I | - | - | G | P | G | R | A | F | Y | T | T | G | E | I | M | G | D | I | R | Q | A | H | C | 24,2 | -10,55 | 3,1 | R5 |
| X1bp | · | A | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | 9,3 | -10,35 | 3,1 | R5 |
| X2bp | · | · | · | · | · | · | K | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | 2,7 | -8,09 | 4,1 | R5 |
| X3bp | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | K | · | · | · | · | · | · | · | · | · | · | 5,0 | -6,27 | 5,1 | X4 |
| X4bp | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | R | · | · | · | 6,8 | -9,32 | 4,1 | R5 |
| X5bp | · | · | · | · | · | · | S | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | 8,5 | -9,88 | 3,1 | R5 |
| X6bp | · | · | · | · | · | · | D | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | 7,8 | -9,19 | 2,1 | R5 |
| X7bp | · | · | · | · | · | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | 5,3 | -8,45 | 3,1 | R5 |
| cBaL | · | · | · | · | · | · | · | · | · | · | S | · | · | · | · | · | · | · | · | · | · | · | L | · | · | · | · | · | I | · | · | · | · | · | · | · | · | 51,8 | -9,79 | 3,1 | R5 |
| cHXB2 | · | · | · | · | · | · | · | · | · | · | R | · | R | · | Q | R | · | · | · | · | · | · | V | · | I | · | K | · | I | · | N | M | · | · | · | · | · | 0,0 | 3,27 | 10,0 | X4 |

We investigated viral replicative capacity of these variants by cloning of the patient derived gp120-V3 loop sequence in the backbone of the CXCR4-tropic HXB2 reference strain. The dominant CCR5-predicted and five of the seven CXCR4-predicted strains among which the single viral variant predicted to be CXCR4-tropic by all four algorithms (X3bp) were replication competent (Figure 1a).

Subsequently, co-receptor usage was assessed in MAGI cell lines expressing either CCR5$^{+}$ or CXCR4$^{+}$ as co-receptor[9]. The dominant CCR5-predicted strain from the patient

(Rbp) and the CCR5-tropic control virus (cBaL) were able to infect the cells expressing CCR5 but not those expressing CXCR4 (Figure 1b). The CXCR4-tropic reference strain (cHXB2) was only able to infect CXCR4 expressing cells reflecting a full CXCR4-phenotype. Remarkably, all patient-derived CXCR4-predicted variants, including X3bp, replicated only in cells expressing CCR5, demonstrating CCR5 dependence. (Figure 1b).

As co-receptor preference may differ in cell lines versus natural target cells that express both co-receptors we also assessed co-receptor usage in PBMCs[6]. We used maraviroc (MVC) a CCR5 antagonist to mimic the CCR5Δ32/Δ32 phenotype. Likewise we used AMD-3100 to block CXCR4 co-receptor binding. As expected, the dominant patient-derived CCR5-predicted strain and cBaL were fully inhibited by MVC and not by AMD-3100 (Figure. 3c), reflecting a full CCR5 phenotype. The control virus cHXB2 was completely inhibited by AMD-3100 and no effect of MVC was observed (Figure. 1c). Interestingly, the CXCR4-predicted variants were not inhibited by AMD-3100 but demonstrated CCR5 dependence in natural target cells, as shown by complete inhibition of replication by MVC (Figure. 1c).

Finally, we investigated whether the viral constructs could replicate in post-transplant derived CCR5-Δ32/Δ32 PBMCs of the Berlin Patient. These experiments showed that the Berlin Patient's new target cells can be infected by a CXCR4-tropic reference strain, but not with the pre-SCT patient-derived viruses. (Figure 1d).

Given the presence of long-lived CCR5 positive macrophages[2] we explored the potential to evolve towards CXCR4-usage by performing cell

selection experiments. The CCR5-tropic control virus cBaL and the patient-derived Rbp, X2bp and X3bp were cultured in the presence of increasing percentages of CXCR4-expressing cells (0-90%). After 10 weeks of selection no co-receptor switch was observed, suggesting a relatively high genetic barrier towards CXCR4-usage.

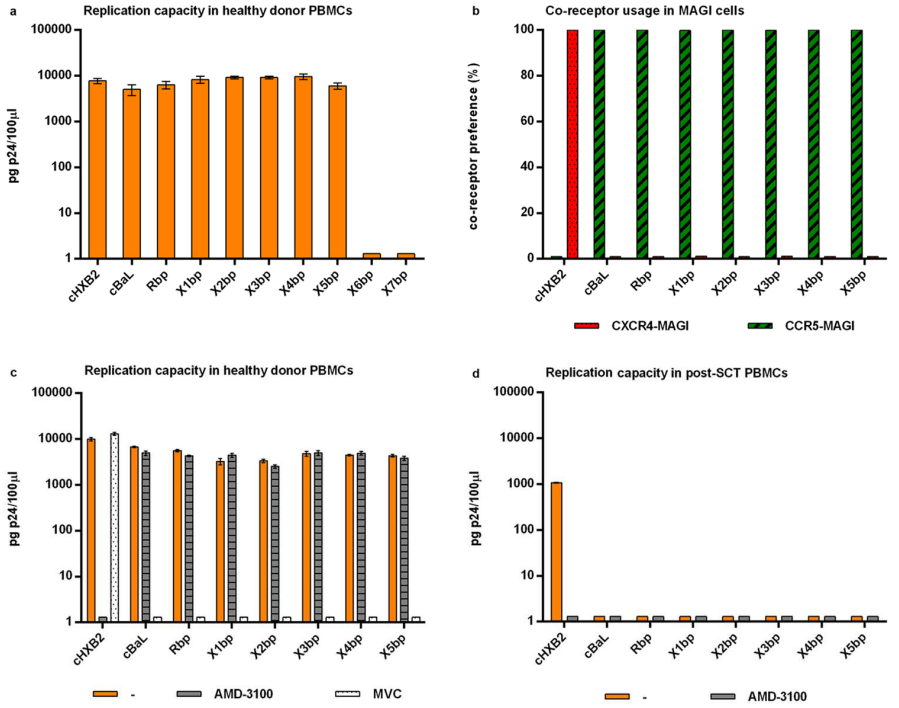



**Figure 1. Replication capacity and phenotypic co-receptor usage.** (a) Replication capacity analysis in PBMCs from healthy donors expressing both co-receptors CCR5 and CXCR4 (picogram p24/100µL supernatant at day 7). (b) Co-receptor usage was determined by expressing the percentage of viral entry in CXCR4 or CCR5 U373-MAGI cells. (c) Replication capacity in PBMCs from healthy donors with 1 µM MVC (CCR5 antagonist) or 10 µM AMD-3100 (CXCR4 antagonist) or without inhibitor (picogram p24/100µL supernatant at day 7). (d) Replication capacity in patient derived PBMCs obtained after allogeneic homozygous CCR5-Δ32 SCT as tested with 10 µM AMD-3100 or without inhibitor (picogram p24/100µL supernatant at day 7). cHXB2 and cBal were used as control viruses. Rbp is the dominant CCR5-predicted viral construct and X1bp-X5bp are the viable Geno2Pheno$_{[coreceptor]}$ X4-predicted viral constructs. Error bars are s.d. of quadruplicate wells. All experiments were performed at least two times and the figures are based on one representative experiment.

**Discussion**

The Berlin Patient is the first patient with a cured HIV infection. Prior to SCT a minority population of viruses was predicted to use the alternative CXCR4 co-receptor[1]. Considering the normal levels of CXCR4 co-receptor expression on the donor-derived cells and susceptibility of these cells for CXCR4-tropic virus it was remarkable that HIV did not rebound post-SCT in the absence of cART[2]. Our study demonstrates that CXCR4-predicted minority viruses present prior to transplantation were unable to rebound after transplantation due to their dependence on CCR5 for replication and high genetic barrier towards CXCR4-usage.

The main determinant of co-receptor usage is the gp120-V3 loop. Genotypic tropism algorithms use this region to predict co-receptor tropism. In clinical practice conservative cut-offs which show good correlations with virological outcome during CCR5-inhibitor based therapy are generally applied to prevent underestimation of the presence of CXCR4-tropic viruses (FPR cut-offs 3.5-10%)[7,11]. We have previously shown that viral variants with an even lower FPR (<2% ) are selected during CCR5-inhibitor therapy failure, suggesting that only viruses with an extremely low FPR use the CXCR4 co-receptor *in vivo*[12]. These data are in line with the observed CCR5 dependence of the viral clones of the Berlin Patient prior to SCT (lowest FPR 2.7%)[12]. Our observations suggest that in case of a CCR5Δ32-SCT procedure a more lenient FPR and/or phenotypic testing should be applied.

Envelope regions outside the gp120-V3 sequence can modulate co-receptor affinity[13]. Unfortunately, prior to SCT full envelope sequences were not generated from the Berlin Patient and no additional samples were stored. Although there is only limited evidence that the envelope background may impact actual co-receptor preference we cannot exclude that the patient envelope background could have influenced co-receptor usage. In absence of patient derived full envelope sequences we decided to clone the gp120-V3 loop sequences of the Berlin Patient in the background of a CXCR4-tropic HIV-1 lab strain to limit bias for CCR5 usage.

In the Berlin Patient, CCR5-tropic permissive cells could still be detected for at least 5.5 months in the colon and proviral-DNA was observed two months after SCT[1,2]. Recently, more than five years post-SCT, very low levels of HIV DNA and RNA were intermittently detected using very sensitive assays[5]. Given the volatile combination of long-lived CCR5-expressing cells and a potential CCR5-tropic reservoir, HIV replication may have continued. Residual replication of CCR5-tropic viruses in the setting of increasing numbers of $CCR5^{-}$ $CXCR4^{+}$ cells could result in evolution towards CXCR4 usage. We tested the evolution potential for the most CXCR4-predicted patient-derived viruses and

did not observe viral evolution towards CXCR4 usage, suggesting a relatively high barrier for co-receptor switch.

Aside from absence of CXCR4-tropic virus, the lack of a rebound of CCR5-tropic virus immediately post-SCT in the absence of cART indicates that transplantation of CCR5Δ32/Δ32 stem cells was pivotal to the apparent cure of HIV of the Berlin Patient. Though, a recent study in which two HIV infected heterozygote CCR5Δ32 patients with a small viral reservoir received CCR5WT/WT SCT demonstrated that also prolonged cART might be sufficient to clear the HIV infection[14]. Additional studies are warranted to investigate the different factors involved in HIV eradication after SCT.

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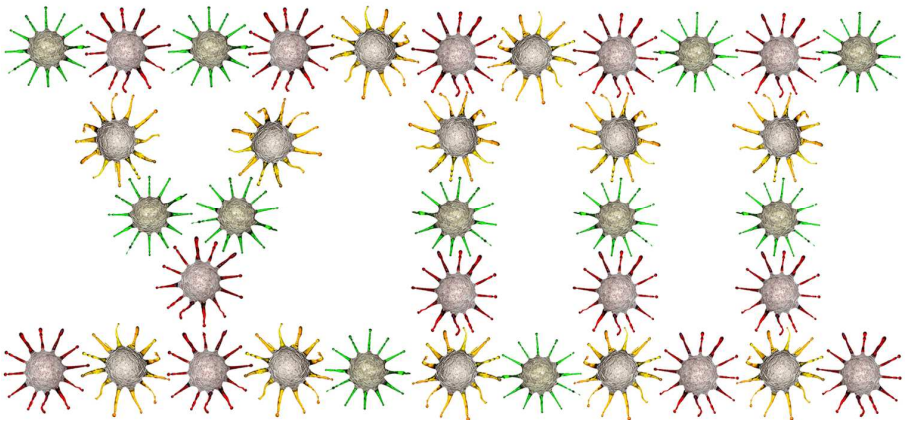
**Chapter 8**

**Summary, general discussion and future perspectives**



The introduction of combination antiretroviral therapy (cART) in 1996 has significantly reduced HIV related morbidity and mortality in the Western world[1]. Recent advances in antiretroviral treatment have resulted in a life expectancy of effectively treated HIV infected patients, comparable to those having other chronic diseases[2,3].

There is a group of patients that have a slow progression towards AIDS if they are untreated. The presence of a heterozygous 32 nucleotide deletion in one of the CCR5 co-receptor genes is often seen in these long term non-progressors[4,5]. This CCR5Δ32 results in a truncated gene product which is not expressed at the cellular surface. Homozygous CCR5Δ32 individuals are healthy and are largely resistant to HIV infection[4,5]. These observations lead to the development of CCR5 antagonists as antiretroviral drugs. Maraviroc (MVC) is the first antiretroviral drug in clinical practice that targets this host chemokine receptor and only inhibits viral entry of CCR5 using HIV[6]. One important consequence of the administration of CCR5 antagonists is the risk of selection of viral variants capable of using the alternative CXCR4 co-receptor, therefore, co-receptor tropism should be assessed before clinical use of the inhibitor[7,8]. Furthermore, given the redundancies in the immune system, CCR5 is not critical for normal immune function and CCR5 based HIV curative strategies are now widely investigated[9].

In this thesis we investigated the effect of inhibiting HIV binding to the CCR5 co-receptor on HIV treatment, pathogenesis and HIV cure. In this chapter the impact of these findings for CCR5 based antiretroviral therapy and HIV cure strategies are discussed in a broader perspective.

**Predicting clinical outcome of MVC containing therapy and the added value of genotypic tropism testing performed in triplicate**

We assessed the clinical outcome of MVC containing regimens in a heterogeneous, heavily pre-treated cohort of HIV-1 infected patients in **Chapter 2**. Treatment with MVC was well tolerated and resulted in a good virological and immunological response. To our knowledge, this is the first study in which three different HIV co-receptor tropism assays have been evaluated on the predictive value of clinical outcome. The two phenotypic tropism assays (OTA/ESTA and MT-2 assay) and genotypic tropism testing had comparable predictive value for virological and immunological outcome (89-100%). In the phenotypic MT-2 assay there is no positive control[10]. Only a result of syncytia inducing gives a solid indication, whereas with a non-syncytia inducing result assay failure cannot be excluded. Furthermore, our data confirms that genotypic population tropism testing is an excellent alternative for predicting HIV co-receptor tropism in clinical practice.

Population based genotypic tropism prediction is based on Sanger sequencing[11]. Sanger sequencing based methods cannot detect variants that

are below 15-20% of the viral population[12]. Therefore, predicted X4-tropic minority variants may remain undetected. Independent genotypic tropism testing performed in triplicate may be better in capturing the relatively high levels of variation in HIV *envelope* and increase the detection of minority variants. In a prospective analysis we evaluated the added value of triplicate versus single testing on genotypic tropism prediction in routine clinical practice, **Chapter 3**. In this study we performed genotypic tropism prediction using the web based algorithm geno2pheno$_{[coreceptor]}$[13] applying different false positive rate (FPR) cut-offs for tropism prediction[14,15].

Genotypic tropism testing in triplicate compared to single testing (10% FPR cut-off[8]) resulted in 4% of viral populations reclassified from a predicted R5-tropic to a predicted X4-tropic viral population. Genotypic tropism testing performed in triplicate rendered these patients ineligible for MVC therapy. Failure to detect predicted X4-tropic minorities increases the risk of selection of X4-tropic viral variants[16]. Since the ability to detect a predicted X4-tropic minority is increased in a triplicate procedure we recommend, if population based genotypic tropism testing is applied, to be performed in an independent triplicate amplification and sequencing procedure.

**The inhibitory effect and the selective pressure of MVC**

In about 50% of HIV infected individuals the viral population switches from CCR5-usage to CXCR4-usage and this event is associated with accelerated CD4$^+$ T-cell decline and progressive HIV disease[17-22]. Recently emerged CXCR4-using variants generally maintain the ability to use CCR5. Viral variants that have the ability to use both co-receptors are dual-tropic. Only as few as one or two mutations in the V3-region are necessary to confer CXCR4 usage *in vitro*[23-27]. However, it was observed that *in vivo* the newly emerged CXCR4 using variants differed in more amino acid substitutions from the coexisting R5-tropic variants. This suggests that the *in* vivo evolution of CXCR4 using virus from the parental CCR5 strain is gradual[28-31].

The inhibitory effect of MVC on dual-tropic variants was unknown. We assessed this effect in detail in a dual-tropic HIV infected patient in **Chapter 4**. Prior to start of MVC, the patient harboured a dominant viral population that was able to utilise the CCR5 and CXCR4 co-receptor in cell-based assays. The dominant viral population could not infect natural target cells (PBMCs) in the presence of MVC. These variants preferred to utilize CCR5 for viral entry and were called dual-R5-tropic. This study demonstrated that MVC not only inhibited R5-tropic viruses but also a range of dual-tropic variants.

*In vitro* cell selection experiments in which several R5-tropic reference strains were cultured in increasing presence of CXCR4 expressing U87-MAGI cells demonstrated that R5-tropic variants evolved to utilize CXCR4[32]. The first variants that were able to infect CCXCR4 U87-MAGI cells were not able to infect MT-2 cells or PBMCs with a dysfunctional CCR5 co-receptor[32]. The authors

reason that the high density of the CXCR4 co-receptor on U87-MAGI cells enabled these variants to utilize CXCR4[32]. In our study the dual-R5 tropic variants were capable of infecting MT-2 cells. Of note, the density of CXCR4 on MT-2 cells is about 10-fold higher compared to PBMCs[33].

As discussed above, the phenotypic MT-2 assay and genotypic tropism testing were excellent in predicting clinical outcome of MVC containing therapy (**Chapter 2**). Nonetheless, both the genotypic tropism assay and a phenotypic result from a cell based assay could falsely classify virus as true CXCR4 using. As a consequence, MVC could be withheld from patients that would benefit from CCR5-antagonists in combination with an active backbone. Unfortunately, in our patient the absence of an active backbone regimen rapidly facilitated expansion of an X4-tropic minority variant.

The observed rapid selection of X4-tropic variants raised the question of what the selective pressure of MVC was in patients infected with a viral population with a mixed tropism (R5-tropic, dual-tropic and X4-tropic variants). We longitudinally investigated and quantified the selective pressure of MVC on the dynamics and composition of the viral population in four HIV mixed tropism infected patients who had experienced MVC treatment failure (**Chapter 5**). Using 454 deep sequencing methods to predict co-receptor usage, viral tropism was examined in plasma samples of patients that were infected with a viral population predicted to be able to use CXCR4. Alongside the suppression of predicted R5-tropic variants, X4-tropic populations expanded rapidly. In our study, MVC therapy favoured the selection of predicted X4-tropic variants with an extremely low FPR (below 2%). These results together with the observation of full inhibition by MVC of dual-R5 tropic variants (**Chapter 4**) suggest that MVC may be effective in a broader range of HIV variants than currently assumed.

The predicted X4-tropic variants were present in the viral population prior to start of MVC and were rapidly selected after addition of MVC to the cART regimen. Interestingly, the X4-tropic variants expanded to an extent that the total viral load remained nearly constant. The observation of rapid replacement of the R5-tropic viral population by X4-tropic variants after the start of MVC might suggest competition between R5-tropic and X4-tropic variants in the absence of CCR5 antagonists. Competition for a cellular reservoir seems unlikely since a greater proportion of $CD4^+$ T-cells express CXCR4 compared to CCR5 with an approximate ratio of 4:1[34,35]. R5-tropic variants reside mainly in memory ($CD45RO^+$) $CD4^+$ T-cells and X4-tropic variants reside in both naïve ($CD45RA^+$) and memory $CD4^+$ T-cells[36,37]. Even though, R5-tropic and X4-tropic variants seem to have separate cellular reservoirs, two distinct studies have observed that coexisting R5-tropic and CXCR4 using variants undergo frequent recombination events[38,39]. Of note, co-receptor tropism of these variants was assessed in MT-2 or U87-MAGI cells and therefore these variants might represent variants still able to utilise CCR5[38,39]. The abundant high viral load of R5-tropic virions prior to start of MVC may out

compete X4-tropic variants for the binding of the CD4 receptor on target cells even if these do not express CCR5. It was suggested that dual-tropic viral variants or CXCR4-tropic variants have a lower *in vivo* viral fitness due to a lower replication capacity, decreased co-receptor binding affinity and higher susceptibility for inhibition by co-receptor ligands[32]. *In vivo* viral fitness is also influenced by the level of antibody neutralisation. It has been postulated that X4-tropic variants are more sensitive to neutralisation by antibody binding to the CD4 binding site of gp120[40-42]. However, a higher sensitivity to neutralising antibodies would likely result in a lower viral setpoint of the selected X4-tropic variants. In this study the X4-tropic variants replicated to a similar extent in the presence of MVC as the R5-tropic variants prior to MVC. In future studies it would be of interest to investigate the difference in antibody neutralizing capability and co-receptor ligand sensitivity of R5-tropic variants and X4-tropic variants that are selected during MVC therapy failure. The factors that are involved in the competition between R5-tropic and X4-tropic variants remain elusive and are in need of further investigation.

The emergence of CXCR4-using HIV variants has long been hypothesized to be the cause of accelerated immune deterioration. In our study the X4-tropic variants were present prior to MVC therapy but only expanded rapidly when MVC therapy was initiated. This suggests that X4-tropic variants are not driving disease progression but are able to emerge due to environmental changes within the host. In two MVC trials remarkable observations were seen concerning co-receptor tropism switch in support of this hypothesis. MVC failure was observed in the MOTIVATE 1 and 2 trials, which assessed MVC efficacy in 1049 treatment experienced patients[44]. In these trials 63 patients failed with a virus able to utilize CXCR4 which were all present prior to MVC initiation[44]. After the cessation of MVC the viral population in 68% of the patients reverted to R5-tropic dominance[45]. Similar observations were made in a clinical trial with the CCR5 antagonist vicriviroc[45]. This data suggest that although CXCR4-using variants can replicate and reach similar viral loads during therapy failure the environment in the hosts at that time favoured the infection with a R5-tropic viral population.

**The virological effect of MVC intensification in immunologic non-responders**

Inhibition of CCR5 by MVC does not only inhibit R5-tropic viral entry in the cell but also inhibits binding and signalling of the CCR5 ligands MIP-1α, MIP-1β and RANTES of many CCR5 positive cells such as $CD4^+$ T-cells, dendritic cells, monocytes and macrophages[46]. Given the redundancies in the immune system interference with the CCR5 pathway may have consequences for different cellular pathways. The CCR5 ligands can also bind and signal through CCR1, CCR3 and CCR4[47-49]. Augmented signalling through these receptors might have an influence on cellular functions and ultimately virological parameters such as HIV transcription. Our study was focused on the effects of

MVC intensification on virological parameters. The 15 patients included participated in a larger placebo controlled trial of 85 patients determining the effect of MVC intensification on immunological parameters in immunologic non-responders. Over the 48 weeks of this study no overall effect was seen in $CD4^+$ T-cell change or cell activation by MVC intensification[52]. In **Chapter 6** we investigated in detail the effect of MVC intensification on virological parameters in these 15 patients using cutting-edge ultra-sensitive digital droplet PCR techniques. Overall, during the entire study length of 48 weeks of intensification we observed no virological effect of MVC versus intensification with placebo. Of interest, during the first eight weeks we observed a significant difference between the MVC group and placebo group of the study in relative HIV RNA expression with a slight increase in the MVC group and a slight decrease in the placebo group. Augmented signalling mainly through CCR4 may increase intracellular NF-κB levels and thereby induce HIV transcription[50]. However, this does not fully explain the demonstrated effects in the first eight weeks of the study. The observed increase in MIP-1β by Hunt *et al.* plateaued at week 4 and remained high until the end of the study at week 24[51]. Our findings in the first eight weeks warrants further analysis in the early effects on MVC intensification on the in the larger trial with 85 patients.

**HIV co-receptor usage explains absence of X4-tropic viral rebound in the first patient cured of HIV**

The Berlin Patient is the first patient with a cured HIV infection. After diagnosis of acute myeloid leukaemia the patient underwent a stem cell transplantation with homozygous CCR5Δ32 stem cells[53]. Cells derived from these stem cells are therefore resistant to infection with a R5-tropic HIV-1 variant[54]. Directly following SCT the Berlin Patient stopped cART[53,54]. Prior to SCT the patient harboured a minority population (2.9%) of viruses predicted to use the CXCR4 co-receptor[53]. Considering the susceptibility of donor derived cells for CXCR4-tropic virus it was remarkable that HIV did not rebound post-SCT in the absence of cART[53-55]. We investigated the role of co-receptor tropism in lack of predicted X4-tropic viral rebound in the Berlin Patient. Our study, described in **Chapter 7**, demonstrates that predicted X4-tropic minority viruses (FPR 2.7-9.3%) present prior to transplantation were unable to rebound after transplantation due to their dependence on CCR5 for replication. To assess co-receptor tropism we generated recombinant viruses based on the V3-loop sequence of the seven predicted X4-tropic strains and the dominant predicted R5-tropic strain. The main determinant of co-receptor usage is the V3-loop[56,57]. Nevertheless, other regions outside this V3 loop such as the overall positive charge of the gp120, the number of potential N-linked glycosylation sites (PNGS) can influence co-receptor affinity. It is suggested that increased positive charge and fewer PNGS enhances co-receptor binding affinity due to the reduced repulsion between gp120 and negatively charged

cellular membrane[58-60]. To avoid an introduced bias for the usage of CCR5 we used the pure X4-tropic HXB2 reference strain as a background for the patient-derived V3-loop sequences. Of the seven X4-predicted strains, five were replication competent in our system. It is unknown if the HXB2 reference strain was compatible with the V3-loops of the two replication incompetent predicted X4-tropic strains. Unfortunately, we could not phenotypically assess one of these replication incompetent X4-tropic strain which harboured aspartic acid at position seven which would result in the loss of the glycosylation site g15 at the stem of the V3-loop. Loss of this glycosylation site is associated with increased CXCR4 binding affinity[61].

Besides the lack of CXCR4 using variants in the Berlin Patient several other hypotheses for cure in the Berlin Patient have been postulated. Firstly, myeloablative conditioning with chemotherapy and total body irradiation could have contributed to the cure of HIV by destroying the latent reservoir and reducing the numbers of susceptible activated $CD4^+$ T cells[62]. It was hypothesised that the number of residual CCR5-expressing $CD4^+$ cells was too low after SCT to support replication of the R5-tropic variants and therefore evolution toward CXCR4 usage[62]. On top of that, we demonstrated an intrinsic high genetic barrier for co-receptor switch of the predicted X4-tropic strains. In a cell selection assay, these predicted X4-tropic viral variants were cultured in increasing amounts of CXCR4 expressing cells and no co-receptor switch was demonstrated (**Chapter 7**). The Berlin Patient experienced a relapse of myeloid leukaemia and the patient underwent a second SCT demonstrating that myeloblative conditioning was not absolute[54]. Secondly, ongoing graft-versus-host disease (GHVD) may have acted to clear latently infected cells and residual target cells[62]. Nonetheless, HIV proviral DNA could still be detected 61 days post initial SCT and CCR5 expressing HIV target cells were still seen in the gut half a year post SCT[53,54]. The contributions of these factors to cure are in need of further investigation. However, it seems likely that in the absence of antiretroviral treatment the CCR5Δ32/Δ32 genotype of the stem cell and the lack of CXCR4 using HIV were pivotal in the cure of the Berlin Patient.

***Future perspectives***

**Co-receptor tropism prediction**

Although MVC is relatively well tolerated and resulted in a good virological and immunological response (**chapter 2**), MVC is only registered for therapy experienced patients in Europe. The MERIT trial compared the safety and efficacy of MVC versus efavirenz in R5-tropic HIV infected drug naïve patients. In the original analysis MVC did not meet the non-inferiority criteria[63]. However, co-receptor tropism was analysed with the original trofile

assay (OTA) and reanalysis of baseline viral co-receptor tropism with the more sensitive trofile assay (ESTA) or 454 ultra-deep sequencing would have excluded about 15% of the patients and MVC would have been non-inferior to efavirenz[64]. This example stresses the use of an accurate phenotypic or genotypic co-receptor tropism assay.

We demonstrated in **chapter 4** that some dual-tropic viral variants can utilize CXCR4 in cell lines but are dependent on CCR5 for viral entry in natural target cells. To accurately determine if MVC would be of benefit in patients that harbour these viral variants, the phenotype has to be determined in natural target cells. In a standard diagnostic setting this is not feasible since patient derived PBMCs need to be co-cultured with healthy donor derived PBMCs over a period of three weeks, after which $TCID_{50}$ needs to be determined and subsequently replication capacity in the presence of CCR5 and CXCR4 antagonists needs to be assessed. This method, however, will give insight into phenotypic co-receptor usage which can then be correlated to the genotypic prediction. On the other hand, the phenotypic assays used in **chapter 2** were excellent in predicting clinical outcome of MVC containing antiretroviral therapy.

Genotypic tropism testing demonstrated excellent predictive value in MVC clinical outcome (**chapter 2**). Furthermore, when performed in triplicate it increases the likelihood of identification of an X4-tropic minority variant (**chapter 3**). Still, it is unknown to what extent the triplicate procedure can detect minority variants. The presence of as little as 2% X4-tropic virus (FPR <3.5%) in the population increases the risk of MVC therapy failure[65,66]. A different approach of genotypic tropism testing is sequencing of the V3-loop via ultra-deep sequencing methods. Ultra-deep sequencing can detect minorities below 1% of the viral population[67,68]. It has been suggested that ultra-deep sequencing increases the detection of minority variants predicted to be X4-tropic compared to population based genotypic tropism testing in triplicate[69]. However, the sample size in this study was relatively small (N=12)[69] making it difficult to draw general conclusions from this study. Studies that compare genotypic tropism testing in triplicate and ultra-deep sequencing with clinical outcome of CCR5 antagonist based therapy are warranted to determine superiority of one or the other method. Currently, ultra-deep sequencing has several disadvantages over population based sequencing in a routine diagnostic setting. It is associated with higher cost and higher technical difficulty compared to population based Sanger sequencing. However, this is rapidly changing and since ultra-deep sequencing enables analysis of minority variants at exceptional resolution which could shed light on the clinical significance of detecting minute minority predicted X4-tropic variants and dynamics of the viral population within the host when an antiretroviral drug is initiated. Ultra-deep sequencing will likely be more generally applied in predicting HIV co-receptor tropism in the near future in a research and diagnostic setting. Furthermore, the optimal cut-of for R5-classification in the generally used

geno2pheno$_{[coreceptor]}$ web based algorithm in not fully explored[8]. Our observations in **chapter 5** suggest that the FPR cut-off could be lower than currently used which could lead to a broader use of CCR5 antagonists.

**Co-receptor switch in untreated patients**

Given the high viral turn over and high error rate of reverse transcriptase a rapid emergence of CXCR4 using viral variants could be expected, especially considering that only about 20% of $CD4^+$ T-cells express CCR5 whereas 90% express CXCR4[33,35]. However, a co-receptor switch was typically observed around eight years after seroconversion in about 50% of untreated patients[70,71]. The co-receptor switch is thought to be a gradual process with dual-tropic variants as intermediates between the parental R5-tropic strain and the X4 tropic strain. It has been observed that dual-tropic variants have decreased CCR5 and CXCR4 binding affinity[32,72]. Furthermore, dual-tropic variants were more susceptible to competitive inhibition of viral entry by natural ligands of these co-receptors[72-74]. The natural course of HIV disease is often accompanied with high levels of immune activation which is associated with high levels of CCR5 ligand expression[5,76]. Additionally, at the primary site of HIV infection, secondary lymphoid structures, there are high levels of CXCR4 ligand expression[77-79]. Since dual-tropic variants are more sensitive to inhibition by natural co-receptor ligands there is high inhibitory pressure against these variants to emerge. It has been shown that ongoing immune erosion and scarification of lymph nodes reduces the level of CXCR4 ligand expression[78]. This suggest that the emergence of CXCR4 using variants is an effect of immune deterioration.

**Co-receptor switch during MVC containing therapy**

Co-receptor switch from CCR5 to CXCR4 usage has been observed during CCR5 based antiretroviral therapy (**chapter 5**). Interestingly, it has been demonstrated in an *in vitro* analysis that dual-tropic variants were more susceptible to CCR5 inhibitors and CXCR4 inhibitors[32]. Our results and that of others suggest that therapy failure with X4-tropic variants during CCR5 antagonist based therapy is not due to the generation of novel X4-tropic variants but merely results from the selective outgrowth of a pre-existing X4-tropic reservoir[43,44,80]. Several studies suggested that the loss in replication capacity *in vivo* is too high of a burden for the parental R5-strain to evolve towards CXCR4 usage during CCR5 antagonist containing antiretroviral treatment (Figure 1)[81-82].

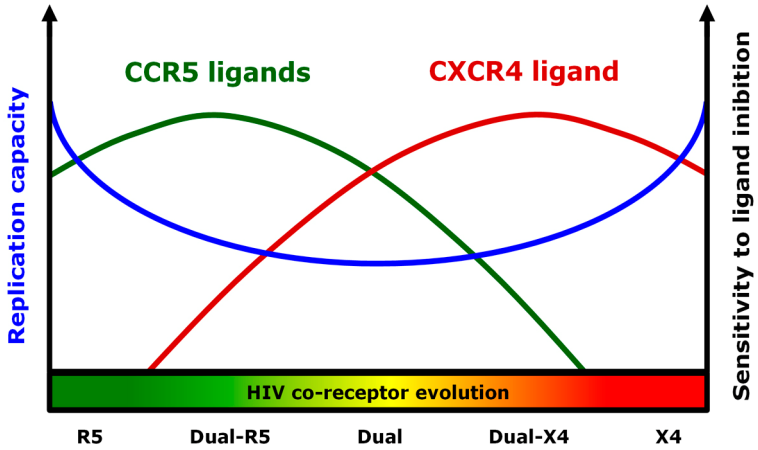



**Figure 1.** Schematic over view of gradual co-receptor tropism and replication capacity in blue and sensitivity to CCR5 ligands in green and CXCR4 ligand in dark red.

HIV can also escape CCR5 antagonists by selection of mutations in the gp160 molecule that enable utilisation of the CCR5-antagonist bound complex[83], although this is less frequently observed compared to failure with an X4-tropic variant. In the MOTIVATE trials 1 and 2 which included 1049 treatment experienced patients, 133 patients experienced therapy failure in the MVC arm of which the majority failed with an X4-tropic viral population[43,44]. Of the patients with therapy failure, 17% had an R5-tropic MVC resistant strain at failure. These variants tended to emerge later compared to X4-tropic variants which are thought to emerge from pre-existing reservoirs[44].

It is tempting to speculate that a viral population can more easily accommodate the evolution towards R5-tropic MVC resistance than evolution towards CXCR4 usage. The drop in replication capacity is less of a burden for evolution towards R5-tropic MVC resistant variants than to evolve towards CXCR4 usage during CCR5 antagonist based therapy.

Interestingly, two independent studies observed that about 7% of CCR5 antagonist naïve patients harboured mutations in the V3-loop associated with R5-tropic resistance to MVC[84,85]. The most abundant mutation observed was the 26V mutation which is strongly associated with R5-tropic resistance to MVC[85]. Furthermore, this mutation was more frequently detected in patients that where infected longer and therapy experienced but CCR5 antagonist naïve[85]. The clinical relevance of these pre-existing mutations is hard to determine since the variable loop is highly polymorphic. Since X4-tropic variants and the main mutation associated with R5-tropic resistance arise relatively late during the course of infection it might be more sensible to consider to start MVC earlier during HIV infection.

**CCR5 in HIV curative strategies**

Current HIV treatment strategies are not curative and several alternative approaches including stem cell transplantation, gene therapy and anti-HIV latency drugs are investigated as an approach towards HIV cure. Based on our observations from **chapter 6** it is tempting to speculate that increased HIV RNA transcription, possibly induced by MVC[87], may result in an increased presentation of HLA-class I HIV epitopes. This could enhance recognition of HIV infected cells by cytotoxic T-lymphocytes[88-90]. In support of this hypothesis, a significant increase of $CD38^{+}HLA\text{-}DR^{+}$ rectal $CD8^{+}$ T-cells in patients receiving MVC was observed in a small subanalysis of a larger trial performed by Hunt *et al.*[52]. Unfortunately, no virological analysis was performed in rectal tissue from these patients and the increase $CD8^{+}$ T-cell activation could not be associated with increased HIV transcription[52]. The observed *in vitro* upregulation of CD69[91] together with our observation of a difference in relative HIV RNA transcription and observed increased $CD8^{+}$ T-cell activation[52] could possibly indicate an HIV anti-latency side effect of MVC and warrants further investigation.

The Berlin Patient is the first patient who was cured of an HIV infection[53-55]. The CCR5Δ32/Δ32 stem cells that this patient received were fully resistant to the viral population prior to SCT and were pivotal in this curative strategy in which antiretroviral therapy was stopped at the moment of SCT (**chapter 7**). Recently two other patients have been described who underwent stem cell therapy and are currently reported to be off cART[92,93]. There are several important differences between these patients in the approach to cure of the malignancy (Table 1). The two patients named the Boston Patients underwent SCT with wild type CCR5 stem cells, CCR5WT/WT[92]. Moreover, cART was continued during the entire procedure and they remained on cART for 2-5 years post SCT[92]. Although it is premature to call these patients cured it has been reported that they did not experience a virological rebound shortly after cessation of cART[93]. A common feature among the Berlin and the Boston patients is that they themselves were heterozygous for CCR5Δ32. Prior to SCT both Boston patients had a relatively low viral reservoir (<150 total HIV DNA cp/$10^{6}$ PBMCs)[92], comparable to the levels detected at baseline in the four CCR5WT/Δ32 patients in our maraviroc intensification study (**chapter 6**). The levels of HIV DNA prior to SCT were not reported for the Berlin Patient. The possible low levels of HIV DNA in these patients could have played a major part in the lack of viral rebound immediately after SCT. The importance of CCR5WT/Δ32 in CCR5 based curative strategies is further underscored by the observation in a phase I clinical trial of CCR5-zinc-finger nucleases. Patients were treated with a CCR5-zinc finger nuclease (SB-728-T)[94]. Six patients underwent a 12 week cART interruption. One patient, heterozygous for CCR5Δ32, reached undetectable HIV RNA plasma levels by the end of treatment interruption[94]. Interestingly, in this particular patient the

highest levels of CCR5 modification was attained[94]. CCR5 based curative strategies so far have shown potential in heterozygous CCR5Δ32 infected individuals. This deletion is present in 20% of the Caucasian population but hardly present in people of African or Asian descent[95]. Collectively, these observations warrant further investigation in the role of the human CCR5Δ32 status with respect to CCR5 based curative strategies.

**Table 1. Characteristics of three HIV infected patients who received allogeneic SCT. Berlin Patient and the two Boston Patients.**

| Patients | Indication for SCT | Route of HIV-1 infection | Treatment years prior to SCT | CCR5 genotype patients | Stem cell therapy | CCR5 genotype stem cells | Conditioning therapy | GVHD prophylaxis and treatment | GVHD | Therapy cessation after initial SCT |
|---|---|---|---|---|---|---|---|---|---|---|
| Berlin Patient | Acute myeloid leukaemia | Sexual | 4 | **CCR5WT/Δ32** | allogeneic HSCT | **CCR5Δ32/Δ32** | ATG, CX, TBI | MMF, CS | Yes | Day 0 |
| Boston patient A | Hodgkin's Lymphoma | Perinatal | 3-4 | **CCR5WT/Δ32** | allogeneic HSCT | **CCR5WT/WT** | ICE | MTX, SIR, TAC. PRED, EXP | Severe | 5 years |
| Boston patient B | B-cell Lymphoma Hodgkin's disease | Sexual | 7 | **CCR5WT/Δ32** | sibling donor RIC allogeneic HSCT | **CCR5WT/WT** | ICE | MTX, SIR, TAC. PRED | Yes | 2 years |

Abbreviations SCT = stem cell transplantation, GVHD = graft versus host disease, HSCT = hematopoietic stem cell transplantation, ATG = rabbit antithymocyte globuline, CX = amsacrine, fludarabine, cytarabine and cyclophosphamide, TBI = total body irradiation, MMF = mycophenonlate mofetil, CS = cyclosporine, RIC = reduced intensity conditionin, ICE = ifosfamide, carboplatin, and etoposide, MTX = minimethotrexate, SIR = sirolimus, TAC = tacrolimus, PRED = prednisone and EXP = extracorporeal photopheresis

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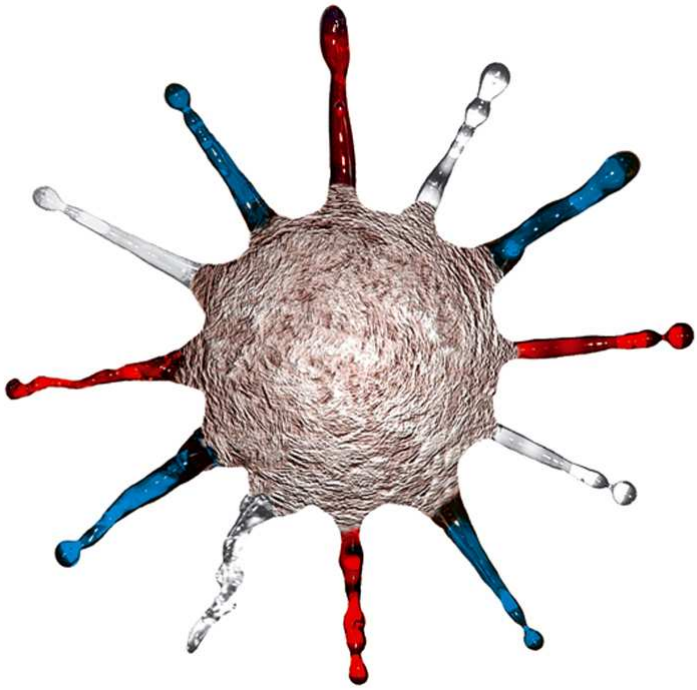
# Nieuwe inzichten in het effect van CCR5 inhibitie op HIV behandeling, pathogenese en genezing



Het humaan immunodeficiëntie virus (HIV) is een retrovirus en de veroorzaker van AIDS. Meer dan 35 miljoen mensen zijn overleden ten gevolge van een HIV infectie sinds de ontdekking van het virus in 1983 en tegenwoordig leven er ongeveer 35.3 miljoen mensen met een HIV infectie. HIV infectie zorgt er in het algemeen voor dat $CD4^+$ T-lymfocyten afnemen, dit resulteert in het verlies van cellulaire immuniteit en de ontwikkeling van AIDS.

Om een cel te infecteren moet HIV via het glycol-eiwit gp120 binden aan receptoren van de cel. Vervolgens fuseert het virus deeltje met het celmembraan en wordt de inhoudt van HIV in de cel gedeponeerd. In de cel wordt het HIV genoom, dat bestaat uit enkelstrengs RNA, omgezet in dubbelstrengs DNA door het virale eiwit reverse transriptase. Dit dubbelstrengs HIV DNA wordt samen met het virale eiwit integrase naar de celkern getransporteerd waar het integrase het HIV DNA in het menselijk genoom integreert. Als de cel is geactiveerd, wordt er van het HIV DNA in het genoom nieuw HIV RNA afgeschreven. Dit HIV RNA wordt omgezet in een poly-eiwit door de cellulaire machinerie. Twee nieuw afgeschreven HIV genomisch RNA kopieën komen samen met de replicatie eiwitten en structurele eiwitten bij het celmembraan en worden omvat in een niet volledig gevormd capside. Vervolgens komt het onvolwassen virus- deeltje vrij van de cel en worden poly-eiwitten geknipt door het virale protease wat resulteert in een volwassen virusdeeltje dat een nieuwe cel kan infecteren.

**HIV co-receptor tropisme**

De eerste essentiële stap in de HIV levenscyclus is het binnendringen van het virus in de cel. Dit is een meerstappenproces dat afhankelijk is van de chronologische interactie met twee gastheerreceptoren. De binding van gp120 aan de CD4 receptor van de cel induceert een conformatieverandering in gp120 waardoor de co-receptor bindings- plaats uitsteekt. Deze bestaat uit de variabele V1V2 lus, een overbruggingsstuk en de hypervariabele V3 lus. De V3 lus bindt aan de co-receptor. De zeven Transmembraan C-C Chemokine Receptor 5 (CCR5) en C-X-C Chemokine Receptor type 4 (CXCR4) zijn de belangrijkste co-receptoren voor HIV. Viraal co-receptor tropisme wordt gedefinieerd door het gebruik van de co-receptor. HIV dat de CCR5 co-receptor gebruikt, wordt R5-troop genoemd en X4-troop HIV als het CXCR4 gebruikt. HIV dat beide co-receptoren gebruikt, wordt duaal-troop genoemd.
De V3 lus bestaat gewoonlijk uit 35 aminozuren en de aminozuren op positie 11 en 25 hebben de meeste invloed op co-receptor gebruik. Als deze aminozuren positief geladen zijn, bindt de V3 lus beter aan de negatief geladen extracellulaire lus 2 van de CXCR4 co-receptor.

**Co-receptor tropisme gedurende HIV ziekte progressie**

De klinische progressie van HIV ziekte is goed gedocumenteerd en bestaat uit drie fases: de acute fase, de klinische latente fase en AIDS. Uit onderzoek is gebleken dat R5-troop HIV preferentieel wordt over gedragen. Dit blijkt onder

andere uit dat mensen die homozygoot zijn voor een defect in de CCR5 co-receptor grotendeels beschermd zijn tegen een HIV infectie. R5-troop HIV is voornamelijk geassocieerd met de acute fase. In sommige gevallen is er sprake van R5-troop en X4-troop HIV transmisse. Het is echter gedemonstreerd dat de X4-trope HIV varianten verdwenen gedurende de acute infectie. De sterke negatieve selectie voor X4-troop HIV komt bijvoorbeeld doordat cellen in de primaire plekken voor infectie voornamelijk CCR5 tot expressie brengen en er veel ligand van CXCR4 wordt afgeschreven. Binding van CXCL12, CXCR4 ligand, aan de co-receptor inhibeert de binding van X4-troop HIV aan CXCR4.
In de klinische latente fase is continue virale replicatie geassocieerd met een persisterend hoog niveau van immuunactivatie en vernietiging van de lymfeklier- structuur. Dit zorgt ervoor dat er minder CXCL12 wordt afgeschreven in de lymfklieren. Gedurende progressie van HIV ziekte verandert het co-receptor gebruik van R5-troop naar X4-troop in ongeveer 50% van HIV geïnfecteerden die niet behandeld worden. De verandering in co-receptor gebruik wordt geassocieerd met een versnelde afname van $CD4^+$ T-cellen. De derde fase, AIDS, komt op als de T-cellen zoveel afnemen dat opportunistische pathogenen een infectie kunnen veroorzaken.

**Het voorspellen van HIV co-receptor gebruik**

De ontdekking van de twee co-receptoren gaf de moleculaire verklaring voor de verschillende HIV phenotypes. HIV dat CXCR4 kan gebruiken, kan syncytia induceren in cellijnen maar R5-troop virus kan dit niet. Deze eigenschap kan gebruikt worden om het tropisme van een HIV populatie phenotypisch te bepalen.

Aangezien de V3 lus het meest bepalend is in co-receptor gebruik, kan men een genotypisch onderscheid maken tussen R5-troop en X4-troop HIV. Genotypische tropisme-testen die in diagnostische settings worden gebruikt, zijn gebaseerd op de amplificatie en het 'sequensen' van de V3 lus. Met behulp van de gegenereerde V3 lus sequenties wordt via een computerprogramma het HIV tropisme voorspeld. De meest gebruikte programma's zijn PSSMX4-R5 en $\text{geno2pheno}_{[\text{coreceptor}]}$. Het resultaat van $\text{geno2pheno}_{[\text{coreceptor}]}$ wordt gegeven in een vals positieve waarde (FPR); dit geeft de kans weer om een HIV variant vals te voorspellen als een X4-troop virus. De lage waarde voor de grens van R5 of X4 voorspelling verhoogt de specificiteit maar verlaagt de gevoeligheid van een X4-trope voorspelling.

**Inhibitie van HIV binding aan de CCR5 co-receptor**

CCR5 inhibitors zijn de eerste antivirale medicijnen die aan een gastheerreceptor binden. Verschillende CCR5 inhibitoren zoals vicriviroc (VVC), TAK-220 en cenicriviroc, een CCR2/CCR5 antagonist, worden bestudeerd in klinische trials. Tot nu toe is maraviroc (MVC) de enige CCR5 antagonist die beschikbaar is voor klinisch gebruik. MVC bindt specifiek en selectief aan de transmembraan helices van CCR5 waardoor R5-troop HIV niet meer kan binden aan de co-receptor.

In Europa en de Verenigde Staten is MVC toegestaan in reeds behandelde patiënten. In de Verenigde Staten mag MVC ook worden voorgeschreven aan patiënten die nog niet behandeld zijn. Voor beide groepen is het expliciet gesteld dat het virale tropisme eerst moet worden geëvalueerd en alleen kan worden voorgeschreven aan patiënten die alleen geïnfecteerd zijn met een R5-trope HIV populatie.

**Behandeling met MVC en therapie falen**

In verschillende klinische trials is gebleken dat MVC effectief het niveau van R5-troop HIV-1 RNA in plasma verlaagt. Maar MVC heeft nauwelijks virologisch effect in patiënten die met HIV varianten waren geïnfecteerd die CXCR4 konden gebruiken. Ook is er gebleken dat de patiënten die MVC kregen in deze trials een snellere toename van $CD4^+$ T-cellen hadden.

HIV-1 heeft een hoge mutatie snelheid van ongeveer $3.4 \times 10^{-5}$ mutaties per base- paar per replicatiecyclus. Hierdoor kan de HIV populatie zich snel aanpassen aan de omgeving binnen de gastheer. Er zijn twee mechanismen waardoor HIV aan CCR5 inhibitie kan ontsnappen. Ten eerste kan HIV evolueren om de andere co-receptor, CXCR4, te gaan gebruiken om cellen te infecteren. Ten tweede is er een mogelijkheid voor HIV om te evolueren zodat hij het CCR5-antagonist complex kan gebruiken als co-receptor.

In deze thesis hebben we gekeken naar het effect van CCR5 inhibitie van HIV behandeling, pathogenese en genezing.

**Het effect van CCR5 inhibitie op HIV behandeling**

In **hoofdstuk 2** hebben we laten zien dat MVC goed getolereerd wordt door HIV geïnfecteerde patiënten en dat MVC effectief virale replicatie onderdrukt. Ook bleek uit dit onderzoek dat zowel phenotypische als genotypische technieken om het tropisme van de HIV populatie te bepalen uitstekend waren om de uitkomst van MVC therapie te voorspellen. Aangezien het genotypisch voorspellen van HIV co-receptor tropisme makkelijker, sneller en goedkoper is dan phenotypische testen worden er in Europa genotypische testen voor co-receptor gebruik aangeraden. Bij het sequensen van een HIV populatie kan er een minderheidspopulatie gedetecteerd worden als het meer dan ongeveer 15% van de totale HIV populatie uitmaakt. Omdat het missen van X4-trope varianten het risico met zich mee brengt dat MVC therapie niet effectief zal zijn, moet de gevoeligheid van het detecteren van minderheidspopulaties verbeterd worden. Het uitgevoerd Het in drievoud uitvoeren van genotypisch testen van tropisme verhoogt de kans om een X4-trope populatie te detecteren met 4% zoals gezien in **hoofdstuk 3**. Hierdoor is er minder kans op therapie falen. Op basis van dit onderzoek wordt er door ons aangeraden om genotypische co-receptor tropisme testen uit te voeren in drievoud.

**Het effect van CCR5 inhibitie op HIV pathogenese**

CCR5 antagonisten inhiberen per definitie HIV dat CCR5 gebruikt. Het effect van MVC op duaal-troop HIV was nog onbekend. Wij hebben dit detail onderzocht in een HIV geïnfecteerde patiënt in **hoofdstuk 4**. Voor de start van MVC was de patiënt geïnfecteerd met een dominante HIV populatie die beide co-receptoren kon gebruiken in cellijnen. Dit virus wordt dus gedefinieerd als duaal-troop. Maar in aanwezigheid van MVC kon dit virus geen natuurlijke targetcellen infecteren. Het blijkt dat dit virus *in vivo* het gebruik van CCR5 prefereert boven CXCR4 en daarom wordt het duaal-R5 troop genoemd. Deze studie demonstreert dat MVC niet alleen R5-trope virussen inhibeert maar ook duaal-R5 trope virussen. Dit heeft de consequentie dat MVC misschien niet zal worden gegeven aan patiënten die wel degelijk baat zouden hebben met MVC en een actieve achtergrond- therapie. Helaas had de patiënt in deze studie geen goede achtergrondtherapie door een extensief resistentiepatroon van de HIV populatie. In deze patiënt werd de dominante duaal-R5-trope populatie snel vervangen door een X4-trope HIV populatie. De snelle vervanging riep de vraag op wat de selectieve druk was van MVC in patiënten die waren geïnfecteerd met een HIV populatie met een gemengd tropisme (R5-trope, duaal-trope en X4-trope varianten). We hebben in **hoofdstuk 5** longitudinaal de selectieve druk van MVC op de dynamiek en compositie van de virale populatie onderzocht en gekwantificeerd in vier patiënten die MVC therapiefalen ondergingen. We deden dit met ultradiepe sequentiemethoden om co-receptor tropisme te voorspellen. Het bleek dat er naast de suppressie van het niveau van R5-trope HIV varianten een snelle expansie was van X4-trope varianten. In onze studie bleek dat tijdens MVC therapiefalen de HIV populatie een selectie onderging met een uitgroei van extreme X4-trope varianten, met een vals positieve waarde beneden de 2%. Samen met de resultaten uit **hoofdstuk 4** lijkt het erop dat MVC effectiever is in het inhiberen van HIV varianten dan voorheen werd gedacht. Opvallend in deze studie was dat de virale lading in plasma na een initiële afname weer snel toenam naar het naar het niveau van voor MVC start. Er lijkt competitie plaats te vinden tussen R5-trope en X4-trope varianten. Aangezien meer T-lymphocyten CXCR4 tot expressie brengen dan CCR5, lijkt competitie voor cellen onwaarschijnlijk. Een van de andere mogelijkheden is de competitie voor CD4 op T-lymphocyten. R5-troop virus kan de CD4 receptor blokkeren op T-cellen die geen CCR5 tot expressie brengen zodat X4-troop virus deze cellen niet kan infecteren. MVC blokkeert de R5-troop HIV replicatie waardoor het niveau van R5-troop virus daalt en X4-troop CD4 kan binden en cellen kan infecteren.

Het opkomen van HIV dat CXCR4 kan gebruiken, werd lang als een van de oorzaken gezien van versnelde immuunverslechtering. In onze studie blijkt het dat er alleen een expansie van X4-trope varianten werd gezien bij inhibitie van R5-trope varianten. Dit suggereert dat niet X4-trope varianten de drijvende kracht achter ziekte- progressie zijn maar pas opkomen door omgevingsveranderingen in de gastheer. Deze hypothese wordt ondersteund door het feit dat na MVC therapiefalen met X4-trope varianten deze varianten

verdwijnen en worden vervangen door R5-trope varianten als MVC wordt gestopt. De omgeving in de gastheer op dat moment prefereert een infectie met R5-trope HIV.

Aangezien MVC aan de CCR5 receptor bindt, heeft het ook invloed op cellulaire functies. Het inhibeert niet alleen R5-troop HIV maar ook de natuurlijke liganden (MIP-1α, MIP-1β en RANTES) van binding aan CCR5. Deze liganden kunnen ook binden aan andere chemokine receptoren zoals CCR4 en CCR3. Verhoogde signalering door deze receptoren zou cellulaire functies mogelijk HIV transcriptie kunnen beïnvloeden en. Wij hebben het effect van MVC intensivering onderzocht in 15 patiënten met een sub-optimale immunologische respons in **hoofdstuk 6**. Gedurende de gehele studie van 48 weken werd geen verschil gezien in virologische parameters zoals HIV transcriptie. Sommige van onze patiënten hadden een laag HIV reservoir. Deze patiënten hadden een heterozygoot CCR5Δ32 genotype. De deletie van 32 nucleotiden in de CCR5 co-receptor zorgt ervoor dat dit type CCR5 niet tot expressie komt op het cellulaire oppervlak. Het was opmerkelijk dat we wel een significant verschil vonden in de eerste acht weken in HIV transcriptie tussen de placebogroep en de MVC groep. In de placebogroep werd een lichte afname gezien van HIV RNA en in de MVC groep een lichte toename in HIV RNA transcriptie. Verhoogde signalering via CCR4 kan leiden tot een toename in transcriptie- factoren zoals NF-κB en kan daardoor HIV transcriptie induceren. Onze resultaten pleiten voor verder onderzoek naar het effect van MVC op cellulaire functies.

**HIV co-receptor gebruik in de eerste van HIV genezen patiënt**

De Berlijnse Patiënt is de eerste patiënt die is genezen van een HIV infectie. De patiënt onderging een stamcel transplantatie met homozygote CCR5Δ32 cellen en stopte met antivirale therapie. De CCR5Δ32 mutatie zorgt ervoor dat deze cellen resistent zijn tegen een infectie met R5-troop HIV. De virale populatie in de Berlijnse Patiënt bestond voor stamcel transplantatie vooral uit R5-trope varianten. Echter, 2.9% van de virale populatie was genotypisch voorspeld om CXCR4 te gebruiken. De cellen die afkomstig waren van de stamcellen hadden normale niveaus van CXCR4 expressie en konden worden geïnfecteerd met X4-troop HIV. Gezien deze feiten was het zeer opmerkelijk dat X4-troop HIV niet terug kwam na stamcel transplantatie. Wij hebben de rol van HIV co-receptor tropisme in de genezing van de Berlijnse Patiënt onderzocht in **hoofdstuk 7**. Omdat er geen biologisch materiaal of volledige envelope sequentie van de voorspelde X4-trope minderheidspopulatie beschikbaar was, hebben wij de V3 lus van deze varianten gekloneerd in een X4-trope referentie variant. Uit onze studie bleek dat de minderheidspopulatie waarvan was voorspeld dat hij CXCR4 zou gebruiken, volledig afhankelijk was van CCR5 voor infectie. De voorspelde X4-trope varianten konden zowel cellijnen, als T-lymphocyten van gezonde donoren en cellen afkomstig van de Berlijnse Patiënt alleen infecteren via CCR5.

Behalve het feit dat er geen virus aanwezig was voor stamcel transplantatie dat CXCR4 kon gebruiken, zijn er een aantal hypotheses voor de genezing van HIV infectie gepostuleerd. Ten eerste, myeloablatieve conditionering met chemotherapie en totale lichaamsbestraling kan hebben bijgedragen aan genezing door het vernietigen van het latente reservoir en het verlagen van geactiveerde $CD4^+$ T-cellen. De hypothese was dat na stamcel transplantatie te weinig $CD4^+$ cellen CCR5 tot expressie brachten om replicatie van R5-troop HIV te onderhouden en dat daardoor geen evolutie naar CXCR4 gebruik mogelijk was. Bovendien, hebben wij laten zien dat de intrinsieke genetische barrière voor co-receptor omschakeling naar CXCR4 door de voorspelde X4-trope varianten hoog was. In een celselectie experiment, waarin deze voorspelde X4-trope varianten gekweekt werden in een steeds grotere aanwezigheid van $CXCR4^+$ cellen, werd geen co-receptor omschakeling gezien. Verder was er een terugval van myeloide leukemie in de Berlijnse Patiënt wat aantoonde dat myeloablatieve conditionering niet volledig was. Ten tweede is er gepostuleerd *dat* graft-versus-host ziekte latent geïnfecteerde en overgebleven $CCR5^+CD4^+$ cellen verwijderd heeft. Niettemin kon 61 dagen na stamcel transplantatie HIV DNA nog steeds worden gedetecteerd. Ook werden er een half jaar na stamcel transplantatie nog HIV gevoelige cellen gezien die CCR5 tot expressie brachten. De rol van myeloablatieve conditionering en graft-versus-host ziekte in de genezing van de Berlijnse Patiënt moeten verder worden onderzocht. Het lijkt er op dat in de afwezigheid van antivirale therapie en HIV dat CXCR4 kon gebruiken het CCR5Δ32/Δ32 genotype van de stamcellen het belangrijkst was voor genezing van HIV infectie in de Berlijnse Patiënt.

**Co-receptor omschakeling in onbehandelde patiënten en tijdens therapie falen**

Gegeven het feit dat HIV snel repliceert en de vele fouten die reverse transcriptase maakt, zou men een snelle evolutie verwachten van R5-troop naar X4-troop virus, zeker als men in aanmerking neemt dat maar 20% van de $CD4^+$ T-cellen CCR5 tot expressie brengt en 90% CXCR4. Omschakeling van co-receptor gebruik wordt echter meestal pas acht jaar na infectie gezien en maar in 50% van onbehandelde patiënten. Men denkt dat de evolutie van een R5 naar X4-troop HIV een gradueel proces is met duaal-troop HIV als een tussenvorm van tropisme. Verschillende studies hebben geobserveerd dat duaal-troop HIV een verminderde bindingsaffiniteit heeft voor zowel CCR5 als CXCR4 ten opzichte van R4 of X4-trope varianten. Ook is duaal-troop HIV gevoeliger voor competitieve inhibitie van de natuurlijke liganden van de co-receptoren. Gedurende het natuurlijke ziekteverloop van de HIV infectie is er een constant verhoogd niveau van immuunactiviteit. Dit is geassocieerd met een toename in CCR5 ligand expressie. Bovendien is er veel CXCR4 ligand expressie in secundaire lymfeklieren die de belangrijkste plaats zijn van HIV

infectie. Aangezien duaal-trope varianten gevoeliger zijn voor ligand inhibitie is er een grote negatieve selectiedruk tegen het ontstaan van deze varianten uit R5-troop HIV. Uit studies is gebleken dat continue erosie van het immuunsysteem en littekenvorming van lymfeklieren het niveau van CXCR4 ligand expressie significant doet afnemen waardoor X4-varianten zouden kunnen ontstaan. Dit suggereert dat de ontwikkeling van HIV dat CXCR4 gebruikt een effect is van immuunsysteem achteruitgang.

Co-receptor omschakeling van CCR5 naar CXCR4 is geobserveerd gedurende CCR5 antagonist gebaseerde antivirale therapie. Naast verhoogde gevoeligheid voor inhibitie van natuurlijke co-receptor liganden zijn duaal-trope varianten ook gevoeliger voor inhibitie door CCR5 en CXCR4 antagonisten. Onze resultaten in **hoofdstuk 5** en dat van andere studies suggereert dat therapiefalen met X4-trope varianten niet komt door het genereren van nieuw X4-troop HIV maar het resultaat is van selectieve uitgroei van een al bestaand X4-troop reservoir. Verschillende studies suggereren dat het verlies in virale replicatie capaciteit *in vivo* te hoog is om van een R5-trope variant te evolueren naar een variant dat CXCR4 kan gebruiken tijdens CCR5 antagonist gebaseerde antivirale therapie.

HIV kan ook CCR5 antagonist werking omzeilen door selectie van mutaties te ondergaan in envelop die het mogelijk maken om het CCR5-antagonist complex te gebruiken voor infectie. Deze varianten komen later op in vergelijking met X4-trope varianten die uitgroeien uit een bestaand X4-troop reservoir tijdens therapiefalen. Het is verleidelijk om te speculeren dat een virale populatie gemakkelijker evolueert richting R5-trope MVC resistentie dan richting CXCR4 gebruik tijdens MVC therapie. Twee onafhankelijke studies hebben laten zien dat ongeveer 7% van CRR5 antagonist naïeve patiënten mutaties in de V3 lus hadden die geassocieerd zijn met R5-trope MVC resistentie. De belangrijkste van deze mutaties 26V verschijnt pas relatief laat tijdens de HIV infectie. Sinds X4-trope varianten en de belangrijkste R5-trope MVC resistentie mutatie pas laat tijdens de HIV infectie opkomen, is het misschien beter om MVC eerder tijdens HIV infectie voor te schrijven aan patiënten.

**De rol van CCR5 in genezing van HIV**

Huidige strategieën van HIV behandeling zijn niet genezend en verschillende alternatieve benaderingen zoals stamcel transplantatie, gentherapie en antilatentie worden onderzocht als therapie die leidt tot genezing. Op basis van onze resultaten uit hoofdstuk 6 is het aantrekkelijk om te speculeren dat verhoogde HIV transcriptie, mogelijk geïnduceerd door MVC, zou kunnen resulteren in verhoogde presentenstatie van HLA-klasse I HIV epitopen. Dit zou de herkenning van geïnfecteerde cellen door het immuun-systeem kunnen doen toenemen en uiteindelijk de grootte van het HIV reservoir doen afnemen. Ter ondersteuning van deze hypothese hebben verschillende studies een lichte activatie van T-cellen, waaronder cytotoxische

T-lymfocten, gezien onder de invloed van MVC. Het geobserveerde effect van MVC zou misschien een antilatentie effect kunnen zijn en moet verder worden onderzocht.

De Berlijnse Patient is de eerste die genezen is van een HIV infectie. De CCR5Δ32/Δ32 stam cellen waren volledig resistent tegen R5-troop HIV. Recentelijk zijn twee andere HIV patiënten uit Boston beschreven die ook een stamcel transplantatie hebben ondergaan en tegenwoordig leven zonder antivirale therapie. Deze twee patiënten zijn in een aantal opzichten verschillend van de Berlijnse Patiënt. Ten eerste kregen zij therapie tijdens en tot minimaal twee jaar na stamcel transplantatie. Ook kregen zij donor stamcellen met een CCR5WT/WT genotype. De cellen hebben een normale CCR5 expressie. Een zeer belangrijke overeenkomst is dat al deze patiënten zelf een CCR5WT/Δ32 genotype hadden. Voor stamcel transplantatie hadden de twee patiënten uit Boston een laag HIV DNA reservoir zoals gezien bij CCR5WT/Δ32 patiënten uit **hoofdstuk 6**. Helaas is dit niet gemeten in de Berlijnse Patiënt. Lage niveaus van HIV DNA geassocieerd met CCR5WT/Δ32 zouden een belangrijke factor kunnen zijn voor genezing door stamcel transplantatie. Het belang van CCR5ΔWT/Δ32 in behandeling naar genezing wordt verder onderstreept door observaties uit een klinische fase I studie. In deze studie ondergingen HIV geïnfecteerde patiënten gentherapie met een CCR5-zinc vinger nuclease. Dit nuclease zorgt ervoor dat een stuk van het CCR5 gen uit het menselijk genoom wordt geknipt waardoor er geen CCR5 expressie is. Bij zes patienten werd antivirale therapie 12 weken lang gestopt. Een patiënt, heterozygoot voor CCR5Δ32, haalde ondetecteerbare HIV RNA niveaus in plasma. Bovendien werden in deze patiënt de meeste gemodificeerde cellen gezien. Strategieën voor genezing gebaseerd op CCR5 hebben potentie laten zien in heterozygote CCR5Δ32 HIV geïnfecteerde individuen. Deze deletie komt ongeveer 20% voor als hetrozygote mutatie in de Kaukasische populatie maar nauwelijks in personen van Afrikaanse of Aziatische afkomst. Collectief pleiten deze observaties voor verder onderzoek naar de rol van de menselijke CCR5Δ32 status met het oog op CCR5 genezing strategieën.

## Publication list

*Publications*

R. A. McGovern, **J. Symons**, A. F. Y. Poon, P. R. Harrigan, S. F. L. van Lelyveld, A. I. M. Hoepelman, P. M. van Ham, W. Dong, A. M. J. Wensing, M. Nijhuis. Maraviroc treatment in non-R5-HIV-1 infected patients results in the selection of extreme CXCR4-using variants with limited effect on the total viral setpoint. Journal of Antimicrobial Chemotherapy, 2013; 68(9):2007-2014.

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E. G. M Berkhoff, E. De Wit, M. M. Geelhoed-Mieras, A. C. M. Boon, **J. Symons**, R. A. M. Frouchier, A. D. M. E. Osterhaus, G. F. Rimmelzwaan. Fitness costs limit escape from cytotoxic T lymphocytes by influenza A viruses. Vaccine, 2006; 24(44):6594-6596.

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*Publications in preparation*

S.F.L. Van Lelyveld, **J. Symons**, J. R. van Bergen, P. M. van Ham, M. Nijhuis, A. M. J. Wensing, A. I. M. Hoepelman. Clinical outcome of maraviroc containing therapy in HIV-1 infected patients. In preparation.

**J. Symons**, W. de Spiegelaere, A. M. J. Wensing, P. M. van Ham, J. Drylewicz, J. A. M. Borghans, A. I. M. Hoepelman, K. Tesselaar, L. Vandekerckhove, S. F. L. van Lelyveld, M. Nijhuis. Impact of maraviroc therapy intensification on virological parameters in HIV-1 infected patients with a poor immunological recovery. In preparation.

**J. Symons**, L. Vandekerckhove, G. Hütter, A. M. J. Wensing, P. M. van Ham, S. G. Deeks, M. Nijhuis. Dependence on the CCR5 co-receptor for viral replication explains the lack of rebound of CXCR4-predicted HIV-variants in the Berlin patient. Submitted

**Dankwoord**

Het zit erop!!! Op naar een nieuw avontuur!!!

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## Curriculum Vitea

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**Name:** Jori Symons
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*EDUCATION*

**PhD** Title: Novel insights into the effect of CCR5 inhibition on HIV treatment, pathogenesis and cure
Thesis submission: October 2013, thesis defence: January 20, 2014
Department Virology, Medical microbiology, University Medical Center Utrecht, The Netherlands
Supervised by Dr. M. Nijhuis, Dr. A.M.J. Wensing and Prof.dr. E.J.H. Wiertz

**MSc** Biomedical Sciences, Vrije Universiteit Amsterdam, The Netherlands degree awarded in 2009
*Major*: Studying cytotoxic T-lymphocyte escape of influenza A
Supervised by Prof.dr. A. D. M. E. Osterhaus and Dr. G. F. Rimmelzwaan, at Erasmus University Rotterdam,
The Netherlands

*Minor*: Improvement of the single-chain transactivators of the Tet-On gene expression system.
Supervised by Prof.dr. B. Berkhout and Dr. A. T. Das, at Amstredam Medical Center, The Netherlands

*MSc Thesis*: Hepatitis C viral escape from cytotoxic T-lymphocyte recognition; from acute to chronic.
Supervised by Prof.dr. J. M. A. Lange and Prof.dr. Y. van Kooyk, at Amstredam Medical Center, The Netherlands

*PRESENTATIONS*

**Invited lectures**
**2013:** MOMA conference, University Maastricht, The Netherlands

**Oral at conference**
**2013:** 20th International HIV Dynamics & Evolution Utrecht, The Netherlands
**2012:** 19th International AIDS conference, 2012, Washington DC, USA
Royal Dutch Assoc. for Microbiology, annual spring meeting, The Netherlands
**2011:** Royal Dutch Assoc. for Microbiology, annual spring meeting, The Netherlands

**Poster at conference**

| | |
|---|---|
| **2013:** | 20th International HIV Dynamics & Evolution, Utrecht, The Netherlands |
| **2012:** | 19th International AIDS conference, Wachington DC, USA<br>19th Conference on Retroviruses and Opportunistic Infections, Seattle, USA<br>6th NCHIV in Amsterdam, The Netherlands |
| **2011:** | 3rd **Frontiers of Retrovirology, Amsterdam, The Netherlands**<br>5th NCHIV in Amsterdam, The Netherlands |
| **2010:** | 8th European HIV Drug Resistance Workshop, Sorrento, Italy<br>17th Conference on Retroviruses and Opportunistic Infections, 2010, San Francisco, USA<br>4th NCHIV in Amsterdam, The Netherlands |

| | |
|---|---|
| **Awards** | Young Investigator award, 19th Conference on Retroviruses and Opportunistic Infections, 2012, Seattle, USA<br>Young Investigator award, 17th Conference on Retroviruses and Opportunistic Infections, 2010, San Francisco, USA |

*TEACHING EXPERIENCE*

Teaching techniques to research technicians of the virology diagnostics department
Supervision of Bachelor of Life Science student (5 months)
Supervision of Master of Life Science student (9 months)
Supervision of Medical student (5 months)
Supervision of two Master of Biomedical Science students (6 months)