Genotypic and phenotypic characterization of HIV-1 from cerebrospinal fluid and blood compartments in patients with cryptococcal meningitis

By

Katlego Sojane

Supervisor

Professor Thumbi Ndung'u

Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal.

2017

Declaration 1: Plagiarism

I, Katlego Sojane, declare that

I. The research reported in this thesis, except otherwise indicated, is my original work.

II. This thesis has not been submitted for any degree or examination at any other university.

III. This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

IV. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. Their words have been re-written but the general information attributed to them has been referenced.

   b. Where their exact words have been used, their writing has been placed inside quotation marks and referenced.

   c. Where I have reproduced a publication of which I am an author, co-author or editor, I have indicated in detail which part of the publication was written by myself alone and have fully referenced such publications.

V. The thesis does not contain text, graphics or tables copied and pasted from the internet unless specifically acknowledged, and the source being detailed in the thesis and in the references section.

Signed:  **Date:** 25 AUGUST 2017

Declaration 2: Manuscripts

The manuscripts that constitute this thesis are listed below, as well as the contributions that I and other co-authors made to each manuscript are declared here:

Manuscript 1: Sojane, K., Kangethe, R. T., Chang, C. C., Moosa, M-Y. S., Lewin, S. R., French, M. A, and Ndung'u, T., 2017. Individuals with HIV-1 subtype C infection and cryptococcal meningitis exhibit viral genetic intermixing of HIV-1 between plasma and cerebrospinal fluid, and a high prevalence of CXCR4-using variants. AIDS Research and Human Retroviruses. Submitted (AID-2017-0209).

Authors' contributions: Prof. Ndung'u, Prof. Sharon R Lewin, Prof. Martyn A French, Prof. Mahomed Yunus S Moosa and Dr. Christina C Chang were part of the study-cohort protocol steering committee. Prof. Ndung'u and Dr. Christina C Chang conceived this study. Dr. Richard T Kangethe and I designed the experiments. I performed all of the laboratory experiments, data analysis and wrote the manuscript. All of the authors approved of the manuscript before it was submitted for publication.

Manuscript 2: Sojane, K., Roche, M., Chang, C. C., Lewin, S. R., French, M. A, Gorry, P., and Ndung'u, T., 2017. CXCR4 usage by HIV-1 subtype C is common in plasma but not the cerebrospinal fluid of individuals with cryptococcal meningitis.

Authors' contributions: Prof. Ndung'u, Prof. Sharon R Lewin, Prof. Martyn A French and Dr. Christina C Chang were part of the study-cohort protocol steering committee. Prof. Ndung'u and I conceived the current study. Dr. Michael Roche, Prof. Paul Gorry, Prof. Ndung'u and I designed the experiments. I performed all of the laboratory experiments, data analysis and wrote the manuscript.

Manuscript 3: Sojane, K., Roche, M., Chang, C. C., Lewin, S. R., French, M. A, Gorry, P., and Ndung'u, T., 2017. Usage of CCR3 by HIV-1 subtype C is common in patients with cryptococcal meningitis.

Authors' contributions: Prof. Ndung'u, Prof. Sharon R Lewin, Prof. Martyn A French and Dr. Christina C Chang were part of the study-cohort protocol steering committee. Prof. Ndung'u and I conceived the current study. Dr. Michael Roche, Prof. Paul Gorry, Prof. Ndung'u and I designed the experiments. I performed all of the laboratory experiments, data analysis and wrote the manuscript.

Katlego Sojane (student): K Sojane **Date:** 25 AUGUST 2017

Thumbi Ndung'u (supervisor): **Date:** 24 AUGUST 2017

Oral presentation

1. Katlego Sojane, Richard T. Kangethe, Christina C. Chang, Omarjee, S., Mahomed-Yunus S. Moosa, Sharon R. Lewin, Martyn A. French, and Thumbi Ndung'u. Cryptococcal meningitis/HIV co-infection is characterized by viral genotypic intermixing between the cerebrospinal fluid and blood compartments, and predominance of CCR5-tropic variants. Virology Africa 2015, 1st to 3rd December 2015, Cape Town (South Africa).

Poster Presentations

1. Katlego Sojane, Michael Roche, Anne Ellet, Christina C. Chang, Sharon R. Lewin, Paul R. Gorry and Thumbi Ndung'u. Identification of functional HIV-1 C envelope variants containing a phenylalanine residue at position 309 of the hypervariable region 3 in matched peripheral blood and cerebrospinal fluid. The 21st International AIDS Conference (AIDS 2016), 18th to 22nd July 2016, Durban (South Africa). Poster number: TUPEA008.

2. Katlego Sojane, Michael Roche, Christina C. Chang, Richard T. Kangethe, Martyn A. French, Sharon R. Lewin, Paul Gorry and Thumbi Ndung'u. HIV-1 subtype C may possess extended coreceptor utilization in peripheral blood and the central nervous system of patients experiencing cryptococcal meningitis. The 9th International AIDS Society Conference (IAS) on HIV Science, 23rd to 26th July 2017, Paris (France). Poster number: MOPEA0022.

Dedication

For Ntate Modise Sojane and Mme Sarah Masekela who raised me with love, patience, and unconditional support.

Acknowledgements

Sincere thanks to my supervisor, Prof. Thumbi Ndung'u (HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal) for motivation, guidance and support with this project. I am grateful that I was afforded the opportunity to learn and work in the HIV Pathogenesis Programme laboratory.

Dr. Christina Chang, Dr. Kamini Gounder and Dr. Michael Roche for their advice and assistance in the laboratory.

Members of the HIV Pathogenesis Programme Laboratory and the HIV Molecular Pathogenesis laboratory (Burnet Institute, Melbourne) for training and support.

The HIV Research Trust (Cheshire, U.K) and the University of KwaZulu-Natal for financial support.

The study participants for their samples.

Many thanks to my family members and friends for their prayers, support and encouragement. To my sister Bonolo Sojane, thank you for motivating me. To my uncles, Moses Masekela and Kaizer Masekela, thank you for keeping me under your wings. To my best friend, Rhameez Herbst, much love brother, you have carried me through everything good and bad life has thrown at me. To my late friend, Vuyo Makapela, your faith in me and my abilities strengthened me. To my late

grandmothers, Koko Mirriam Masekela and Koko Zipporah Sojane thank you for caring for me and being my greatest supporters.

Table of contents

List of figures

List of tables

List of abbreviations

AIDS: Acquired immune deficiency syndrome

ARV: AIDS-associated retrovirus

BBB: Blood-brain barrier

cART: Combination antiretroviral therapy

CNS: Central nervous system

CM: Cryptococcal meningitis

CPAs: Coreceptor usage prediction algorithms

CRF: Circulating recombinant form

CSF: Cerebrospinal fluid

DNA: Deoxyribonucleic acid

Env: Envelope glycoprotein

HAD: HIV-associated dementia

HAND: HIV-associated neurological disorders

HIV: Human immunodeficiency virus

HTLV-III: Human T-cell lymphotropic virus

ID: Identity

kD: Kilodalton

kb: Kilobases

LAV: Lymphadenopathy-associated virus

LTR: Long terminal repeat

MDM: Monocyte-derived macrophage

mL: Millilitre

NK: Natural killer

NNRTIs: Non-nucleoside reverse transcriptase inhibitors

nm: Nanometer

NRTIs: Nucleoside reverse transcriptase inhibitors

NSI: Non-syncytium inducing

NtRTIs: Nucleotide reverse transcriptase inhibitors

PDB: Protein databank

RLU: Relative light unit

RNA: Ribonucleic acid

RT: Reverse transcriptase

SI: Syncytium inducing

SIV: Simian immunodeficiency virus

TAR: Transacting response element

T/F: Transmitter/founder

URF: Unique recombinant forms

µL: Microlitre

Abstract

The combination of HIV and cryptococcal meningitis (CM) is a major cause of morbidity and mortality in sub-Saharan Africa. The phylogenetic relatedness of HIV-1 subtype C (HIV-1C) variants in peripheral blood and the central nervous system (CNS) compartments of individuals with CM is unknown. Additionally, the major and alternative coreceptor usage of HIV-1C in those compartments are not clear and these have implications for the pathogenesis of the virus, and the use of coreceptor blocking therapies.

For genotypic studies, 16 antiretroviral therapy naïve individuals with CM were randomly selected. We conducted single-genome, or bulk PCR amplification and sequencing of full-length HIV-1 *env* genes from plasma and/or cerebrospinal fluid (CSF) of the participants. Additionally, we estimated the prevalence of CXCR4-using variants in our cohort using coreceptor usage prediction algorithms (CPAs). Next, we evaluated the usage of CCR3, CCR5 and CXCR4 expressed on NP2/U87-CD4 cells by HIV-1C Envs derived from the plasma and/or CSF of 14 of the 16 participants.

In all 11 of the 16 participants with HIV-1C *env* sequences available from matched plasma and CSF samples, we observed genetic intermixing of viral variants between compartments using a neighbor-joining phylogenetic tree. In two participants, CM089 and CM112, with multiple sequences collected from both compartments using single-genome amplification and sequencing exclusively, compartmentalized variants were not observed in CM089 and CM112 using the Slatkin-Maddison test (*p*-value 0.19 and 0.35, respectively), and this result was confirmed by the Hudson nearest-neighbor test (*p*-value 0.34 and 0.54, respectively). Using a combination of publicly available coreceptor usage prediction algorithms (Geno2pheno, PhenoSeq and WebPSSM subtype C sinsi), ~44% of participants were predicted to possess CXCR4-using variants.

HIV-1C in plasma and/CSF samples of 14 of the 16 participants was then evaluated for the ability to use CCR3, CCR5 and CXCR4. We showed that HIV-1C is capable of using CCR5, CXCR4 or

CCR3 alone, or in combination with the other receptors. Overall, our results have improved the understanding of HIV-1C pathogenesis in the peripheral blood and CNS compartments of individuals with end-stage infection and CM, and provides clinically relevant information for therapies including coreceptor antagonists in this setting.

Chapter 1: Introduction

1.1. HIV: The etiological agent of the AIDS pandemic

Acquired immune deficiency syndrome (AIDS) observed in a series of cases among homosexual men in the year 1981 marked the emergence of a new epidemic (1-3). The human immunodeficiency virus type 1 (HIV-1) which was implicated as the cause of the AIDS epidemic had been characterized initially by three different research groups by the end of the year 1984. Each group, however, had named the virus in their own way. Researchers at the Pasteur Institute (Paris, France) named the virus "lymphadenopathy-associated virus" (LAV), whereas researchers at the National Cancer Institute (Bethesda, USA) named it "human T-cell lymphotropic virus type III" (HTLV-III), and a group at the University of California (California, USA) named it "AIDS-associated retrovirus" (ARV) (4-7).

There are two types of human immunodeficiency virus (HIV), 1 (HIV-1) and 2 (HIV-2), which belong to a family of primate lentiviruses that includes the simian immunodeficiency virus (SIV) (8). Notably, HIV-1 arose from a form of SIV which infected chimpanzees and was able to establish itself in a human host through cross-species transmission. SIV, unlike HIV-1 or HIV-2, infects non-human primates, whereas HIV-1 and HIV-2 are adapted to humans as hosts (9-11). HIV-1 and HIV-2 are not equally pathogenic (12), nor are they equally distributed globally. HIV-1 is composed of groups M (major), N (non-M, non-O), O (outlier) and P, though it is group M which is responsible for the global epidemic, contributing approximately 60 million infections and 25 million deaths to date (13). It is not known for sure how the first individual(s) were infected with HIV, but it is suspected that they were exposed to ape blood products containing the virus in West Africa (Kinshasa, formerly Leopoldville), and that the virus entered their body cutaneously or through exposed membranes (14, 15).

1.2. HIV-1 exhibits high genetic diversity

Within HIV-1 group M there are subtypes A-K, circulating recombinant forms (CRFs) and unique recombinant forms (URFs) which are unevenly distributed across the globe (Figure 1.1) (16). HIV-1 subtype C (HIV-1C) is responsible for 48% of infections globally, followed by subtype A (HIV-1A; 12%), subtype B (HIV-1B; 11%), CRF02_AG (8%), CRF01_AE (5%), subtype G (5%) and D (HIV-1D; 2%). Other CRFs and URFs each account for 4% of infections, while the remaining subtypes account for less than 1% of all infections (17, 18). Sequence differences within subtypes may be between 15-20%, but may be up to 30% between subtypes (19). When HIV-1 is transmitted from a donor to a recipient, a severe genetic bottleneck selects for only a single or very limited number of variants to establish infection. Transmitted viruses generally have low genetic diversity, with one viral variant being sufficient to establish infection in approximately 80% of infected individuals (20, 21).

The high genetic diversity which arises eventually within and between individuals is due to the high replication rate of the virus, the error-prone nature of its reverse transcriptase (RT) enzyme which has little or no proof-reading ability (22), and the intense immune pressure exerted by the host (23-26). In effect, a "swarm" of diverse viruses (better known as the quasispecies) forms within an infected individual, with viral variants in the population displaying varied genetic and phenotypic properties (27, 28).

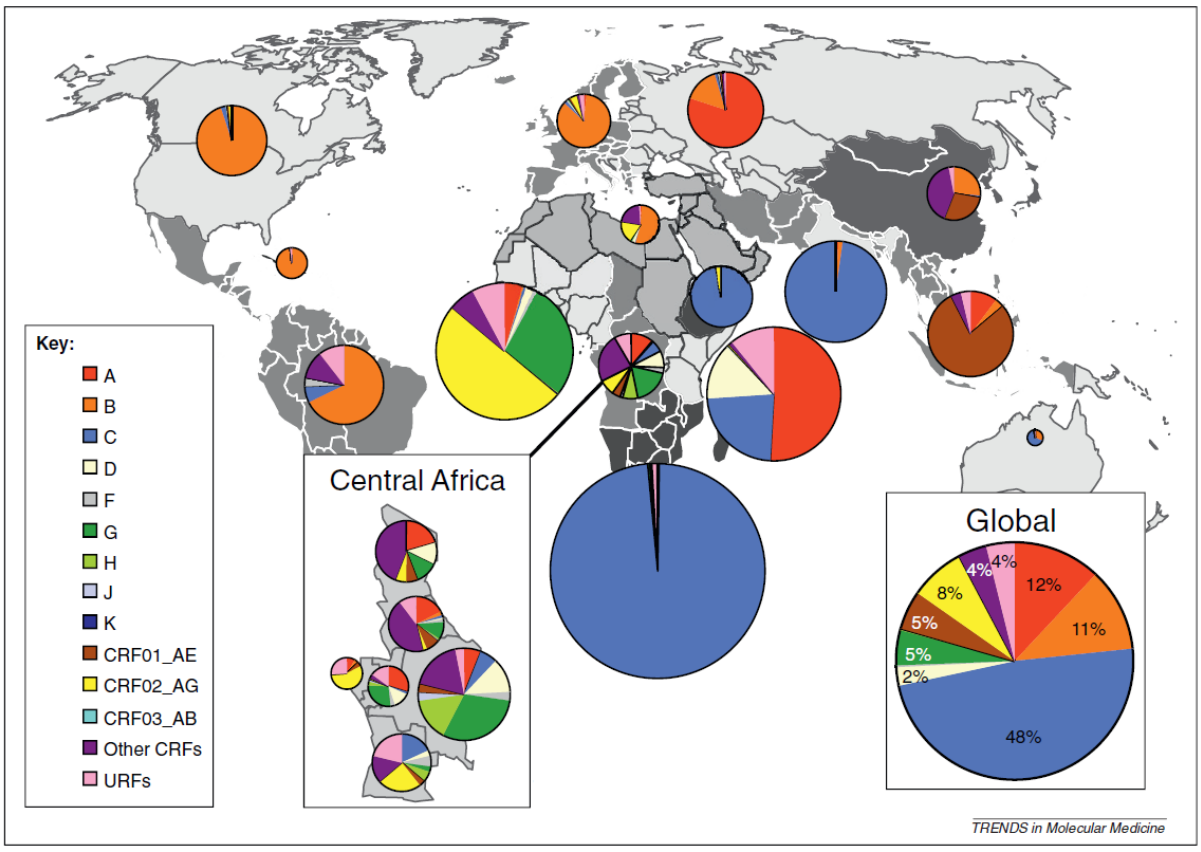

Figure 1.1: The distribution of HIV-1 subtypes across the globe.

In the main figure, pie-charts show the share of infections each HIV-1 subtype is responsible for in a specific geographical region. The size of each pie-chart reflects the total number of infections in a region relative to other pie-charts. The legend on the left-side of the main figure shows the subtypes corresponding to the colors shown in each pie-chart. In the inserts of the main figure, the distribution of HIV-1 subtypes in Central Africa and the world are shown. Reproduced from Hemelaar (2012) (13).

1.3. The genomic composition of HIV-1

The genome of HIV-1 is approximately 10 kilobases (kb) long (29). Different genes are responsible for coding for different structures of the virus (Figure 1.2). The *gag* gene codes for the p17, p24, and the p7/p9 proteins. The *pol* gene codes for the protease, RT and integrase enzymes. The *env* gene codes for the precursor gp160 protein molecule which is cleaved by a cellular protease to produce non-covalently associated gp120 and gp41 heterodimers. Regulatory and accessory proteins are encoded by the *tat*, *rev*, *vif*, *vpu*, *vpr*, *rev*, as well as *nef* gene. The ends of the HIV genome have the untranslated long terminal repeat (LTR) sequences.

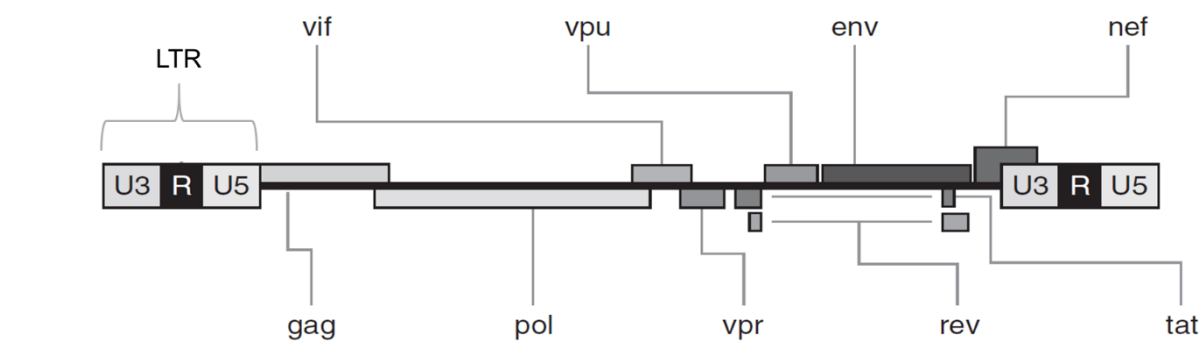

Figure 1.2. The genome structure of HIV-1.

The organization of the genes coding for the different gene products of HIV-1 is shown. The open reading frame for each genomic region is shown as a rectangle. Reproduced with modifications from Cassis-Ghavami *et al* (2009) (30).

1.4. The arrangement of major HIV-1 proteins

The mature HIV-1 particle is between 110 and 128 nanometers (nm) in diameter (31). The core of the virus is cone-shaped (32) and composed of a major capsid protein (p24) and nucleocapsid protein (p7/p9) which encase two single-stranded RNA genome copies, protease, RT and integrase enzymes. The core itself is surrounded by the matrix protein (p17), while the envelope glycoproteins (Envs) (composed of gp41 and gp120 heterodimers) coat the outside of the virus, as they are embedded in a lipid bilayer derived from the host (Figure 1.3).

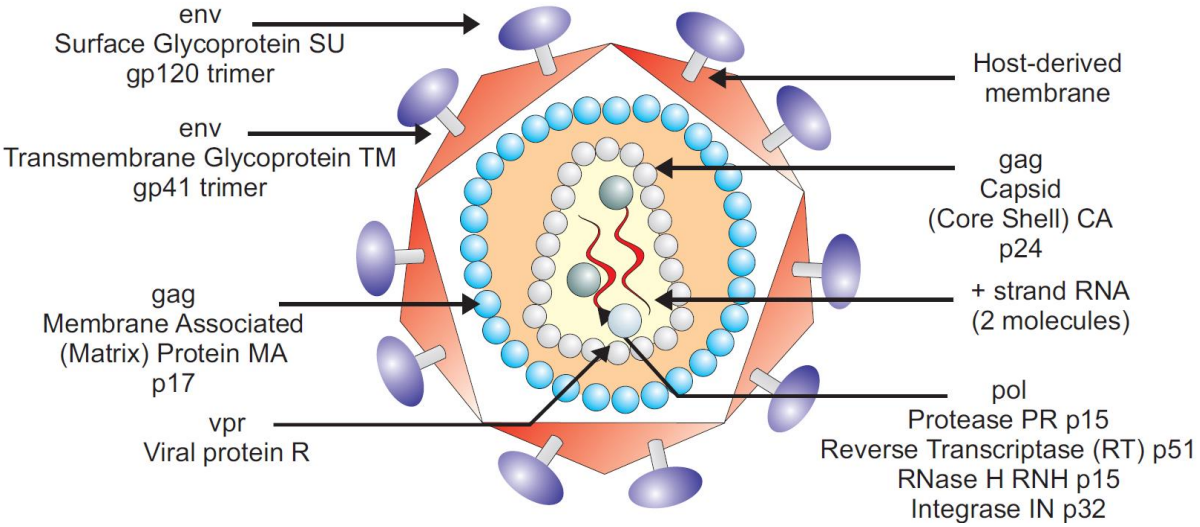

Figure 1.3. The arrangement of HIV-1 proteins and RNA in a virion.
Reproduced from Stevens and Papathanasopoulos (2009) (33).

1.5. The functions of the major HIV-1 proteins

HIV-1 Env is responsible for attachment of the virus to the host cell, and facilitates the fusion of the viral membrane with the host membrane (34, 35). The RT enzyme, a key component of the retrovirus, converts the RNA genome into DNA to form what is known as the provirus (22). The integrase enzyme facilitates integration of the reverse-transcribed viral genome into the host genome (36, 37). The Gag polyprotein (composed of the p7/p9, p17 and p24 proteins) allows for the assembly of mature virus particles (38). HIV protease processes the Gag and Gag-Pol polyprotein into individual proteins (39). The Tat protein regulates virus transcription initiation (40), and the Rev protein facilitates the export of HIV RNA, spliced and unspliced, from the nucleus to the cytoplasm (41). Nef downregulates cell-surface receptors such as the CD4 and major histocompatibility complex class I molecules (42, 43), Vpr enhances virus replication (44, 45), Vif regulates the infectivity of virions (46-48), and Vpu improves the release of virions from the host cell (49-51).

1.5.1 The structure of HIV-1 Envs

The Env protein of HIV-1 is initially synthesized as a 160 kilodalton (kD) precursor protein (Figure 1.4) which is subsequently cleaved to yield a gp120 portion (120 kD in size) that is non-covalently associated to a gp41 portion (41 kD in size) (52). Three gp120-gp41 heterodimers associate non-covalently with each other on the surface of the virus, forming a trimer or spike (Figure 1.5). There are an estimated 14 spikes on average on each virus particle (53). The gp120 portion is typically heavily glycosylated and possesses five constant (C1-C5) and five variable regions (V1-V5). It is divided into three distinct sub-domains; the inner, outer and bridging sheet. Notably, this protein has a site which binds to CD4 and another site which binds to a chemokine coreceptor, though the amino acids responsible for binding at either site are discontinuous (54). The gp41 portion is composed of an extracellular domain, a membrane-spanning domain, and a cytoplasmic tail. The extracellular domain is composed of a fusion peptide, an N-terminal heptad repeat (NHR), a loop region, C-terminal heptad repeat (CHR), and a membrane-proximal external region (55).

1.5.2 The main function of HIV-1 Envs

Virus attachment to host cells is initiated with the engagement of cell-surface CD4 by HIV-1 gp120 of Env (34), followed by a number of conformational changes within gp120 which permit the engagement of a chemokine receptor (54, 56). The major coreceptors usually engaged by HIV-1 are CCR5 and CXCR4; both are seven-pass G-protein coupled chemokine receptors which act as cofactors for entry for most variants (57-59). Conformational changes in gp41 then arise which allow its fusion peptide to penetrate the host cell membrane while a pre-hairpin fusion intermediate of the NHR trimer forms. Three CHR molecules then pack tightly together in an anti-parallel fashion into the hydrophobic grooves of the NHR trimer. This causes the viral and host membranes to come closer to each other and fuse (Figure 1.6) (55).

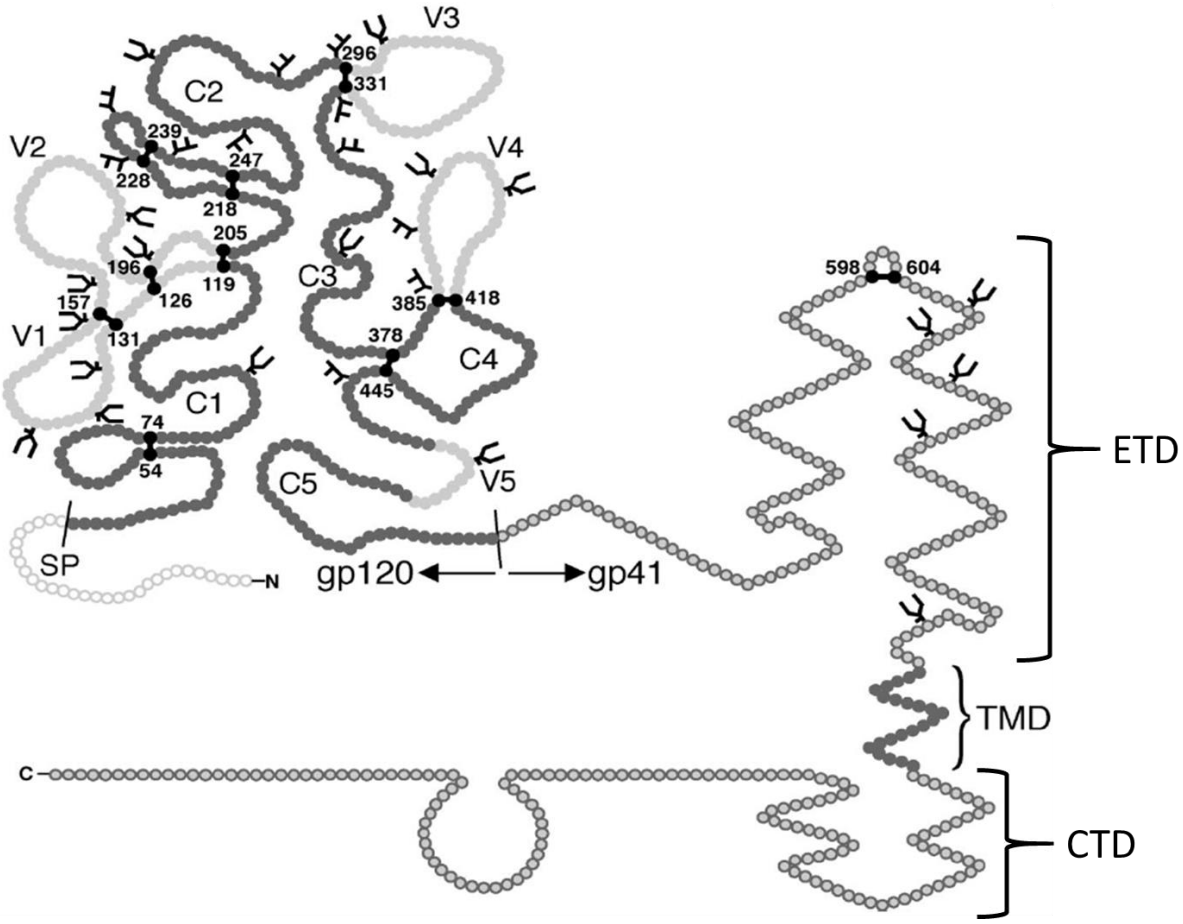

Figure 1.4. The HIV-1 gp160 precursor protein.
The residues shown as black spheres indicate the location of disulphide bonds. SP, ETD, TMD and CTD refer to the gp120 signal peptide, gp41 ectodomain, transmembrane domain and cytoplasmic tail domain, respectively. Reproduced from van Anken *et al* (2008) (60).

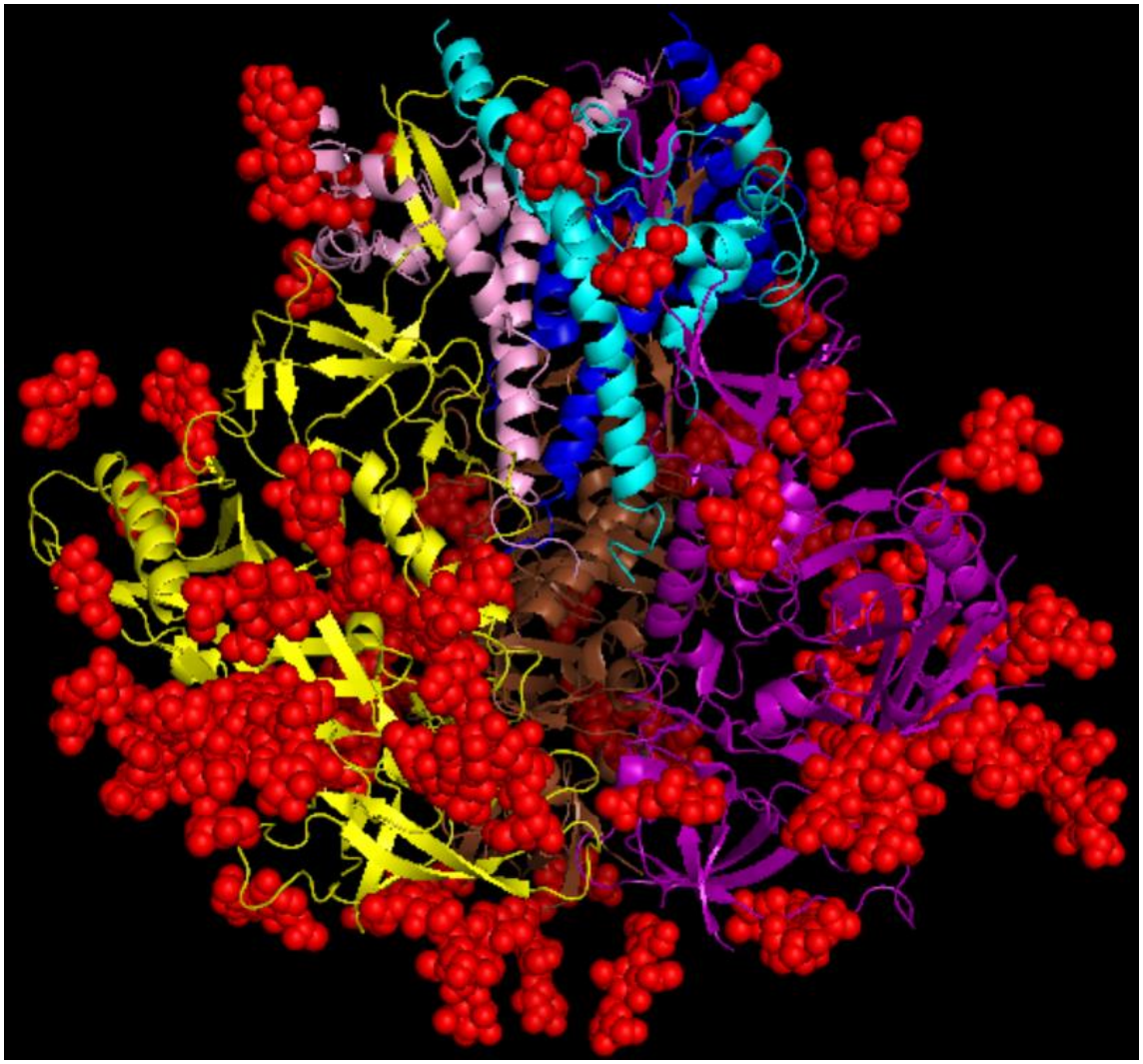

Figure 1.5: The HIV-1 envelope trimer
The three glycosylated gp120-gp41 heterodimers which form one HIV-1 envelope trimer were crystalized by Lee *et al* (2015; PDB ID: 5ACO) (61). The yellow, purple and brown ribbon structures represent gp120 portions. The pink, cyan and blue ribbon structures represent gp41 portions. The red spheres represent the glycan structures. The image was generated using The PyMOL Molecular Graphics System, evaluation version 1 Schrödinger, LLC.

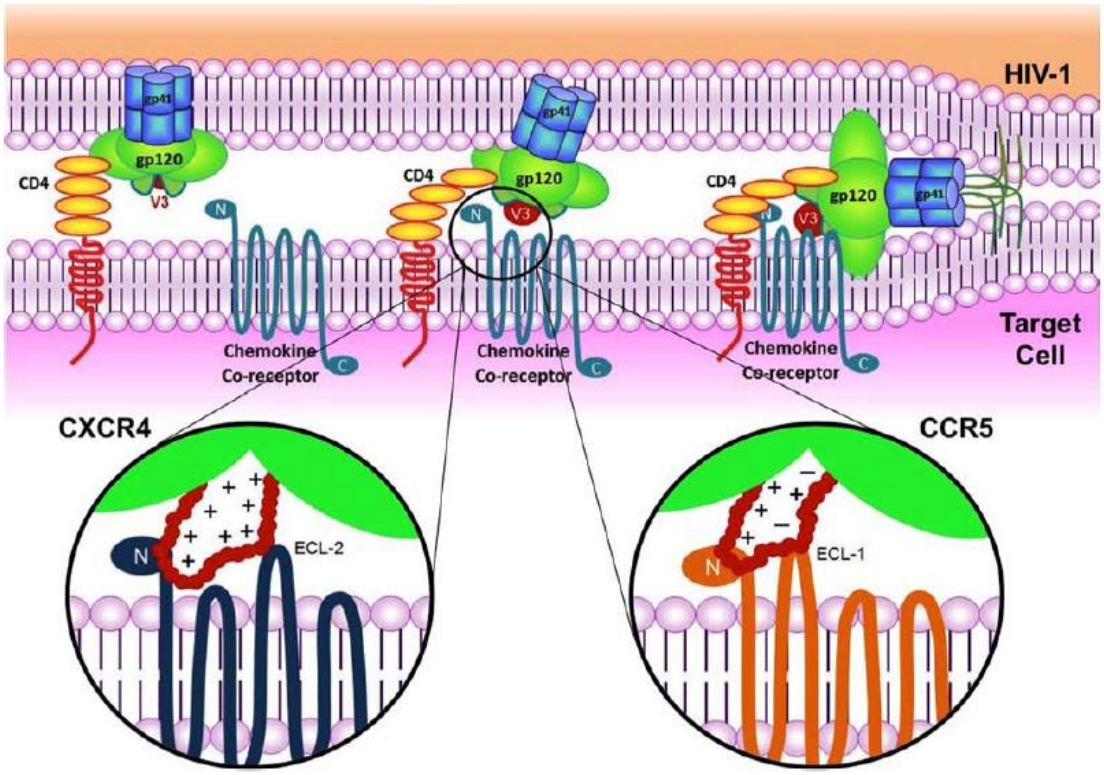

Figure 1.6: The attachment and fusion steps of HIV-1 entry

The attachment of HIV-1 to the host cell requires the initial engagement of the gp120 portion of the envelope protein (Env) with cell-surface CD4. Conformational changes within gp120 arise, including the exposure of the V3 loop by the V1/V2 loops of Env. The coreceptor binding site of Env which includes the V3 loop then binds a chemokine coreceptor, usually CCR5 or CXCR4. Viruses which use CCR5 preferentially typically have a low net charge of the V3 loop, and dock onto the N-terminus and extracellular loop 1 (ECL-1) of CCR5. CXCR4-using viruses typically have high net charge of the V3 and dock onto the N-terminus and ECL-2 of CXCR4. After the engagement of the coreceptor, more conformational changes in gp120 expose the gp41 portion of Env. Gp41 facilitates the fusion of the virus and host membrane, affording the virus an opportunity to translocate its core into the host cell. Reproduced from Aiamkitsumrit *et al* (2014) (62).

1.5.2.1 The HIV-1 Env V3

The third variable region (V3), amino acids 296-331 according to HXB2 numbering, of HIV-1 Env is typically 35 (range: 31-39) amino acids in length. It plays an important role during the attachment step of the HIV-1 life-cycle, as it forms part of the coreceptor binding site which determines which coreceptor, usually CCR5 or CXCR4, will be used for entry (Figure 1.6 and Figure 1.7) (reviewed by Hartley *et al*., 2005) (63). It is composed of three structural regions; a base, flexible stem, and a β-hairpin tip (Figure 1.8) (64).

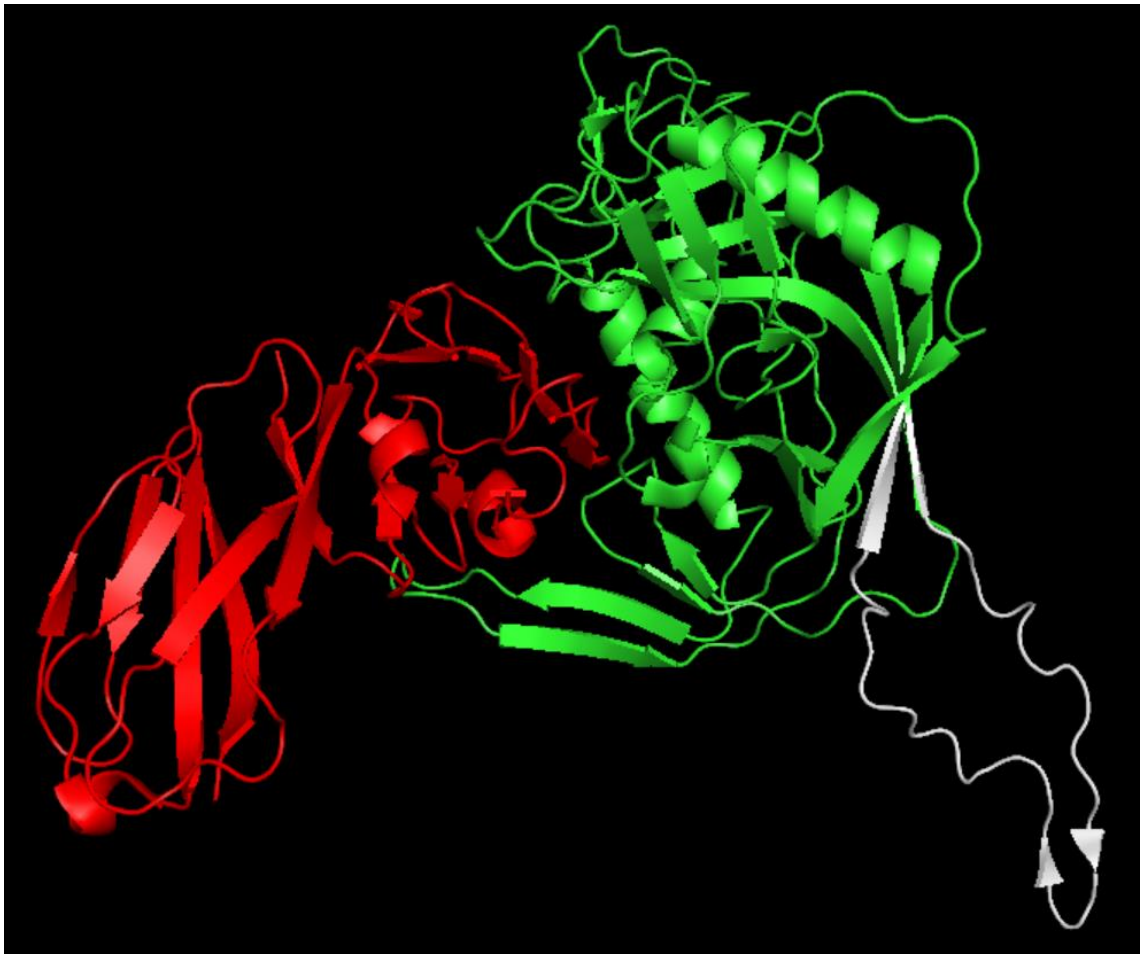

Figure 1.7: The HIV-1 gp120 core with V3 region bound to CD4.
The crystal structure of soluble two-domain CD4 (red) in complex with the JR-FL gp120 core (green) is shown (PDB ID: 2B4C). The intact V3 region (white) which is part of the gp120 protein, and interacts with a chemokine coreceptor, typically CCR5 or CXCR4, is also shown. Reproduced from Huang *et al* (2005) (64).

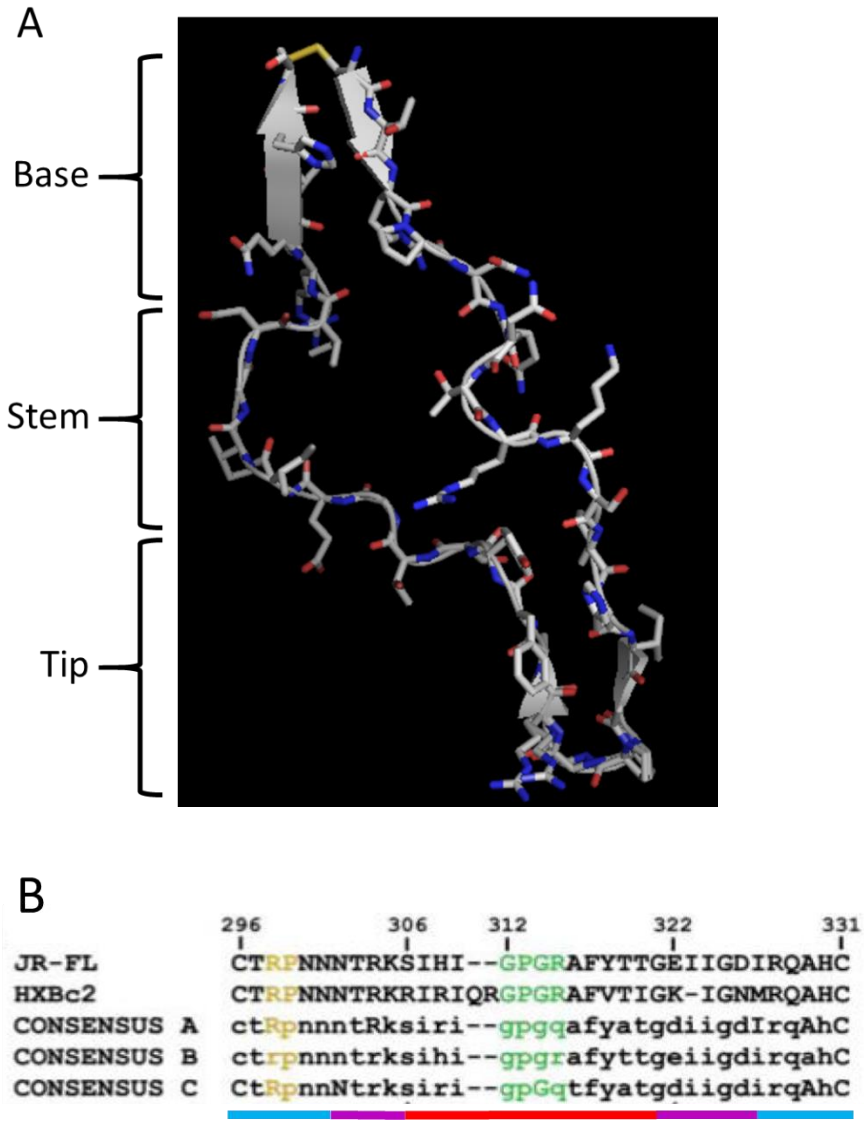

Figure 1.8: The structure and protein sequence of the HIV-1 Env V3.
(**A**) The crystal structure of the HIV-1 JR-FL Env V3 region (color code: grey, carbon atoms; blue, nitrogen atoms; red, oxygen atoms; and orange, disulfide bond) (PDB ID: 2B4C). The three sub-regions of the V3 (the base, stem and β-hairpin tip) are shown. (**B**) The Env V3 protein sequences of JR-FL and HXB2 are shown along with those of consensus subtype A, B and C viruses. Uppercase letters in the consensus sequences show conserved residues, while lowercase letter show variable residues. The conserved arginine-proline (yellow) and crown (green) motifs are indicated. The blue, magenta, and red line below the sequences correspond to the base, stem and β-hairpin tip sub-regions of the V3. Reproduced from Huang *et al* (2005) (64).

The Env V3 region was initially shown to determine the cytopathicity, cellular tropism and infectivity of HIV-1 (65-67). After the discovery of coreceptors which act as cofactors for HIV-1 entry, mutational analyses of the V3 showed that it was the major determinant of CCR5 and/or CXCR4 usage (68). Within the V3, specific residues were observed only in viruses which bound to CCR5. A serine or glycine (uncharged residues) at position 11, and glutamic acid or aspartic acid (negatively charged residues) at position 25 was found in CCR5-using isolates, but an arginine (positively charged residue) or glutamine (uncharged) at one or both positions conferred the ability to use other coreceptors (69). Other modifications of the V3 which were shown to alter coreceptor usage for HIV-1B and HIV-1C isolates were the overall net charge and the number of potential N-linked glycosylation sites (PNGS), where an increased overall net charge and reduced number of PNGS, individually, conferred usage of CXCR4. These results suggested that the requirements for coreceptor switching for HIV-1B and HIV-1C were the same (70). Later studies showed that there were other determinants of CXCR4 usage. The sequence variability of R5 and CXCR4-using viruses was different, with R5 viruses having lower variability than CXCR4-using viruses (71). The crown motif sequence of the V3, GPGQ, is characteristic of R5 viruses (72), but CXCR4-using viruses frequently have alterations of the motif and/or a two-amino acid insertion beside the motif (73-77).

Although the V3 is the major determinant of coreceptor usage, other regions of Env have been shown to contribute to usage of coreceptors. Modifications in the V1-V2 region were shown to contribute to a switch of major coreceptor (68, 78-80). The observation that certain dualtropic Envs share an identical V3 sequence with R5 Envs (81), and that a single amino acid mutation in the gp41 protein may confer tropism for CXCR4 (82), confirms that the V3 is the major but not the sole determinant of coreceptor switching.

1.6. The life-cycle of HIV-1

The HIV-1 Env protein mediates the attachment of the virus to the host cell, and fusion of the virus and host cell membranes. Thereafter, the virus is able to transfer its core, including the nucleocapsid, into the cytosol of the host cell. The RNA genome strands of HIV-1 encased by the nucleocapsid are then exposed and associate with RT, integrase, Vpr, and an array of host proteins to form a pre-integration complex which migrates to the nucleus of the cell (83). Reverse transcription is performed by the RT enzyme, and the remaining RNA template is degraded by the RNAse H portion of RT. Because RT has poor proof-reading ability, mutations are incorporated into the proviral DNA genome of HIV-1, thereby increasing the genetic diversity of the virus population over time (22, 84).

For replication, HIV-1 must insert a copy of its genome into the host genome. After reverse transcription, the integrase enzyme supported by other proteins facilitates the integration of the proviral (double-stranded) DNA genome into the host genome (85-87). In some cases auto-integration occurs where the linear proviral DNA forms 2-LTR circles, or 1-LTR circle after the recombination of the LTR ends (86).

The transcription of balanced amounts of spliced and unspliced proviral DNA is required for efficient viral replication. The integrated proviral genome of HIV-1 may occupy resting cells (hence the persistence of latent HIV-1 reservoirs despite antiretroviral therapy) or active cells. In both cases, the Tat protein of HIV-1 along with other host cellular factors initiate transcription from the transacting response element (TAR) in the LTR for the generation of mRNA molecules (88). From one proviral genome more than 30 mRNA molecules may be generated through alternative splicing; unspliced products include the approximately 10 kilobase transcript, or transcripts of varying sizes after splicing (89).

The translation of HIV-1 mRNA transcripts leads to the synthesis of Gag, Gag-Pol, Vpr and Vif proteins along with two viral RNA genome molecules, which are all assembled at the plasma membrane of the host cell to make a virion (90). Host proteins such as tumor suppressor gene 101

are diverted from their normal roles to work in combination with viral proteins to assemble virions (91, 92). The budding of virions occurs preferentially in micro-domains of the plasma membrane where cholesterol is richly deposited (93), and is assisted by the Vpu protein of the virus. So important is Vpu that viruses with a defective version of the protein are unable to be released from the host cell (94). Notably, HIV-1 may be released from an infected cell at cell-to-cell membrane sites where intercellular adhesion molecules such as ICAM-1 are concentrated. The incorporation of host adhesion molecules in the virion, and the co-localization of budding virions and host adhesion molecules during syncytium formation are important for viral spread (95). Once the virion is released from the cell it is able to infect new cellular targets in a cell-free manner, or spread from the infected cell to an uninfected cell; the latter occurring via a virological synapse which spreads HIV more efficiently (96).

1.6.1 Inhibiting steps of the HIV-1 life-cycle

Different steps of the life-cycle of HIV-1 may be targeted using combination antiretroviral therapy (cART) (Figure 1.9). More than 25 compounds have been approved by the Food and Drug Administration (FDA) for use as anti-HIV compounds, as reviewed by De Clercq (2009) (97). The different anti-HIV compounds fall into one of five classes based on the targets they block or the step of the HIV life-cycle they stop from proceeding. Nucleoside analogue reverse transcriptase inhibitors (NRTIs) and nucleotide reverse transcriptase inhibitors (NtRTIs) form one class, while non-nucleoside reverse transcriptase inhibitors (NNRTIs) form a second class, though both classes describe reagents which target the reverse transcription step by inhibiting the function of the RT enzyme. Protease inhibitors are a third class of reagents which target HIV protease and prevent it from performing its proteolytic processing function. The strand-transfer reaction catalyzed by the integrase enzyme is blocked from proceeding by integrase inhibitors. Attachment and fusion inhibitors form their own class of drugs as they prevent entry of HIV into the cell: the fusion of viral and host membranes is inhibited by Enfuvirtide (also known as T-20), a 36 amino

acid peptide fusion inhibitor which binds to the gp41 protein (98). The use of CCR5 by HIV Env is prevented by maraviroc, a CCR5 antagonist which blocks entry (99).

Despite the ability of monotherapy or cART to reduce the viral load, boost CD4 cell count and slow disease progression (100, 101), the efficacy of the treatment is undermined by its high toxicity and the ability of HIV-1 to mount resistance (102, 103). It was initially recommended in the year 2006 that a NNRTI (or a protease inhibitor boosted with ritonavir) be used in combination with two NRTIs, unless the patient profile required otherwise, only after the CD4 cell count of an individual dropped to 350 or 200 cells/μL (104). By the year 2012, new recommendations were provided, advising that two NRTIs (Tenofovir/Emtricitabine or Abacavir/Lamivudine), a NNRTI (Efavirenz), a ritonavir-boosted protease inhibitor (Atazanavir or Duranavir) or an integrase inhibitor (Raltegravir) be used by patients, unless their clinical profile required otherwise. Additionally, it was recommended that all patients use cART regardless of their CD4 cell count and viral load (105). The benefits of early initiation of cART were confirmed by the Strategic Timing of Antiretroviral Treatment (START) Study Group. The group showed that it was immediate treatment of HIV-1, when individuals had a CD4 count higher than 500 cell/μL and not when the CD4 count had declined to 350 cells/μL, that provided better clinical outcomes for infected individuals (106).

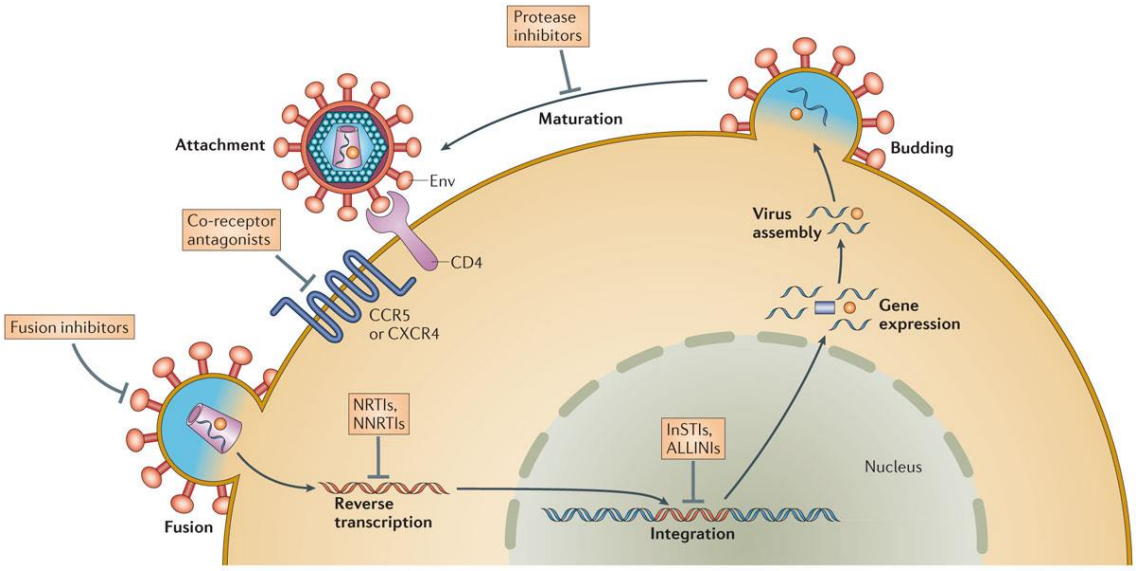

Figure 1.9: The life-cycle of HIV-1 and the inhibition of steps thereof
HIV-1 enters a host cell after its envelope protein (Env) binds to CD4 and a chemokine coreceptor, usually CCR5 or CXCR4. Conformational changes in Env facilitate the fusion of the viral and host cell membranes which lead to the transfer of the viral core into the host cell. In the cytosol, HIV-1 RNA is reverse transcribed and the pre-integration complex is formed before it is targeted to the nucleus. Inside the nucleus, the double-stranded DNA provirus is integrated into the host genome. New viral RNA genomes, core proteins and enzymes are then generated from the integrated provirus. New virions are made and released after a process of budding and maturation. Nucleoside analogue reverse transcriptase inhibitors (NRTIs) as well as non-nucleoside reverse transcriptase inhibitors (NNRTIs) block reverse transcription from proceeding. Integrase strand transfer inhibitors (InSTIs) and allosteric integrase inhibitors (ALLINIs) block the process of integration facilitated by the viral integrase enzyme. Protease inhibitors prevent the protease enzyme from maturing the assembled virion. Coreceptor antagonists and fusion inhibitors block the entry of viruses into the host cells. Reproduced from Laskey and Siliciano (2014) (107).

1.7. HIV-1 disease progression

Although there is person to person variation, the $CD4^+$ T-cell count and viral load dynamics during the course of untreated infection are consistent (Figure 1.10). In a review by Coffin and Swanstrom (2013) (108), the authors describe the clinical response of individuals from the time they are infected with HIV-1 until they die of AIDS. In the first two weeks after infection (the eclipse phase), viremia is not readily detectable in blood though the virus is replicating unchecked and spreading from the initial site of infection to other tissues and organs which also support viral replication. At this stage the host immune response is ignorant of the presence of the virus and no symptoms are presented by the infected individual. Between the second and fourth week of infection (primary/acute infection), the HIV viral load spikes to as high as 10^7 RNA copies/milliliter (mL) in the blood while the $CD4^+$ T-cell count declines transiently. Concurrently, the host immune response recruits $CD8^+$ T-cells to target viral antigens expressed on infected cells and antibodies are produced which attempt to neutralize or control virus replication. Towards the end of this phase of infection the viral load declines due to the action of the $CD8^+$ T-cells and possibly antibodies and innate immune responses. Symptoms presented by individuals in this stage of infection include fever and enlarged lymph nodes, similar to influenza infections. Clinical latency follows the acute infection stage and may last approximately one to 20 years. Here, the viral load is relatively stable, and the $CD4^+$ T-cell count recovers from its lowest point but does not returned to pre-infection levels. Typically, infected individuals are asymptomatic and not aware that they harbor HIV. Nevertheless, $CD4^+$ T-cells are persistently killed due to the infection and steadily decline in number (108). When the $CD4^+$ T-cell count number drops to approximately 200 cells/microlitre (µL), and the immune system is unable to control the infection, opportunistic infections start to appear. The HIV viral load spikes to exceedingly high levels once more, reminiscent of the acute stage of infection. In this end-stage of disease individuals are diagnosed as having AIDS and die if left untreated. The prognosis for untreated AIDS patients is poor as the mortality rate is above 95% (108). Common opportunistic infections experienced by individuals

with AIDS are *Mycobacterium tuberculosis* or *avium* complex, cryptococcal meningitis, *Pneumocystis jirovecii* pneumonia (formerly known as *Pneumocystis carinii*), diarrhea-associated diseases, candidiasis, cytomegalovirus retinitis, histoplasmosis, progressive multifocal leukoencephalopathy, Kaposi's sarcoma and non-Hodgkin lymphoma (109).

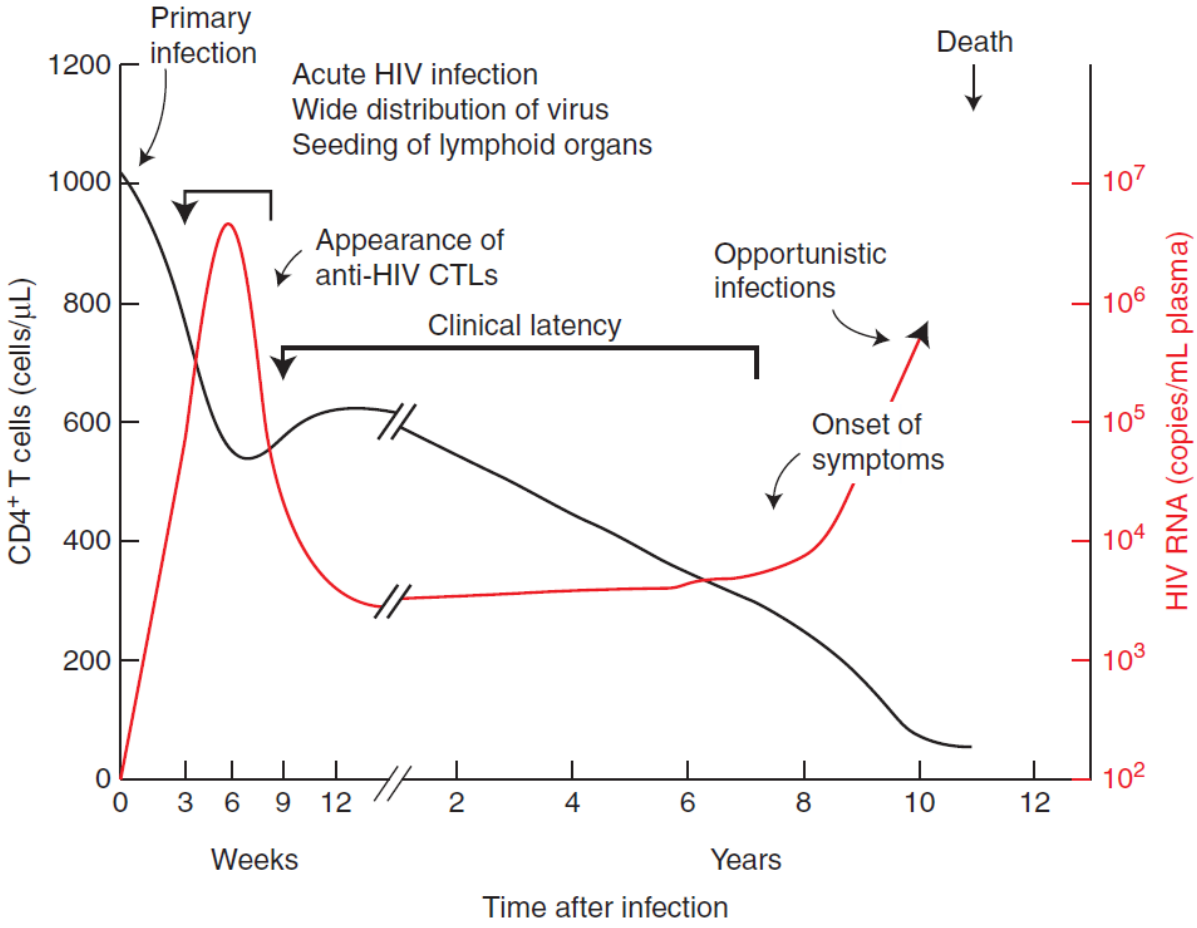

Figure 1.10. The HIV disease progression of the average individual not on antiretroviral therapy.
Early in acute infection, the HIV RNA load increases drastically while the $CD4^+$ T-cell count drops slightly. Cytotoxic T-lymphocytes aid in the partial control of virus replication and reduce it to a set point, allowing the partial recovery of the $CD4^+$ T-cell count and the onset of clinical latency. The set point of the HIV RNA load is correlated with the rate of disease progression. After clinical latency and the deterioration of the immune system, the HIV RNA load rises drastically once more while the $CD4^+$ T-cell count of the infected individual drops to below 200 cells/μL, allowing opportunistic infection to arise. Reproduced from Coffin and Swanstrom (2013) (108).

1.7.1 Cryptococcal meningitis in persons living with HIV-1

Cryptococcal meningitis (CM) is an opportunistic infection commonly experienced by immunosuppressed individuals with end-stage HIV infection (110). The causative agents of CM, *Cryptococcus neoformans* (*C. neoformans*) and *gattii* (*C. gattii*), are the two *Cryptococcus* species which infect humans (111), though *C. neoformans* is responsible for the majority of central nervous system (CNS) cryptococcosis events in individuals living with HIV/AIDS. In patients presenting with CM, inflammation of the meninges due to *C. neoformans* infection, may present with varying forms and degrees of neurological deterioration, manifesting as neck stiffness, headaches, confusion, altered levels of consciousness, visual impairments and encephalitis (112, 113). Without rapid treatment including amphotericin B, flucytocine or fluconazole, 100% of individuals with CM will die (114). The distribution of CM cases globally is not even, with sub-Saharan African accounting for approximately 720,000 of the 957,900 (~75.2%) total cases annually. HIV-related CM disease is associated with poor clinical outcome with as high as 624,725 deaths recorded annually; 504,000 of those deaths were in sub-Saharan Africa (115).

Although individuals with advanced HIV infection and CM may harbor CXCR4-using HIV-1 (116), thus precluding them from using cART regimes including maraviroc, an accurate estimation of the prevalence of CXCR4-using HIV-1 in individuals with CM is unavailable. If the prevalence is high, patients may need to be screened for the presence of CXCR4-using HIV-1 before initiating regimens including maraviroc.

1.7.1.1 The life-cycle of Cryptococcus neoformans

Reviews by Idnurm *et al* (2005) and Kwon-Chung *et al* (2014) have discussed the life-cycle of *C. neoformans* (117, 118). *C. neoformans* is a heterothallic organism: two compatible partners of opposite mating types (*MAT*α and *MAT*a) are required for mating to occur. Before strains of opposing mating types come into contact with each other, haploid *C. neoformans* cells bud asexually to propagate themselves. Once the *MAT*α and *MAT*a mating types of *C. neoformans*

come into contact with each other, they each release unique pheromones. Thereafter, the growth of the *MAT*a strain becomes isotropic (uniform in all directions) while the *MAT*α strain forms conjugation tubules. Fusion of the two strains by virtue of conjugation tubules produces dikaryotic hyphae with fused connections (119). During the mating process, mitochondria of the *MAT*α strain are eliminated and only those of the *MAT*a strain are preserved and inherited by progeny produced after mating (118, 120). The nuclei of the two mating strains fuse to making a diploid nucleus that undergoes one round of meiotic division which results in the generation of four haploid nuclei. After that, the products of the meiotic division subsequently undergo mitotic divisions and budding to produce chains of basidiospores (sexual spores) consisting of *MAT*α and *MAT*a type spores (118).

*MAT*α strains may reproduce unisexually and produce basidiospores which are morphologically identically to basidiospores produced by opposing mating types (monokaryotic fruiting). However, this form of reproduction generates unfused clamp connections (117, 118). Furthermore, the mitochondria are inherited from *MAT*a strains only (121). It is thought that this form of reproduction may provide a survival advantage under harsh or changing conditions such as nutrient limitations. In favorable conditions, mating and monokaryotic fruiting may be stimulated due to darkness, nitrogen starvation, desiccation, and mating pheromones (117).

1.7.1.2 The pathogenesis of Cryptococcus

Infection occurs when the spores of *Cryptococcus* or desiccated yeast cells which circulate in the air are inhaled by an individual and then deposit themselves in the alveoli of the lung. In the presence of a strong cell-mediated immune response, *Cryptococcus* may be cleared. However, if it is not cleared, it may be controlled by the immune system through the development of granuloma (117). Immunosuppressed individuals are unable to control or clear *Cryptococcus* which subsequently migrates through the blood, reaches the blood-brain barrier (BBB) and passes through it to enter the CNS. The main mechanism of crossing the BBB may be by transcytosis

(122), and phagosomal extrusion (also known as the "Trojan-horse mechanism") (123). A third mechanism of entry into the CNS may be through the tight junctions which hold together the brain microvascular endothelial cells that make up the BBB, but clear evidence of this has not been shown (122). In the CNS, *Cryptococcus* reaches the meninges and causes inflammation thereof as it replicates rapidly, leading to the development of meningoencephalitis or CM.

1.8. The main target cells of HIV-1

HIV-1 is able to infect different types of cells usually expressing CD4 on their surface. The cells targeted by HIV-1 include dendritic cells, activated and inactivated $CD4^+$ T-cell lymphocytes, macrophages, and cells of the CNS such as astrocytes and microglia. *In vivo*, the preferred target of HIV-1 is the activated or HIV-specific memory T-cell (124-128). The cause of T-cell death is mostly due to necrosis, lysis, pyroptosis or cytopathic effects (129-132). Additionally, the death of cells via bystander effects, where uninfected cells in the vicinity of infected cells die has also been observed (133). $CD4^+$ T-cells live for a short period of time, constituting short-lived reservoirs, whereas macrophages and dendritic cells are long-lived reservoirs which can sustain HIV for weeks or months (127, 134). HIV replicates extremely well, and completes its life-cycle in approximately 2.5 days, generating over 10 billion virus particles daily (135).

1.9. The biological phenotype of HIV-1

HIV-1 isolates were initially characterized as non-syncytium-inducing (NSI) or syncytium-inducing (SI) for cytopathology characterizations, and slow/low or rapid/high when characterized according to its replication. Furthermore, NSI and SI isolates were associated with the slow/low and the rapid/high phenotypes, respectively (136-138). After CCR5 and CXCR4 were identified to act as cofactors for entry of HIV-1 into host cells (57, 58, 139), NSI and SI isolates were identified to use these coreceptors differently. NSI isolates predominantly used CCR5, whereas

SI isolates used CXCR4 alone or in combination with CCR5. Additionally, the slow/low replication phenotypes was associated with the usage of CCR5, whereas the rapid/high phenotype was observed in isolates that used CCR5 and other coreceptors including CXCR4 (140). The observation that all SI isolates infect lymphocytes from donors who do not express functional CCR5, and that NSI isolates are dependent on CCR5 for entry, created certain assumptions about the biological phenotype of HIV-1. The assumptions were that NSI isolates were macrophage-tropic and had the slow/low rate of replication, while SI isolates are T-cell tropic and have the rapid/high replication phenotype (69). Because of the inconsistencies observed when characterizing the biological phenotype of HIV-1 according to replication rate, syncytium inducing ability, and tropism for macrophages or $CD4^+$ T cell, a new way of characterizing the phenotype of HIV-1 was devised based on the usage of major coreceptors exclusively. Viruses which used CCR5 exclusively were termed R5, those that used CXCR4 exclusively were termed X4, and viruses which used CCR5 and CXCR4 (dualtropic) were termed R5X4 (141).

Besides CCR5 and CXCR4, some variants of HIV-1 are capable of using alternative coreceptors for entry. Using cells lines induced to express selected coreceptors on their surface, HIV-1 has been shown to enter cells using the following alternative coreceptors: APJ, CCR1, CCR2b, CCR3, CCR8, CX3CR1, CXCR6, CMKLR1, D6, FPRL1, GPR15, RDC-1 and STRL33 (142-150). Usage of certain alternative coreceptors is not consistent between subtypes as HIV-1B variants were shown to have robust usage of CCR3, whereas HIV-1A and HIV-1C variants preferred using FPRL1 over CCR3, and HIV-1D was unable to use CCR3 or FPRL1 (146). Normally, variants use alternative coreceptors along with CCR5 and/or CXCR4, but one report showed that a rare transmitted/founder HIV-1B variant was able to enter cell lines expressing GPR15, APJ and FPRL1 while it did not use CCR5 or CXCR4 (151). A subset of HIV-1B isolates are able to use CCR5 as well as CCR3 to enter microglia, major target cells of HIV-1 in the CNS (152-154), while some variants depend on CCR5 primarily for entry (155). Blocking of CCR3 and CCR5

with antibodies, however, does not prevent infection of microglia by some viruses as other receptors may be used by HIV-1 (156). Although alternative coreceptors may be used to enter primary cells such as PBMCs (144, 157, 158), it is unclear what the importance of alternative coreceptor usage is for disease progression as individuals in end-stage disease may have variants which do not use alternative coreceptors (72). It is suggested, however, that the additional usage of alternative coreceptors may contribute to the pathogenicity of HIV-1 (159).

In one study, HIV-1C from the central nervous system (CNS) has been shown to use CXCR6 frequently unlike other subtypes, suggesting that they may be a subtype-specific preference of alternative coreceptor usage in the CNS (160). CCR3 is an alternative coreceptor previously shown to support infection of microglia (153), a major target of HIV-1 in the CNS, though it is not known whether HIV-1C from the CNS is capable of using this coreceptor for entry. If HIV-1C in the CNS is capable of using CCR3, this may impact the pathogenesis of the virus in the CNS compartment and potentially undermine the efficacy of maraviroc used to treat CCR5-using viruses in that compartment. Further investigations about the alternative coreceptor usage of HIV-1C in the CNS are required to understand the pathogenesis of the virus in that compartment and to optimize therapies which include entry inhibitors.

1.9.1 Major coreceptor usage and disease progression

Individuals who inherit a homozygous defect of the CCR5 gene, which results in a 32 base pair deletion of the gene, are not susceptible to infection by viruses despite repeated exposure, providing evidence that R5 variants establish HIV infection (161, 162). The $CD4^+$ T-cells of these individuals are, however, infected *in vitro* by viral variants which use CXCR4 for entry (163). Individuals who are heterozygous for the defective gene have slower disease progress than those with intact copies of the CCR5 gene (164).

The switch of major coreceptors by HIV-1 from exclusive usage of CCR5 to the additional or exclusive usage of CXCR4 is often accompanied by the usage of alternative coreceptors (165,

166). Approximately 50% of individuals with chronic stage HIV-1B harbor viruses which switch to using CXCR4, and this is associated with progression to AIDS (167, 168). However, a coreceptor switch is not compulsory for disease progression or the development of AIDS, as individuals with R5 variants exclusively may develop AIDS without CXCR4-using variants. In fact, some cohorts did not have a single individual infected with a HIV-1C CXCR4-using, as CCR5-using variants dominated irrespective of the stage of disease progression (72, 169). Other studies, however, debunked the assumption that HIV-1C is incapable of using CXCR4, as they noted rare usage of CXCR4 by HIV-1C (170, 171). In contrast, a few studies indicate that usage of CXCR4 by HIV-1C is not uncommon and may be observed in up to 30% of antiretroviral therapy naïve individuals, albeit those with advanced or end-stage HIV disease progression of AIDS (116, 172, 173). It was suggested that HIV-1C may be evolving the ability to use CXCR4 more frequently over time (173), but it is incompletely clear what the causes or requirements of enhanced CXCR4 usage by HIV-1C are.

1.9.2 Cellular tropism

The vast majority of HIV-1 isolates infect $CD4^+$ T-cells at all stages of disease (124, 174). However, some strains of HIV-1 have the ability to infect macrophages and to sustain replication in this cell type longer than in $CD4^+$ T-cells (175, 176). Viruses adapted to replicate in cells of the lymphoid lineage (T-cell tropic; T-tropic) often are unable to infect macrophages productively. On the other hand, viruses which productively infect macrophages (macrophage-tropic; M-tropic) have better host-cell range, as they are able to additionally infect $CD4^+$ T-cells efficiently (177).

A population bottleneck is associated with transmitted HIV-1 variants, where a single R5 transmitted/founder (T/F) virus typically establishes infection (21). The R5 T/F viruses infect $CD4^+$ T-cell efficiently, and have modest capacity to infect monocyte-derived macrophages (MDMs) which are a surrogate of primary macrophages (178). Unlike prototypic M-tropic strains of HIV-1 which efficiently infect MDMs, such as HIV-$1_{Ba\text{-}L}$, T/F viruses have significantly lower

infectivity in MDMs, suggesting that macrophage tropism (M-tropism) is not a requirement for transmission (179).

The development and determinants of M-tropism are different from individual to individual, as reviewed by Arrildt *et al* (2012) (180), however, what is consistent is that determinants of M-tropism are within Env, where single amino acids or regions have been associated with M-tropism (181-186). M-tropic viruses are linked with the ability to enter cells expressing low levels of CD4 on their surface (187-190). Given that M-tropic viruses were frequently observed in individuals with advanced/end stages of infection, it was suggested that HIV-1 may become M-tropic when the host becomes immunosuppressed and target cells expressing CCR5 are depleted (191). Furthermore, the absence of M-tropic viruses in individuals with acute and chronic infection (147) supported information suggesting that M-tropism arises in end stage disease, and may be enriched in anatomical compartments where the cells express low levels of CD4 on their surface. Although M-tropic viruses were frequently isolated from the brain of individuals experiencing severe forms of HAND (181, 192, 193), these viruses have also been identified circulating in the blood (194).

The majority of HIV-1 variants circulating in the blood, lymph nodes and semen are unable to infect macrophages (190). In contrast, macrophages and microglia resident in the CNS are susceptible to infection by HIV-1, and this may ultimately lead to the development of HIV-associated neurological disorders (HAND) (195). CCR5 is the main coreceptor used for entry in macrophages and microglia (153, 154, 156, 196), and supports HIV-1 infection in more than one part of the brain (192, 197). However, a subset of X4 viruses have the capacity to infect macrophages and microglia (198, 199). Besides the major coreceptors, alternative coreceptors appear important for entry into microglia also. CCR3, which is expressed on microglia alongside CCR5 and CXCR4, and may be used by some strains of HIV-1 to infect this cell type (152-154). Taken together, cellular tropism is not restricted by coreceptor usage. Instead, M-tropism of HIV-1 is predictive of neurotropism (192).

The ability of HIV-1 to infect MDMs has been used as a standard to determine whether it was M-tropic or T-tropic (reviewed by Arrildt *et al*., 2012 and Josephs *et al*., 2015) (180, 200). It was recently established that M-tropic viruses infect MDMs on average ~28-fold more efficiently than T-tropic viruses (201). A limitation of this method is that MDMs vary in their ability to support infection, and with M-tropic viruses having various efficiencies of infection, M-tropism is therefore not well defined. Thus, due to variability between donor samples and sampling time-points, or the preparation of the assay, some T-tropic viruses would be qualified as M-tropic, while M-tropic viruses would be classified as T-tropic (reviewed by Arrildt *et al*., 2012 and Joseph *et al*., 2015) (180, 200). A new, accurate method for characterizing M-tropism involves the evaluation of HIV-1 entry in Affinofile 293 cells (202). The levels of CD4 and CCR5 on the surface of Affinofile 293 cells may be induced independently and simultaneously, such that they reflect the phenotype of cells from the myeloid (expressing low densities of CD4) or lymphoid (expressing high densities of CD4) lineage (194, 201-203). With this method, M-tropic virus have been identified to efficiently enter Affinofile 293 cells expressing as low as ~1,200-1,400 CD4 receptors per cell, while T-tropic viruses from the same individual are at least 15% less efficient at infecting the cells using the same number of receptors (203).

1.10. HIV in the central nervous system

1.10.1 Trafficking of HIV-1 into the CNS

The blood-brain barrier (BBB) is a biological, physical and immunological separator of the peripheral blood and CNS compartments (204). Essentially, it is composed of brain microvascular endothelial cells (BMECs) held together by tight junction proteins, which determine the permeability of the BBB also. Below the BMECs lie the astrocytes, perivascular macrophages, microglia and neurons which regulate the function of the BMECs. There are three main ways which HIV-1 uses to gain entry into the CNS from peripheral blood (Figure 1.11) (205). The first

mechanism is by diapedesis of infected $CD14^+/CD16^+$ monocytes or macrophages which are major reservoirs of HIV-1 and contribute to the establishment of virus in the CNS. This mode of viral trafficking is also known as the "Trojan-horse mechanism". One study showed that HIV-1 infected $CD14^+/CD16^+$ monocytes circulating in peripheral blood may be recruited into the CNS to perform immune surveillance, thus trafficking HIV-1 across the BBB and into the CNS (206). Like the $CD14^+/CD16^+$ monocytes, infected macrophages from the periphery may also enter the CNS and introduce glia to HIV infection. Replication of HIV-1 in the CNS leads to the added recruitment of activated $CD14^+/CD16^+$ monocytes from the periphery via cytokine-chemokine networks (207). HIV-1 infected cells and bystander cells in the CNS secrete pro-inflammatory cytokines, chemokines and other compounds which may be neurotoxic, leading to neuronal dysfunction (208). A second way of HIV-1 trafficking into the CNS may involve the tight junctions which hold together the BMECs that compose the BBB and keep the transport of cells and substances regulated. The loss of tight junction integrity increases the permeability of the BBB allowing monocytes to migrate into the CNS (209, 210). HIV-1 gp120 and Tat proteins, individually, have the ability to affect the integrity of tight junctions by regulating their expression and distribution (211). A third mechanism of viral trafficking is by transcytosis, where BMECs are permissively infected by HIV-1 in a non-cytolytic way. Infected BMECs may allow leakage of cytokines and chemokines from the CNS to peripheral blood, thus affecting the regulated transport of substances across the BBB and also increasing the inflow of infected leukocytes into the CNS from the periphery. Naturally, CNS cells intimately associated with the infected BMEC are then exposed and infected by HIV-1 as well (212).

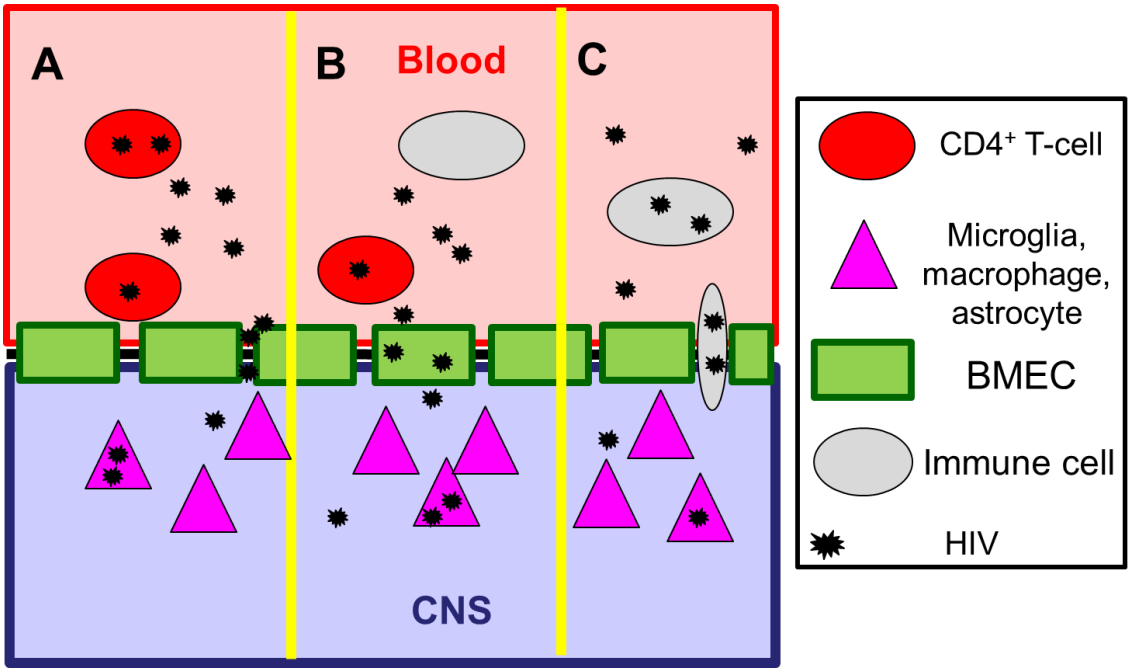

Figure 1.11: The trafficking of HIV-1 from peripheral blood to the central nervous system (CNS)

The blood-brain barrier (BBB) is a physical barrier composed of brain microvascular endothelial cells (BMECs) which regulates the movement of cells and substances between the peripheral blood and CNS compartments. The predominant target cells of HIV-1 in peripheral blood are the $CD4^+$ T-cells. In the CNS, microglia, perivascular macrophages and astrocytes are targeted by HIV-1. There are three main ways HIV-1 may enter the CNS from the periphery: The virus may enter the CNS through disrupted tight junctions which hold together the BMECs (**A**). HIV-1 in the periphery may enter BMECs via transcytosis (a non-cytolytic processes) and then be introduced to target cells in the CNS (**B**). HIV-1 infected mononuclear phagocytes ($CD14^+/CD16^+$ monocytes or macrophages) in the periphery may be introduced into the CNS via diapedesis. Thereafter, HIV-1 in these cells may escape to infect other CNS resident cells. This mode of trafficking is also known as the "Trojan-horse mechanism" (**C**). Adapted from Strazza *et al* (2011) (205).

1.10.2 Compartmentalization of HIV-1 in the CNS

HIV-specific antibodies identified in the central nervous system (CNS), and not the periphery, in advanced disease suggested there was active infection of the brain by HIV-1, but it was not known when, how or why the virus enters the CNS (213). Early studies found HIV-1 antigens in individuals who had not yet seroconverted showing that HIV-1 was able to infiltrate the CNS soon after primary infection and persists in all stages of diseases (214). While it was recognized

that people living with HIV frequently developed HIV-associated neurological disorders (HAND) including HIV-associated dementia (HAD), pathogenic mechanisms to explain the observation were minimal. Autopsies revealed that HIV-1 could infect tissues of the brain (215-217). More specifically, the virus was confirmed to have the capacity to infect and replicate in the choroid plexus, astrocytes, neurons and microglia in chronic stages of disease before AIDS (218-222).

Because isolating HIV-1 from the brain-tissues of living study participants is an invasive procedure and not possible, HIV-1 in the cerebrospinal fluid (CSF) is studied as a surrogate of CNS viruses instead (223-231). HIV-1 was observed to exhibit a CNS-specific replication or biological phenotype, but it was not clear why, when and how the virus accomplished this form of compartmentalization (232). Individuals with HAND were found to have elevated CSF HIV RNA levels (223, 233), and a discordant biological phenotype of HIV-1 between plasma and CSF, suggesting that HIV may evolve differently in the CNS (223). The concept of CNS compartmentalization of HIV-1 was proposed by Ellis *et al* (1997). They proposed that in the pre-AIDS period HIV-1 is trafficked to the CNS through mononuclear cells and this is how the viral load seen in the CSF is determined. Additionally, in AIDS, independent CNS replication of HIV-1 is unmasked when the $CD4^+$ target cells are depleted, and this is linked to the HAND observed (233). A number of studies thereafter showed that peripheral blood-derived viruses can be genetically different to viruses in the CSF or brain-tissue (226-228, 231, 234, 235), confirming that an enrichment of compartmentalized CNS-exclusive variants arise when individuals present with HAD. Furthermore, R5 variants were responsible for the development of HAD in the majority of individuals, while it was unclear whether CXCR4-using variants contributed the development of HAD, as they were rarely identified in the CSF or CNS compartment (224).

Cells of the monocyte-macrophage lineage or immune pressures in the brain were thought to constrain the genetic and functional diversity of HIV-1 in a way that is different to other cells (236, 237). It was proved that some well-established R5 variants specific to the CNS may have the added ability to amplify themselves clonally in MDMs, which express low densities of CD4 on their surface, while other variants can only infect lymphoid-like cells which express high

densities of CD4 on their surface (194, 238, 239). In individuals with neurological deterioration it is likely that cells of the myeloid lineage replace cells of the lymphoid lineage as the dominant host for HIV-1 replication in the CNS, especially in the context of limited pleocytosis and CD4 counts (239).

Few studies have focused on the neurotropism of HIV-1C and its ability to infect myeloid-like cells. One study revealed that half of children under the age of 18 month may have incomplete equilibration of viral variants between peripheral blood and the CNS compartment, whereas half of children older than 18 months of age had compartmentalized viral variants in the CNS. HIV-1C is introduced into the CNS compartment early after primary infection or is recruited to the CNS later during the course of infection. Furthermore, the authors proposed four different ways HIV-1 may be compartmentalized in the CNS: Firstly, the virus may be trafficked to the CNS early after primary infection with little selectivity. Secondly, certain HIV-1 variants may be selected to replicate in the CNS early after primary infection while others are not. Thirdly, limited or minor compartmentalization in the CNS may arise early after primary infection without a significant increase of viral load in that compartment. Lastly, import of virus into the CNS may lead to distinct compartmentalization of variants which are adapted to replicate in myeloid-like cells, with an increased viral load concurrently (228). An extension of the study of HIV-1 neurotropism by the same authors, designed to factor in the effects of pleocytosis, revealed four states to describe the viral populations between the blood and CNS in early primary infection (Figure 1.12). The first state observed was minimal CNS replication of HIV-1 and pleocytosis; viral populations in blood were similar to those in the CNS, and any independent viral replication was undetectable. The second state observed was equilibration of viral variants between compartments, high virus copy numbers in both compartments and high levels of pleocytosis: the source of virus in the CNS was $CD4^+$ T-cells trafficked from the blood. The third state observed was clonally amplified HIV-1 in the CNS with low genetic diversity; R5 virus was introduced recently into the CNS but relied on cells expressing high amounts of CD4 to expand. The last

state observed was persistent CNS replication of complex viral populations which are compartmentalized (231).

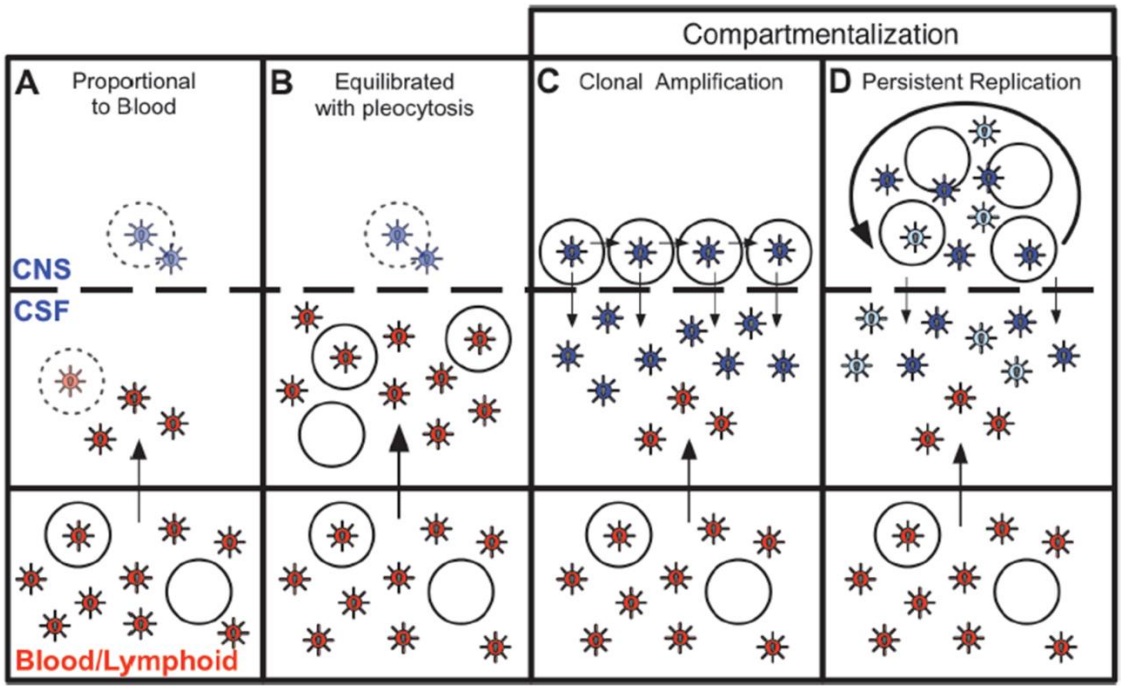

Figure 1.12: The relationship of viral populations between the blood and CNS compartments in early primary infection.
The blood and CSF/CNS compartments are shown; blood plasma and CSF/CNS-produced HIV-1 variants are red and blue color, respectively; solid and dotted circles represent $CD4^+$ T-cells producing detectable and undetectable HIV-1 populations, respectively, and arrows show the movement of viral variants between compartments. The first state observed is when the viral load in the CSF/CNS compartment is low, pleocytosis is low and the viral variants in both compartments are similar (**A**). The second state observed is when viral populations between compartments are equilibrated and pleocytosis is high. Viruses seen in the CSF are likely produced from $CD4^+$ T-cells infiltrating the CSF, and local CNS replication of virus is obscured if occurring (**B**). The third state observed is when low diversity of clonally amplified HIV-1 in the CSF/CNS compartment persists. Low diversity is shown as viruses with a single shade of blue color (**C**). The fourth and last state is when genetically complex HIV-1 populations in the CNS persist; ongoing local viral replication is observed, and the viral population is diverse, shown as variants with different shades of blue color (**D**). Reproduced from Sturdevant *et al* (2015) (231).

1.10.3 Cryptococcal meningitis (CM) affects compartmentalization of HIV-1

Opportunistic infections of the CNS infections affect HIV-1 RNA concentrations in the CNS. Studies of individuals with HIV-1 and CM co-infection revealed an association between HIV-1 RNA concentrations in the CSF and the number of CSF white blood cells (240). However, because the HIV-1 RNA concentration in the CSF did not correlate with that of the plasma, this was indication of intrathecal HIV-1 replication, and that the blood and CNS compartments may behave differently (241, 242). Although it was not clear initially why there was a lymphocytic infiltrate into CNS, it was thought that intrathecal cytokine production may recruit already infected lymphocytes into the CNS and enhance local inflammation. The consequences of that would be that more lymphocytes would be recruited into the CNS, further breakdown of the BBB and increased viral replication. Subsequent studies confirmed that pro- and anti-inflammatory cytokines produced locally in the CNS contribute to lymphocytic infiltrates into the CNS (243). A recent study shed more light on compartmentalized immune responses in matched blood and the CNS of patients with CM. It was shown that natural killer (NK) cells and monocytes in the CSF have different markers of activation and function to those in the blood. Immunoregulatory NK cells in the CSF were in higher proportions than in blood, suggesting that they there were recruited to the CNS by pro-inflammatory cytokines (244). Further evidence that the CNS is a unique environment relative to the blood is the observation that HIV-1 RNA concentration in the CSF is significantly lower than in peripheral blood in patients with CM. However, it is unclear whether this is because the viral genotypes and phenotypes in the two compartments are different (245).

Core research problems and their significance

The CNS is an anatomical compartment distinct from other compartments of the body. The immunological milieu and targets for HIV-1 in the CNS are different compared to the blood. The majority of the studies of HIV-1 genetic or phenotypic compartmentalization between the blood and CNS were focused on HIV-1B to understand HAND in the absence of co-infections (223, 226, 229, 235, 239, 246-250). Furthermore, two studies of HIV-1C compartmentalization between the blood and CNS were focused on young children with HAND (228) and individuals in the chronic stage of HIV-1 disease (160). Even less is known about the effect of co-infection on HIV-1 compartmentalization, though one case study suggested that CNS co-infection with varicella-zoster virus may increase the replication of HIV-1 (251). In the context of HIV-1 and CM co-infection, it has been shown that HIV-1 RNA concentrations between the plasma and CSF may be compartmentalized (242, 245). However, it is not clear whether this is due a difference of HIV-1 genotypes and/or phenotypes between the blood and CNS compartments.

Given that *Cryptococcus neoformans*, the fungal pathogen which causes CM, is capable of crossing the blood-brain barrier (BBB) and may alter the immunological environment in the CNS, this may affect the trafficking of HIV-1 between the blood and CNS as well. Therefore, clarifying whether or not CM patients have compartmentalized HIV-1C will be important for understanding the role of co-infections in HIV-1 compartmentalization and informing the therapeutic management of HIV-1 in the CNS.

The switch of major coreceptor usage by HIV-1, from exclusive usage of CCR5 to the additional or exclusive usage of CXCR4, usually occurs in the chronic stage of infection for individuals harboring HIV-1B (165, 167), and the switch is associated with an accelerated rate of disease progression (166, 168). There is no consensus on the prevalence of HIV-1C major coreceptor switching, although it appears to be less frequent than in HIV-1B. The switch of major coreceptor usage has been observed largely in individuals with end-stage HIV disease; when they already

have a low $CD4^+$ T-cell count and an AIDS-defining illness (116, 172, 173). However, other studies have recorded rare switching occurring (170, 171, 252, 253), while some noted an absence of coreceptor switching in end-stage disease (72, 169). Therefore, the context and requirements of HIV-1C coreceptor switching are unclear. If usage of CXCR4 by HIV-1C is on the rise, then an accurate prevalence of individuals with CXCR4-using HIV-1C is important as it will indicate the number of individuals who are likely to progress to AIDS faster, and will inform which individuals are eligible for maraviroc, a therapeutic CCR5 antagonist.

HIV-1C is capable of using the major coreceptors CCR5 and CXCR4 individually or in combination (74, 77, 116, 173), and may even use alternative coreceptors (116, 146, 147, 158-160, 170). The usage of alternative coreceptors by HIV-1C has been frequently reported in individuals with chronic or end-stage disease, however, those were viruses were not from peripheral blood predominantly. An evaluation of the major and alternative coreceptor usage of HIV-1 in the CNS revealed that HIV-1C uses CCR5 predominantly while it may also have a preference for the CXCR6 coreceptor (160). However, it is not clear which receptors other than CCR5 or CXCR6 may be used by HIV-1C in the CNS. CCR3 is expressed on the surface of microglia and macrophages in the CNS, and may be used by HIV-1B isolates (152-154). However, it is unclear whether CCR3 is used by HIV-1C isolated from the CNS compartment. Evaluating the usage of this alternative coreceptor by HIV-1C will improve our understanding of HIV-1 phenotypes in end stage disease and the pathogenesis of HIV-1C in the CNS compartment.

The motivation for this research study

A comprehensive genotypic and phenotypic characterization of HIV-1C in individuals presenting with CM is lacking. Here, the overall aim of our research study was to characterize the genotypic and phenotypic properties of HIV-1C circulating in cerebrospinal fluid and plasma of antiretroviral therapy naïve individuals presenting with CM. We designed our study to contribute to the understanding of the following:

- HIV-1C genotypes, and gene flow between the peripheral blood and CNS compartments.
- HIV-1C major coreceptor usage phenotypes in end-stage disease, and the eligibility of individuals for using maraviroc.
- The usage of an alternative coreceptor, CCR3, by HIV-1C in peripheral blood and CNS.

For the genotypic characterization of HIV-1C, we had the following sub-aims:

1. To determine whether there was genetic intermixing of HIV-1C variants between the peripheral blood and CNS compartments.
2. To determine whether cART naïve patients living with HIV-1C and CM are eligible for treatment with the CCR5 antagonist maraviroc, which is now in clinical use only for individuals with R5 viruses only.

For the phenotypic characterizations of HIV-1, we had the following sub-aims:

1. To confirm the functionality and major coreceptor usage of HIV-1C in CM patients.
2. To determine the accuracy of CPAs which predict the major coreceptor usage of HIV-1C.
3. To evaluate the usage of an alternative coreceptor by HIV-1C in end-stage disease.

The organization of the thesis

This thesis is organized in the following way:

1. Chapter 1 is the introduction of the thesis. It includes the background of topics relevant to this thesis, the aims and objectives for our studies.
2. Chapter 2 is the first results chapter. It was structured in accordance with the specifications of the journal it was submitted to for publication.
3. Chapter 3 is the second results chapter.
4. Chapter 4 is the third results chapter.
5. Chapter 5 is the discussion for the three results chapters.
6. Chapter 6 is the conclusions section for the three results chapters.
7. Chapter 7 consists of recommendations relating to the results chapters.
8. Chapter 8 consists of the references used for the introduction and discussion chapters.
9. Chapter 9 consists of appendices.

The background and literature review presented in Chapter 1 highlighted that the CNS may provide a unique tissue reservoir for HIV-1, and that the trafficking of viral variants from the blood to the CNS has implications for disease persistence and progression. Additionally, it was indicated that the frequency reported of CXCR4 usage by HIV-1C is inconsistent, though it may be high in individuals with end stage disease. In Chapter 2, we present a study conducted to establish whether or not HIV-1C variants in peripheral blood are genetically segregated from those in the CNS of individuals with CM. Additionally, we provide an estimated frequency of CXCR4-using HIV-1C in individuals with end stage infection.

Chapter 2: Individuals with HIV-1 subtype C infection and cryptococcal meningitis exhibit viral genetic intermixing of HIV-1 between plasma and cerebrospinal fluid, and a high prevalence of CXCR4-using variants

Katlego Sojane,[1] Richard T. Kangethe,[1] Christina C. Chang,[2] Mahomed-Yunus S. Moosa,[3] Sharon R. Lewin,[2,4] Martyn A. French,[5,6] and Thumbi Ndung'u[1,7,8,9]

[1]HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [2]Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, Australia; [3]Department of Infectious Diseases, King Edward VIII Hospital, University of KwaZulu-Natal, Durban, South Africa; [4]The Peter Doherty Institute for Infection and Immunity, University of Melbourne and Royal Melbourne Hospital, Melbourne, Australia; [5]Medical School and School of Biomedical Sciences, University of Western Australia, Perth, Australia; [6]Department of Clinical Immunology, Royal Perth Hospital and PathWest Laboratory Medicine, Perth, Australia; [7]Africa Health Research Institute, Durban, South Africa; [8]Ragon Institute of MGH, MIT and Harvard University, Cambridge, MA 02139, USA; [9]Max Planck Institute for Infection Biology, Berlin, Germany.

Running title: HIV-1 *env* in cryptococcal meningitis patients

Keywords: Cerebrospinal fluid, cryptococcal meningitis, HIV-1, plasma.

2.1 Abstract

The genotypic properties of HIV-1 subtype C in individuals presenting with cryptococcal meningitis (CM) are not well established. Employing single-genome amplification as well as bulk PCR, cloning and sequencing strategies, we evaluated the genetic properties of HIV-1 subtype C *env* in 16 antiretroviral therapy naïve study participants with CM. Eleven of the 16 participants had matched blood plasma and cerebrospinal fluid (CSF) evaluated, with the rest having either a plasma or CSF sample evaluated. Before antiretroviral therapy initiation, matched plasma and cerebrospinal fluid-derived *env* sequences of all 11 participants displayed genetic intermixing between the two compartments. Overall, seven of the 16 (~43.8%) participants harbored CXCR4-using variants in plasma and/or CSF, according to coreceptor usage prediction algorithms. This study suggests that HIV-1 subtype C genetic intermixing between peripheral blood and the central nervous system is common in individuals presenting with CM, and that CXCR4 usage is present in one or both compartments in approximately 44% of individuals.

2.2 Introduction

To initiate infection, the human immunodeficiency virus type 1 (HIV-1) attaches itself to a target cell by binding to cell-surface CD4 and a chemokine receptor, usually CCR5 or CXCR4 (1-3). The ability of HIV-1 to use a particular coreceptor for entry is determined primarily by the properties of the envelope protein (Env) V3 loop (4, 5), though determinants outside of the V3 loop may also contribute (6-9). Variants which use CCR5 only (R5-tropic) predominate early in infection generally, however, variants with expanded coreceptor usage ability may arise over time (10, 11). Approximately 50% of individuals infected with HIV-1 subtype B (HIV-1B) have variants which use CCR5 and CXCR4 (R5X4-tropic) or CXCR4 only (X4-tropic) during the chronic stage of disease, and the emergence of these variants is associated with an accelerated decline of CD4+ T-cell count (12, 13). In contrast, the prevalence of CXCR4 usage for entry by HIV-1 subtype C (HIV-1C) is unclear. Some studies have reported an absence or low prevalence of CXCR4 usage by HIV-1C (14-18), while others have detected 15% to 30% of individuals with advanced or end-stage disease harboring CXCR4-using variants (19-21). Further studies are needed to clarify the frequency and circumstances of CXCR4 usage by HIV-1C isolates, so that individuals' eligibility for use of coreceptor blocking drugs such as maraviroc, a CCR5 antagonist, may be accurately determined.

HIV-1 is detectable in the central nervous system (CNS) in primary infection (22-24), where its presence may ultimately cause HIV-associated neurological disorders (25, 26). Studies of CNS compartmentalization of HIV-1 are limited because obtaining brain biopsies is not possible, however, the cerebrospinal fluid (CSF) has been used often as a surrogate of the CNS compartment (27-29). Early studies of HIV-1B env genes from matched plasma and CSF showed that variants unique to the CSF could arise (30), and that this genetic compartmentalization of variants was most pronounced in individuals with HIV-associated dementia (HAD) (31, 32). Later studies combining genotypic and/or phenotypic analyses confirmed clonal amplification of HIV-1B and HIV-1C variants in the CNS, indicating that the CNS can act as a compartment of unique

HIV persistence relative to peripheral blood (24, 33, 34). In a limited number of studies, a discordant entry phenotype of HIV-1B between the blood and CNS was reported in individuals with chronic stage HIV-1 disease (27, 35-37). It is unclear how common genotypic discordance of HIV-1C between plasma and CSF is in individuals with advanced HIV infection and a co-infection.

Individuals harboring R5 HIV-1 only are eligible for combination antiretroviral therapy (cART) containing maraviroc, a CCR5 antagonist, while those harboring R5X4 or X4-tropic viruses are precluded from using this drug (38). There are multiple genotypic tools or algorithms used to determine the coreceptor usage of HIV-1 in order to determine eligibility for use of maraviroc (39). One approach is to use coreceptor usage prediction algorithms (CPAs) such as Geno2pheno, WebPSSM and PhenoSeq which utilize the whole V3 loop sequences of HIV-1 Envs to predict whether a virus will be R5 or CXCR4-using (using CCR5 and CXCR4, or CXCR4 alone) (40-42). CPAs, therefore, are attractive diagnostic tools, as they are relatively inexpensive and less laborious to use than determinations by in vitro phenotypic assays.

HIV-related cryptococcal meningitis (CM) is an acquired immune deficiency syndrome (AIDS) defining illness which is associated with high acute mortality rates, and is most prevalent in sub-Saharan Africa (43-45). Previous studies by our group revealed that compartmentalization of immune responses may arise between the blood and CNS compartment in individuals with CM, where pro-inflammatory natural killer cells, non-classical monocytes, chemokines and cytokines are enriched in CSF relative to blood (46). We were interested in establishing whether or not HIV-1 genetic properties are compartmentalized in individuals with HIV-related CM. We hypothesized that localized inflammation in the CNS caused by *Cryptococcus neoformans* (or its antigens) provides an immunological milieu that recruits HIV-1C variants into the CNS, causing viral genetic mixing between peripheral blood and the CNS. We therefore explored whether CM was associated with HIV-1C genetic compartmentalization between blood plasma and the CSF or not, and whether in this setting there was evidence of CXCR4 usage by HIV-1 subtype C variants.

2.3 Materials and methods

2.3.1 Study participants

The participants for this sub-study were part of a larger prospective clinical study of Cryptococcosis-associated immune reconstitution inflammatory syndrome (C-IRIS) conducted in Durban, South Africa between August 2009 and September 2011, as described in detail elsewhere (47). Written informed consent was provided by each participant or their next-of-kin. The ethics review boards of the University of KwaZulu-Natal (reference number BF053/09), Monash University (2009001224) and University of Western Australia (RA/4/1/2541) granted ethics approval for the study. For the current study, 16 antiretroviral therapy naïve participants infected with HIV-1 and presenting with CM for the first time at the clinic were randomly selected based on the criteria of detectable HIV-1 RNA in the plasma and CSF according to the COBAS TaqMan HIV-1 test (Hoffmann-La Roche, Basel, Switzerland) with a lower limit of 34 copies/mL, a $CD4^+$ T-cell count less than 200 cells/µL, and either a positive CSF cryptococcal antigen (CrAg) or Indian ink test result.

2.3.2 Plasma and CSF sample processing

The cell-free HIV-1 RNA levels in matched plasma and CSF samples were measured in the same polymerase chain reaction (PCR) assay run using the COBAS TaqMan HIV-1 test as described previously (48). Routine cell count analyses including the enumeration of white blood cell subsets in the CSF and $CD4^+$ T-cells in peripheral blood were performed by standard clinical laboratory assays in an accredited laboratory (47).

2.3.3 RNA extraction and the synthesis of cDNA

The extraction of HIV-1 RNA from the plasma and CSF was performed using the QIAamp Viral RNA Mini kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions.

SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) was used for cDNA synthesis as previously described (49). Briefly, 10 µL of extracted RNA was added to a mixture of 1 mM of deoxynucleoside triphosphates and 2 µM of primer OFM19 (5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3'; HXB2 positions 9,604-9,632). The mixture was first heated to 65 °C for five minutes and cooled to 4 °C for one minute. The following reagents were combined and then added to the initial mixture for a final volume of 20 µL per sample: 1X reverse transcriptase (RT) buffer, 0.1 M dithiothreitol, 40 units of RNaseOUT (Invitrogen), and 200 units of SuperScript III Reverse Transcriptase enzyme. The complete reaction mixture was incubated at 50 °C for 60 minutes, 55 °C for 60 minutes to commence cDNA synthesis, incubated at 70 °C for 15 minutes to inactivate the reverse transcriptase enzyme, and cooled to 4 °C to end cDNA synthesis.

2.3.4 Single genome amplification of plasma and CSF-derived variants

Unique viral variants were purified by single-genome amplification (SGA) and Sanger sequencing as described previously (49). Synthesized cDNA was serially diluted, and a minimum of 10 PCR reactions for each dilution were performed. The dilution which presented less than or equal to 30% positive amplification reactions represented the end-point dilution, where each positive amplification reaction contained a unique viral template (50). Both rounds of PCR were performed using the Platinum Taq DNA Polymerase High Fidelity (Invitrogen) as described previously (49, 51). The first round of PCR included the forward primer VIF1 (5'-GGGTTTATTACAGGGACAGCAGAG-3'; HXB2 positions 4,900-4,923), reverse primer OFM19, and 1 µL of diluted cDNA. The second round of PCR included forward primer ENVA (5'-GCTTAGGCATCTCCTATGGCAGGAAGAA-3'; HXB2 positions 5,945-5,982), reverse primer ENVN (5'-CTGCCAATCAGGGAAGTAGCCTTGTGT-3'; HXB2 positions 9,145-9,171) and 2 µL of first round PCR product. The cycling conditions for both rounds of PCR were as follows: 94 °C for 4 minutes; 35 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds, 68 °C

for 4 minutes; a final extension step of 68 °C step for 20 minutes followed by a hold at 4 °C. Positive PCR reactions were detected by agarose gel electrophoresis, and amplicons from end-point dilution reactions were sequenced directly from the second round PCR products.

2.3.5 Bulk PCR and cloning

Using a modified, nested bulk PCR and cloning protocol, 2.1 kb KpnI-to-BamHI *env* gene fragments were amplified from undiluted cDNA as described previously (52-54). Briefly, the forward VIF1 primer, reverse OFM19 primer, the Platinum Taq DNA Polymerase High Fidelity (Invitrogen), and 1 µL of undiluted cDNA were included in the first round PCR reaction mixture. In the second round of PCR, the forward Env-KpnI primer (5'-GTCTATTATGGGGTACCTGTGTGG-3'; HXB2 positions 6,336-6,359), reverse Env-BamHI primer (5'-GCTAAGGATCCGTTCACTAATCGT-3'; HXB2 position 8,463-8,485), the Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), and 2 µL of PCR product from the first round of PCR were included. PCR products from second round reactions were analyzed by agarose gel electrophoresis, and detected amplicons were gel purified. Purified amplicons were cloned into the pSVIII-Env plasmid for the purposes of sequencing (52, 54, 55).

2.3.6 Sequencing of HIV-1 env genes

SGA-derived amplicons from second round PCR products and recombinant pSVIII-Env vectors were sequenced using the ABI Prism Big Dye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA), incorporating multiple forward and reverse primers that covered the entire HIV-1 *env* gene (56). Sequences were resolved using the ABI 3130 XL genetic analyzer and contigs composing *env* genes were assembled and manually edited with Sequencher version 5.4.1 (Genecodes, Ann Arbor, MI). Sequencing chromatograms with multiple overlapping peaks were discarded along with duplicate sequences.

2.3.7 Phylogenetic analyses

All participant-derived *env* genes were aligned with a panel of reference sequences from different subtypes available in the Los Alamos National Laboratory HIV database (https://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html) using Clustal W. With MEGA 7 software (57), a neighbor-joining phylogenetic tree of 1,000 bootstrap replicates was constructed using the V1 to V5 *env* gene regions to determine the subtype, detect inter-participant sequence contamination, and identify inter-compartment mixing of HIV-1 variants. Evolutionary distances were computed using the maximum-likelihood composite method, where all positions containing gaps or missing data were excluded in the analysis as described previously (15).

2.3.8 Compartmentalization analyses

For two participants, CM112 and CM089, there were a minimum of 10 SGA-derived sequences from the plasma and CSF each, allowing for evaluation of the degree of HIV-1 genetic compartmentalization between the two compartments as described previously (33, 58). Maximum-likelihood phylogenetic trees showing the intra-participant phylogenetic relatedness of plasma and CSF-derived *env* genes were generated using MEGA 7 (57) and PhyML software (59) with the following parameters: HK85 nucleotide substitution model, four substitution rate categories, a transition/transversion ratio of four, estimated gamma distribution parameter and 1,000 bootstrap replicates. Compartmentalization of CSF sequences was determined using the Slatkin-Maddison (tree-based) and Hudson nearest-neighbor tests (distance-based) with 10,000 permutations, in HYPHY as previously described (33, 58, 60). A *p*-value equal to or lower than 0.05 signified CSF compartmentalization of sequences.

2.3.9 Prediction of coreceptor usage

The publicly available coreceptor usage prediction algorithms (CPAs), Geno2pheno with a false positive rate (FPR) of 5% (http://coreceptor.bioinf.mpi-inf.mpg.de/), WebPSSM subtype C sinsi (WebPSSM; https://indra.mullins.microbiol.washington.edu/webpssm) and PhenoSeq (www.burnet.edu.au/phenoseq) were used to predict the major coreceptor usage of participant-derived sequences (40-42). All three tools predict the major coreceptor usage of Env V3 sequences, however, they are not optimized to distinguish R5X4-tropic variants from X4-tropic variants. Here, we designated variants as R5 if they were predicted to use CCR5, and R5X4/X4 if they were predicted to use CXCR4 in combination with CCR5 or alone. Where the algorithms differed in predictions, we characterized an Env as R5 or R5X4/X4 based on two concordant algorithms.

2.3.10 Identification of N-linked glycosylation sites in the Env V3 loop sequences

Using the N-GlycoSite tool (https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html), the location and number of putative N-linked glycosylation sequons (tripeptide N-X-S and N-X-T, where X is any amino acid except proline) in V3 loop sequences was recorded (61).

2.3.11 Net charge calculation

Positively charged amino acids (lysine, arginine, and histidine) were scored as +1, and negatively charged amino acids (aspartate and glutamate) were scored as -1. The net charge of the Env V3 was the sum of all positive and negative charges within the V3 sequence.

2.3.12 Statistical analyses

Two-tailed, unpaired nonparametric t-tests were performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA) unless otherwise stated.

2.4 Results

2.4.1 Clinical features of individuals harboring HIV-1 and presenting with CM

The detailed demographic and clinical characteristics of HIV-1 infected individuals with CM in the parent study have been described in detail elsewhere (47). Here, we randomly selected eight males and eight females infected with HIV-1 and presenting with CM, with matched plasma and CSF samples available in our laboratory for HIV-1 genotypic analyses. The samples were collected prior to initiation of cART. Demographic and clinical characteristics of these study participants are summarized in Table 2-1. The median age of the participants was 29.5 years (range: 23-45), with no significant difference between the median age of males and females (p = 0.46), and the median $CD4^+$ T-cell count was 27.5 (interquartile range: 10-72 cells/µL). The median plasma and CSF HIV-1 viral load of the participants were 5.1 (interquartile range: 4.72-5.2) $\log_{10}$ copies/mL and 4.73 (range: 4.3-4.7) $\log_{10}$ copies/mL, respectively, and were not significantly different (p = 0.13). There was no significant difference between male and female plasma HIV-1 viral load (p = 0.23), and no significant difference between male and female CSF HIV-1 viral load (p = 0.88). Two participants, CM032 and CM117, did not have detectable white blood cells in the CSF, whereas all other participants exhibited pleocytosis (>5 CSF white blood cells/µL). We observed a positive correlation between the $CD4^+$ T-cell count and white blood cell count in the CSF (spearman r = 0.7, p = 0.0026).

Table 2-1: Demographic and clinical characteristics of the study participants.

ID[a]	Age	Sex	CD4 count[b]	Plasma VL[c]	CSF VL[d]	CSF WBC[e]
CM019	45	M	7	5.15	4.33	28
CM021	30	F	134	4.34	4.34	40
CM029	33	F	121	5.22	5.99	424
CM032	34	F	5	4.94	2.52	0
CM041	26	F	58	4.51	4.93	102
CM050	23	F	16	4.92	4.8	6
CM052	24	F	172	4.62	4.83	36
CM054	23	M	7	5.2	4.2	14
CM070	25	M	11	5.04	4	18
CM089	27	M	114	5.67	5.85	154
CM094	29	F	35	5.15	4.03	84
CM098	40	M	14	4.76	5.27	66
CM108	27	M	20	5.16	5.02	28
CM112	34	F	39	5.52	5.62	6
CM117	38	M	1	5.72	4.66	0
CM132	34	M	53	4.58	4.43	38

[a]The identification number of the study participant. [b]The $CD4^+$ T-cell count was recorded as cells/μL. [c,d]The cell-free HIV-1 RNA viral load (VL) in plasma and CSF was recorded as $\log_{10}$ copies/mL. [e]The CSF white blood cell (WBC) count was recorded as cells/μL.

2.4.2 The phylogenetic relationships of HIV-1 variants

We amplified and sequenced a total of 251 unique *env* genes from the plasma and CSF compartments of 16 participants, using either single-genome amplification (SGA) and sequencing, or bulk PCR, cloning and sequencing methods. The number of sequences generated per compartment of each participant using the two methods is summarized in Table 2-2.

Phylogenetic analysis, performed as described in Materials and methods, revealed that all the sequences generated were subtype C. As expected, plasma and/or CSF sequences of each individual participant formed a unique cluster. All of the eleven participants with available matched plasma and CSF sequences had evidence of inter-compartment mixing of *env* sequences (Figure 2.1).

Genetic compartmentalization between anatomical compartments is more accurately determined using SGA-derived sequences than bulk PCR-derived sequences, as the former limits or eliminates taq-induced recombination, nucleotide misincorporation, template resampling and cloning bias (49). We sought to establish whether there was genetic compartmentalization of HIV-1C sequences between the plasma and CSF or not of two participants (CM089 and CM112) where we generated at least 10 sequences from both of their compartments by SGA. For both participants, inter-compartment mixing of *env* sequences was evident (Figure 2.2). The Slatkin-Maddison test revealed seven and 10 inter-compartment migrations in participants CM089 and CM112, respectively. Additionally, neither CM089 nor CM112 demonstrated compartmentalization of *env* sequences (*p*-value 0.19, and 0.35 respectively). This result was confirmed with the Hudson nearest-neighbor test (*p*-value 0.34 and 0.58 for CM089 and CM112, respectively).

Table 2-2: The number of sequences generated from the plasma and CSF of the study participants.

ID[a]	Plasma[b]		CSF[c]		Total
	Cloning	**SGA**	**Cloning**	**SGA**	
CM019	11	17	1	0	29
CM021	1	2	2	13	18
CM029	0	0	5	6	11
CM032	3	-	1	-	4
CM041	-	4	-	0	4
CM050	2	1	1	9	13
CM052	3	6	3	11	23
CM054	3	-	0	-	3
CM070	5	-	0	-	5
CM089	-	10	-	19	29
CM094	0	-	3	-	3
CM098	11	-	16	-	27
CM108	9	-	8	-	17
CM112	-	16	-	16	32
CM117	12	-	9	-	21
CM132	-	8	1	3	12
Total					251

[a]The identification number of the study participant. [b,c]Sequences were generated by the single-genome amplification (**SGA**) and sequencing, or bulk PCR, **cloning** and sequencing method. Dashes indicate where we did not attempt to generate sequences for this sample using this method, and zeros indicate where we were unable to amplify cDNA before the sample was completely consumed or we could not amplify an *env* gene.

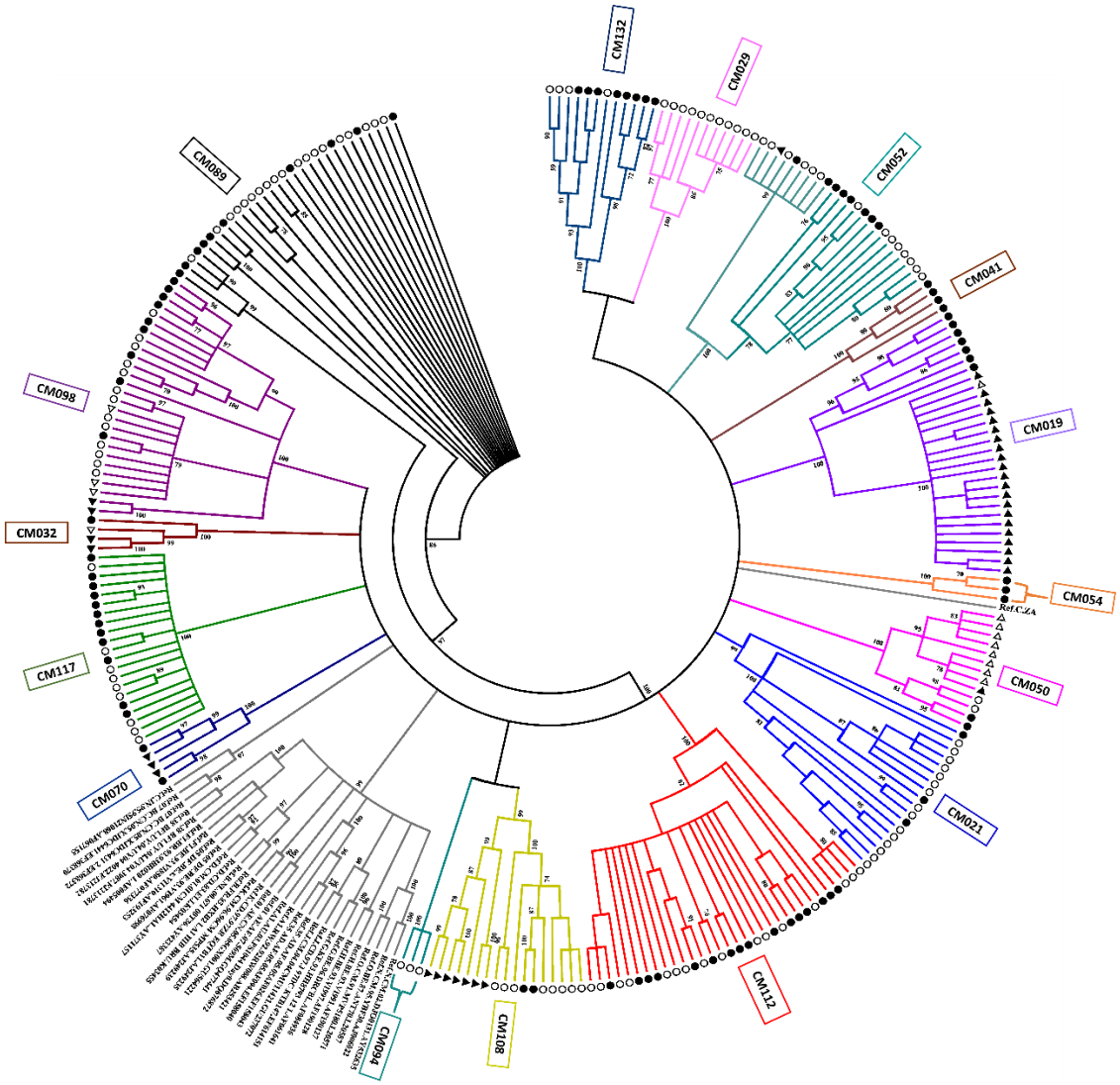

Figure 2.1: A neighbor-joining phylogenetic tree of participant-derived HIV-1 envelope gene sequences and a panel of reference sequences from different subtypes.

All of the sequences from individual study participants share the same color branches, and reference subtype sequences have grey branches. Closed circles and triangles at the tip of each branch indicate predicted R5 and CXCR4-using plasma-derived sequences, respectively. Open circles and triangles indicate predicted R5 and CXCR4-using cerebrospinal fluid-derived sequences, respectively. No inter-participant mixing of sequences was observed, suggesting an absence of inter-participant sequence contamination. Furthermore, all of the participant-derived sequences clustered with reference subtype C sequences while all other reference sequence subtypes clustered together but separately from subtype C sequences.

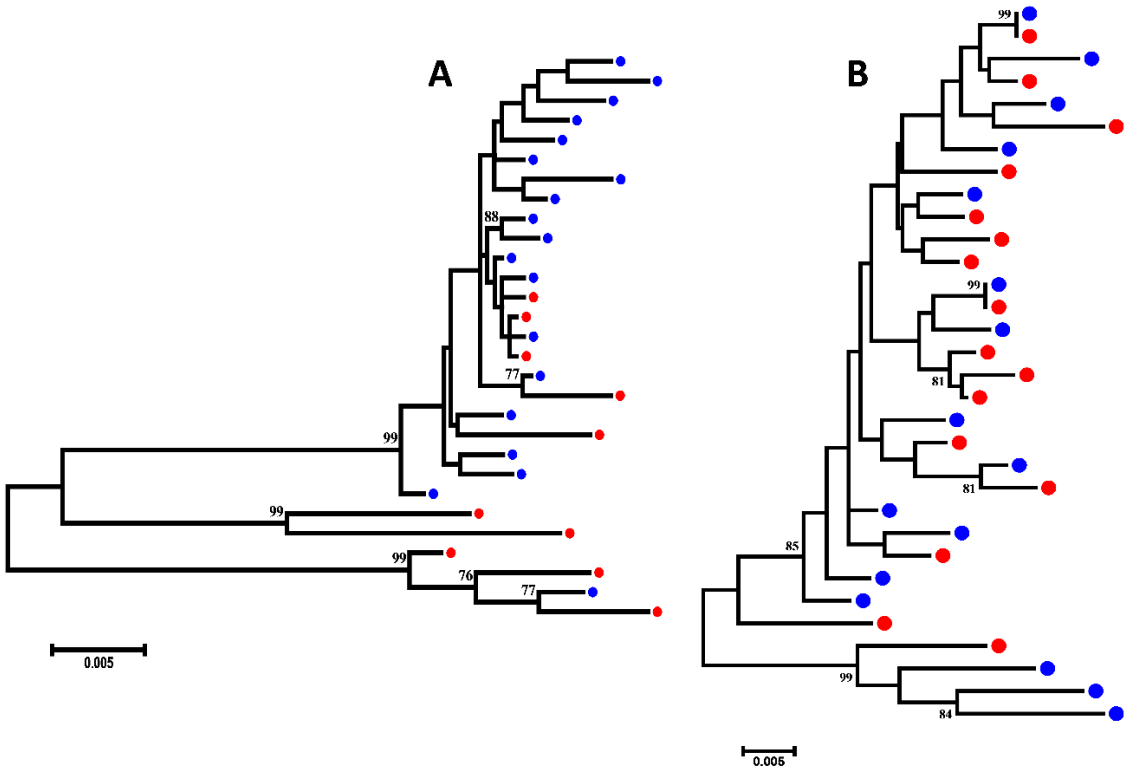

Figure 2.2: A maximum-likelihood phylogenetic tree of HIV-1 subtype C envelope variants in study participant CM089 (A) and CM112 (B).
Envelope gene sequences labeled with red and blue circles were derived from plasma and cerebrospinal fluid, respectively. Bootstrap values greater than 70% are shown at their respective nodes and the genetic distance scale bar reflects the number of nucleotide substitutions per site.

2.4.3 Predicted coreceptor usage of HIV-1C in plasma and CSF

To get better insight into the predicted coreceptor usage by HIV-1 subtype C variants in end-stage disease, we used three publicly available CPAs (Geno2pheno, WebPSSM and PhenoSeq) to generate a consensus prediction of the coreceptor usage of unique V3 sequences obtained in this study (Table 2-3). We also assessed whether predicted R5 variants predominate over CXCR4-using variants in plasma and CSF samples (Figure 2.3). Assessing plasma samples first, we identified that seven of 14 (50%) participants harbored predicted R5 variants only, whereas one participant (7.1%) harbored only predicted CXCR4-using variants, and six participants (42.9%) harbored a combination of predicted R5 and CXCR4-using variants. Thus, predicted R5 variants featured in 13 participants (92.9%) and predicted CXCR4-using variants were detected in seven

participants (50%). When we assessed the predicted coreceptor usage profile of variants in the 13 CSF samples, we identified that nine of 13 (69.2%) CSF samples harbored predicted R5 variants only, whereas two (15.4%) samples each harbored predicted R5 and CXCR4-using, and CXCR4-using variants only. Therefore, predicted R5 variants featured in 11/13 (84.6%) samples, while predicted CXCR4-using variants were in 4/13 (30.8%) samples. These results suggest that predicted R5 variants predominate in plasma as well as CSF samples, and that CXCR4 usage is more common in plasma than in the CSF of HIV-1C infected participants with CM. Overall, seven of the 16 (~43.8%) participants included in this analysis harbored predicted CXCR4-using variants in plasma and/or CSF.

We next assessed whether matched plasma and CSF samples have HIV-1C variants with concordant predicted coreceptor usage or not (

). Seven of 11 (63.6%) participants with matched plasma and CSF sequences displayed concordant predicted coreceptor tropism of HIV-1C. A further two of 11 (18.2%) participants had discordance of predicted coreceptor tropism between plasma and CSF, where both predicted R5 and CXCR4-using were detected in plasma but only predicted CXCR4-using or R5 variants were detected in CSF. In two of 11 (18.2%) participants (CM019 and CM032), the concordance of coreceptor usage between the plasma and CSF was indeterminate as we only had one CSF sequence compared to multiple plasma sequences. Our results show that concordant predicted coreceptor tropism between matched plasma and CSF compartments predominates over discordance in individuals with CM.

Table 2-3: V3 loop properties of HIV-1C envelopes.

ID[a]	Compart.	Sequence[b]	G2P	PhenoSeq	PSSM-C	Consensus	Net charge	Pos. 11, 25	PNGS	Length
CM019	CSF	CM019C.B (4:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	5	SD	0	37
	Plasma	CM019P.2 (7:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	5	SD	0	37
		CM019P.B.3 (7:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	5	SD	0	37
		CM019P.E (5:0)	R5	R5X4/X4	R5	R5	4	SD	1	35
		CM019P.I.2 (0:0)	R5	R5	R5	R5	4	SD	1	35
		CM019P_SGA14 (0:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	6	SD	0	37
CM021	CSF	CM021C.7.3 (2:14)	R5	R5	R5	R5	3	SD	1	34
	Plasma	CM021P_SGA22 (0:0)	R5	R5	R5	R5	4	SD	1	35
CM029	CSF	CM029C.B.3 (0:5)	R5	R5	R5	R5	4	SD	1	35
		CM029C.D.2 (0:1)	R5	R5	R5	R5	4	SD	1	35
		CM029C.E.2 (0:1)	R5	R5	R5	R5	5	SD	1	35
		CM029C_SGA01 (0:0)	R5	R5	R5	R5	4	SD	1	35
CM032	CSF	CM032C.6.4 (0:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	5	SR	1	35
	Plasma	CM032P.10 (1:0)	R5	R5X4/X4	R5X4/X4	R5X4/X4	4	SR	1	35

ID[a]	Compart.	Sequence[b]	G2P	PhenoSeq	PSSM-C	Consensus	Net charge	Pos. 11, 25	PNGS	Length
		CM032P.4 (0:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	9	HR	0	35
CM041	plasma	CM041P_SGA01 (3:0)	R5	R5	R5	R5	4	SD	1	35
CM050	CSF	CM050C.5 (2:1)	R5	R5X4/X4	R5	R5	5	GD	1	35
		CM050C_SGA01 (1:7)	R5	R5X4/X4	R5X4/X4	R5X4/X4	6	S-	1	34
CM052	CSF	CM052C.1 (5:7)	R5	R5	R5	R5	3	SD	1	35
		CM052C_SGA04B (0:0)	R5	R5	R5	R5	4	SD	1	35
		CM052C_SGA10B (2:4)	R5	R5	R5	R5	4	SD	1	35
	Plasma	CM052P_SGA03 (0:0)	R5X4/X4	R5	R5X4/X4	R5X4/X4	4	SD	1	35
		CM052P_SGA06 (0:0)	R5	R5	R5	R5	5	SD	1	35
CM054	plasma	CM054P.10.5 (2:0)	R5	R5	R5	R5	4	SD	1	35
CM070	Plasma	CM070P.1 (1:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	7	SK	1	37
		CM070P.F.4 (1:0)	R5	R5	R5	R5	5	SD	1	35
		CM070P.I.1 (0:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	8	NK	1	37
CM089	CSF	CM089C_SGA01 (5:13)	R5	R5	R5	R5	4	SD	1	35
		CM089C_SGA07 (0:2)	R5	R5	R5X4/X4	R5	5	SG	1	35

ID[a]	Compart.	Sequence[b]	G2P	PhenoSeq	PSSM-C	Consensus	Net charge	Pos. 11, 25	PNGS	Length
		CM089C_SGA16 (0:0)	R5	R5	R5	R5	5	SG	1	35
		CM089C_SGA21 (3:0)	R5	R5	R5	R5	4	SD	1	35
	Plasma	CM089P_SGA05 (0:0)	R5	R5	R5	R5	5	SD	1	35
		CM089P_SGA09 (0:0)	R5	R5	R5	R5	5	SD	1	35
CM094	CSF	CM094C.11.2 (0:2)	R5	R5	R5	R5	4	SD	1	34
CM098	CSF	CM098C.1 (2:6)	R5	R5X4/X4	R5	R5	3	SE	1	35
		CM098C.C.2 (0:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	3	SE	1	28
		CM098C.F.1 (5:0)	R5	R5	R5	R5	5	SA	1	35
		CM098C.F.2 (0:1)	R5	R5X4/X4	R5X4/X4	R5X4/X4	3	NE	1	35
		CM098C.H.1 (2:3)	R5	R5	R5	R5	4	SA	1	35
		CM098C.J.4 (0:0)	R5	R5	R5	R5	4	SA	1	35
	Plasma	CM098P.A.1 (1:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	6	SK	1	37
CM108	CSF	CM108C.7.12 (0:6)	R5	R5	R5	R5	4	SD	1	35
		CM108C.8.9 (0:0)	R5	R5	R5	R5	3	SD	1	35
	Plasma	CM108P.1 (1:0)	R5X4/X4	R5X4/X4	R5X4/X4	R5X4/X4	7	RD	1	35

ID[a]	Compart.	Sequence[b]	G2P	PhenoSeq	PSSM-C	Consensus	Net charge	Pos. 11, 25	PNGS	Length
		CM108P.D.5 (4:0)	R5	R5X4/X4	R5X4/X4	R5X4/X4	5	SD	1	35
		CM108P.K.1 (1:0)	R5	R5	R5X4/X4	R5	3	SD	1	35
CM112	CSF	CM112C_SGA01 (11:11)	R5	R5	R5	R5	4	SD	1	35
		CM112C_SGA11 (1:0)	R5	R5	R5	R5	4	SD	1	35
		CM112C_SGA12 (0:1)	R5	R5	R5	R5	4	SD	1	35
		CM112C_SGA13 (0:0)	R5	R5	R5	R5	3	SD	1	35
	Plasma	CM112P_SGA02 (1:0)	R5	R5	R5	R5	3	SD	1	35
		CM112P_SGA10 (0:0)	R5	R5	R5	R5	4	SE	1	35
		CM112P_SGA11 (0:0)	R5	R5	R5	R5	4	SD	1	35
CM117	CSF	CM117C.A (12:7)	R5	R5	R5	R5	2	SD	1	35
		CM117C.H.1 (0:0)	R5	R5	R5	R5	2	SD	1	35
CM132	CSF	CM132C.1 (3:2)	R5	R5	R5	R5	3	SD	1	35
		CM132C_SGA01 (0:0)	R5	R5	R5	R5	4	SD	1	35
	Plasma	CM132P_SGA02 (4:0)	R5	R5	R5	R5	4	SD	1	35

[a]The identification number of the study participant. [b]The name of a unique sequence; the first and second number in the round brackets indicates the number of other sequences with the same V3 in plasma and CSF, respectively, of the participant. The predicted coreceptor usage was determined using

Geno2Pheno (**G2P**, with false positive rate of 5%), **PhenoSeq** and WebPSSM subtype C sinsi (**PSSM-C**). **Consensus** predicted tropism was determined based on the agreement of three, or two of three coreceptor usage prediction algorithms (CPAs). The amino acids occupying positions 11 and 25 (**Pos. 11, 25**) were identified using PSSM-C; a dash indicates a gap at that position. The number of potential N-linked glycosylation sites (**PNGS**) in the V3 sequence was counted using N-GlycoSite.

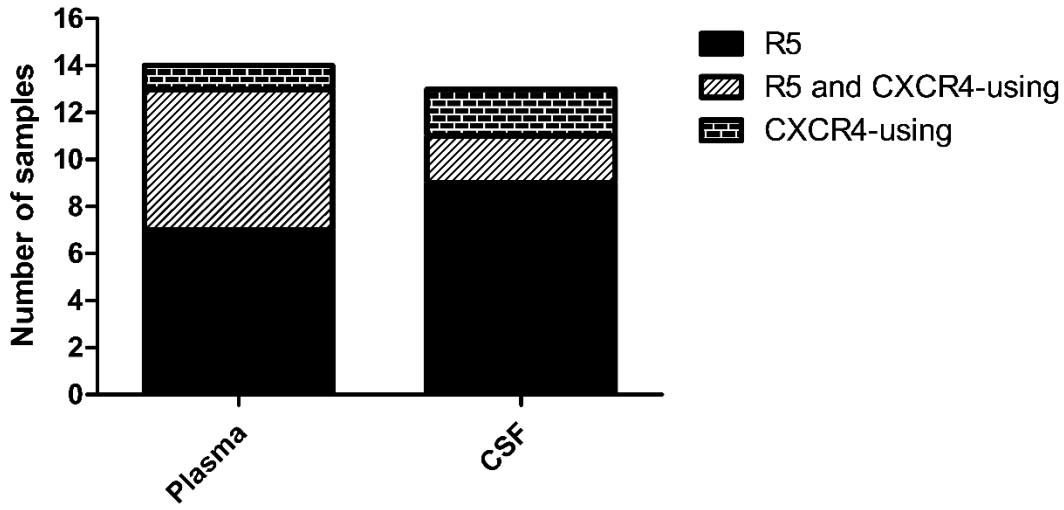

Figure 2.3: The coreceptor usage in plasma and CSF samples.
The overall predicted coreceptor usage, based on the consensus of three CPAs, in each of the 14 plasma and 13 CSF samples across 16 participants was determined. The number of plasma and CSF samples harboring predicted R5, R5 and CXCR4-using, or only CXCR4-using variants are shown as stacked columns.

Table 2-4: The overall coreceptor usage of HIV-1 subtype C in the plasma and CSF of each participants with CM.

ID[a]	Coreceptor usage	
	Plasma	**CSF**
CM019	R5, R5X4/X4	R5X4/X4
CM021	R5	R5
CM029	n.d	R5
CM032	R5X4/X4	R5X4/X4
CM041	R5	n.d
CM050	R5, R5X4/X4	R5, R5X4/X4
CM052	R5, R5X4/X4	R5
CM054	R5	n.d
CM070	R5, R5X4/X4	n.d
CM089	R5	R5
CM094	n.d	R5
CM098	R5, R5X4/X4	R5, R5X4/X4
CM108	R5, R5X4/X4	R5
CM112	R5	R5
CM117	R5	R5
CM132	R5	R5

[a]The identification number of the study participant. The abbreviation "n.d" (not determined) indicates where were did not generate a sequence for the sample.

2.4.4 Coreceptor usage determinants of HIV-1C Env

Understanding the determinants of CCR5 or CXCR4 usage by HIV-1C is of clinical relevance given that a coreceptor antagonist, maraviroc, is FDA approved and available to individuals who harbor R5 variants only (38, 62). Previously, it was shown that HIV-1C CXCR4-using variants

have specific Env third variable loop (V3) properties which distinguish them from R5 variants. CXCR4-using HIV-1C variants have a V3 loop with greater amino acid variability, increased net charge, increased length, increased frequency of insertions, GPGQ crown motif substitutions, lower glycosylation site numbers, and charged residues at position 11 and/or 25 of the V3 (15, 21, 63-66). Given that we identified a high prevalence of predicted CXCR4 usage by variants in this study, we sought to identify genetic properties of the major determinant of coreceptor usage, the V3 loop, which confer the ability to use CCR5 and/or CXCR4.

We determined the following additional properties of the V3 of all the sequences generated in our study: the overall net charge, number of potential N-linked glycosylation sites (PNGS), residues at position 11 and 25, and length (Table 2-3). Although 251 unique *env* genes were available in this study, only 58 unique V3 sequences were identified and aligned with a reference South African HIV-1C sequence (Figure 2.4). The three CPAs all agreed on the predicted coreceptor usage for 48 of 58 (82.8%) unique V3 sequences, but in 10 of 58 (17.2%) instances only two of the three CPAs agreed with each other.

As expected, the net charge of variants predicted to use CXCR4 (median 5; range 3-9) was significantly higher ($p< 0.0001$) than that of variants predicted to use CCR5 only (median 4; range 2-5) (Figure 2.5). CXCR4-using variants of participants CM019, CM050 and CM052 did not have a positively charged residue at position 11 and/or 25 of the V3, whereas participants CM032, CM070, CM098 and CM108 harbored at least one variant with a histidine, arginine or lysine at position 11 and/or 25. No variant predicted as R5 possessed a charged residue at position 11 and/or 25. This suggests that a charged residue at position 11 and/or 25 may be used to confer usage of CXCR4 for some CXCR4-using variants. Additionally, all CXCR4-using variants (except three from participant CM032 and one from CM052) had a positively charge residue at position 16 and/or 18 of the V3, within the crown motif. R5 variants maintained a GPGQ crown motif generally.

We then identified multiple sequences from participant CM019 and one from participant CM032 which did not have PNGS and were predicted to use CXCR4, while every other sequence had one PNGS only at position 6 of the V3. This suggested that an absence of a PNGS in the V3 may contribute to the usage of CXCR4, but is not compulsory for CXCR4 usage. The range of V3 length for CXCR4-using and CCR5 variants was 28-37 and 34-35 amino acids, respectively, both with a median of 35 (Figure 2.5).

2.4.5 Rare mutation of the Env V3 detected

The V3 loop of HIV-1 Env is a region important for function and maintains signature sequences according to subtype. The isoleucine at position 14 of the V3 (position 309 according to HXB2 numbering or I309) is important for the structure of the V3 loop, and is conserved in approximately 99% of HIV-1C sequences (67). I309 also modulates the exposure of the CD4 binding site (68). Notably, five unique V3 loop sequences of participant CM108 had a rare replacement of I309 with a phenylalanine residue, while every other sequence analyzed had an isoleucine at that position including the reference South African HIV-1C sequence (Ref.C.ZA) (Figure 2.4). The I309F residue was not specific to variants predicted to use CCR5, as it was present in variants predicted to use CXCR4 as well.

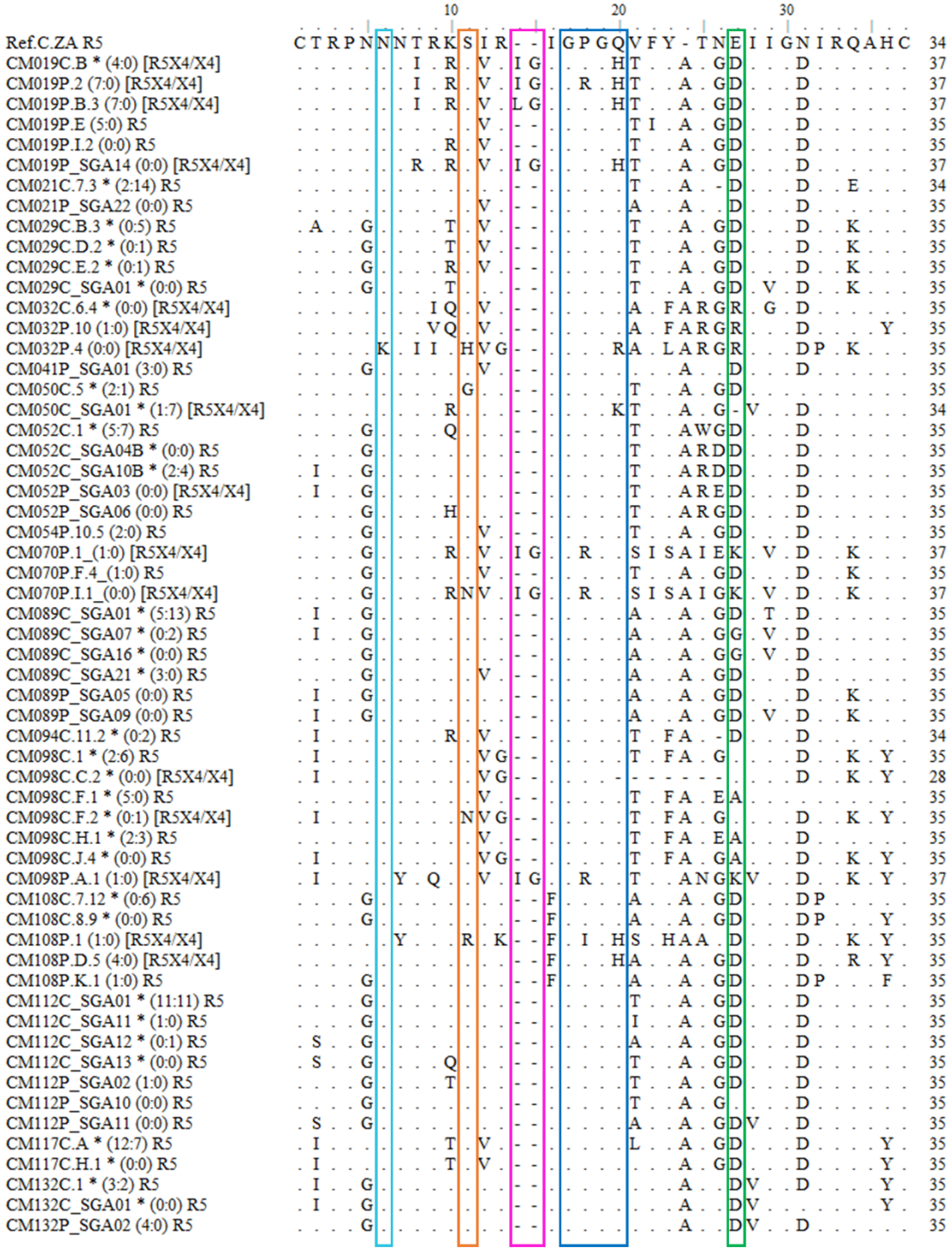

Figure 2.4: An amino acid sequence alignment of unique HIV-1C envelope V3 sequences from plasma and cerebrospinal fluid-derived viruses of antiretroviral therapy naïve individuals living with cryptococcal meningitis (CM).

A reference South African HIV-1C envelope (Ref.C.ZA) V3 sequence was aligned with CM participant-derived sequences using MUSCLE (79). A star beside the sequence name indicates a variant that circulates in CSF, whereas the absence of a star beside the sequence name indicates a variant that circulates in plasma. The first and second number in the round brackets indicates

the number of identical V3 sequences in other variants circulating in plasma and CSF, respectively. The predicted coreceptor usage of each V3 sequence is indicated in square brackets: "R5" indicates it was predicted to use CCR5 only, and "R5X4/X4" indicates it was predicted to use CXCR4 in combination with CCR5 or alone. Amino acids identical to the reference sequence are shown as dots, disagreements are indicated as bold lettering, dashes indicate deletions, and the number to the right of the amino acid sequence indicates the length of the V3. The light blue bar shows position 6 of the V3 where potential N-linked glycosylation sites are located, the orange bar shows amino acids at position 11, the pink bar shows where two-amino acid insertions occur, the dark blue bar shows the crown motif sequences and the green bar shows amino acids at position 25 of the V3.

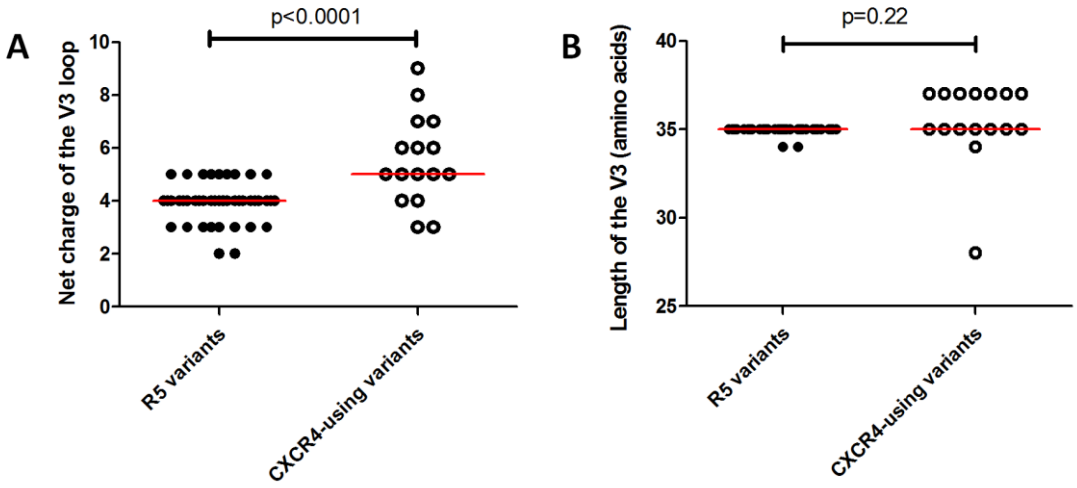

Figure 2.5: The net charge and length of HIV-1 subtype C envelope V3.
The net charge (**A**) and length (**B**) of the V3 sequence of 42 unique R5 (closed circles) and 16 CXCR4-using (open circles) HIV-1C variants in this study was compared. The sequences from plasma and CSF sharing the same predicted tropism were pooled for this analysis. The red line indicates the median for each group.

2.5 Discussion

Although others have studied HIV-1C *env* genes in individuals with advanced HIV disease and AIDS-defining illnesses (15, 17, 64, 69), genetic information of HIV-1C in matched blood and the CNS compartments of individuals with CM is sparse. In this study, we characterized the phylogenetic relatedness, and predicted coreceptor usage of plasma and CSF-derived HIV-1 variants in individuals with CM.

The sampling of HIV-1 from the CSF has been shown to be an adequate surrogate of sampling virus from the brain (29, 70). Equilibration, partial or complete, and compartmentalization of viral sequences between plasma and CSF have been described previously (24, 30, 33, 34). The occurrence of extreme genotypic compartmentalization of HIV-1 in the CNS, in particular, is suggestive of independent replication and evolution of viruses relative to the blood, and this is strongly associated with the development of neurocognitive impairment (31-33). The implications of equilibration or compartmentalization of HIV-1 sequences between blood and the CNS are unclear for individuals with co-infections. Here, we observed frequent intermixing of HIV-1C plasma and CSF-derived sequences in all 11 participants, such that plasma and CSF sequences did not form separate clusters but were phylogenetically interspersed among each other. Additionally, using the Slatkin-Maddison and Hudson nearest-neighbor compartmentalization algorithms on sequences from two participants CM089 and CM112, we observed an absence of compartmentalization of CSF-derived sequences in both participants. The majority of the participants in this study exhibited pleocytosis, a marker of CNS inflammation which is associated with HIV-1 trafficking between peripheral blood and the CNS (71), therefore it is probable that *Cryptococcus neoformans* and/or HIV-1C co-infection led to a breach of the blood-brain barrier which allowed movement of viruses between the peripheral blood and CNS compartments. Participants CM032 and CM117 did not display pleocytosis, however, though they had inter-compartment mixing of sequences suggesting that pleocytosis is not a prerequisite for genetic intermixing between blood and the CNS. Because we did not evaluate longitudinal samples, we

also could not rule out a previous pleocytosis event that may had led to an absence of viral genetic segregation in these participants. Nevertheless, these results supported our hypothesis where we expected that inter-compartment mixing would arise in some individuals with CM. It is possible that immunological compartmentalization was a recent event associated with the development of active cryptococcosis, and therefore did not allow enough time for detectable independent viral evolution.

R5 variants establish infection in the CNS compartment (23, 24), but R5, R5X4 and X4 have been isolated from the brain later in disease (72). We observed that concordance of predicted HIV-1 coreceptor usage predominated in matched plasma and CSF samples. Our results were in agreement with other studies where phenotypic assays (35, 36) and CPAs (37) were employed, showing that concordance of HIV-1 coreceptor usage predominated but discordance of HIV-1 between the plasma and CSF occurred in 10.9% to 35.7% of matched samples. Additionally, we showed that the plasma may harbor CXCR4-using variants in combination with R5 variants while the matched CSF harbors R5 variants only (participants CM108 and CM052), as described previously (36, 37). These results suggest that discordance of coreceptor usage between compartments was reflective of the functional compartmentalization of HIV-1, as others have shown that *env* sequences in the CNS may be compartmentalized (73).

Previously, it was shown that CXCR4-using HIV-1C has greater amino acid variability, increased net charge, increased length, increased frequency of insertions, GPGQ crown motif substitutions, lower glycosylation site numbers, and charged residues at position 11 and/or 25 of the V3 relative to R5 variants (21, 63-66). In this study, we investigated whether the HIV-1C R5 and CXCR4-using variants predicted by CPAs possessed the same determinants of coreceptor usage as variants from previous studies. We identified that many previously described V3 properties specific to HIV-1C R5 and CXCR4-using variants are consistent with variants in our study. In summary, the predicted CXCR4-using variants in our study had, compared to R5 variants, V3 sequences with

higher net charge, two amino acids insertions upstream of the crown motif, an absence of PNGS, a charged residue at position 11/and 25, and alterations of the GPGQ crown motif.

Changes in position 14 of the V3 (position 309 according to HXB2 numbering) affect the structure of the loop (67), and the exposure of the CD4 binding site (68). We identified that sequences from participant CM108 possessed a change of the conserved isoleucine at V3 position 14 (I309) to phenylalanine (F309). Furthermore, the F309 residue was present in variants predicted as R5 and CXCR4-using, suggesting that this substitution was not unique to any particular coreceptor usage phenotype. Given that I309 was shown to influence the structure of the V3 and modulate the exposure of the CD4 binding site, it suggests that variants in participant CM108 may have a unique entry and immune evasion profile compared to variants with the I309 residue harbored by other individuals with CM.

Reliable estimates of the prevalence of CXCR4 by HIV-1 are required to understand the evolution of the HIV-1C epidemic and to inform effective therapeutic interventions. HIV-1B has been shown to expand its usage of coreceptors in chronic and the end stages of disease (10-13). On the other hand, HIV-1C has been observed to switch major coreceptor usage less frequently (14, 15, 19-21). In some cases, a complete absence of CXCR4 usage by HIV-1C was observed in individuals with advanced disease, suggesting that the ability to switch major coreceptors for this subtype is rare or not essential (17, 18). We showed that R5 variants predominate in both plasma as well as CSF samples, but that at least one CXCR4-using variant was present in plasma and/or CSF in ~43.8% of study-participants. Although we expected to detect CXCR4 usage by HIV-1C in some individuals in this study, considering that all had an AIDS defining condition, we observed a much higher frequency of CXCR4 usage than expected based on previous studies. It may be that the CPAs overestimated the predicted CXCR4 usage as described previously (74-77).

Coreceptor usage tests *in vitro* may be employed in future to corroborate our findings. It remains unclear, however, whether CXCR4-using variants are enriched in individuals depending on the co-infection they have, or that HIV-1C has evolved to use CXCR4 more over time. We also showed that 4/13 (30.8%) CSF samples harbored predicted CXCR4-using HIV-1C variants, indicating that different phenotypes besides R5 variants may circulate in the CNS in chronic to end-stage disease. The clinical importance of our findings may require further longitudinal evaluation considering that genetic intermixing between peripheral blood and the CNS may afford HIV-1 the ability to target cells of the CNS using CXCR4.

Our study was limited to a small cohort of 16 participants with highly similar clinical characteristics. We did not have a control group of participants infected with HIV-1 but not presenting with CM. We generated a low number of sequence for one or both compartments of some participants, therefore we may have missed minority lineages that are specific to the peripheral blood or CNS compartment. Another limitation of the study which may have impacted the results of our study was the use of bulk PCR amplification and cloning together with SGA to generate sequences. SGA has been shown to provide a better measure of viral variants, especially minority ones, in a quasispecies compared to bulk PCR and cloning, as described previously (49). On the other hand, a different study showed that bulk PCR and cloning may adequately determine viral diversity as SGA does (78).

Future longitudinal studies assessing differences of HIV-1 in blood and the CNS of individuals with and without CM will improve our understanding of the pathogenesis of HIV-1. Our results suggest that trafficking of HIV-1 variants between blood and the CNS is common, though the mode and the effect on HIV-1 neurotropism is not completely clear. We also established using CPAs that maraviroc use would not be suitable for approximately 44% of individuals with CM, suggesting an alternative mode of therapy must be optimized for them if standard regimens fail.

Acknowledgements

This research was funded by the Howard Hughes Medical Institute, the South African Department of Science and Technology/National Research Foundation Research Chairs Initiative and the Victor Daitz Foundation. Partial funding was provided by the Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE), a DELTAS Africa Initiative [grant # DEL-15-006]. The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust [grant # 107752/Z/15/Z] and the UK government. The views expressed in this publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government. We extend our gratitude to Professor Paul Gorry, Head of the HIV Molecular Pathogenesis Laboratory (Burnet Institute), for providing us with the pSVIII-Env plasmid. Finally, we thank and acknowledge the study participants.

Sequence data

Nucleotide sequences are available in GenBank with the following accession numbers: MF284812-MF284921 and MF284922-MF285062.

Supporting information

The neighbor-joining tree is reproduced in FigShare (https://figshare.com/s/603ccb67a7d1f3709a4e).

Competing interests

The authors have no competing interests.

2.6 References

1. **Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955-1958.
2. **Feng Y, Broder CC, Kennedy PE, Berger EA.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872-877.
3. **Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661-666.
4. **Xiao L, Owen SM, Goldman I, Lal AA, deJong JJ, Goudsmit J, Lal RB.** 1998. CCR5 coreceptor usage of non-syncytium-inducing primary HIV-1 is independent of phylogenetically distinct global HIV-1 isolates: delineation of consensus motif in the V3 domain that predicts CCR-5 usage. Virology **240:**83-92.
5. **Verrier F, Borman AM, Brand D, Girard M.** 1999. Role of the HIV type 1 glycoprotein 120 V3 loop in determining coreceptor usage. AIDS Res Hum Retroviruses **15:**731-743.
6. **Cashin K, Sterjovski J, Harvey KL, Ramsland PA, Churchill MJ, Gorry PR.** 2014. Covariance of charged amino acids at positions 322 and 440 of HIV-1 Env contributes to coreceptor specificity of subtype B viruses, and can be used to improve the performance of V3 sequence-based coreceptor usage prediction algorithms. PLoS One **9:**e109771.

7. **Cho MW, Lee MK, Carney MC, Berson JF, Doms RW, Martin MA.** 1998. Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4. J Virol **72:**2509-2515.

8. **Smyth RJ, Yi Y, Singh A, Collman RG.** 1998. Determinants of entry cofactor utilization and tropism in a dualtropic human immunodeficiency virus type 1 primary isolate. J Virol **72:**4478-4484.

9. **Huang W, Toma J, Fransen S, Stawiski E, Reeves JD, Whitcomb JM, Parkin N, Petropoulos CJ.** 2008. Coreceptor tropism can be influenced by amino acid substitutions in the gp41 transmembrane subunit of human immunodeficiency virus type 1 envelope protein. J Virol **82:**5584-5593.

10. **Scarlatti G, Tresoldi E, Bjorndal A, Fredriksson R, Colognesi C, Deng HK, Malnati MS, Plebani A, Siccardi AG, Littman DR, Fenyo EM, Lusso P.** 1997. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat Med **3:**1259-1265.

11. **Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR.** 1997. Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med **185:**621-628.

12. **Koot M, Vos AH, Keet RP, de Goede RE, Dercksen MW, Terpstra FG, Coutinho RA, Miedema F, Tersmette M.** 1992. HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. AIDS **6:**49-54.

13. **Koot M, Keet IP, Vos AH, de Goede RE, Roos MT, Coutinho RA, Miedema F, Schellekens PT, Tersmette M.** 1993. Prognostic value of HIV-1 syncytium-

inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Annals of internal medicine **118:**681-688.

14. **Abebe A, Demissie D, Goudsmit J, Brouwer M, Kuiken CL, Pollakis G, Schuitemaker H, Fontanet AL, Rinke de Wit TF.** 1999. HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS. AIDS **13:**1305-1311.

15. **Jakobsen MR, Cashin K, Roche M, Sterjovski J, Ellett A, Borm K, Flynn J, Erikstrup C, Gouillou M, Gray LR, Saksena NK, Wang B, Purcell DF, Kallestrup P, Zinyama-Gutsire R, Gomo E, Ullum H, Ostergaard L, Lee B, Ramsland PA, Churchill MJ, Gorry PR.** 2013. Longitudinal Analysis of CCR5 and CXCR4 Usage in a Cohort of Antiretroviral Therapy-Naive Subjects with Progressive HIV-1 Subtype C Infection. PLoS One **8:**e65950.

16. **Choge I, Cilliers T, Walker P, Taylor N, Phoswa M, Meyers T, Viljoen J, Violari A, Gray G, Moore PL, Papathanosopoulos M, Morris L.** 2006. Genotypic and phenotypic characterization of viral isolates from HIV-1 subtype C-infected children with slow and rapid disease progression. AIDS Res Hum Retroviruses **22:**458-465.

17. **Morris L, Cilliers T, Bredell H, Phoswa M, Martin DJ.** 2001. CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. AIDS Res Hum Retroviruses **17:**697-701.

18. **Cecilia D, Kulkarni SS, Tripathy SP, Gangakhedkar RR, Paranjape RS, Gadkari DA.** 2000. Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India. Virology **271:**253-258.

19. **Connell BJ, Michler K, Capovilla A, Venter WD, Stevens WS, Papathanasopoulos MA.** 2008. Emergence of X4 usage among HIV-1 subtype C: evidence for an evolving epidemic in South Africa. AIDS **22:**896-899.

20. **Lin NH, Smeaton LM, Giguel F, Novitsky V, Moyo S, Mitchell RM, Makhema J, Essex M, Lockman S, Kuritzkes DR.** 2011. Prevalence and clinical associations of CXCR4-using HIV-1 among treatment-naive subtype C-infected women in Botswana. J Acquir Immune Defic Syndr **57:**46-50.

21. **Cilliers T, Nhlapo J, Coetzer M, Orlovic D, Ketas T, Olson WC, Moore JP, Trkola A, Morris L.** 2003. The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C. J Virol **77:**4449-4456.

22. **Sonnerborg A, Nybom R, Britton S, Ehrnst A, Forsgren M, Larsson PH, Strannegard O, Andersson J.** 1989. Detection of cell-free human immunodeficiency virus in cerebrospinal fluid by using immune scanning electron microscopy. J Infect Dis **159:**1037-1041.

23. **Schnell G, Price RW, Swanstrom R, Spudich S.** 2010. Compartmentalization and clonal amplification of HIV-1 variants in the cerebrospinal fluid during primary infection. J Virol **84:**2395-2407.

24. **Sturdevant CB, Joseph SB, Schnell G, Price RW, Swanstrom R, Spudich S.** 2015. Compartmentalized replication of R5 T cell-tropic HIV-1 in the central nervous system early in the course of infection. PLoS Pathog **11:**e1004720.

25. **Brew BJ, Rosenblum M, Cronin K, Price RW.** 1995. AIDS dementia complex and HIV-1 brain infection: clinical-virological correlations. Annals of neurology **38:**563-570.

26. **Ellis RJ, Gamst AC, Capparelli E, Spector SA, Hsia K, Wolfson T, Abramson I, Grant I, McCutchan JA.** 2000. Cerebrospinal fluid HIV RNA originates from both local CNS and systemic sources. Neurology **54:**927-936.

27. **Di Stefano M, Monno L, Fiore JR, Buccoliero G, Appice A, Perulli LM, Pastore G, Angarano G.** 1998. Neurological disorders during HIV-1 infection correlate with viral load in cerebrospinal fluid but not with virus phenotype. AIDS **12:**737-743.

28. **Price RW, Staprans S.** 1997. Measuring the "viral load" in cerebrospinal fluid in human immunodeficiency virus infection: window into brain infection? Annals of neurology **42:**675-678.

29. **Strain MC, Letendre S, Pillai SK, Russell T, Ignacio CC, Gunthard HF, Good B, Smith DM, Wolinsky SM, Furtado M, Marquie-Beck J, Durelle J, Grant I, Richman DD, Marcotte T, McCutchan JA, Ellis RJ, Wong JK.** 2005. Genetic composition of human immunodeficiency virus type 1 in cerebrospinal fluid and blood without treatment and during failing antiretroviral therapy. J Virol **79:**1772-1788.

30. **Pillai SK, Pond SL, Liu Y, Good BM, Strain MC, Ellis RJ, Letendre S, Smith DM, Gunthard HF, Grant I, Marcotte TD, McCutchan JA, Richman DD, Wong JK.** 2006. Genetic attributes of cerebrospinal fluid-derived HIV-1 env. Brain : a journal of neurology **129:**1872-1883.

31. **Ritola K, Robertson K, Fiscus SA, Hall C, Swanstrom R.** 2005. Increased human immunodeficiency virus type 1 (HIV-1) env compartmentalization in the presence of HIV-1-associated dementia. J Virol **79:**10830-10834.

32. **Harrington PR, Schnell G, Letendre SL, Ritola K, Robertson K, Hall C, Burch CL, Jabara CB, Moore DT, Ellis RJ, Price RW, Swanstrom R.** 2009. Cross-sectional characterization of HIV-1 env compartmentalization in cerebrospinal fluid over the full disease course. AIDS **23:**907-915.

33. **Schnell G, Joseph S, Spudich S, Price RW, Swanstrom R.** 2011. HIV-1 replication in the central nervous system occurs in two distinct cell types. PLoS Pathog **7:**e1002286.

34. **Sturdevant CB, Dow A, Jabara CB, Joseph SB, Schnell G, Takamune N, Mallewa M, Heyderman RS, Van Rie A, Swanstrom R.** 2012. Central nervous system compartmentalization of HIV-1 subtype C variants early and late in infection in young children. PLoS Pathog **8:**e1003094.

35. **Spudich SS, Huang W, Nilsson AC, Petropoulos CJ, Liegler TJ, Whitcomb JM, Price RW.** 2005. HIV-1 chemokine coreceptor utilization in paired cerebrospinal fluid and plasma samples: a survey of subjects with viremia. J Infect Dis **191:**890-898.

36. **Karlsson U, Antonsson L, Repits J, Medstrand P, Owman C, Kidd-Ljunggren K, Hagberg L, Svennerholm B, Jansson M, Gisslen M, Ljungberg B.** 2009. Mode of coreceptor use by R5 HIV type 1 correlates with disease stage: a study of paired plasma and cerebrospinal fluid isolates. AIDS Res Hum Retroviruses **25:**1297-1305.

37. **Soulie C, Tubiana R, Simon A, Lambert-Niclot S, Malet I, Canestri A, Brunet C, Murphy R, Katlama C, Calvez V, Marcelin AG.** 2009. Presence of HIV-1 R5 viruses in cerebrospinal fluid even in patients harboring R5X4/X4 viruses in plasma. J Acquir Immune Defic Syndr **51:**60-64.

38. **Fatkenheuer G, Pozniak AL, Johnson MA, Plettenberg A, Staszewski S, Hoepelman AI, Saag MS, Goebel FD, Rockstroh JK, Dezube BJ, Jenkins TM, Medhurst C, Sullivan JF, Ridgway C, Abel S, James IT, Youle M, van der Ryst E.** 2005. Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. Nat Med **11:**1170-1172.

39. **Aiamkitsumrit B, Dampier W, Antell G, Rivera N, Martin-Garcia J, Pirrone V, Nonnemacher MR, Wigdahl B.** 2014. Bioinformatic analysis of HIV-1 entry and pathogenesis. Curr HIV Res **12:**132-161.

40. **Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, He HX, McLaughlin S, Shankarappa R, Margolick JB, Mullins JI.** 2003. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. J Virol **77:**13376-13388.

41. **Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R.** 2007. Bioinformatics prediction of HIV coreceptor usage. Nat Biotechnol **25:**1407-1410.

42. **Cashin K, Gray LR, Harvey KL, Perez-Bercoff D, Lee GQ, Sterjovski J, Roche M, Demarest JF, Drummond F, Harrigan PR, Churchill MJ, Gorry PR.** 2015. Reliable genotypic tropism tests for the major HIV-1 subtypes. Scientific reports **5:**8543.

43. **Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM.** 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23:**525-530.

44. **Jarvis JN, Bicanic T, Loyse A, Namarika D, Jackson A, Nussbaum JC, Longley N, Muzoora C, Phulusa J, Taseera K, Kanyembe C, Wilson D,**

Hosseinipour MC, Brouwer AE, Limmathurotsakul D, White N, van der Horst C, Wood R, Meintjes G, Bradley J, Jaffar S, Harrison T. 2014. Determinants of mortality in a combined cohort of 501 patients with HIV-associated Cryptococcal meningitis: implications for improving outcomes. Clin Infect Dis **58:**736-745.

45. **Mwaba P, Mwansa J, Chintu C, Pobee J, Scarborough M, Portsmouth S, Zumla A.** 2001. Clinical presentation, natural history, and cumulative death rates of 230 adults with primary cryptococcal meningitis in Zambian AIDS patients treated under local conditions. Postgrad Med J **77:**769-773.

46. **Naranbhai V, Chang CC, Durgiah R, Omarjee S, Lim A, Moosa MY, Elliot JH, Ndung'u T, Lewin SR, French MA, Carr WH.** 2014. Compartmentalization of innate immune responses in the central nervous system during cryptococcal meningitis/HIV coinfection. AIDS **28:**657-666.

47. **Chang CC, Dorasamy AA, Gosnell BI, Elliott JH, Spelman T, Omarjee S, Naranbhai V, Coovadia Y, Ndung'u T, Moosa MY, Lewin SR, French MA.** 2013. Clinical and mycological predictors of cryptococcosis-associated immune reconstitution inflammatory syndrome. AIDS **27:**2089-2099.

48. **Chang CC, Kangethe R, Omarjee S, Hiramen K, Gosnell B, Sojane K, Moosa MS, Lewin SR, French MA, Ndung'u T.** 2017. Relationship of Human Immunodeficiency Virus Viral Load in Cerebrospinal Fluid and Plasma in Patients Co-infected With Cryptococcal Meningitis. Open forum infectious diseases **4:**ofx032.

49. **Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J,**

Anderson JA, Swanstrom R, Haynes BF, Athreya GS, Korber BT, Sharp PM, Shaw GM, Hahn BH. 2008. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. J Virol **82:**3952-3970.

50. **Palmer S, Kearney M, Maldarelli F, Halvas EK, Bixby CJ, Bazmi H, Rock D, Falloon J, Davey RT, Jr., Dewar RL, Metcalf JA, Hammer S, Mellors JW, Coffin JM.** 2005. Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. J Clin Microbiol **43:**406-413.

51. **Archary D, Gordon ML, Green TN, Coovadia HM, Goulder PJ, Ndung'u T.** 2010. HIV-1 subtype C envelope characteristics associated with divergent rates of chronic disease progression. Retrovirology **7:**92.

52. **Gao F, Morrison SG, Robertson DL, Thornton CL, Craig S, Karlsson G, Sodroski J, Morgado M, Galvao-Castro B, von Briesen H, Beddows S, Weber J, Sharp PM, Shaw GM, Hahn BH.** 1996. Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization. J Virol **70:**1651-1667.

53. **Roche M, Jakobsen MR, Sterjovski J, Ellett A, Posta F, Lee B, Jubb B, Westby M, Lewin SR, Ramsland PA, Churchill MJ, Gorry PR.** 2011. HIV-1 escape from the CCR5 antagonist maraviroc associated with an altered and less-efficient mechanism of gp120-CCR5 engagement that attenuates macrophage tropism. J Virol **85:**4330-4342.

54. **Ohagen A, Devitt A, Kunstman KJ, Gorry PR, Rose PP, Korber B, Taylor J, Levy R, Murphy RL, Wolinsky SM, Gabuzda D.** 2003. Genetic and functional analysis of full-length human immunodeficiency virus type 1 env genes derived from brain and blood of patients with AIDS. J Virol **77:**12336-12345.

55. **Sterjovski J, Churchill MJ, Ellett A, Gray LR, Roche MJ, Dunfee RL, Purcell DF, Saksena N, Wang B, Sonza S, Wesselingh SL, Karlsson I, Fenyo EM, Gabuzda D, Cunningham AL, Gorry PR.** 2007. Asn 362 in gp120 contributes to enhanced fusogenicity by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with AIDS. Retrovirology **4:**89.

56. **Rousseau CM, Birditt BA, McKay AR, Stoddard JN, Lee TC, McLaughlin S, Moore SW, Shindo N, Learn GH, Korber BT, Brander C, Goulder PJ, Kiepiela P, Walker BD, Mullins JI.** 2006. Large-scale amplification, cloning and sequencing of near full-length HIV-1 subtype C genomes. J Virol Methods **136:**118-125.

57. **Kumar S, Stecher G, Tamura K.** 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular biology and evolution **33:**1870-1874.

58. **Kumar SB, Handelman SK, Voronkin I, Mwapasa V, Janies D, Rogerson SJ, Meshnick SR, Kwiek JJ.** 2011. Different regions of HIV-1 subtype C env are associated with placental localization and in utero mother-to-child transmission. J Virol **85:**7142-7152.

59. **Guindon S, Gascuel O.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic biology **52:**696-704.

60. **Pond SL, Frost SD, Muse SV.** 2005. HyPhy: hypothesis testing using phylogenies. Bioinformatics **21:**676-679.

61. **Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, Korber B.** 2004. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology **14:**1229-1246.

62. **Gulick RM, Lalezari J, Goodrich J, Clumeck N, DeJesus E, Horban A, Nadler J, Clotet B, Karlsson A, Wohlfeiler M, Montana JB, McHale M, Sullivan J, Ridgway C, Felstead S, Dunne MW, van der Ryst E, Mayer H, Teams MS.** 2008. Maraviroc for previously treated patients with R5 HIV-1 infection. N Engl J Med **359:**1429-1441.

63. **Cashin K, Gray LR, Jakobsen MR, Sterjovski J, Churchill MJ, Gorry PR.** 2013. CoRSeqV3-C: a novel HIV-1 subtype C specific V3 sequence based coreceptor usage prediction algorithm. Retrovirology **10:**24.

64. **Coetzer M, Cilliers T, Ping LH, Swanstrom R, Morris L.** 2006. Genetic characteristics of the V3 region associated with CXCR4 usage in HIV-1 subtype C isolates. Virology **356:**95-105.

65. **Zhang H, Tully DC, Zhang T, Moriyama H, Thompson J, Wood C.** 2010. Molecular determinants of HIV-1 subtype C coreceptor transition from R5 to R5X4. Virology **407:**68-79.

66. **Lin NH, Becerril C, Giguel F, Novitsky V, Moyo S, Makhema J, Essex M, Lockman S, Kuritzkes DR, Sagar M.** 2012. Env sequence determinants in CXCR4-using human immunodeficiency virus type-1 subtype C. Virology **433:**296-307.

67. **Stanfield RL, Gorny MK, Zolla-Pazner S, Wilson IA.** 2006. Crystal structures of human immunodeficiency virus type 1 (HIV-1) neutralizing antibody 2219 in complex with three different V3 peptides reveal a new binding mode for HIV-1 cross-reactivity. J Virol **80:**6093-6105.

68. **Lynch RM, Rong R, Li B, Shen T, Honnen W, Mulenga J, Allen S, Pinter A, Gnanakaran S, Derdeyn CA.** 2010. Subtype-specific conservation of isoleucine 309 in the envelope V3 domain is linked to immune evasion in subtype C HIV-1 infection. Virology **404:**59-70.

69. **Michler K, Connell BJ, Venter WD, Stevens WS, Capovilla A, Papathanasopoulos MA.** 2008. Genotypic characterization and comparison of full-length envelope glycoproteins from South African HIV type 1 subtype C primary isolates that utilize CCR5 and/or CXCR4. AIDS Res Hum Retroviruses **24:**743-751.

70. **McArthur JC, McClernon DR, Cronin MF, Nance-Sproson TE, Saah AJ, St Clair M, Lanier ER.** 1997. Relationship between human immunodeficiency virus-associated dementia and viral load in cerebrospinal fluid and brain. Annals of neurology **42:**689-698.

71. **Smith DM, Zarate S, Shao H, Pillai SK, Letendre SL, Wong JK, Richman DD, Frost SD, Ellis RJ, Group H.** 2009. Pleocytosis is associated with disruption of HIV compartmentalization between blood and cerebral spinal fluid viral populations. Virology **385:**204-208.

72. **Gorry PR, Bristol G, Zack JA, Ritola K, Swanstrom R, Birch CJ, Bell JE, Bannert N, Crawford K, Wang H, Schols D, De Clercq E, Kunstman K, Wolinsky SM, Gabuzda D.** 2001. Macrophage tropism of human

immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. J Virol **75:**10073-10089.

73. **Korber BT, Kunstman KJ, Patterson BK, Furtado M, McEvilly MM, Levy R, Wolinsky SM.** 1994. Genetic differences between blood- and brain-derived viral sequences from human immunodeficiency virus type 1-infected patients: evidence of conserved elements in the V3 region of the envelope protein of brain-derived sequences. J Virol **68:**7467-7481.

74. **Ceresola ER, Nozza S, Sampaolo M, Pignataro AR, Saita D, Ferrarese R, Ripa M, Deng W, Mullins JI, Boeri E, Tambussi G, Toniolo A, Lazzarin A, Clementi M, Canducci F.** 2015. Performance of commonly used genotypic assays and comparison with phenotypic assays of HIV-1 coreceptor tropism in acutely HIV-1-infected patients. J Antimicrob Chemother **70:**1391-1395.

75. **Mulinge M, Lemaire M, Servais JY, Rybicki A, Struck D, da Silva ES, Verhofstede C, Lie Y, Seguin-Devaux C, Schmit JC, Bercoff DP.** 2013. HIV-1 tropism determination using a phenotypic Env recombinant viral assay highlights overestimation of CXCR4-usage by genotypic prediction algorithms for CRF01_AE and CRF02_AG [corrected]. PLoS One **8:**e60566.

76. **Raymond S, Delobel P, Mavigner M, Cazabat M, Encinas S, Souyris C, Bruel P, Sandres-Saune K, Marchou B, Massip P, Izopet J.** 2010. CXCR4-using viruses in plasma and peripheral blood mononuclear cells during primary HIV-1 infection and impact on disease progression. AIDS **24:**2305-2312.

77. **Seager I, Travers SA, Leeson MD, Crampin AC, French N, Glynn JR, McCormack GP.** 2014. Coreceptor usage, diversity, and divergence in drug-

naive and drug-exposed individuals from Malawi, infected with HIV-1 subtype C for more than 20 years. AIDS Res Hum Retroviruses **30:**975-983.

78. **Jordan MR, Kearney M, Palmer S, Shao W, Maldarelli F, Coakley EP, Chappey C, Wanke C, Coffin JM.** 2010. Comparison of standard PCR/cloning to single genome sequencing for analysis of HIV-1 populations. J Virol Methods **168:**114-120.

79. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32:**1792-1797.

In Chapter 2, we determined the predicted coreceptor usage of HIV-1C variants in peripheral blood and the CNS of individuals with CM using CPAs. However, we did not conduct experiments to confirm the *in vitro* usage of CCR5 or CXCR4 by those variants. In Chapter 3, we present the *in vitro* ability of HIV-1C from plasma and the CSF of individuals with CM to infect cell lines expressing CCR5 or CXCR4. Additionally, the accuracy of three publicly available CPAs, and the determinants of CCR5 and CXCR4 usage are also presented.

Chapter 3: CXCR4 usage by HIV-1 subtype C is common in plasma but not the cerebrospinal fluid of individuals with cryptococcal meningitis

Katlego Sojane,[1] Michael Roche,[2] Christina C Chang,[3] Sharon R Lewin,[2,3] Martyn A French,[4,5] Paul Gorry[6] and Thumbi Ndung'u[1,7,8,9]

[1]HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [2]The Peter Doherty Institute for Infection and Immunity, University of Melbourne and Royal Melbourne Hospital, Melbourne, Australia; [3]Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, Australia; [4]School of Pathology and Laboratory Medicine, University of Western Australia, Perth, Australia; [5]Department of Clinical Immunology, Royal Perth Hospital and PathWest Laboratory Medicine, Perth, Australia; [6]School of Health and Biomedical Research, RMIT University, Melbourne, Australia; [7]Africa Health Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [8]Ragon Institute of MGH, MIT and Harvard University, Cambridge, MA 02139, USA; [9]Max Planck Institute for Infection Biology, Berlin, Germany.

3.1 Abstract

The coreceptor usage of HIV-1 subtype C (HIV-1C) in patients with clinical end-stage infection and AIDS defining opportunistic infections such as cryptococcal meningitis (CM) is not well understood. To better understand HIV-1 subtype C pathogenesis and eligibility for maraviroc, a CCR5 receptor antagonist, in patients with advanced AIDS, we characterized the major coreceptor usage of HIV-1C in 10 and 12 plasma and cerebrospinal fluid samples, respectively, across 14 participants with CM. Eleven out of 12 (~91%) CSF samples harbored R5 viruses only, while the remaining sample had an R5X4 virus detected. In contrast, nine out of 10 (90%) plasma samples harbored R5 viruses, though three (30%) of those samples additionally had R5X4 and/or X4 viruses, while the remaining plasma sample had only X4 virus detected. Overall, four out of 14 (~28.6%) participants harbored CXCR4-using viruses in plasma and/or cerebrospinal fluid. Our results suggest that HIV-1C envelope proteins in advanced AIDS use CCR5 and/or CXCR4 frequently in peripheral blood but CCR5 almost exclusively in the central nervous system.

Keywords: Cryptococcal meningitis, cerebrospinal fluid, HIV-1, plasma, maraviroc.

3.2 Introduction

The envelope protein (Env) of the human immunodeficiency virus type 1 (HIV-1) engages the primary receptor, cell-surface CD4, and a chemokine receptor, normally CCR5 or CXCR4, which serves as a coreceptor for the virus to attach to the target cell and infect it (1-4). In primary HIV-1 infection, CCR5 is the coreceptor used for target cell entry by almost all strains transmitted through the mucosal route (5, 6). In later stages of infection, the virus may switch coreceptor usage from CCR5 to CXCR4 and additional coreceptors (7), and the switch is associated with an accelerated rate of disease progression (8, 9). The mechanisms that underlie coreceptor switching by HIV-1 as disease progresses are not completely understood, and it has been reported that the extent of coreceptor switching is not consistent across different subtypes (10). Therefore, knowing the requirements and mechanisms of coreceptor switching is essential for understanding HIV disease progression.

Recent studies of individuals in end-stage infection have yielded conflicting data on the prevalence of HIV-1C CXCR4 usage, though current evidence weighs towards higher frequency of CXCR4 usage than previously thought. In a small study of 20 participants, CXCR4-using HIV-1C was detected in 30% of infected participants, whereas a larger cohort study of 148 participants showed CXCR4-using HIV-1C in approximately 15% of the participants. Both studies, however, highlighted that CXCR4 usage by HIV-1C may have increased in the general population over time, though better estimates of CXCR4 usage are required (11, 12). CXCR4 usage by HIV-1C has been observed in individuals presenting with various AIDS-defining illnesses (13-15), though it is unclear what the true prevalence is of CXCR4 usage by HIV-1C in larger cohorts with specific comorbidities or coinfections. Clearly, CXCR4 usage by HIV-1C is not insignificant, has relevance for disease pathogenesis and the applicability of CCR5 inhibitors which are now in clinical use.

Because it is invasive to obtain a biopsy of the brain to study the properties of HIV-1 potentially resident there, cerebrospinal fluid (CSF) samples are often used as a surrogate (16). Although the

coreceptor usage by HIV-1 isolates in peripheral blood has been investigated thoroughly, much less is known about viral strains in the central nervous system (CNS). Studies have shown that it is primarily R5 T-cell tropic viruses that establish infection in the CNS (17), but viruses which use CXCR4 alone or in combination with CCR5 have also been observed in the CSF of individuals in the chronic stage of infection (18, 19).

Coreceptor usage predictive algorithms (CPAs), which predict whether an isolate is R5 or CXCR4-using, have been designed to potentially replace in vitro characterizations of HIV-1 coreceptor usage which are laborious, costly and time-consuming. There are three publicly available CPAs, Geno2pheno, PhenoSeq and WebPSSM subtype C sinsi (WebPSSM) that were developed using different training sets of HIV-1 Env V3 sequences and distinct algorithms for predicting coreceptor usage. All have been shown to be highly sensitive and specific for the detection of R5 and CXCR4-using viruses, respectively (20-25). However, at present, none of the CPAs are so accurate that they may replace in vitro coreceptor usage characterizations entirely. This is largely because determinants of coreceptor usage are not completely restricted to the V3 loop, thus reducing reliability of CPAs (23, 26-29). Additionally, the majority of sequences used in the training sets for the development of CPAs were derived from viruses circulating in plasma and it is unclear whether the predictions are generalizable to viruses circulating in other anatomical compartments. An examination of the accuracy of CPAs with viruses derived from other anatomical compartments such as the central nervous system (CNS) has not been performed.

One of the most common opportunistic infections in people with advanced HIV/AIDS infection in sub-Saharan Africa is cryptococcal meningitis (CM). Approximately 504,000 individuals die annually due to HIV-associated CM, making this coinfection a major public health problem in sub-Saharan Africa (30). Previously, we detected HIV-1C predicted to use CXCR4 in seven of 16 patients who presented with CM with the aid of CPAs (Chapter 2). In that study, variants predicted to use CXCR4 were detected in plasma and cerebrospinal samples, though we did not conduct phenotypic tests to confirm that those variants were functional and indeed use CXCR4

in vitro. Here, we sought to confirm the coreceptor usage of those Envs using in vitro phenotypic assays, and to evaluate the accuracy of publicly available CPAs. Our results will contribute to our understanding of HIV-CM disease, the coreceptor tropism of HIV-1C in the CNS compartment, and the refinement of frequently used CPAs.

3.3 Materials and methods

3.3.1 Study participants

The parent clinical study cohort including the participants selected for this study has been described previously (31). We initially had plasma and/or CSF samples from 16 participants shortlisted for analysis (Chapter 2), however, we only analyzed samples from 14 of those participants by the conclusion of this sub-study. Briefly, all the participants in this study were retrospectively selected based on $CD4^+$ T-cell count, plasma and CSF viral load. At the time of analysis, all the participants were antiretroviral therapy-naïve, with a $CD4^+$ T-cell count lower than 200 cells/µL, enumerated using the Becton Dickinson TruCount Technology (BD, Franklin lakes, NJ). They also had detectable HIV RNA in plasma and CSF according to the COBAS TaqMan HIV-1 test with a lower detection limit of 34 copies/mL (Hoffmann-La Roche, Basel, Switzerland), and experienced their first episode of CM. All but two participants, CM032 and CM117, had pleocytosis (white blood cell count of 5 cells/µL or more).

3.3.2 RNA extraction and first-strand cDNA synthesis

Total cell-free RNA from plasma and CSF was extracted using the QIAmp Viral RNA Mini kit (Qiagen, Dusseldorf, Germany) according to manufacturer's instructions. The synthesis of single-strand HIV cDNA from extracted RNA was performed using the SuperScript III (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, extracted RNA was combined with primer OFM19 (5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3'; HXB2 positions

9,604-9,632) and deoxynucleoside triphosphates, then the reaction mixture was heated to 65 °C for 5 minutes and cooled to 4 °C immediately thereafter. The first-strand synthesis reaction incorporated the following reagents in the reaction mixture: 1X reverse transcriptase buffer with dithiothreitol, RNAseOut (Invitrogen) and SuperScript III. The complete reaction mixture was incubated at 50 °C for 60 minutes, 55 °C minutes for 60 °C, incubated at 70 °C for 15 minutes and cooled to 4 °C (32). To ensure that residual RNA was degraded, RNAse H (Invitrogen) was added to the complete reaction mixture.

3.3.3 HIV-1 env gene amplification and cloning

Full-length HIV-1 *env* genes were amplified by nested PCR, employing the single-genome amplification (SGA) or a bulk-genome amplification method. For the resolution of single-genomes, cDNA was diluted serially, and multiple replicate PCR reactions of each dilution were performed using the Platinum Taq DNA Polymerase High Fidelity (Invitrogen) according to manufacturer's instructions. Dilutions which yielded 30% or less positive amplifications had a high probability of containing single-genomes, according to Poisson's distribution, as described previously (33). The first round PCR included the outer primers VIF1 and OFM19, while the second round PCR included the inner primers ENVA and ENVN (34, 35). The bulk-genome amplification method involved the purification of *env* genes without the dilution of cDNA. The first round of PCR included outer primers VIF1 and OFM19, and the second round of PCR included the inner primers Env-KpnI (5'-GTCTATTATGGGGTACCTGTGTGG-3'; HXB2 positions 6,336-6,359) and Env-BamHI (5'-GCTAAGGATCCGCTCACTAATCG-3'; HXB2 position 8,463-8,485) as described previously (34, 36).

Cloning of the 2.1 kb KpnI-to-BamHI *env* fragment (positions 6,348 to 8,478: HXB2 numbering) has been described previously (36-38). Briefly, 2 µL of first round PCR products were added to a PCR reaction mixture including the following reagents: 1X Phusion HF buffer (New England Biolabs, Ipswich, MA), 10 mM of deoxynucleoside triphosphates, 10 µM of forward primer Env-

KpnI, 10 µM of reverse primer Env-BamHI, 1 U of Phusion High-Fidelity DNA Polymerase (New England Biolabs), and nuclease free water for a final volume of 50 µL. Thermocycling conditions for the amplification of DNA were as follows: 1 cycle at 98 °C for 30 seconds; 35 cycles of 98 °C for 10 seconds, 65 °C for 30 seconds, 72 °C for four minutes; 1 cycle at 72 °C for 10 minutes and a hold at 4 °C. Amplicons were purified, digested using the Acc65I and BamHI restriction enzymes (Invitrogen), and ligated to pSVIII-Env vectors using the Rapid DNA Ligation Kit (Invitrogen). XL10-Gold Ultracompetent Cells (Agilent Technologies, Santa Clara, CA) were transformed with the recombinant pSVIII-Env vectors.

3.3.4 Sequencing of env genes and subtyping

Second round PCR products were sequenced to resolve the DNA sequences of *env* genes amplified by SGA. The *env* genes amplicons generated by bulk PCR were sequenced directly from pSVIII-Env expression plasmids following successful cloning. All *env* genes were sequenced bi-directionally using up to eight forward and reverse primers spanning the entire gene (39). The ABI 3130 genetic analyzer (Applied Biosystems, Waltham, MA) was used to resolve the *env* gene DNA sequences, and the contigs acquired from it were assembled and manually edited using Sequencher (Genecodes, Ann Arbor, MI). Sequence chromatograms which had overlapping peaks and duplicate sequences were discarded. To determine the subtype and confirm absence of inter-patient sequence contamination, the V1-V5 *env* gene regions of sequences from participants were first aligned together with those of sequences from subtypes A-K, group N and O obtained from the Los Alamos HIV sequence database (www.hiv.lanl.gov) using Clustal W. Then, the phylogenetic relatedness of the aligned sequences was inferred using the neighbor-joining method in MEGA 7 (40): all positions containing gaps and missing data were stripped and the final tree was generated after 1,000 bootstrap replicates, as described previously (14).

3.3.5 Coreceptor usage prediction using three algorithms

We used three coreceptor usage prediction algorithms (CPAs) to determine the predicted coreceptor usage of our *env* gene sequences. The publicly available CPAs, Geno2pheno with a 5% false-positive rate (http://coreceptor.bioinf.mpi-inf.mpg.de/), WebPSSM subtype C sinsi (WebPSSM; https://indra.mullins.microbiol.washington.edu/webpssm) and PhenoSeq (www.burnet.edu.au/phenoseq) were used to determine the predicted coreceptor tropism of sequenced *env* genes (20-22). The CPAs use V3 loop sequences to predict whether viruses are CCR5-using only (R5), or CXCR4-using (R5X4 or X4). Where one CPA did not agree with the classification of the other two CPAs, the predicted tropism was determined based on two concordant CPAs.

3.3.6 Identification of potential N-linked glycosylation sites

Potential N-linked glycosylation sites (PNGS) in V3 loop sequences were counted using the publicly available program N-GlycoSite (https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). The program identifies N-X-[S or T] patterns in an amino acid sequence, where "X" can be any amino acid, except proline, and counts them as PNGS (41).

3.3.7 Maintenance of mammalian cells

293T cells were propagated in supplemented Dulbecco's modified Eagle's medium (DMEM; Invitrogen) consisting of 10% fetal bovine serum, 1% GlutaMax (Invitrogen), and 1% penicillin-streptomycin (Biochrom AG, Berlin, Germany). The human glioma cell lines, NP2-CD4 and U87-CD4 cells, transduced to express CCR5 or CXCR4 were propagated in supplemented DMEM with the addition of 1 µg/mL of puromycin and 300 µg/mL of G418 (Sigma-Aldrich, St. Louis, USA). All mammalian cells were incubated at 37 °C and 5% CO_2.

3.3.8 Production of single-round replication luciferase reporter viruses

To produce single-round replication luciferase reporter viruses (pseudoviruses), pSVIII-Env expression plasmids containing patient-derived or lab-strain *env* genes were cotransfected with plasmids pCMVΔP1ΔenvpA and pHIV-1Luc at a ratio of 1:1:3, respectively, into 0.6 X 10^6 293T cells using Lipofectamine 2000 (Invitrogen) or polyethylenimine, as described previously (37). At 48 hours post-transfection, supernatants were collected, passed through a 0.45 µm pore-filter and then stored at -80 °C.

3.3.9 Coreceptor usage screen

In order to determine the coreceptor used by Envs, we measured the ability of pseudoviruses to enter NP2-CD4 [42] or U87-CD4 cells expressing CCR5 or CXCR4 (43) as previously described (14). NP2-CD4 or U87-CD4 cells expressing CCR5 or CXCR4 were seeded at 1 X 10^4 cells per well in 96-well plates. Then, duplicated five-fold serial dilutions of pseudoviruses in 100 µL volumes were incubated with the cells and infections were allowed to proceed over a period over 6-12 hours at 37 °C. Thereafter, fresh culture medium was supplied to the cells and incubation was continued for another 60 hours. Following that, the culture medium was discarded, cells were washed with phosphate-buffered saline (Invitrogen), and lysed using the Luciferase cell culture lysis reagent (Promega, Madison, WI). Pseudovirus entry was measured by assaying luciferase activity in cell lysates using the Luciferase Assay System (Promega), according to manufacturer's instructions. Luminescence readings were acquired using the FLUOStar microplate reader (BMG Labtech, Champigny-sur-Marne, France) or the Modulus Microplate reader (Turner Biosystems, Sunnyvale, CA) and the mean relative units (RLU) were recorded. Pseudoviruses YU2 (R5 tropic), JR-CSF (R5 tropic), 89.6 (R5X4 tropic) or HXB2 (X4 tropic) were included in the panel for comparison and assay quality control, whereas culture medium without pseudovirus was used as a negative control. Pseudovirus entry levels greater than 10-fold that of control levels and/or negative control pseudoviruses indicated the Env used the coreceptor exposed to it, while lower

than 10-fold control levels indicated the Env could not use the coreceptor. Pseudoviruses which used CCR5 only and CXCR4 only were classified as R5 and X4, respectively. Pseudoviruses which used both CCR5 and CXCR4 were classified as R5X4.

3.3.10 Statistical analyses

The unpaired, non-parametric t-test was performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA) unless otherwise stated.

3.4 Results

3.4.1 HIV-1 sequence and phylogenetic analyses

A total of 14 antiretroviral naïve participants with CM were included in this study. The clinical characteristics of the participants are shown in Table 3-1. Across the 14 participants, we analyzed eight matched plasma and CSF samples. Additionally, two and four unmatched plasma and CSF samples, respectively, were also included in our analyses. Thus, we analyzed a total of 10 plasma and 12 CSF samples, respectively.

From the 10 plasma and 12 CSF samples, 26 (median 2, range 1-7 per participant) and 40 (median 2.5, range 1-16 per participant) *env* genes, respectively, were sequenced and cloned as described previously (35, 39). The predicted coreceptor usage of each virus was previously determined (Chapter 2). Notable, a combination of predicted R5 and CXCR4-using Envs, where present, from each compartment of each participant were selected for phenotyping in this sub-study. Based on phylogenetic analyses, all of the *env* genes analyzed in this study were confirmed to belong to subtype C, as they clustered together with reference subtype C sequences. Sequences obtained from the Los Alamos database representative of other subtypes clustered according to subtype, but separately from the study sequences. There was no evidence of inter-patient sequence

contamination as the sequences from each participant clustered together and did not mix with the sequences from other participants (Chapter 2).

Table 3-1: Clinical characteristics and samples analyzed of the study participants.

ID[a]	Age	Sex[b]	CD4 count[c]	Pl VL[d]	CSF VL[e]	CSF WBC[f]	Sample[g]
CM019	45	M	7	5.15	4.33	28	Pl and CSF
CM021	30	F	134	4.34	4.34	40	CSF only
CM029	33	F	121	5.22	5.99	424	CSF only
CM032	34	F	5	4.94	2.52	0	Pl and CSF
CM050	23	F	16	4.92	4.8	6	CSF only
CM052	24	F	172	4.62	4.83	36	Pl and CSF
CM054	23	M	7	5.2	4.2	14	Pl only
CM070	25	M	11	5.04	4	18	Pl only
CM089	27	M	114	5.67	5.85	154	Pl and CSF
CM094	29	F	35	5.15	4.03	84	CSF only
CM098	40	M	14	4.76	5.27	66	Pl and CSF
CM108	27	M	20	5.16	5.02	28	Pl and CSF
CM117	38	M	1	5.72	4.66	0	Pl and CSF
CM132	34	M	53	4.58	4.43	38	Pl and CSF

[a]The identification number of the study participant. [b]The sex of the study participant, where "M" refers to male and "F" refers to female. [c]The CD4 count was recorded as cells/μL. [d,e]The cell-free plasma (Pl) and CSF viral load (VL) was recorded as $\log_{10}$ HIV RNA copies/mL. [f]The CSF white blood cell count (WBC) was recorded as cells/μL. [g]The samples analyzed in this study.

3.4.2 Coreceptor usage of HIV-1C Envs

The sequenced genes from the plasma and/or CSF samples of study participants coding for HIV-1 gp160 were cloned into pSVIII-Env expression plasmids. In total, 66 single-round infection luciferase reporter viruses (pseudoviruses) pseudotyped with Envs from study participants were produced, and their ability to enter NP2-CD4 or U87-CD4 cells expressing CCR5 or CXCR4 was determined. There was good agreement of entry profiles of the HIV-1B pseudovirus infection controls between NP2-CD4 and U87-CD4 cells, and we confirmed that pseudoviruses YU2 and JR-CSF are R5, while HXB2 and NL4.3 are X4, and 89.6 is dual-tropic. Overall, we identified a total of 50 R5, seven X4, four R5X4, and five Envs which did not register usage of CCR5 or CXCR4. The *in vitro* entry profiles of the infection controls and pseudoviruses pseudotyped with participant-derived HIV-1C Envs evaluated in our study are shown in

Figure 3.1: The usage of CCR5 and CXCR4 of HIV-1 subtype C Envs in individuals with cryptococcal meningitis.

, and summarized in Table 3-2.

Eleven out of 12 (~91.7%) CSF samples harbored R5 viruses exclusively, while the remaining CSF sample (~8.3%) from participant CM019 had an R5X4 virus detected in the CSF. Nine out of 10 (90%) plasma samples possessed R5 viruses, though three (30%) of those samples (from CM019, CM098 and CM108) also contained CXCR4-using viruses. Specifically, the plasma sample from participant CM019 had R5, R5X4 and X4 viruses; plasma of CM098 had R5 and X4 Envs, and plasma of CM108 had R5 and R5X4 viruses. The only virus tested for participant CM070 was plasma-derived and X4-tropic. Coreceptor tropism of viruses overlapped between the matched plasma and CSF-derived viruses of all eight (100%) participants. Notably for participants CM098 and CM108, CXCR4-using Envs were not detected in the CNS compartment, suggesting that for these participants there may have been partial compartmentalization of

coreceptor usage, with CXCR4-using variants detected in plasma but not in CSF. Overall, we detected CXCR4-using variants in four out of 14 (~28.6%) participants. These results suggest that HIV-1C in peripheral blood and CNS compartments prefers to use CCR5 predominantly over CXCR4 in individuals with end-stage HIV and CM. Additionally, in individuals with CXCR4-using variants, CXCR4 may be used exclusively or in combination with CCR5.

Figure 3.1 (A)

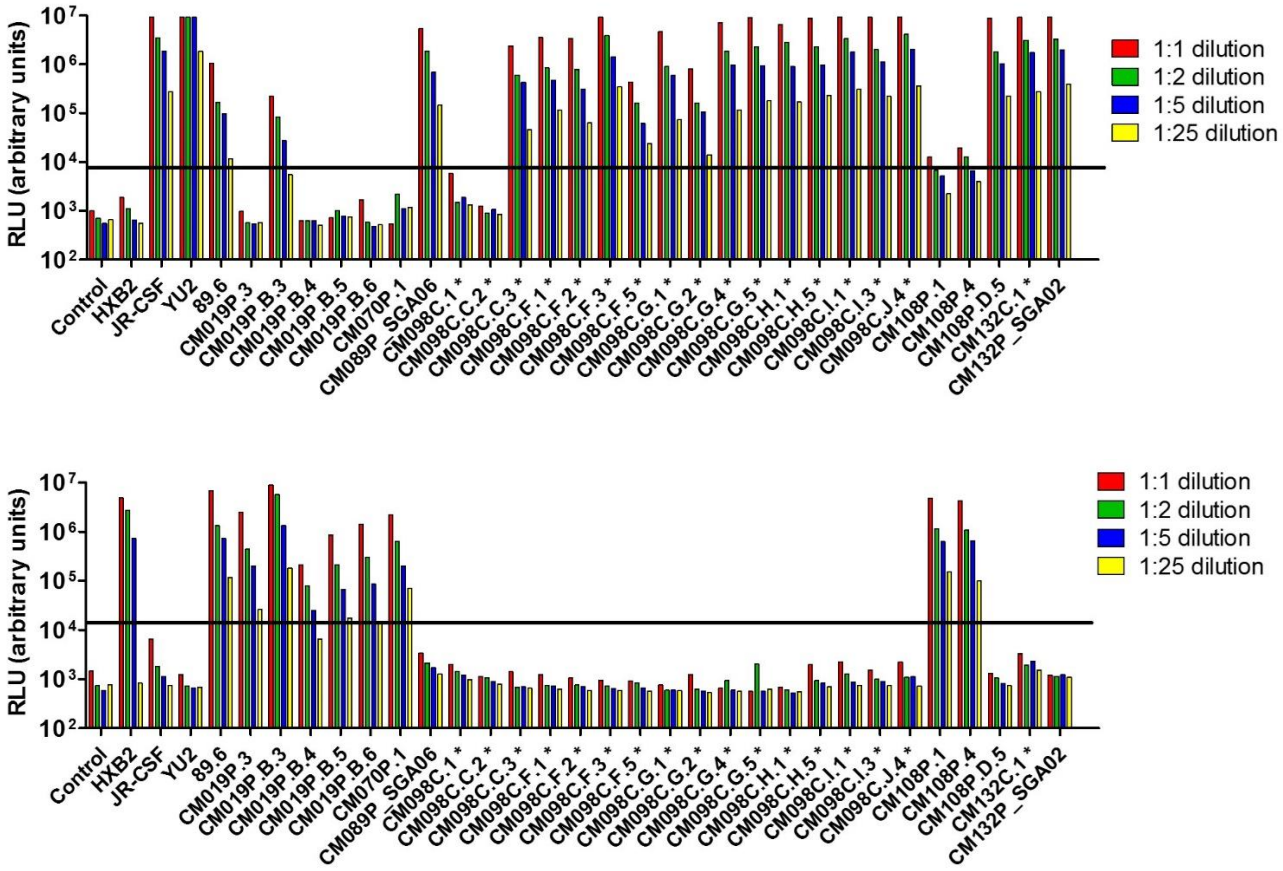
RLU (arbitrary units)
10^2
10^3
10^4
10^5
10^6
10^7
1:1 dilution
1:2 dilution
1:5 dilution
1:25 dilution
Control
HXB2
JR-CSF
YU2
89.6
CM019P.3
CM019P.B.3
CM019P.B.4
CM019P.B.5
CM019P.B.6
CM070P.1
CM089P_SGA06
CM098C.1*
CM098C.C.2*
CM098C.C.3*
CM098C.F.1*
CM098C.F.2*
CM098C.F.3*
CM098C.F.5*
CM098C.G.1*
CM098C.G.2*
CM098C.G.4*
CM098C.G.5*
CM098C.H.1*
CM098C.H.5*
CM098C.I.1*
CM098C.I.3*
CM098C.J.4*
CM108P.1
CM108P.4
CM108P.D.5
CM132C.1*
CM132P_SGA02

Figure 3.1 (B)

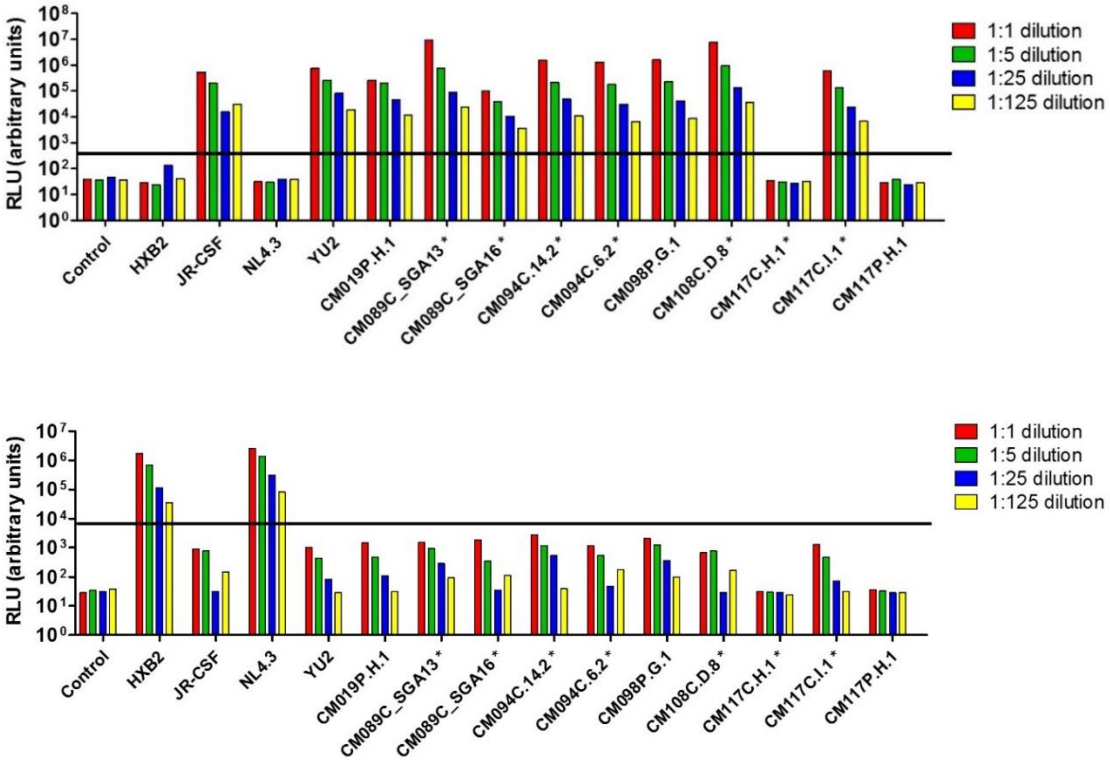
RLU (arbitrary units)
1:1 dilution
1:5 dilution
1:25 dilution
1:125 dilution
Control
HXB2
JR-CSF
NL4.3
YU2
CM019P.H.1
CM089C_SGA13 *
CM089C_SGA16 *
CM094C.14.2 *
CM094C.6.2 *
CM098P.G.1
CM108C.D.8 *
CM117C.H.1 *
CM117C.I.1 *
CM117P.H.1

Figure 3.1 (B) continued

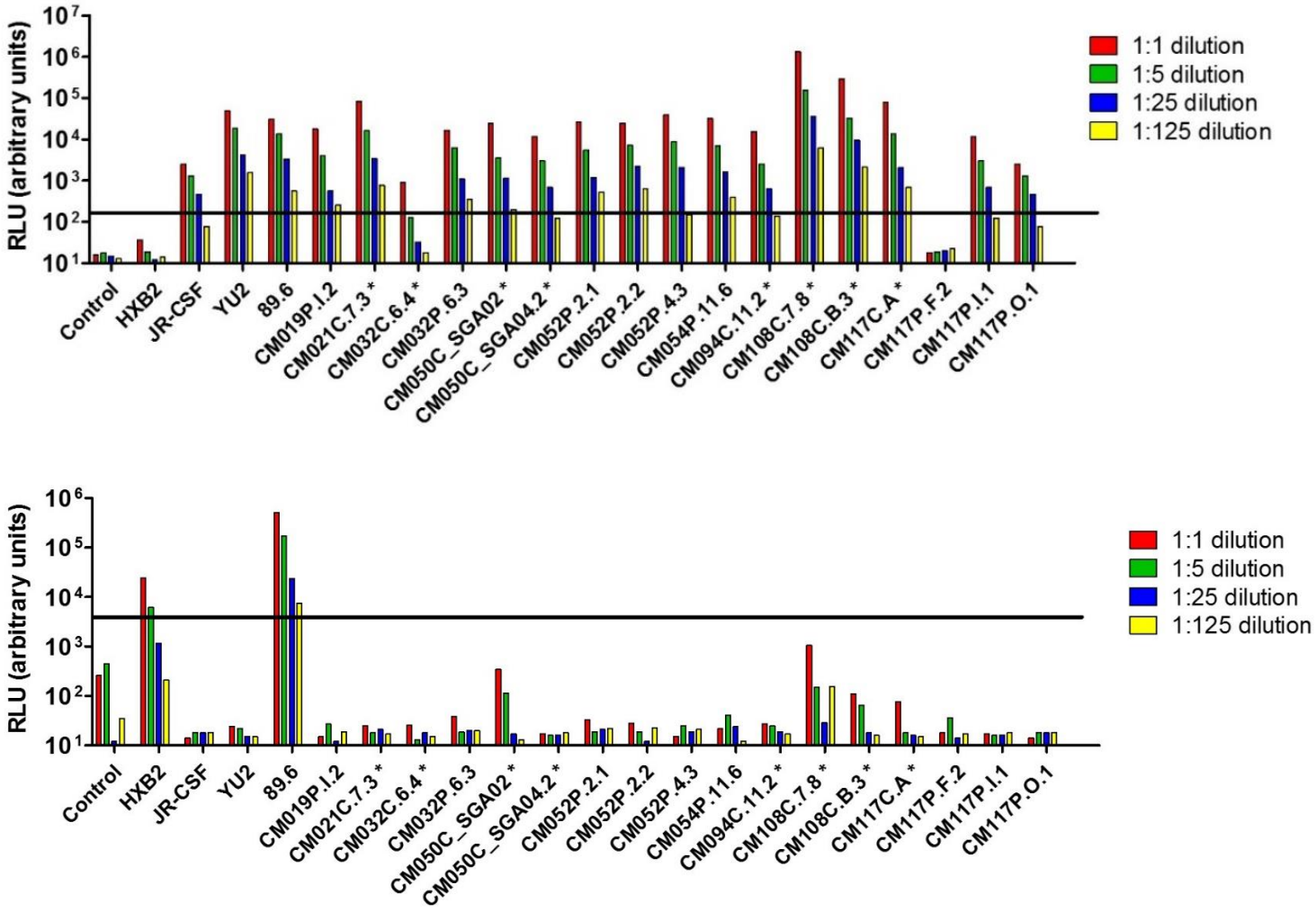

Figure 3.1 (B) continued

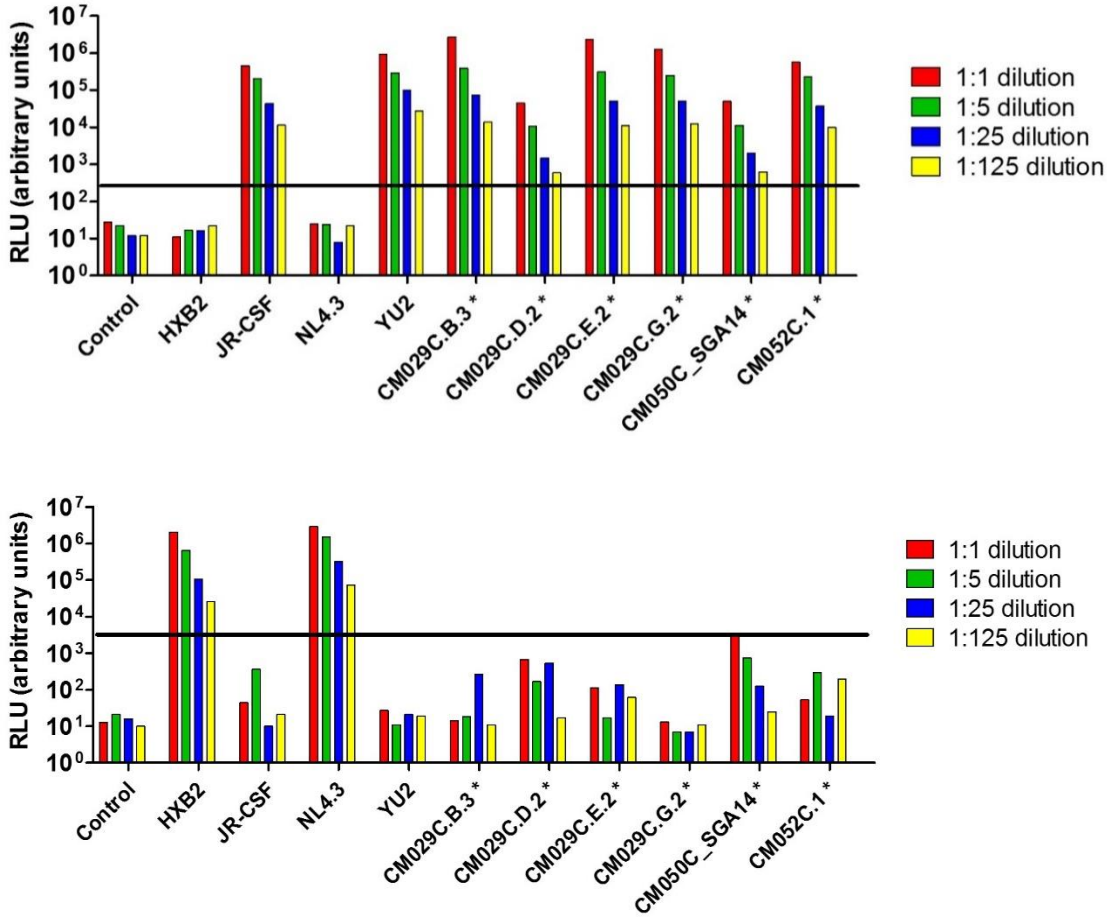

Figure 3.1 (B) continued

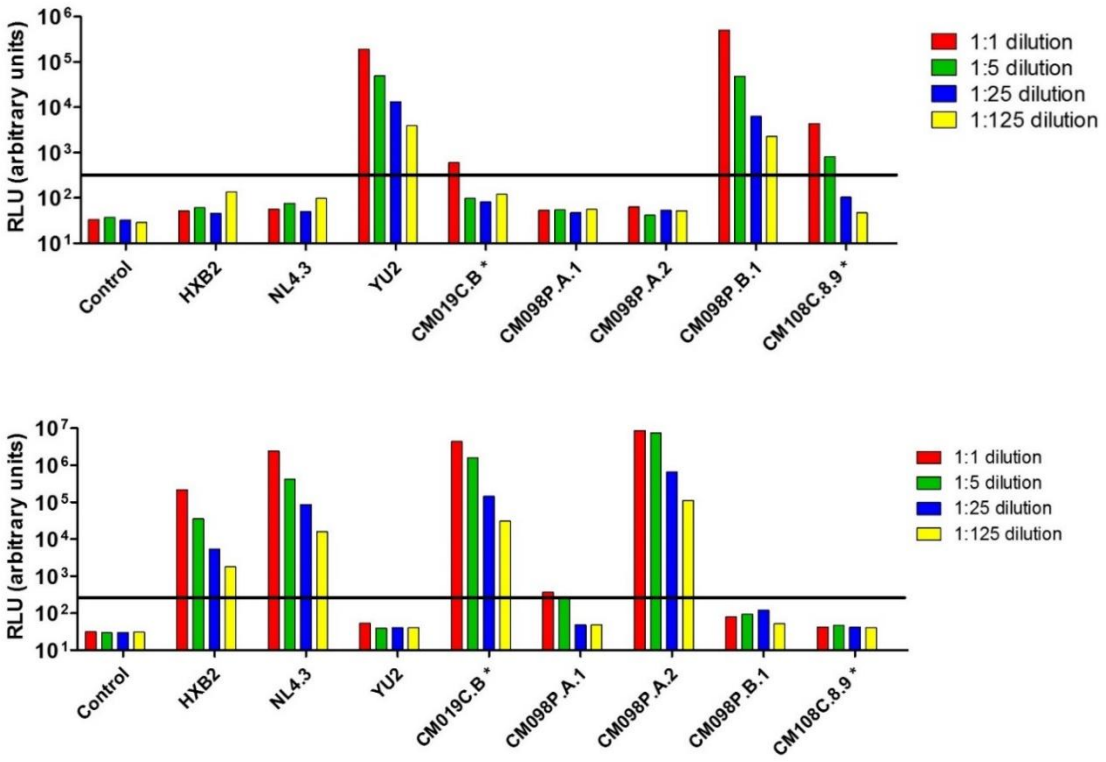

Figure 3.1: The usage of CCR5 and CXCR4 of HIV-1 subtype C Envs in individuals with cryptococcal meningitis.

Single-round infection luciferase reporter viruses (pseudoviruses) pseudotyped with plasma or cerebrospinal fluid-derived HIV-1 subtype C Env (listed on the x-axis) were used to infect NP2-CD4 cells (**A**) expressing CCR5 (top) and CXCR4 (bottom), or U87-CD4 cells (**B**) expressing CCR5 (top) and CXCR4 (bottom), as described in Materials and methods. Pseudoviruses with a star symbol beside their name indicate they were pseudotyped with an Env derived from the CSF, whereas pseudoviruses without a star beside them indicate their Env was derived from plasma. Luciferase activity in cell lysates, measured as relative light units (RLU), was used as a measure of pseudovirus entry. Entry of each pseudovirus at different dilutions (1:1, 1:5, 1:25 and 1:125) was tested in duplicate and the mean values were recorded. Pseudoviruses baring HIV-1B Envs (HXB2, JR-CSF, YU2 and 89.6) were included in the assay as infection controls, as well as culture medium without pseudovirus (negative control). Pseudoviruses recording levels of entry that were greater than 10-fold that of the infection and negative control (black line cutoff) were able to use the coreceptor for entry. Pseudoviruses using CCR5, CXCR4 or both CCR5 and CXCR4 were classified as R5, X4 or R5X4, respectively.

Table 3-2: The predicted and *in vitro* coreceptor usage of HIV-1C Envs in individuals with CM.

ID[a]	Clones[b]	Compartment[c]	G2P[d]	PhenoSeq[e]	PSSM-C[f]	Consensus[g]	*In vitro* tropism[h]
CM019	CM019C.B	CSF	CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	R5X4
	CM019P.3	Plasma	CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	X4
	CM019P.B.3		CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	R5X4
	CM019P.B.4		CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	X4
	CM019P.B.5		CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	X4
	CM019P.B.6		CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	X4
	CM019P.H.1		R5	CXCR4-using	R5	R5	R5
	CM019P.I.2		R5	R5	R5	R5	R5
CM021	CM021C.7.3	CSF	R5	R5	R5	R5	R5
CM029	CM029C.B.3	CSF	R5	R5	R5	R5	R5
	CM029C.D.2		R5	R5	R5	R5	R5
	CM029C.E.2		R5	R5	R5	R5	R5
	CM029C.G.2		R5	R5	R5	R5	R5
CM032	CM032C.6.4	CSF	CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	R5

ID[a]	Clones[b]	Compartment[c]	G2P[d]	PhenoSeq[e]	PSSM-C[f]	Consensus[g]	*In vitro* tropism[h]
	CM032P.6.3	Plasma	R5	CXCR4-using	CXCR4-using	CXCR4-using	R5
CM050	CM050C_SGA02	CSF	R5	CXCR4-using	CXCR4-using	CXCR4-using	R5
	CM050C_SGA04.2		R5	CXCR4-using	CXCR4-using	CXCR4-using	R5
	CM050C_SGA14		R5	CXCR4-using	CXCR4-using	CXCR4-using	R5
CM052	CM052C.1	CSF	R5	R5	R5	R5	R5
	CM052P.2.1	Plasma	R5	R5	R5	R5	R5
	CM052P.2.2		R5	R5	R5	R5	R5
	CM052P.4.3		R5	R5	R5	R5	R5
CM054	CM054P.11.6	Plasma	R5	R5	R5	R5	R5
CM070	CM070P.1	Plasma	CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	X4
CM089	CM089C_SGA13	CSF	R5	R5	R5	R5	R5
	CM089C_SGA16		R5	R5	R5	R5	R5
	CM089P_SGA06	plasma	R5	R5	R5	R5	R5
CM094	CM094C.11.2	CSF	R5	R5	R5	R5	R5
	CM094C.14.2		R5	R5	R5	R5	R5

ID[a]	Clones[b]	Compartment[c]	G2P[d]	PhenoSeq[e]	PSSM-C[f]	Consensus[g]	*In vitro* tropism[h]
	CM094C.6.2		R5	R5	R5	R5	R5
CM098	CM098C.1	CSF	R5	CXCR4-using	R5	R5	-
	CM098C.C.2		CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	-
	CM098C.C.3		R5	CXCR4-using	R5	R5	R5
	CM098C.F.1		R5	R5	R5	R5	R5
	CM098C.F.2		R5	CXCR4-using	CXCR4-using	CXCR4-using	R5
	CM098C.F.3		R5	CXCR4-using	R5	R5	R5
	CM098C.F.5		R5	CXCR4-using	CXCR4-using	CXCR4-using	R5
	CM098C.G.1		R5	CXCR4-using	R5	R5	R5
	CM098C.G.2		R5	CXCR4-using	R5	R5	R5
	CM098C.G.4		R5	CXCR4-using	R5	R5	R5
	CM098C.G.5		R5	CXCR4-using	R5	R5	R5
	CM098C.H.1		R5	R5	R5	R5	R5
	CM098C.H.5		R5	R5	R5	R5	R5
	CM098C.I.1		R5	R5	R5	R5	R5

ID[a]	Clones[b]	Compartment[c]	G2P[d]	PhenoSeq[e]	PSSM-C[f]	Consensus[g]	*In vitro* tropism[h]
	CM098C.I.3		R5	R5	R5	R5	R5
	CM098C.J.4		R5	R5	R5	R5	R5
	CM098P.A.1	Plasma	CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	X4
	CM098P.A.2		CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	X4
	CM098P.B.1		R5	CXCR4-using	R5	R5	R5
	CM098P.G.1		R5	R5	R5	R5	R5
CM108	CM108C.7.8	CSF	R5	R5	R5	R5	R5
	CM108C.8.9		R5	R5	R5	R5	R5
	CM108C.B.3		R5	R5	R5	R5	R5
	CM108C.D.8		R5	R5	R5	R5	R5
	CM108P.1	Plasma	CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	R5X4
	CM108P.4		CXCR4-using	CXCR4-using	CXCR4-using	CXCR4-using	R5X4
	CM108P.D.5		R5	CXCR4-using	CXCR4-using	CXCR4-using	R5
CM117	CM117C.A	CSF	R5	R5	R5	R5	R5
	CM117C.H.1		R5	R5	R5	R5	-

ID[a]	Clones[b]	Compartment[c]	G2P[d]	PhenoSeq[e]	PSSM-C[f]	Consensus[g]	*In vitro* tropism[h]
	CM117C.I.1		R5	R5	R5	R5	R5
	CM117P.F.2	Plasma	R5	R5	R5	R5	-
	CM117P.H.1		R5	R5	R5	R5	-
	CM117P.I.1		R5	R5	R5	R5	R5
	CM117P.O.1		R5	R5	R5	R5	R5
CM132	CM132C.1	CSF	R5	R5	R5	R5	R5
	CM132P_SGA02	plasma	R5	R5	R5	R5	R5

[a]The patient identification number. [b]The name of the unique pseudotyped Env. [c]The compartment which is represented by the Env-pseudotyped pseudovirus. [d,e,f]The predicted coreceptor tropism of the pseudovirus according to Geno2pheno (G2P), PhenoSeq and WebPSSM subtype C sinsi (PSSM-C), respectively. [g]The consensus predicted coreceptor tropism of the pseudovirus based on the agreement of any two CPAs. [h]The coreceptor tropism of the Env based on its ability to mediate entry into NP2-CD4/U87-CD4 cells expressing CCR5 or CXCR4. Dashes indicate envelopes unable to mediate entry using CCR5 and CXCR4.

3.4.3 The accuracy of publicly available coreceptor usage prediction algorithms

The predictions of coreceptor usage for the Envs in this study are summarized in Table 3-2. The three CPAs agreed with each other generally, sharing the same prediction on 50 out of 66 (~75.8%) occasions. We evaluated the accuracy of the individual CPAs to identify which was the most reliable. The three CPAs were 100% accurate when predicting CXCR4 usage, but had predicted multiple false positives for CXCR4 use. Geno2pheno was the most accurate, accurately predicting 49 out of 50 (98%) R5 viruses. PSSM-C was less accurate, predicting 42 out of 50 (84%) R5 viruses accurately. PhenoSeq was the least accurate, predicting 34 out of 50 (68%) R5 viruses accurately. Next, we evaluated whether or not using the CPAs in combination would provide better accuracy, so we determined the overall predicted coreceptor usage of each virus based on the agreement of at least two CPAs. For 15 out of 61 (~24.6%) viruses which used CCR5 and/or CXCR4, however, one CPA did not agree with the prediction of the other two. Overall, using three CPAs provided an expected 100% accuracy when predicting CXCR4-using viruses. Forty-two out of 50 (84%) R5 viruses were predicted accurately using the CPAs, suggesting that using a combination of CPAs to determine coreceptor usage may not be superior to using Geno2pheno or WebPSSM alone, but it is more accurate than using PhenoSeq alone. Also, three participants (CM032, CM050 and CM098) with CXCR4-using variants predicted to circulate in the CSF had, in fact, R5 viruses in the CSF only. This shows that CPAs may overestimate the presence of CXCR4-using viruses in some samples.

3.4.4 V3 properties of R5 and CXCR4-using viruses

Although a total of 66 unique *env* genes were sequenced, cloned and used to prepare pseudoviruses, there were only 35 unique V3 loop sequences between them. Combining the results of coreceptor usage determinations *in vitro* and sequencing information, we were able to identify well-known determinants of CXCR4 usage based on Env V3 loop properties (Figure 3.2). Most of the R5 viruses in this study had a Env V3 crown motif consisting of a GPGQ sequence,

except for three viruses, predicted to use CXCR4, from participant CM050 and one from CM108 (Env CM108P.D.5 had a GPGH, and the Envs from CM050 had a GPGK motif), whereas all of the CXCR4-using Envs had a positively charged residue at position 16 and/or 18 of the V3 within the crown motif.

Statistically significantly length differences between R5 and some CXCR4-using Env V3s were not previously identified (Chapter 2). However, the V3 length of some CXCR4-using viruses from participants CM019, CM070P and CM098 was longer (37 amino acids) than the majority of viruses in the study due to a two-amino acid insertion (isoleucine-glycine or leucine-glycine) upstream of the crown motif. All of the R5 viruses, on the other hand, had a V3 that was 35 amino acids long. A longer V3 loop was not compulsory for usage of CXCR4, however, as V3 sequences of CXCR4-using viruses from CM108 were 35 amino acids long.

Only one PNGS was identified in the V3 of all R5 viruses at position 6, whereas multiple CXCR4-using viruses from participant CM019 lacked a V3 PNGS. CXCR4-using viruses from CM070, CM098 and CM108 maintained a PNGS at position 6 like R5 viruses suggesting that the absence of a PNGS alone does not account entirely for usage of CXCR4.

CXCR4-using viruses in this study possessed a significantly higher ($p<0.0001$) net charge of the Env V3 (median +5; range 5-7) than R5 viruses (median +4; range 2-6) (data not shown), and this was consistent with observations in Chapter 2. Although the CXCR4-using viruses of participants CM070, CM098 and CM108 had charged residues at position 11 and/or 25 of the V3 which has been previously been associated with CXCR4 usage, the R5 viruses from participant CM032 also had charged residues at position 25 of the V3.

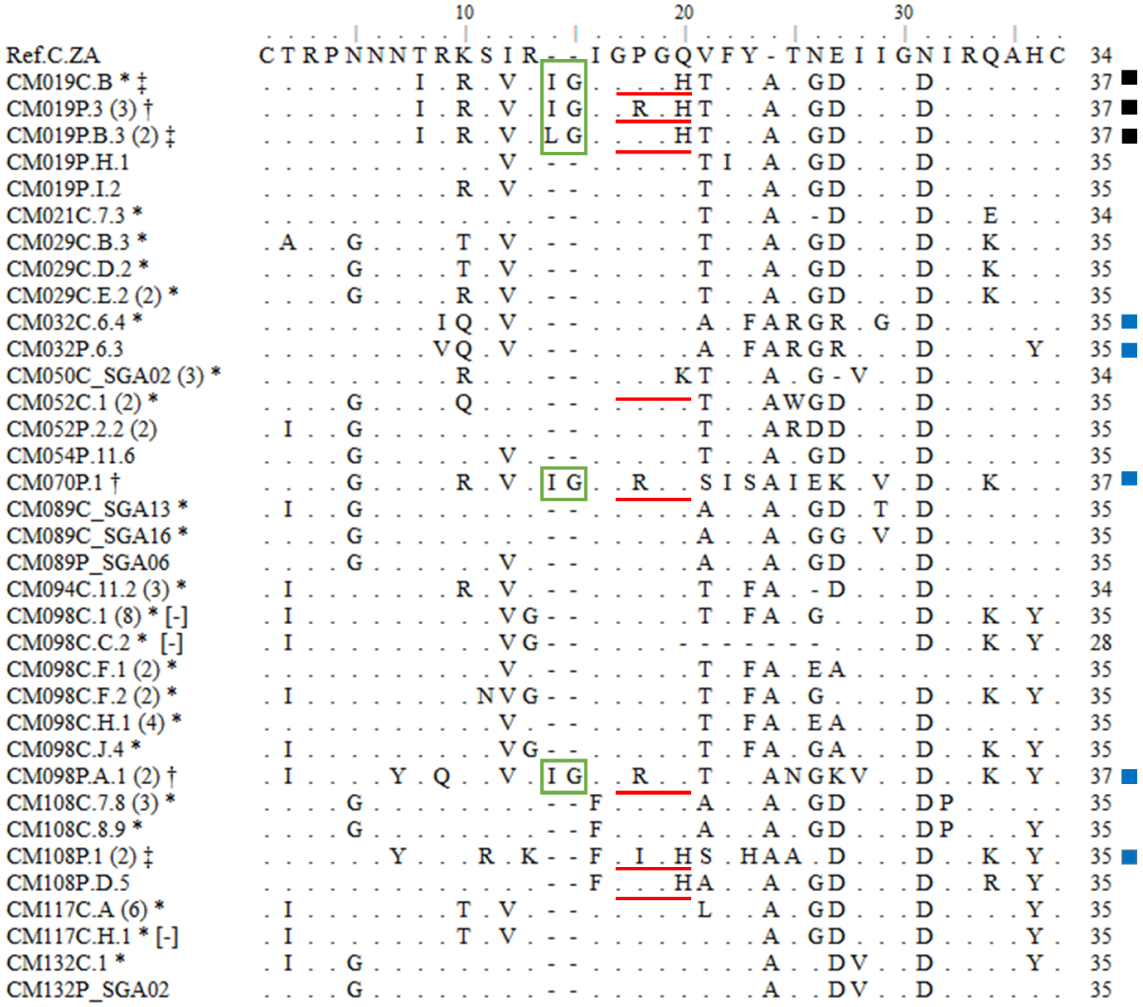

Figure 3.2: An amino acid alignment of unique HIV-1C Env V3 sequences showing the determinants of CXCR4 usage.

A reference South African HIV-1C Env V3 sequence (Ref.C.ZA) was aligned with HIV-1C Env V3 sequences derived from the plasma and/or CSF of study participants with cryptococcal meningitis using MUSCLE. The number in the round brackets next to the sequence name indicates the total number of Envs in the study which shared the same V3 sequence; the absence of a bracketed number indicates that only that Env had the sequence. A star symbol indicates an Env that was in the CSF, and the absence of a star symbol indicates the Env was from plasma. A dagger symbol indicates an X4 Env, a double-dagger symbol indicates an R5X4 Env and the absence of a dagger and double-dagger indicates the Env was R5. A dash between square brackets indicates an Env which did not utilize CCR5 or CXCR4 for entry. Two-amino acid insertions are indicated within green boxes, a red underlining shows a crown motif sequence different to GPGQ. Black squares show V3 sequences without a PNGS at position 6, and blue squares show V3s with a positively charged residue at position 11 and/or 25. The length of the V3 is shown on the right-hand side of the protein sequence.

3.4.5 Some Envs may be functionally defective or do not use CCR5 or CXCR4

We identified five Envs (two CSF-derived Envs from participant CM098; one CSF-derived, and two plasma-derived Envs from participant CM117) which were unable to use CCR5 or CXCR4 for entry. This suggests that they were functionally defective or used alternative coreceptors. We noted, however, that parts of the V3 and other regions of the Envs may be responsible for these observations. Env CM098C.C.2 (from participant CM098) had a 7 amino acid deletion within the V3, commencing at position 18 and ending at position 24, which may have affected its usage of CCR5 and CXCR4. Env CM098C.1 (from participant CM098), CM117P.F.2 and CM117P.H.1 (from participant CM117) shared the entire V3 loop sequence with other R5 Envs, suggesting that the difference of coreceptor usage or functional defect was not in the V3 but elsewhere in Env. We were unable to identify a possible reason why CM117C.H.1 was unable to use CCR5 or CXCR4.

3.4.6 Rare mutation at position 14 of the V3 loop of HIV-1C Envs from one participant

Position 14 of the V3 loop (immediately upstream of the crown motif) of all Envs sequenced in this study was occupied by a conserved isoleucine residue, except for Envs from participant CM108. Plasma as well as CSF-derived Envs of participant CM108 had a phenylalanine residue occupying position 14 of the V3 in place of an isoleucine. The occurrence of this rare residue at that position did not, however, make the pseudoviruses defective as all the Envs with the rare residue were able to use CCR5 and/or CXCR4 for entry.

3.5 Discussion

Overall, we showed that antiretroviral naïve HIV-infected individuals experiencing CM harbor HIV-1C which predominantly uses CCR5 for entry in peripheral blood and the CNS compartment. We were also able to show that the CNS compartment predominantly consists of R5 viruses but not X4 viruses, while R5, R5X4 and X4 viruses are found in peripheral blood. Our appraisal of CPAs revealed that Geno2pheno, PhenoSeq and WebPSSM generally provide the same prediction of HIV-1C coreceptor usage. Though all the three CPAs were 100% accurate with predicting CXCR4-usage, Geno2pheno was better with predicting R5 virus accurately compared with the other two CPAs. With the combination of phenotypic and sequence information, we were able to show that R5 viruses have Env V3 properties which are different to CXCR4-using viruses.

Many studies regarding the coreceptor tropism of HIV-1C were relevant to the peripheral blood compartment (11, 13-15, 44-51), whereas few have characterized viruses from the CNS compartment (18, 52, 53). Our study was uniquely designed to characterize the major HIV-1C coreceptor tropism in the peripheral blood and CNS compartment of individuals with end-stage HIV disease and CM.

Our results are consistent with previous observations that for South African HIV-1C, at advanced stages of disease, the infection is still dominated principally by R5 tropic viruses (11, 13, 54). We also show that HIV-1C is capable of using CCR5 or CXCR4 exclusively, and CCR5 in combination with CXCR4 in end-stage, as described previously (13, 46). More specifically, we identified that R5 viruses predominated in 90% of plasma samples, but noted that R5X4 and X4 viruses were also detectable in 20% and 30% of samples, respectively. In contrast, approximately 91% of CSF samples possessed R5 viruses only, another ~8.3% of samples harbored R5X4 viruses and no sample had X4 viruses. Our results suggest that the usage of CCR5 by HIV-1 is required for entry in the CNS, as previously described (55-58). Additionally, we showed that

HIV-1C R5 viruses generally occupy the CSF, that R5X4 viruses are rare and X4 viruses are absent in that compartment, as described previously (18).

All eight participants with Envs tested from both plasma and CSF compartments displayed coreceptor tropism overlap between the two compartments: at least one Env from plasma had the same tropism as at least one Env in the CSF. R5 tropism overlapped between compartments in seven participants, though R5X4 tropism overlap was also detected in one participant. For two participants with inter-compartment overlap of R5 viruses, CM098 and CM108, we additionally identified CXCR4-using viruses in plasma but not the CSF suggesting there may be partial compartmentalization of tropism. Previous studies shared similar results to ours, identifying that discordance of coreceptor usage may arise between plasma and CSF isolates (18, 19).

A study by Sturdevant *et al* (2012) highlighted that the usage of CCR5 by HIV-1C Envs in plasma and CSF may be different, and that macrophage tropic viruses may arise specifically in the CNS compartment (17). We did not determine the efficiency of CCR5 usage of viruses in our study and may have missed tissue tropism differences of viruses between plasma and CSF.

Because the majority of our sequences were obtained using bulk PCR, cloning and Sanger sequencing, we may have lost the opportunity to detect minor variants or coreceptor usage combinations in both compartments. We also collected a small number of viruses per participant which may have limited our ability to resolve phenotypic compartmental differences of HIV-1C Envs. Previously, other studies were able to accurately determine the composition of the quasispecies in plasma and CSF using repeated SGA which allows for the detection of minor variants (17, 53, 59, 60). However, for even greater sensitivity in detecting variants that are not detected in a quasispecies by bulk PCR techniques, deep sequencing approaches may be considered in future (61).

Our small collection of sequences allowed us to determine the predictive accuracy of publicly available CPAs. Geno2pheno, PhenoSeq and WebPSSM provided high accuracy when predicting R5 tropism or CXCR4 usage as previously observed (20-22, 24, 25). In our study, Geno2pheno was the best at predicting R5 tropism accurately, followed by WebPSSM and then PhenoSeq. This suggests that Geno2pheno is applicable and best for subtype C sequences despite that it was designed using subtype B sequences predominantly (24, 25, 62). All of the CPAs showed equivalent, high accuracy in their predictions of CXCR4 usage. We did note, however, that CPAs may incorrectly call viruses as CXCR4-using which may lead to an overestimation of the prevalence of CXCR4 usage, as described previously (63-65). The CPAs require refinement if they are to be used in the clinic as replacements of *in vitro* phenotypic assays, especially to eliminate the identification of false positive CXCR4 usage. The identification of false positive CXCR4-using variants would incorrectly disqualify patients from accessing therapeutic CCR5 antagonists. Our results contribute to the refinement of current and future CPAs.

Through the analysis of V3 loop sequences, we were able to identify determinants of CCR5 and CXCR4 usage. CXCR4-using viruses had at least two of the following features of the Env V3 loop: one or two positively charged residues in the crown motif, a two-amino acid insertion upstream of the crown motif, a positively charged residue at position 11 and/or 25, an absence of a PNGS at position 6, or a net charge of +5 and above. The determinants of CXCR4 usage which we observed were consistent with those observed by others regarding HIV-1C Env (13, 14, 46, 66, 67). Features of the V3 loop unique to R5 viruses such as low net charge, shorter length, and the GPGQ crown motif were also identified as described previously (54, 68).

Many studies have shown CXCR4 usage by HIV-1 in plasma, but few have shown CNS-derived Envs using CXCR4 *in vitro* (18, 52, 69, 70). We confirmed that CXCR4 usage is not always

limited to the viruses circulating in plasma, as we identified one participant with a R5X4 CSF-derived virus. Unexpectedly, we identified three participants with plasma viruses which used CXCR4 exclusively, suggesting that HIV-1C may persist without using CCR5 in end-stage disease. We did not, however, confirm the usage of CCR5 or CXCR4 by Envs in this study using coreceptor antagonists such as Maraviroc or AMD3100. Future studies may also explore site-directed mutagenesis as a tool for revealing novel determinants of CXCR4 usage.

The residue at position 14 of the envelope V3 loop (position 309 HXB2 numbering), immediately upstream of the crown motif, is highly conserved, displaying an isoleucine in 99% of HIV-1C sequences found in the Los Alamos sequence database (https://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html). When an isoleucine does not occupy that position, a leucine residue is preferred by HIV-1C albeit in less than 1% of the sequences in the database. Because the V3 crown motif consists of antiparallel β-strands forming β-hairpins, changes at position 14 alter the conformation of the V3, and may affect envelope recognition by antibodies by masking the CD4 site (71). Although the I309L mutation does not alter virus replication and spread observably, it does enhance the ability of HIV-1C to infect monocyte-derived macrophages and a cell line expressing low levels of CD4, suggesting the mutation affects exposure of the CD4 binding site and usage of CD4 for entry (72). A recent study showed that a functional R5 HIV-1C may bear an envelope M309 residue (46). In our study, we identified multiple functional plasma and CSF-derived viruses from one participant bearing an unusual F309 in the V3 loop. Furthermore, F309 was not specific to CCR5 or CXCR4-using viruses, as R5 and R5X4 viruses had the residue. We did not, however, evaluate the functional importance of the residue through site-directed mutagenesis. The biological impact of F309 may be evaluated by substituting it with isoleucine and testing the ability of those viruses to enter $CD4^+$ T-cells and monocyte-derived macrophages. If F309 confers a biological advantage or

disadvantage to HIV-1C, it may influence future vaccine design strategies involving antibodies which target Env.

All the participants in our cross-sectional study were classified as having AIDS, because they generally had high viral load counts, a CD4 count lower than 200 cells/μL and CM. We did not include other control groups or longitudinal samples in our study to better understand the impact of HIV-1C in HIV-CM co-infection. A control group of antiretroviral naïve HIV-1C infected individuals, with low CD4 count but without co-infection, would have been advantageous for understanding coreceptor usage of HIV-1C in advanced stages of HIV-1C infection. A second control group of participants with healthy CD4 counts would have improved our understanding of HIV coreceptor tropism before accelerated disease progression commences. Nevertheless, our study provided valuable insight into the coreceptor usage of HIV-1C in the peripheral blood and CNS compartments of patients experiencing CM. These data also highlight that the use of therapeutic CCR5 antagonists may be beneficial for targeting HIV-1C in most individuals with CM. However, due to the propensity of HIV-1C to use CXCR4 in end-stage disease, phenotypic assays to determine coreceptor usage of viruses ought to be conducted before CCR5 antagonists are administered.

Acknowledgements

We thank H. Hoshino for permission to use the NP2 cells lines. We also would like to thank the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH for the 293T, and U87 cell lines.

Competing interests

The authors of this manuscript have no competing interests.

3.6 References

1. **Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955-1958.

2. **Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661-666.

3. **Feng Y, Broder CC, Kennedy PE, Berger EA.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872-877.

4. **Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:**667-673.

5. **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM.** 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci U S A **105:**7552-7557.

6. **Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH, Shaw GM.** 2009. Genetic identity, biological phenotype, and evolutionary

pathways of transmitted/founder viruses in acute and early HIV-1 infection. J Exp Med **206:**1273-1289.

7. **Scarlatti G, Tresoldi E, Bjorndal A, Fredriksson R, Colognesi C, Deng HK, Malnati MS, Plebani A, Siccardi AG, Littman DR, Fenyo EM, Lusso P.** 1997. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat Med **3:**1259-1265.

8. **Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR.** 1997. Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med **185:**621-628.

9. **Xiao L, Rudolph DL, Owen SM, Spira TJ, Lal RB.** 1998. Adaptation to promiscuous usage of CC and CXC-chemokine coreceptors in vivo correlates with HIV-1 disease progression. AIDS **12:**F137-143.

10. **Tscherning C, Alaeus A, Fredriksson R, Bjorndal A, Deng H, Littman DR, Fenyo EM, Albert J.** 1998. Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. Virology **241:**181-188.

11. **Connell BJ, Michler K, Capovilla A, Venter WD, Stevens WS, Papathanasopoulos MA.** 2008. Emergence of X4 usage among HIV-1 subtype C: evidence for an evolving epidemic in South Africa. AIDS **22:**896-899.

12. **Lin NH, Smeaton LM, Giguel F, Novitsky V, Moyo S, Mitchell RM, Makhema J, Essex M, Lockman S, Kuritzkes DR.** 2011. Prevalence and clinical associations of CXCR4-using HIV-1 among treatment-naive subtype C-infected women in Botswana. J Acquir Immune Defic Syndr **57:**46-50.

13. **Cilliers T, Nhlapo J, Coetzer M, Orlovic D, Ketas T, Olson WC, Moore JP, Trkola A, Morris L.** 2003. The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C. J Virol **77:**4449-4456.

14. **Jakobsen MR, Cashin K, Roche M, Sterjovski J, Ellett A, Borm K, Flynn J, Erikstrup C, Gouillou M, Gray LR, Saksena NK, Wang B, Purcell DF, Kallestrup P, Zinyama-Gutsire R, Gomo E, Ullum H, Ostergaard L, Lee B, Ramsland PA, Churchill MJ, Gorry PR.** 2013. Longitudinal Analysis of CCR5 and CXCR4 Usage in a Cohort of Antiretroviral Therapy-Naive Subjects with Progressive HIV-1 Subtype C Infection. PLoS One **8:**e65950.

15. **Abebe A, Demissie D, Goudsmit J, Brouwer M, Kuiken CL, Pollakis G, Schuitemaker H, Fontanet AL, Rinke de Wit TF.** 1999. HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS. AIDS **13:**1305-1311.

16. **Resnick L, Berger JR, Shapshak P, Tourtellotte WW.** 1988. Early penetration of the blood-brain-barrier by HIV. Neurology **38:**9-14.

17. **Sturdevant CB, Joseph SB, Schnell G, Price RW, Swanstrom R, Spudich S.** 2015. Compartmentalized replication of R5 T cell-tropic HIV-1 in the central nervous system early in the course of infection. PLoS Pathog **11:**e1004720.

18. **Karlsson U, Antonsson L, Repits J, Medstrand P, Owman C, Kidd-Ljunggren K, Hagberg L, Svennerholm B, Jansson M, Gisslen M, Ljungberg B.** 2009. Mode of coreceptor use by R5 HIV type 1 correlates with disease stage: a study of paired plasma and cerebrospinal fluid isolates. AIDS Res Hum Retroviruses **25:**1297-1305.

19. **Spudich SS, Huang W, Nilsson AC, Petropoulos CJ, Liegler TJ, Whitcomb JM, Price RW.** 2005. HIV-1 chemokine coreceptor utilization in paired cerebrospinal fluid and plasma samples: a survey of subjects with viremia. J Infect Dis **191:**890-898.

20. **Cashin K, Gray LR, Harvey KL, Perez-Bercoff D, Lee GQ, Sterjovski J, Roche M, Demarest JF, Drummond F, Harrigan PR, Churchill MJ, Gorry PR.** 2015. Reliable genotypic tropism tests for the major HIV-1 subtypes. Scientific reports **5:**8543.

21. **Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, He HX, McLaughlin S, Shankarappa R, Margolick JB, Mullins JI.** 2003. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. J Virol **77:**13376-13388.

22. **Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R.** 2007. Bioinformatics prediction of HIV coreceptor usage. Nat Biotechnol **25:**1407-1410.

23. **Cashin K, Sterjovski J, Harvey KL, Ramsland PA, Churchill MJ, Gorry PR.** 2014. Covariance of charged amino acids at positions 322 and 440 of HIV-1 Env contributes to coreceptor specificity of subtype B viruses, and can be used to improve the performance of V3 sequence-based coreceptor usage prediction algorithms. PLoS One **9:**e109771.

24. **Gupta S, Neogi U, Srinivasa H, Shet A.** 2015. Performance of genotypic tools for prediction of tropism in HIV-1 subtype C V3 loop sequences. Intervirology **58:**1-5.

25. **Crous S, Shrestha RK, Travers SA.** 2012. Appraising the performance of genotyping tools in the prediction of coreceptor tropism in HIV-1 subtype C viruses. BMC Infect Dis **12:**203.

26. **Cho MW, Lee MK, Carney MC, Berson JF, Doms RW, Martin MA.** 1998. Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4. J Virol **72:**2509-2515.

27. **Huang W, Toma J, Fransen S, Stawiski E, Reeves JD, Whitcomb JM, Parkin N, Petropoulos CJ.** 2008. Coreceptor tropism can be influenced by amino acid substitutions in the gp41 transmembrane subunit of human immunodeficiency virus type 1 envelope protein. J Virol **82:**5584-5593.

28. **Thielen A, Sichtig N, Kaiser R, Lam J, Harrigan PR, Lengauer T.** 2010. Improved prediction of HIV-1 coreceptor usage with sequence information from the second hypervariable loop of gp120. J Infect Dis **202:**1435-1443.

29. **Boyd MT, Simpson GR, Cann AJ, Johnson MA, Weiss RA.** 1993. A single amino acid substitution in the V1 loop of human immunodeficiency virus type 1 gp120 alters cellular tropism. J Virol **67:**3649-3652.

30. **Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM.** 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23:**525-530.

31. **Chang CC, Dorasamy AA, Gosnell BI, Elliott JH, Spelman T, Omarjee S, Naranbhai V, Coovadia Y, Ndung'u T, Moosa MY, Lewin SR, French MA.** 2013. Clinical and mycological predictors of cryptococcosis-associated immune reconstitution inflammatory syndrome. AIDS **27:**2089-2099.

32. **Salazar-Gonzalez JF, Salazar MG, Learn GH, Fouda GG, Kang HH, Mahlokozera T, Wilks AB, Lovingood RV, Stacey A, Kalilani L, Meshnick SR, Borrow P, Montefiori DC, Denny TN, Letvin NL, Shaw GM, Hahn BH, Permar SR, Center for HIVAVIA.** 2011. Origin and evolution of HIV-1 in breast milk determined by single-genome amplification and sequencing. J Virol **85:**2751-2763.

33. **Palmer S, Kearney M, Maldarelli F, Halvas EK, Bixby CJ, Bazmi H, Rock D, Falloon J, Davey RT, Jr., Dewar RL, Metcalf JA, Hammer S, Mellors JW, Coffin JM.** 2005. Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. J Clin Microbiol **43:**406-413.

34. **Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J, Anderson JA, Swanstrom R, Haynes BF, Athreya GS, Korber BT, Sharp PM, Shaw GM, Hahn BH.** 2008.

Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. J Virol **82:**3952-3970.

35. **Archary D, Gordon ML, Green TN, Coovadia HM, Goulder PJ, Ndung'u T.** 2010. HIV-1 subtype C envelope characteristics associated with divergent rates of chronic disease progression. Retrovirology **7:**92.

36. **Sterjovski J, Churchill MJ, Ellett A, Gray LR, Roche MJ, Dunfee RL, Purcell DF, Saksena N, Wang B, Sonza S, Wesselingh SL, Karlsson I, Fenyo EM, Gabuzda D, Cunningham AL, Gorry PR.** 2007. Asn 362 in gp120 contributes to enhanced fusogenicity by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with AIDS. Retrovirology **4:**89.

37. **Roche M, Jakobsen MR, Sterjovski J, Ellett A, Posta F, Lee B, Jubb B, Westby M, Lewin SR, Ramsland PA, Churchill MJ, Gorry PR.** 2011. HIV-1 escape from the CCR5 antagonist maraviroc associated with an altered and less-efficient mechanism of gp120-CCR5 engagement that attenuates macrophage tropism. J Virol **85:**4330-4342.

38. **Ohagen A, Devitt A, Kunstman KJ, Gorry PR, Rose PP, Korber B, Taylor J, Levy R, Murphy RL, Wolinsky SM, Gabuzda D.** 2003. Genetic and functional analysis of full-length human immunodeficiency virus type 1 env genes derived from brain and blood of patients with AIDS. J Virol **77:**12336-12345.

39. **Rousseau CM, Birditt BA, McKay AR, Stoddard JN, Lee TC, McLaughlin S, Moore SW, Shindo N, Learn GH, Korber BT, Brander C, Goulder PJ, Kiepiela P, Walker BD, Mullins JI.** 2006. Large-scale amplification, cloning and sequencing of near full-length HIV-1 subtype C genomes. J Virol Methods **136:**118-125.

40. **Kumar S, Stecher G, Tamura K.** 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular biology and evolution **33:**1870-1874.

41. **Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, Korber B.** 2004. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology **14:**1229-1246.

42. **Soda Y, Shimizu N, Jinno A, Liu HY, Kanbe K, Kitamura T, Hoshino H.** 1999. Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line. Biochem Biophys Res Commun **258:**313-321.

43. **Whitcomb JM, Huang W, Fransen S, Limoli K, Toma J, Wrin T, Chappey C, Kiss LD, Paxinos EE, Petropoulos CJ.** 2007. Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob Agents Chemother **51:**566-575.

44. **Cashin K, Jakobsen MR, Sterjovski J, Roche M, Ellett A, Flynn JK, Borm K, Gouillou M, Churchill MJ, Gorry PR.** 2013. Linkages between HIV-1 specificity for CCR5 or CXCR4 and in vitro usage of alternative coreceptors during progressive HIV-1 subtype C infection. Retrovirology **10:**98.

45. **Isaacman-Beck J, Hermann EA, Yi Y, Ratcliffe SJ, Mulenga J, Allen S, Hunter E, Derdeyn CA, Collman RG.** 2009. Heterosexual transmission of human immunodeficiency virus type 1 subtype C: Macrophage tropism, alternative coreceptor use, and the molecular anatomy of CCR5 utilization. J Virol **83:**8208-8220.

46. **Lin NH, Becerril C, Giguel F, Novitsky V, Moyo S, Makhema J, Essex M, Lockman S, Kuritzkes DR, Sagar M.** 2012. Env sequence determinants in CXCR4-using human immunodeficiency virus type-1 subtype C. Virology **433:**296-307.

47. **Nedellec R, Coetzer M, Shimizu N, Hoshino H, Polonis VR, Morris L, Martensson UE, Binley J, Overbaugh J, Mosier DE.** 2009. Virus entry via the alternative coreceptors CCR3 and FPRL1 differs by human immunodeficiency virus type 1 subtype. J Virol **83:**8353-8363.

48. **Singh A, Page T, Moore PL, Allgaier RL, Hiramen K, Coovadia HM, Walker BD, Morris L, Ndung'u T.** 2009. Functional and genetic analysis of coreceptor usage by dualtropic HIV-1 subtype C isolates. Virology **393:**56-67.

49. **van Rensburg EJ, Smith TL, Zeier M, Robson B, Sampson C, Treurnicht F, Engelbrecht S.** 2002. Change in co-receptor usage of current South African HIV-1 subtype C primary isolates. AIDS **16:**2479-2480.

50. **Ndung'u T, Renjifo B, Essex M.** 2001. Construction and analysis of an infectious human Immunodeficiency virus type 1 subtype C molecular clone. J Virol **75:**4964-4972.

51. **Grisson RD, Chenine AL, Yeh LY, He J, Wood C, Bhat GJ, Xu W, Kankasa C, Ruprecht RM.** 2004. Infectious molecular clone of a recently transmitted pediatric human immunodeficiency virus clade C isolate from Africa: evidence of intraclade recombination. J Virol **78:**14066-14069.

52. **Karlsson U, Antonsson L, Ljungberg B, Medstrand P, Esbjornsson J, Jansson M, Gisslen M.** 2012. Dual R3R5 tropism characterizes cerebrospinal fluid HIV-1 isolates from individuals with high cerebrospinal fluid viral load. AIDS **26:**1739-1744.

53. **Sturdevant CB, Dow A, Jabara CB, Joseph SB, Schnell G, Takamune N, Mallewa M, Heyderman RS, Van Rie A, Swanstrom R.** 2012. Central nervous system compartmentalization of HIV-1 subtype C variants early and late in infection in young children. PLoS Pathog **8:**e1003094.

54. **Morris L, Cilliers T, Bredell H, Phoswa M, Martin DJ.** 2001. CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. AIDS Res Hum Retroviruses **17:**697-701.

55. **He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, Gabuzda D.** 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature **385:**645-649.

56. **Gabuzda D, He J, Ohagen A, Vallat AV.** 1998. Chemokine receptors in HIV-1 infection of the central nervous system. Seminars in immunology **10:**203-213.

57. **Ghorpade A, Xia MQ, Hyman BT, Persidsky Y, Nukuna A, Bock P, Che M, Limoges J, Gendelman HE, Mackay CR.** 1998. Role of the beta-chemokine receptors CCR3 and CCR5 in human immunodeficiency virus type 1 infection of monocytes and microglia. J Virol **72:**3351-3361.

58. **Albright AV, Shieh JT, Itoh T, Lee B, Pleasure D, O'Connor MJ, Doms RW, Gonzalez-Scarano F.** 1999. Microglia express CCR5, CXCR4, and CCR3, but of these, CCR5 is the principal coreceptor for human immunodeficiency virus type 1 dementia isolates. J Virol **73:**205-213.

59. **Schnell G, Joseph S, Spudich S, Price RW, Swanstrom R.** 2011. HIV-1 replication in the central nervous system occurs in two distinct cell types. PLoS Pathog **7:**e1002286.

60. **Evering TH, Kamau E, St Bernard L, Farmer CB, Kong XP, Markowitz M.** 2014. Single genome analysis reveals genetic characteristics of Neuroadaptation across HIV-1 envelope. Retrovirology **11:**65.

61. **Gega A, Kozal MJ, Chiarella J, Lee E, Peterson J, Hecht FM, Liegler T, St John EP, Simen BB, Price RW, Spudich SS.** 2015. Deep sequencing of HIV-1 variants from paired plasma and cerebrospinal fluid during primary HIV infection. Journal of virus eradication **1:**264-268.

62. **Raymond S, Delobel P, Mavigner M, Ferradini L, Cazabat M, Souyris C, Sandres-Saune K, Pasquier C, Marchou B, Massip P, Izopet J.** 2010. Prediction of HIV type 1 subtype

C tropism by genotypic algorithms built from subtype B viruses. J Acquir Immune Defic Syndr **53:**167-175.

63. **Ceresola ER, Nozza S, Sampaolo M, Pignataro AR, Saita D, Ferrarese R, Ripa M, Deng W, Mullins JI, Boeri E, Tambussi G, Toniolo A, Lazzarin A, Clementi M, Canducci F.** 2015. Performance of commonly used genotypic assays and comparison with phenotypic assays of HIV-1 coreceptor tropism in acutely HIV-1-infected patients. J Antimicrob Chemother **70:**1391-1395.

64. **Mulinge M, Lemaire M, Servais JY, Rybicki A, Struck D, da Silva ES, Verhofstede C, Lie Y, Seguin-Devaux C, Schmit JC, Bercoff DP.** 2013. HIV-1 tropism determination using a phenotypic Env recombinant viral assay highlights overestimation of CXCR4-usage by genotypic prediction algorithms for CRF01_AE and CRF02_AG [corrected]. PLoS One **8:**e60566.

65. **Raymond S, Delobel P, Mavigner M, Cazabat M, Encinas S, Souyris C, Bruel P, Sandres-Saune K, Marchou B, Massip P, Izopet J.** 2010. CXCR4-using viruses in plasma and peripheral blood mononuclear cells during primary HIV-1 infection and impact on disease progression. AIDS **24:**2305-2312.

66. **Coetzer M, Cilliers T, Ping LH, Swanstrom R, Morris L.** 2006. Genetic characteristics of the V3 region associated with CXCR4 usage in HIV-1 subtype C isolates. Virology **356:**95-105.

67. **Zhang H, Tully DC, Zhang T, Moriyama H, Thompson J, Wood C.** 2010. Molecular determinants of HIV-1 subtype C coreceptor transition from R5 to R5X4. Virology **407:**68-79.

68. **Cashin K, Gray LR, Jakobsen MR, Sterjovski J, Churchill MJ, Gorry PR.** 2013. CoRSeqV3-C: a novel HIV-1 subtype C specific V3 sequence based coreceptor usage prediction algorithm. Retrovirology **10:**24.

69. **Gorry PR, Bristol G, Zack JA, Ritola K, Swanstrom R, Birch CJ, Bell JE, Bannert N, Crawford K, Wang H, Schols D, De Clercq E, Kunstman K, Wolinsky SM, Gabuzda D.** 2001. Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. J Virol **75:**10073-10089.

70. **Gray L, Roche M, Churchill MJ, Sterjovski J, Ellett A, Poumbourios P, Sherieff S, Wang B, Saksena N, Purcell DF, Wesselingh S, Cunningham AL, Brew BJ, Gabuzda D, Gorry PR.** 2009. Tissue-specific sequence alterations in the human immunodeficiency virus type 1 envelope favoring CCR5 usage contribute to persistence of dual-tropic virus in the brain. J Virol **83:**5430-5441.

71. **Stanfield RL, Gorny MK, Zolla-Pazner S, Wilson IA.** 2006. Crystal structures of human immunodeficiency virus type 1 (HIV-1) neutralizing antibody 2219 in complex with three different V3 peptides reveal a new binding mode for HIV-1 cross-reactivity. J Virol **80:**6093-6105.

72. **Lynch RM, Rong R, Li B, Shen T, Honnen W, Mulenga J, Allen S, Pinter A, Gnanakaran S, Derdeyn CA.** 2010. Subtype-specific conservation of isoleucine 309 in the envelope V3 domain is linked to immune evasion in subtype C HIV-1 infection. Virology **404:**59-70.

In the background and literature review presented in Chapter 1, we indicated that HIV-1 has the capacity to expand its usage of major coreceptors over the course of infection, and that this is associated with the ability of the virus to use alternative coreceptors. Furthermore, while HIV-1C in peripheral blood has been shown to use alternative coreceptors for entry, though it is unclear whether viruses circulating the CSF are also capable of using alternative coreceptors. In Chapter 3, we showed that HIV-1C variants circulating in the blood and the CNS have the ability to use CCR5 alone, CXCR4 alone, or CCR5 and CXCR4. However, we did not evaluate whether or not those variants are capable of entering cell lines using an alternative coreceptor. In Chapter 4, we present the ability of HIV-1C from plasma and CSF samples to enter a cell line which expresses CCR3, an alternative coreceptor.

Chapter 4: Usage of CCR3 by HIV-1 subtype C is common in patients with cryptococcal meningitis

Katlego Sojane,[1] Michael Roche,[2] Christina C Chang,[3] Sharon R Lewin,[2,3] Martyn A French,[4,5] Paul Gorry[6] and Thumbi Ndung'u[1,7,8,9]

[1]HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [2]The Peter Doherty Institute for Infection and Immunity, University of Melbourne and Royal Melbourne Hospital, Melbourne, Australia; [3]Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, Australia; [4]School of Pathology and Laboratory Medicine, University of Western Australia, Perth, Australia; [5]Department of Clinical Immunology, Royal Perth Hospital and PathWest Laboratory Medicine, Perth, Australia; [6]School of Health and Biomedical Research, RMIT University, Melbourne, Australia; [7]Africa Health Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [8]Ragon Institute of MGH, MIT and Harvard University, Cambridge, MA 02139, USA; [9]Max Planck Institute for Infection Biology, Berlin, Germany.

4.1 Abstract

Limited information is available regarding the coreceptor usage of HIV-1 subtype C in the central nervous system. Some evidence indicates that uncultured HIV-1 envelope glycoproteins (Envs) may efficiently use CCR3 as an additional coreceptor for entry. Here, 46 Env proteins, from the plasma and/or cerebrospinal fluid (CSF) of 14 antiretroviral therapy naïve individuals with end-stage HIV-1 disease and cryptococcal meningitis (CM) were evaluated for their ability to use CCR3. Eight of 14 participants had matched plasma and CSF samples analyzed. Four of the eight participants with matched plasma and CSF samples harbored CCR3-using HIV-1C in both compartments, whereas the other four had no evidence of CCR3 usage. Two other participants with only plasma samples analyzed did not possess any CCR3-using viruses, whereas one of four other participants with only CSF samples analyzed harbored CCR3-using viruses. Overall, a total of five (~35.8%) participants harbored CCR3-using viruses in plasma and/or CSF samples. These results show that HIV-1 subtype C in individuals with end-stage disease and CM commonly uses CCR3 as an alternative coreceptor in peripheral blood and the central nervous system.

Keywords: CCR3, Cryptococcal meningitis, cerebrospinal fluid, plasma, envelope, HIV-1, plasma.

4.2 Introduction

In addition to the primary CD4 receptor, transmitted human immunodeficiency virus type 1 (HIV-1) envelope proteins (Envs) typically use the CCR5 chemokine receptor for cell entry (1, 2). However, in chronic to end-stage HIV disease, viruses with the ability to use other coreceptors, particularly CXCR4 arise and this is associated with an accelerated rate of the development of acquired immune deficiency syndrome (AIDS) (3, 4). The change of biological phenotype from non-syncytium-inducing (later associated with CCR5 usage) to syncytium-inducing (associated with CCR5 and CXCR4 usage or CXCR4 usage alone) in chronic stage disease has been observed in 50% of patients harboring HIV-1 subtype B (HIV-1B) (5). In contrast, some studies observed the usage of CXCR4 by HIV-1 subtype C (HIV-1C) to be infrequent or absent, indicating that HIV-1C disease progression is not dependent on a switch of coreceptors (6-12). However, other studies have suggested that CXCR4 usage by HIV-1C is not uncommon in individuals with advanced or end-stage HIV disease and may occur in approximately 15-30% of individuals (13-15).

The implications of alternative coreceptor usage (receptor other than CCR5 and CXCR4) by HIV-1 for disease progression and neurotropism are less clear, though usage of alternative coreceptors may highlight the enhanced pathogenicity of HIV-1. Previous reports suggested that CCR3, which is expressed highly on microglia of the central nervous system (CNS), may act as a coreceptor for the infection of microglia by HIV-1 (16-18). Later studies, however, showed that CCR3-using HIV-1 variants are common in chronic or end-stage disease, and are not restricted to the CNS compartment (19, 20).

HIV-1 subtype C (HIV-1C) Envs were shown previously to be capable of using alternative coreceptors such as CCR3, CCR8, CXCR6, BOB/GPR15 or FPRL1 for entry, but those Envs were derived largely from peripheral blood (21-26). One study of plasma and cerebrospinal (CSF)-derived isolates in individuals with chronic stage HIV-1 disease showed that alternative coreceptor usage discordance between peripheral blood and CNS was common, that HIV-1C may

use CXCR6 (another alternative coreceptor) in the CNS but not peripheral blood, while the usage of other alternative coreceptors was less pronounced. Additionally, one individual was shown to possess HIV-1C Env variants capable of using CCR3 in a CSF sample but not the matched plasma. The study highlighted that conclusions relating to HIV-1 Env phenotypes in peripheral blood may not be representative of the CNS compartment (24).

It has been suggested that *in vitro* co-culture of peripheral blood mononuclear cells (PBMCs) before cloning Env variants for the study of HIV-1 entry phenotypes may under-represent the population of CCR3-using variants, and that direct cloning of HIV-1 variants may provide a more accurate representation of the viral population (27). Here, our aim was to determine the frequency of CCR3 usage by cell-free HIV-1C derived from peripheral blood and/or CSF of in individuals with end-stage HIV-1C disease and presenting with cryptococcal meningitis (CM), an AIDS-defining illness.

4.3 Methods and materials

4.3.1 Study participants

The 14 randomly selected combination antiretroviral therapy (cART)-naïve participants in this study were part of a longitudinal prospective cohort study of CM described previously (28). All participants were CSF positive for cryptococcal antigen (CrAg) or Indian ink test, had a $CD4^+$ T-cell count lower than 200 cells/μL according to Becton Dickinson TruCount Technology (BD, Franklin lakes, NJ), and detectable HIV-1 RNA in plasma and CSF based on the COBAS TaqMan HIV-1 test (Hoffmann-La Roche, Basel, Switzerland) with a lower detection limit of 34 HIV-1 RNA copies/mL. Of note, all but two participants had pleocytosis, a CSF white blood cell (WBC) count of five or more cells per microlitre, suggesting ongoing inflammation in the CNS.

4.3.2 Synthesis of cDNA, the amplification and sequencing of HIV-1 env genes

Cell-free RNA was extracted from the plasma and CSF of all the participants using the QIAmp Viral RNA Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Single-strand HIV cDNA synthesis was performed using the SuperScript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) as described previously (29). Full-length HIV-1 env genes were amplified according to the single-genome amplification (SGA) or the standard (bulk) amplification method as described elsewhere (29), and sequenced bi-directionally using up to eight forward and reverse primers using the Sanger method (Chapter 2) (30, 31).

4.3.3 Identification of HIV-1 env gene subtype

The subtype of the *env* genes was identified by constructing a neighbor-joining phylogenetic tree incorporating the V1-V5 *env* gene fragments of participant-derived and reference HIV-1 subtype sequences, available on the Los Alamos National Laboratory HIV database (https://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html). Sequences alignments and phylogenetic inferences were performed in MEGA 7 (32), as described previously (Chapter 2) (12).

4.3.4 Cloning of HIV-1 env genes and the production of pseudoviruses

The 2.1 kb *env* gene fragments, corresponding to nucleotides 6,348 to 8,478 according to HXB2 numbering were amplified, purified, and cloned into the pSVIII-Env expression vector (19, 33). Single-round infection luciferase reporter viruses (pseudoviruses) with participant-derived Envs were produced in 0.6 X 10^6 293T cells, by co-transfecting 0.8 µg of recombinant pSVIII-Env vectors with 0.8 µg of pCMVΔP1ΔenvpA and 2.4 µg of pHIV-1Luc using polyethylenimine as previously described (33). The co-transfected 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies) containing 10% fetal bovine serum, 1%

of 1X Glutamax (Invitrogen Life Technologies) and 1% penicillin-streptomycin (Biochrom AG, Berlin, Germany) at 37 °C and 5% CO_2. After a 48 hour period, the culture supernatants containing pseudoviruses were collected, passed through a 0.45 µm filter and stored at -80 °C.

4.3.5 Major coreceptor usage of HIV-1C

The major coreceptor usage of HIV-1C Envs in this study was determined previously (Chapter 3). Envs which entered only NP2-CD4 or U87-CD4 cells expressing CCR5 were classified as R5, those entering using CXCR4 only were classified as X4, and those entering the cells using CCR5 and CXCR4 were classified as R5X4.

4.3.6 Evaluation of CCR3 usage by HIV-1 Envs

For the pseudovirus entry assays, five-fold serial dilutions of each pseudovirus were prepared using DMEM. One hundred microlitre of each dilution was incubated with 1 X 10^4 U87-CD4 cells expressing CCR3 in a 96-well plate, in duplicate. The cells were provided DMEM supplemented with 1 µg/mL of puromycin (Sigma-Aldrich, St. Louis, USA) and 300 µg/mL of G418 (Sigma-Aldrich), then infections were allowed to proceed for 6-12 hours at 37 °C and 5% CO_2. The medium was changed thereafter and incubation was continued for another 60 hours. Then, the medium was discarded, the cells were washed with phosphate-buffered saline and lysed with the Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI). The entry of pseudoviruses was evaluated by assaying luciferase activity in cell lysates using the Luciferase Assay System (Promega). Luminescence was quantified using the Modulus Microplate reader (Turner Biosystems, Sunnyvale, CA). A pseudovirus which produced luminescence readings that were equal to or greater than 10-fold that of the culture medium without pseudovirus (control) was considered as able to infect the cells expressing CCR3. For comparison and assay quality control, pseudoviruses pseudotyped with CCR3-using HIV-1 subtype B Envs (HXB2, JR-CSF,

NL4.3 YU2 and 89.6) were assayed in parallel. Envs which mediated entry using CCR3 were classified as R3.

4.4 Results

The study participants with CM included in this sub-study were part of a larger cohort study described previously. The clinical characteristics of the participants in this sub-study are shown in

Table 4-1 (28). Here, we report the usage of the CCR3 coreceptor by HIV-1C Envs in eight randomly selected participants with matched plasma and CSF, and an additional two and four participants with unmatched plasma and CSF samples, respectively. Thus, our analyses were based on total of 10 plasma and 12 CSF samples across 14 participants.

Table 4-1: The clinical characteristics and samples analyzed of study participants with CM.

ID[a]	Age	Sex[b]	CD4[c]	Pl VL[d]	CSF VL[e]	CSF WBC[f]	Sample[g]
CM019	45	M	7	5.15	4.33	28	Pl and CSF
CM021	30	F	134	4.34	4.34	40	CSF only
CM029	33	F	121	5.22	5.99	424	CSF only
CM032	34	F	5	4.94	2.52	0	Pl and CSF
CM050	23	F	16	4.92	4.80	6	CSF only
CM052	24	F	172	4.62	4.83	36	Pl and CSF
CM054	23	M	7	5.20	4.20	14	Pl only
CM070	25	M	11	5.04	4.00	18	Pl only
CM089	27	M	114	5.67	5.85	154	Pl and CSF
CM094	29	F	35	5.15	4.03	84	CSF only
CM098	40	M	14	4.76	5.27	66	Pl and CSF
CM108	27	M	20	5.16	5.02	28	Pl and CSF
CM117	38	M	1	5.72	4.66	0	Pl and CSF
CM132	34	M	53	4.58	4.43	38	Pl and CSF

[a]The identification number of the study participant. [b]The sex of the study participant, where "M" refers to male and "F" refers to female. [c]The CD4 count is provided as cells/µL. [d,e]The cell-free plasma (Pl) and CSF viral load (VL) are presented as $\log_{10}$ HIV RNA copies/mL. [f]The CSF white blood cell (WBC) count was measured as cells/µL. [g]The samples analyzed in this study.

A median of 1.5 (range 1-6) and 1 (range 1-7) plasma and CSF Envs per participant, respectively, were included in this study. In total, the alternative coreceptor usage of 46 unique Envs was evaluated. Previously, the Envs were identified as subtype C as they clustered with reference subtype C sequences (Chapter 2). Overall, of the 46 Envs, 37 (13 plasma; 24 CSF) were R5, three (two plasma; one CSF) were R5X4, and six (all from plasma) were X4 based on their ability to enter NP2 or U87-CD4 cells expressing CCR5 and CXCR4 (determined in Chapter 3, and summarized in Table 4-2). The level of entry mediated into U87-CD4 cells expressing CCR3 by

each Env using the CCR3 coreceptor was also determined (Figure 4.1, and summarized in Table 4-2), while the overall HIV-1C coreceptor usage per compartment of each participant was summarized in

Table 4-3. Two out of 13 (~15.4%) plasma-derived R5 viruses used CCR3, whereas 11 (~84.6%) did not. Seven out of 24 (~29.1%) CSF-derived R5 viruses used CCR3, though 17 out of 24 (~70.8%) did not. Two of the three (~66.6%) R5X4 plasma-derived viruses used CCR3 and one (~33.3%) did not, while the only CSF-derived R5X4 viruses in the study used CCR3. Four out of six (~66.6%) plasma-derived X4 viruses used CCR3, whereas the other two (~33.3%) did not. Overall, four out of 10 (40%) plasma and five out of 12 (41.7%) CSF samples harbored CCR3-using viruses. Notably, with the exception of participant CM094 who did not have a plasma sample available for evaluation, we identified that usage of CCR3 in plasma accompanied by usage of CCR3 in the CSF of CM participants Thus, our results suggest that CCR3 usage by HIV-1C is common in individuals with end-stage HIV disease and CM, and is not restricted to the peripheral blood compartment only.

Figure 4.1 (A)

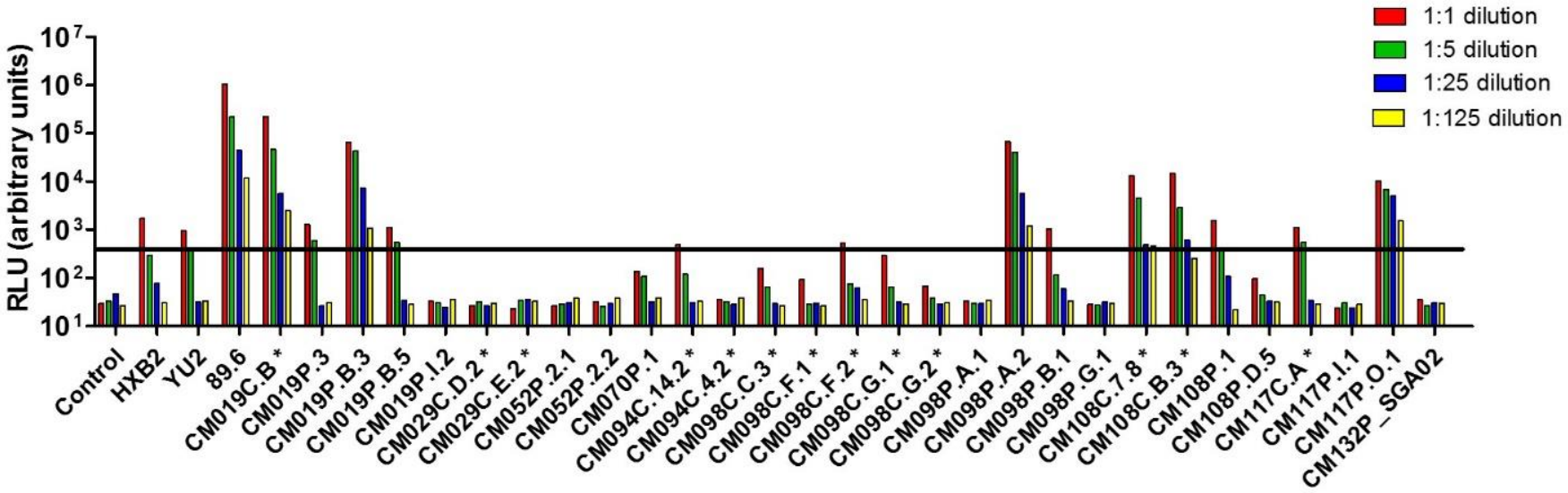
1:1 dilution
1:5 dilution
1:25 dilution
1:125 dilution
RLU (arbitrary units)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Control
HXB2
YU2
89.6
CM019C.B *
CM019P.3
CM019P.B.3
CM019P.B.5
CM019P.I.2
CM029C.D.2 *
CM029C.E.2 *
CM052P.2.1
CM052P.2.2
CM070P.1
CM094C.14.2 *
CM094C.4.2 *
CM098C.C.3 *
CM098C.F.1 *
CM098C.F.2 *
CM098C.G.1 *
CM098C.G.2 *
CM098P.A.1
CM098P.A.2
CM098P.B.1
CM098P.G.1
CM108C.7.8 *
CM108C.B.3 *
CM108P.1
CM108P.D.5
CM117C.A *
CM117P.I.1
CM117P.O.1
CM132P _ SGA02

Figure 4.1 (B)

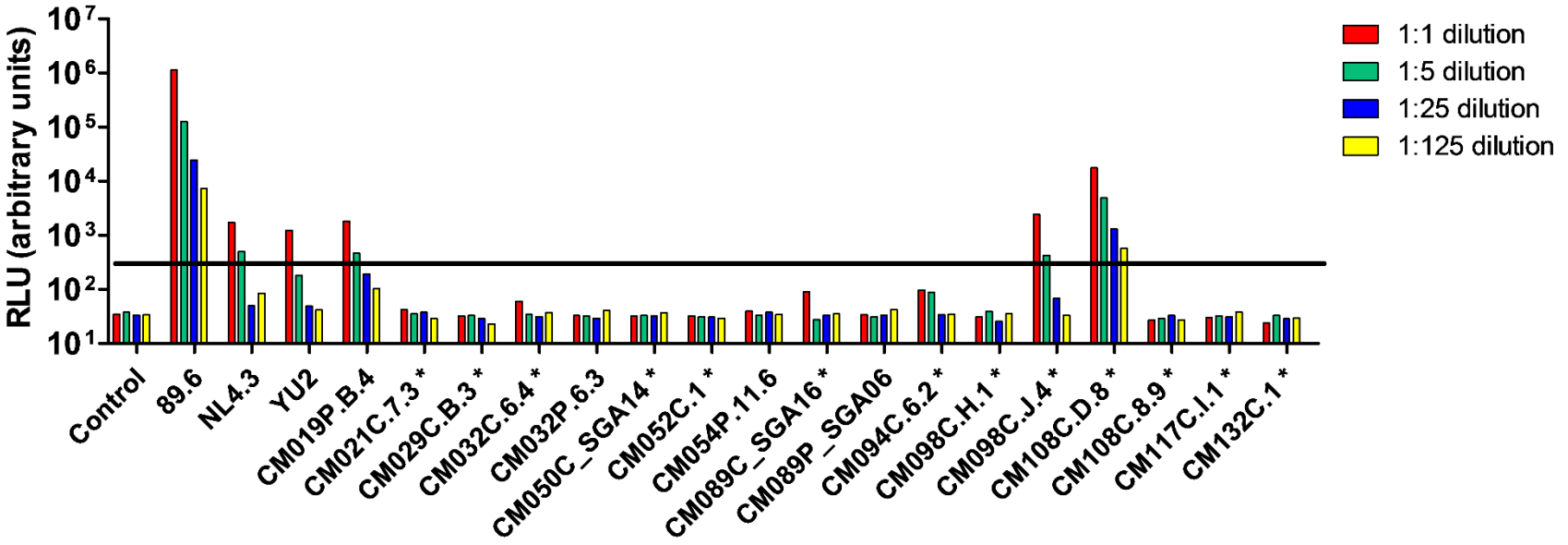

Figure 4.1 (A-B): The entry of HIV-1 subtype C pseudoviruses into U87-CD4 cells expressing CCR3.

HIV-1 subtype C pseudoviruses pseudotyped with participant-derived Envs were engineered to produce luciferase upon successful entry of U87-CD4 cells expressing CCR3 as described in Materials and methods. Pseudoviruses were diluted 1:1, 1:5, 1:25 and 1:125 with supplemented Dulbecco's modified Eagle's medium (DMEM) in 100 µL volumes and incubated with U87-CD4 cells expressing CCR3, in duplicate. The mean luciferase activity 72 hours post-infection in cell lysates was measured as relative light units (RLU). The pseudoviruses with a star symbol beside their name possessed a

CSF-derived Env, whereas those without a star possessed a plasma-derived Env (listed on the x-axis). A background sample consisting of culture medium without pseudovirus (control) was included in the assay to determine which Envs facilitated entry: pseudoviruses recording RLU values which were at least 10-fold greater (black cutoff line) than the control sample were classified as CCR3-using. CCR3-using HIV-1B pseudoviruses pseudotyped with Envs of HXB2, JR-CSF, YU2 and 89.6 were also included in the assay for comparison.

Table 4-2: The major and alternative coreceptor usage of HIV-1C Envs.

ID[a]	Compartment	Env[b]	Major tropism	Alternative tropism[c]
CM019	CSF	CM019C.B	R5X4	R3
	Plasma	CM019P.3	X4	R3
		CM019P.B.3	R5X4	R3
		CM019P.B.4	X4	R3
		CM019P.B.5	X4	R3
		CM019P.H.1	R5	-
		CM019P.I.2	R5	-
CM021	CSF	CM021C.7.3	R5	-
CM029	CSF	CM029C.B.3	R5	-
		CM029C.D.2	R5	-
		CM029C.E.2	R5	-
CM032	CSF	CM032C.6.4	R5	-
	Plasma	CM032P.6.3	R5	-
CM050	CSF	CM050C_SGA14	R5	-
CM052	CSF	CM052C.1	R5	-
	Plasma	CM052P.2.1	R5	-
		CM052P.2.2	R5	-

ID[a]	Compartment	Env[b]	Major tropism	Alternative tropism[c]
CM054	Plasma	CM054P.11.6	R5	-
CM070	Plasma	CM070P.1	X4	-
CM089	CSF	CM089C_SGA16	R5	-
	Plasma	CM089P_SGA06	R5	-
CM094	CSF	CM094C.14.2	R5	R3
		CM094C.6.2	R5	-
CM098	CSF	CM098C.C.3	R5	-
		CM098C.F.1	R5	-
		CM098C.F.2	R5	R3
		CM098C.G.1	R5	-
		CM098C.G.2	R5	-
		CM098C.H.1	R5	-
		CM098C.J.4	R5	R3
	Plasma	CM098P.A.1	X4	-
		CM098P.A.2	X4	R3
		CM098P.B.1	R5	R3
		CM098P.G.1	R5	-
CM108	CSF	CM108C.7.8	R5	R3
		CM108C.8.9	R5	-
		CM108C.B.3	R5	R3
		CM108C.D.8	R5	R3
	Plasma	CM108P.1	R5X4	R3
		CM108P.D.5	R5	-

ID[a]	Compartment	Env[b]	Major tropism	Alternative tropism[c]
CM117	CSF	CM117C.A	R5	R3
		CM117C.I.1	R5	-
	Plasma	CM117P.I.1	R5	-
		CM117P.O.1	R5	R3
CM132	CSF	CM132C.1	R5	-
	Plasma	CM132P_SGA02	R5	-

[a]The identification number of the participant. [b]The name of an individual Env evaluated. [c]The Envs which were able to use CCR3 were classified as R3 tropic, shown as "R3", and those unable to use CCR3 were labelled with a dash.

Table 4-3: The overall coreceptor usage of HIV-1C Envs in each compartment of the study participants.

ID[a]	Plasma[b]	CSF[c]
CM019	R3R5X4, R3X4, R5	R3R5X4
CM021	-	R5
CM029	-	R5
CM032	R5	R5
CM050	-	R5
CM052	R5	R5
CM054	R5	-
CM070	X4	-
CM089	R5	R5
CM094	-	R3R5, R5
CM098	R3R5, R3X4, R5, X4	R3R5, R5,
CM108	R3R5X4, R5	R3R5, R5
CM117	R3R5, R5	R3R5, R5
CM132	R5	R5

[a]The identification number of the study participants. [b,c]The coreceptor usage phenotypes of Envs observed in the plasma and/or CSF samples of the study participants. Dashes indicate where an Env was not available for testing from that compartment. Forty percent of plasma and ~41.7% of CSF samples harbored CCR3-using viruses.

4.5 Discussion

Although usage of alternative coreceptors by HIV-1C has been shown in other studies, those studies focused largely on viruses circulating in plasma (21-23, 25, 26), whereas our study characterized alternative coreceptor of viruses circulating in CSF as well. Limited information regarding the alternative coreceptor usage of HIV-1 in the CNS indicates that HIV-1C prefers to

use CXCR6 in CSF but not matched plasma (24). We showed that matched plasma and CSF of participants with CM harbour HIV-1C variants which use CCR3 for entry, though it was not clear from which compartment CCR3 usage arose. Also, due to the limited number of Envs evaluated, we were unable to determine whether R5 viruses had a propensity to use CCR3 more than R5X4 or X4 viruses.

The CCR3 and CCR5 receptors have been shown to support HIV-1 infection of microglia from the CNS compartment (16-18). It has also been shown that CCR3 and CCR5 co-localize with CD4 at in the plasma membrane of target cells, and that targeting either receptor inhibits HIV-1 infection (34). In our study we observed a variety of CCR3-using HIV-1C viruses (R3R5, R3R5X4 and R3X4) in individuals with CM. This was in contrast with a previous study of HIV-1C in individuals with active tuberculosis, where all the virus there used CCR5 only (10). We did not, however, measure whether or not viruses in this study preferred CCR3 over CCR5 or CXCR4. We also did not evaluate whether CCR3-using viruses in the CNS compartment are capable of entering microglia or monocyte-derived macrophages. Nevertheless, the observation of HIV-1C viruses with expanded coreceptor tropism in the CNS compartment warrants further investigations into the pathogenesis of this subtype in that compartment.

Future studies may include tissue tropism tests to evaluate whether or not HIV-1C Envs with expanded coreceptor tropism have the ability to replicate in the CNS compartment. It is also of interest to evaluate whether certain AIDS defining illnesses enrich for HIV-1C with alternative coreceptor usage, to identify which other alternative coreceptors are used preferentially in the CNS, and to identify determinants of alternative coreceptor usage. Given that HIV-1C is the most prevalent world-wide, understanding the pathogenesis of this subtype further is of great important.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We would like to thank the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH for 293T cells and the U87-CD4 cell line expressing CCR3.

Supporting information

The ability of Envs in this study to use CCR5 and CXCR4 was reported in Chapter 3 and is reproduced in FigShare (https://figshare.com/s/2feeee458f5e685273fa).

4.6 References

1. **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM.** 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci U S A **105:**7552-7557.

2. **Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M,**

Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH, Shaw GM. 2009. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J Exp Med **206:**1273-1289.

3. **Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR.** 1997. Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med **185:**621-628.

4. **Scarlatti G, Tresoldi E, Bjorndal A, Fredriksson R, Colognesi C, Deng HK, Malnati MS, Plebani A, Siccardi AG, Littman DR, Fenyo EM, Lusso P.** 1997. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat Med **3:**1259-1265.

5. **Koot M, Vos AH, Keet RP, de Goede RE, Dercksen MW, Terpstra FG, Coutinho RA, Miedema F, Tersmette M.** 1992. HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. AIDS **6:**49-54.

6. **Ping LH, Nelson JA, Hoffman IF, Schock J, Lamers SL, Goodman M, Vernazza P, Kazembe P, Maida M, Zimba D, Goodenow MM, Eron JJ, Jr., Fiscus SA, Cohen MS, Swanstrom R.** 1999. Characterization of V3 sequence heterogeneity in subtype C human immunodeficiency virus type 1 isolates from Malawi: underrepresentation of X4 variants. J Virol **73:**6271-6281.

7. **Abebe A, Demissie D, Goudsmit J, Brouwer M, Kuiken CL, Pollakis G, Schuitemaker H, Fontanet AL, Rinke de Wit TF.** 1999. HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS. AIDS **13:**1305-1311.

8. **Bjorndal A, Sonnerborg A, Tscherning C, Albert J, Fenyo EM.** 1999. Phenotypic characteristics of human immunodeficiency virus type 1 subtype C isolates of Ethiopian AIDS patients. AIDS Res Hum Retroviruses **15:**647-653.

9. **Cecilia D, Kulkarni SS, Tripathy SP, Gangakhedkar RR, Paranjape RS, Gadkari DA.** 2000. Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India. Virology **271:**253-258.

10. **Morris L, Cilliers T, Bredell H, Phoswa M, Martin DJ.** 2001. CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. AIDS Res Hum Retroviruses **17:**697-701.

11. **Choge I, Cilliers T, Walker P, Taylor N, Phoswa M, Meyers T, Viljoen J, Violari A, Gray G, Moore PL, Papathanosopoulos M, Morris L.** 2006. Genotypic and phenotypic characterization of viral isolates from HIV-1 subtype C-infected children with slow and rapid disease progression. AIDS Res Hum Retroviruses **22:**458-465.

12. **Jakobsen MR, Cashin K, Roche M, Sterjovski J, Ellett A, Borm K, Flynn J, Erikstrup C, Gouillou M, Gray LR, Saksena NK, Wang B, Purcell DF, Kallestrup P, Zinyama-Gutsire R, Gomo E, Ullum H, Ostergaard L, Lee B, Ramsland PA, Churchill MJ, Gorry PR.** 2013. Longitudinal Analysis of CCR5 and CXCR4 Usage in a Cohort of Antiretroviral Therapy-Naive Subjects with Progressive HIV-1 Subtype C Infection. PLoS One **8:**e65950.

13. **Cilliers T, Nhlapo J, Coetzer M, Orlovic D, Ketas T, Olson WC, Moore JP, Trkola A, Morris L.** 2003. The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C. J Virol **77:**4449-4456.

14. **Connell BJ, Michler K, Capovilla A, Venter WD, Stevens WS, Papathanasopoulos MA.** 2008. Emergence of X4 usage among HIV-1 subtype C: evidence for an evolving epidemic in South Africa. AIDS **22:**896-899.

15. **Lin NH, Smeaton LM, Giguel F, Novitsky V, Moyo S, Mitchell RM, Makhema J, Essex M, Lockman S, Kuritzkes DR.** 2011. Prevalence and clinical associations of CXCR4-using HIV-1 among treatment-naive subtype C-infected women in Botswana. J Acquir Immune Defic Syndr **57:**46-50.

16. **He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, Gabuzda D.** 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature **385:**645-649.

17. **Shieh JT, Albright AV, Sharron M, Gartner S, Strizki J, Doms RW, Gonzalez-Scarano F.** 1998. Chemokine receptor utilization by human immunodeficiency virus type 1 isolates that replicate in microglia. J Virol **72:**4243-4249.

18. **Gabuzda D, He J, Ohagen A, Vallat AV.** 1998. Chemokine receptors in HIV-1 infection of the central nervous system. Seminars in immunology **10:**203-213.

19. **Ohagen A, Devitt A, Kunstman KJ, Gorry PR, Rose PP, Korber B, Taylor J, Levy R, Murphy RL, Wolinsky SM, Gabuzda D.** 2003. Genetic and functional analysis of full-length human immunodeficiency virus type 1 env genes derived from brain and blood of patients with AIDS. J Virol **77:**12336-12345.

20. **Peters PJ, Bhattacharya J, Hibbitts S, Dittmar MT, Simmons G, Bell J, Simmonds P, Clapham PR.** 2004. Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplified from brain and lymph

node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. J Virol **78:**6915-6926.

21. **Nedellec R, Coetzer M, Shimizu N, Hoshino H, Polonis VR, Morris L, Martensson UE, Binley J, Overbaugh J, Mosier DE.** 2009. Virus entry via the alternative coreceptors CCR3 and FPRL1 differs by human immunodeficiency virus type 1 subtype. J Virol **83:**8353-8363.

22. **Isaacman-Beck J, Hermann EA, Yi Y, Ratcliffe SJ, Mulenga J, Allen S, Hunter E, Derdeyn CA, Collman RG.** 2009. Heterosexual transmission of human immunodeficiency virus type 1 subtype C: Macrophage tropism, alternative coreceptor use, and the molecular anatomy of CCR5 utilization. J Virol **83:**8208-8220.

23. **Coetzer M, Nedellec R, Cilliers T, Meyers T, Morris L, Mosier DE.** 2010. Extreme genetic divergence is required for coreceptor switching in HIV-1 subtype C. J Acquir Immune Defic Syndr **56:**9-15.

24. **Karlsson U, Antonsson L, Ljungberg B, Medstrand P, Esbjornsson J, Jansson M, Gisslen M.** 2012. Dual R3R5 tropism characterizes cerebrospinal fluid HIV-1 isolates from individuals with high cerebrospinal fluid viral load. Aids **26:**1739-1744.

25. **Cashin K, Jakobsen MR, Sterjovski J, Roche M, Ellett A, Flynn JK, Borm K, Gouillou M, Churchill MJ, Gorry PR.** 2013. Linkages between HIV-1 specificity for CCR5 or CXCR4 and in vitro usage of alternative coreceptors during progressive HIV-1 subtype C infection. Retrovirology **10:**98.

26. **Cilliers T, Willey S, Sullivan WM, Patience T, Pugach P, Coetzer M, Papathanasopoulos M, Moore JP, Trkola A, Clapham P, Morris L.** 2005. Use of alternate coreceptors on primary cells by two HIV-1 isolates. Virology **339:**136-144.

27. **Aasa-Chapman MM, Aubin K, Williams I, McKnight A.** 2006. Primary CCR5 only using HIV-1 isolates does not accurately represent the in vivo replicating quasi-species. Virology **351:**489-496.

28. **Chang CC, Dorasamy AA, Gosnell BI, Elliott JH, Spelman T, Omarjee S, Naranbhai V, Coovadia Y, Ndung'u T, Moosa MY, Lewin SR, French MA.** 2013. Clinical and mycological predictors of cryptococcosis-associated immune reconstitution inflammatory syndrome. AIDS **27:**2089-2099.

29. **Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J, Anderson JA, Swanstrom R, Haynes BF, Athreya GS, Korber BT, Sharp PM, Shaw GM, Hahn BH.** 2008. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. J Virol **82:**3952-3970.

30. **Archary D, Gordon ML, Green TN, Coovadia HM, Goulder PJ, Ndung'u T.** 2010. HIV-1 subtype C envelope characteristics associated with divergent rates of chronic disease progression. Retrovirology **7:**92.

31. **Rousseau CM, Birditt BA, McKay AR, Stoddard JN, Lee TC, McLaughlin S, Moore SW, Shindo N, Learn GH, Korber BT, Brander C, Goulder PJ, Kiepiela P, Walker BD, Mullins JI.** 2006. Large-scale amplification, cloning

and sequencing of near full-length HIV-1 subtype C genomes. J Virol Methods **136:**118-125.

32. **Kumar S, Stecher G, Tamura K.** 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular biology and evolution **33:**1870-1874.

33. **Sterjovski J, Churchill MJ, Ellett A, Gray LR, Roche MJ, Dunfee RL, Purcell DF, Saksena N, Wang B, Sonza S, Wesselingh SL, Karlsson I, Fenyo EM, Gabuzda D, Cunningham AL, Gorry PR.** 2007. Asn 362 in gp120 contributes to enhanced fusogenicity by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with AIDS. Retrovirology **4:**89.

34. **Agrawal L, Maxwell CR, Peters PJ, Clapham PR, Liu SM, Mackay CR, Strayer DS.** 2009. Complexity in human immunodeficiency virus type 1 (HIV-1) co-receptor usage: roles of CCR3 and CCR5 in HIV-1 infection of monocyte-derived macrophages and brain microglia. J Gen Virol **90:**710-722.

Chapter 5: Discussion

5.1 Relatedness of HIV-1C env genes in the plasma and CSF

In this study, we sought to reveal whether *Cryptococcus neoformans*-HIV co-infection was associated with viral genetic intermixing between peripheral blood and the CNS of study participants presenting with CM. We hypothesized that *Cryptococcus neoformans* (or antigens thereof) in the CNS may alter the local immunological milieu and lead to the recruitment of HIV-1 variants into the CNS causing genetic mixing of variants between compartments. We observed viral genetic intermixing in all the participants with multiple sequences collected from plasma and CSF, thus the results supported our hypothesis.

All but two participants (CM032 and CM117) in our study had pleocytosis (a marker of CNS inflammation), and we suspected that this was a contributing cause of viral genetic intermixing between compartments observed in our participants. Pleocytosis was strongly associated with the intermixing of viral variants between the blood and CNS compartments previously (254). Additionally, another study showed that pleocytosis may alter the dynamics of viral populations between the blood and CNS (231). It is, therefore, probable that HIV-1 infected white blood cells were trafficked to the CNS compartment, allowing the virus access to the CNS and causing viral genetic intermixing as shown by Sturdevant *et al* (2015) (231). We did not, however, evaluate the causes of pleocytosis and whether it occurred transiently or not, therefore pleocytosis in participants CM032 and CM117 may have resolved recently and hence genetic mixing was still observed between compartments. An alternative explanation may be that the integrity of the blood-brain barrier may have been compromised due to HIV-1 or *Cryptococcus neoformans* infection, allowing fluid cell-free traffic of virus across compartments. Overall, we established that *Cryptococcus neoformans*-HIV co-infection is associated with HIV-1C genetic intermixing between the peripheral blood and CNS compartments.

A previous study showed that SGA, compared to bulk PCR, minimizes the frequency of taq-induced recombination, nucleotide misincorporation, template switching and template resampling (255). Here, we employed SGA as well as bulk PCR as a method of amplification. A possible limitation of our methods of amplification was the ability to detect minor variants, which are better captured with deep sequencing approaches. Additionally, the low number of sequences we collected for the compartments of some participants may have reduced our capacity to detect compartmentalized sub-populations of viruses.

We did not have a control group of participants without CM or with other co-infections, because our studies were foremost designed to focus on individuals with CM, so future studies will be extended to include adequate control groups to clarify the impact of CM on the viral population dynamics between compartments.

Based on genetic studies of HIV-1 *env* genes of viruses derived from matched blood and CSF, it was shown that the virus may enter the CNS early in primary infection (227, 231). Initial genetic comparisons of plasma and CSF-derived HIV-1 were for the understanding of HIV-1 neurotropism and neurological impairment caused by the virus. They revealed that variants unique to the CNS were probably selected for by target cells and immune pressures in the CNS (235). Following that, studies revealed that CNS compartmentalized variants were implicated as a cause of HAD (224, 226). Employing SGA and sequencing and phylogenetic analyses, a series of studies showed that the viral populations in blood may be distinct from those of the CNS (viral genetic compartmentalization). Phylogenetically, this meant that there were sub-populations of viruses which are represented by CSF-derived viruses only while other populations represented plasma-derived viruses only. Alternatively, the compartmentalization may be incomplete or partial, where there were sub-populations of viruses which were represented by intermixing plasma and CSF-derived variants while other populations represented plasma or CSF-derived variants only. In the absence of compartmentalization, there were no sub-populations specific for plasma or CSF-derived variants and variants from both compartments were homogenously

interspersed or equilibrated (194, 228, 231). These studies were different to ours, however, as they were designed for the understanding of HAD in adults, neurological deterioration in infants, or the timing of HIV-1 entering the CNS.

5.2 Predicted coreceptor usage of HIV-1C Envs

A consistent estimate of the prevalence of CXCR4-using HIV-1C is not available. Some studies have reported an absence or rare usage of CXCR4 by HIV-1C isolates (72, 169-171, 252, 253), while other studies suggest that between 15% and 30% of individuals with end-stage disease harbor these isolates (116, 172, 173). Using CPAs, we evaluated the predicted coreceptor usage of HIV-1C variants circulating in blood and the CNS of participants with end-stage disease and presenting with CM. We observed that predicted R5 variants were present in plasma as well as CSF samples; 92.9% and 84.6% of plasma and CSF samples, respectively, harbored predicted R5 variants. Variants predicted to use CXCR4 were not uncommon in plasma and CSF samples; 50% and 30.8% of plasma and CSF samples, respectively, harbored predicted CXCR4-using variants. Overall, we identified that the prevalence of predicted CXCR4 usage was high, as approximately 44% of participants harbored at least one CXCR4-using variant in plasma and/or CSF. Thus, our results suggest that approximately 44% of individuals presenting with CM would be ineligible for therapy including maraviroc.

We employed three CPAs (Geno2pheno, WebPSSM, and PhenoSeq) to predict coreceptor usage for all variants sequenced in this study (256-258). The three CPAs individually have high sensitivity and specificity for predicting coreceptor usage, but may generate conflicting predictions between them as we and other have seen (75, 259). Thus, we determined predicted coreceptor usage based on the concordance of all three or two of three CPAs in order to maximize the accuracy of our determinations. The limitation of using CPAs for coreceptor usage predictions is that they may overestimate the prevalence of CXCR4-using variants, as previously described (260-264). If the CPAs used overestimated the prevalence of CXCR-using variants, this would

mean that some participants would be incorrectly precluded from regimens including maraviroc, a CCR5 antagonist.

Our results, however, confirm those of previous studies showing that the prevalence of CXCR4-using HIV-1C is high in participants with end-stage disease. Our estimated prevalence of CXCR4 usage was, unexpectedly, much higher that previously reported, where HIV-1C CXCR4 usage was reported in 15% and 30% of participants, according to *in vitro* phenotypic assays (116, 172, 173). Our estimate suggests that HIV-1C is able to use CXCR4 frequently in end-stage HIV disease. Maraviroc shows good antiviral activity against R5, but is ineffective against CXCR4-using strains (99). Additional evidence shows that CXCR4-using viruses may emerge from pre-existing reservoirs under maraviroc treatment (265), and that there is no clinical benefit observed with the administration of maraviroc to patients with non-R5 virus (266). Given that HIV-1C in patients with CM may use CXCR4, we therefore recommend that viral tropism be screened before maraviroc is administered.

Notably, none of the individuals with active tuberculosis in a study by others had isolates which used CXCR4 or alternative coreceptors (72). Although that study had a small sample size of 10 participants, it suggested that active tuberculosis selects for R5 HIV-1C variants. Therefore, it is of interest to establish whether or not certain co-infections select for a higher prevalence of CXCR4 usage than others, and why. Also, as the presence of CXCR4-using HIV-1C sub-populations in the CNS has been reported rarely (160), it is also of interest to employ deep sequencing methods to identify whether they persist as a minority population or not, and whether they are X4 or R5X4 tropic.

5.3 Genotypic concordance of HIV-1 coreceptor usage between plasma and CSF samples predominates

The major coreceptor usage of HIV-1 of viral populations in the blood and CNS compartments has been compared previously to identify whether or not the pathogenesis of HIV-1 in the CNS was different than in the blood compartment, and to ascertain whether coreceptor antagonists like maraviroc would be effective in both compartments (223, 249, 250, 267). In this study, we evaluated whether participants with end-stage disease and presentation of CM have concordance or discordance of HIV-1C predicted coreceptor usage between plasma and CSF samples. We identified that 63.6% of participants displayed concordant coreceptor usage, 18.2% displayed discordance, and a further 18.2% were indeterminate. This result indicated that coreceptor usage in plasma and CSF is not different generally, though discordance is not uncommon, in individuals with CM.

Our results confirm those of previous studies where concordance of coreceptor usage was recorded in 54.5% to 89.1% of participants. However, the previous studies evaluated coreceptor usage in PBMCs/plasma and CSF samples employing *in vitro* phenotypic assays (223, 249, 267), or CPAs (250). Due to the low number of sequences collected for some participants, we may have underestimated the frequency of concordant coreceptor usage between samples.

5.4 *In vitro* usage of CCR5 and/or CXCR4 by HIV-1C

Previously, with the aid of CPAs, we identified that HIV-1C in plasma and CSF of participants presenting with CM was predicted to use CCR5 and/or CXCR4 for entry. CPAs, however, are unable to guarantee biological functionality of Envs, have the potential to overestimate the frequency of CXCR4 usage, and have difficulty differentiating between R5X4 and X4 Envs (260, 262, 264). Thus, we employed *in vitro* phenotypic assays to confirm the biological functionality and coreceptor usage of HIV-1C Envs circulating in the plasma and CSF of participants

presenting with CM. Combining the results of the *in vitro* assays with sequencing information, we then evaluated the determinants of HIV-1C CCR5 and CXCR4 usage to see whether they were consistent with those identified in previous studies.

In total, we evaluated the ability of 66 HIV-1C Envs to use CCR5 and/or CXCR4 expressed on NP2/U87-CD4 cells. Only 61 Envs were able to use CCR5 and/or CXCR4, while five were not able to use the coreceptors. In the 10 plasma samples evaluated, R5, R5X4 and X4 viruses were identified in 90%, 20% and 30% of samples, respectively. In the 12 CSF samples evaluated, R5, R5X4 and X4 viruses were identified in 91.7%, 8.3% and 0% of samples, respectively. These finding suggested that R5, R5X4 and X4 viruses may persist in the blood, but that only R5 and R5X4 persist in the CNS. While multiple studies have shown previously using *in vitro* assays that HIV-1C in the blood (from PBMCs or plasma) is capable of using CCR5 and/or CXCR4 (72, 76, 77, 116, 147, 160, 171, 173, 268), few studies have evaluated the major coreceptor usage of HIV-1C in the in CSF samples. In CSF samples, R5 viruses predominate while R5X4 viruses are rare and X4 viruses have not been recorded (160, 228). Therefore, our results are consistent with those of previous studies. Our results were potentially limited by our conservative cut-offs for pseudovirus entry in cells lines; only pseudoviruses registering luciferase activity values equal to or greater than 10-fold that of controls were qualified as having used CCR5 or CXCR4. Thus, Envs which used CCR5 or CXCR4 albeit inefficiently may have been recorded as unable to use the coreceptors.

5.5 The performance of CPAs

We evaluated the accuracy of Geno2pheno, PhenoSeq and WebPSSM for HIV-1C Envs individually and collectively. For 45 out of 61 (~73.8%) Envs evaluated in *in vitro* coreceptor usage assays, Geno2pheno, PhenoSeq and WebPSSM provided the same prediction of HIV-1C major coreceptor usage. Individually, Geno2pheno was the most accurate of the CPAs when it came to predicting R5 viruses, followed by WebPSSM and then PhenoSeq. When it came to

predicting CXCR4 usage, all three CPAs were 100% accurate. When a consensus prediction was generated, based on the agreement of at least two of three CPAs, this was less accurate for predicting R5 viruses compared to using Geno2pheno alone, but was 100% accurate for predicting CXCR4 usage. Thus, this suggests that predictions of major coreceptor usage based on a consensus of CPAs may not offer more accuracy than using one highly accurate CPA, and that Geno2pheno should be favored over the other CPAs. We also noted that six participants were predicted to harbor CXCR4-using viruses based on a consensus of CPAs, however, only four of those six participants had CXCR4-using viruses based on *in vitro* assays. This highlights that CPAs may overestimate the prevalence of CXCR4 usage, thus some individuals may be precluded from using maraviroc incorrectly.

The three CPAs employed in this study have been shown to be highly accurate, generating predictions with high percentage sensitivity and specificity (62, 75, 269). PhenoSeq, originally named $CoRSeq_{v3\text{-}C}$, was shown to have superior accuracy than Geno2pheno and WebPSSM, but was the least accurate for R5 predictions in our study. Additionally, PhenoSeq was reported to be more accurate for predicting CXCR4 usage than Geno2pheno and WebPSSM, however, the three CPAs were equally accurate in our study (75). Even though Geno2pheno was designed based on HIV-1B sequences (257), it was observed to be the most accurate for predictions of R5 viruses, confirming that it performs well for HIV-1C predictions (73, 259). Given that two participants were incorrectly predicted to harbor CXCR4-using viruses, it shows that CPAs may overestimate the frequency or prevalence of CXCR4, supporting the findings of others (260, 262-264). Nevertheless, we were able to show that CPAs are highly accurate generally and may potentially replace the employment of *in vitro* phenotypic assays in resources-limited settings where the latter may not be feasible.

5.6 Phenotypic discordance of HIV-1 major coreceptor usage between plasma and CSF

Using CPAs, we predicted that the plasma and CSF harbored concordant HIV-1C coreceptor usage phenotypes in participant CM098, but discordant phenotypes in participant CM108 (chapter 2). Employing *in vitro* phenotypic assays (Chapter 3) were able to show that the coreceptor usage phenotypes of HIV-1C in the plasma and CSF of both participant CM098 and CM108 are in fact discordant. Participant CM098 possessed R5 and X4 Envs in plasma but R5 Envs only in CSF, whereas participant CM108 possessed R5 and R5X4 Envs in plasma but R5 Envs only in CSF. This result suggested that functional discordance of Envs may arise between compartments, and that these participants are unlikely to benefit from maraviroc treatment as it targets CCR5-tropic viruses only and is ineffective against CXCR4-using viruses as previously described (99, 265).

Participants CM098 and CM108 had a CSF white blood cell (WBC) count of 66 and 28 cells/μL, respectively, probably caused by inflammation in the CNS. Even though these participants had pleocytosis (a CSF WBC more than 5 cells/μL), different entry phenotypes of HIV-1 between the peripheral blood and the CNS were observed. It is unclear whether this was the case because the CXCR4-using variants established themselves in the blood recently and were not in sufficient concentrations to leak into the CSF. Because we did not sample viruses longitudinally, we were unable to explain why the differences of viral phenotypes between compartments were observed. Pleocytosis has been shown to cause viral intermixing between peripheral blood and the CNS (254), therefore it is possible that CXCR4-using variants may be trafficked to the CNS via infected cells where they may establish themselves alongside R5 viruses. Although HIV-1B isolates from the CNS have been shown to enter macrophages using CXCR4 (186, 192), CNS derived HIV-1C has not been shown to enter macrophages or microglia. We also suspect that discordance may be due to the target cells available for HIV-1C in the CNS, which may preferentially select for variants which use CCR5 alone or in combination with CXCR4. Others

have reported that microglia, major targets for HIV-1 in the CNS, are preferentially infected via the CCR5 coreceptor (152-154, 156).

An early study comparing coreceptor usage phenotypes of HIV-1 in blood (using PBMCs) and the CNS (using CSF) revealed that phenotypic discordance arose when SI variants were observed in the PMBCs while NSI variants were in CSF (223). A separate study then revealed that discordance could arise in one of two ways; when plasma harbored R5 and X4 variants while CSF had R5 variants only, or when plasma harbored R5 variants while CSF had R5 and X4 variants (267). Discordance was also observed when different coreceptors were used or when usage of wild-type CCR5 was different between plasma and CSF. Our studies support those of others showing that discordant coreceptor usage of HIV-1 may arise between plasma and CSF, and that CXCR4-using viruses are observed in the CSF only when they are also present in the plasma of individuals (249). More studies are required to evaluate whether CXCR4-using HIV-1C in the CNS is capable of using CXCR4 for entry into macrophages or microglia, as this has implications for the pathogenesis of HIV in the CNS and the use of regimens including coreceptors antagonists.

5.7 Determinants of CCR5 and CXCR4 usage

Multiple studies have shown that R5 and CXCR4-using HIV-1C have different properties of the V3 loop. V3 loops of CXCR4-using HIV-1C, compared to R5 HIV-1C, have higher net charge and length, but also greater amino acid variability, substitutions of the GPGQ crown motif and a reduced number of PNGS (71, 74-76, 116, 171, 270). Combining the results of the *in vitro* phenotypic assays and sequencing information in our study, we were able to identify determinants of CCR5 and CXCR4 usage for HIV-1C viruses. CXCR4-using viruses in our phenotypic studies were confirmed to have at least two of the following V3 properties: a net charge equal to or greater than +5, 37 amino acid acids length, one or two charged residues in the crown motif, and an absence of PNGS. In contrast, the V3 sequences of R5 viruses were 35 amino acids in length, had a net charge less than +5 and a GPGQ crown motif generally.

Most of the R5 viruses in this study had a Env V3 crown motif consisting of a GPGQ sequence, except for three viruses, predicted to use CXCR4, from participant CM050 and one from CM108 (Env CM108P.D.5 had a GPGH, and the Envs from CM050 had a GPGK motif). Given that the R5 viruses from participant CM108 and CM050 predicted to be CXCR4-using only had one known determinant of CXCR4 usage (an alteration of the GPGQ motif), it is probable that this property alone was not sufficient to confer usage of CXCR4. This highlights that HIV-1C may require at least two unique properties in the V3 to confer usage of CXCR4. Our results were limited in that we did not perform site-directed mutagenesis studies to confirm that the determinants we observed modify usage of major coreceptors.

5.8 Alternative coreceptor usage of HIV-1C Envs

We evaluated whether or not HIV-1C viruses circulating in plasma and CSF were capable of using an alternative coreceptor, specifically CCR3, for entry. For our *in vitro assay*, we measured the ability of HIV-1C Envs to mediate entry into U87-CD4 cells expressing CCR3. Our results showed that R5, R5X4 and X4 viruses may use CCR3, that usage of CCR3 was concordant between plasma and CSF samples, and that approximately 35.7% of participants harbored CCR3-using viruses in plasma and/or CSF.

Multiple previous studies focusing on HIV-1C derived from the blood have shown that HIV-1C virus is capable of using CCR3 (146, 147, 158, 159). In one study including five participants harboring HIV-1C, multiple isolates in the CSF were observed to use CXCR6, while few were able to use GPR1 or CCR3. Furthermore, the use of alternative coreceptors was also limited to the CSF and not observed in plasma suggesting functional compartmentalization of virus (160). Our results confirm those of previous studies indicating that HIV-1C in peripheral blood during end-stage disease uses CCR3, however, we additionally observed that usage of CCR3 was frequent in CSF samples. A possible limitation of this analysis was the selection of a conservative cut-off for CCR3 usage in our *in vitro* phenotypic assay, which may have led to under-reporting

of CCR3 usage. The frequent detection of R3R5 and R5 viruses in CSF samples in our study suggest that CCR5 and CCR3 are important for HIV-1C entry in the CNS. HIV-1 has been shown to enter microglia using CCR5 and CCR3 (152, 154-156), suggesting that viruses in our study may have the ability to enter this cell type.

This analysis was limited by the fact that we did not evaluate whether or not the CCR3-using Envs were capable of mediating infection of monocyte-derived macrophages or microglia using CCR3.

Chapter 6: Conclusions

In summary, our studies elucidated the genotypic and phenotypic properties of HIV-1C in antiretroviral therapy naïve individuals with end-stage HIV disease and CM. We revealed the phylogenetic relatedness of HIV-1C *env* gene sequences from the peripheral blood and CNS compartments, and estimated the prevalence of CXCR4 usage in antiretroviral naïve individuals with CM using CPAs. Additionally, we determined the *in vitro* usage of major coreceptors (CCR5 and CXCR4) and an alternative coreceptor (CCR3) by HIV-1C in peripheral blood and CNS compartments. This allowed us to reveal determinants of CCR5 and CXCR4 usage by HIV-1C viruses when combining our *in vitro* phenotypic assay results with sequencing information. We also were able to clarify the accuracy of publicly available CPAs, individually and in combination. Thus, our studies have improved our understanding of HIV-1C gene flow between the peripheral blood and CNS compartments, revealed the coreceptor usage phenotype of viral variants in individuals with end-stage HIV disease with CM, clarified the eligibility of individuals with CM for using maraviroc and assisted with the refinement of CPAs.

Chapter 7: Recommendations

In this study, we demonstrated that individuals with end-stage HIV-1 disease presenting with CM have trafficking of virus between the blood and CNS compartments. However, the compartmental origins of the viruses we sequenced were unclear; they may have been produced in the blood and trafficked to the CNS or vice versa. Future studies may investigate the identity of the cells which produced the viruses we observed in plasma and the CSF. Additionally, deep sequencing tools may be employed to enhance the detection of minor variants not observed in the two compartments.

We characterized the predicted and *in vitro* coreceptor usage of HIV-1C in our study, but did not measure the efficiency of coreceptor usage or the tissue-tropism of viruses. As the efficiency of coreceptor usage or targeting of tissues may be different between compartments, other types of functional compartmentalization may be investigated in future. Furthermore, it is of interest to investigate whether individuals presenting with CM have macrophage-tropic variants in the blood and/or CNS, and to identify where they originate if so.

Our study showed that HIV-1C may have expanded coreceptor usage, but we only tested the ability of viruses to use one alternative coreceptor, CCR3. Future studies may explore the usage of other alternative coreceptors in the CNS of individuals with end-stage HIV-1 disease, in the absence and presence of CM, and whether or not microglia are infected by HIV-1C. This may also allow us to identify the determinants of alternative coreceptor usage and the pathogenesis of HIV-1C in the CNS. Additionally, the implications of expanded coreceptor usage of HIV-1C for disease progression require further investigation.

Chapter 8: References

1. **Centers for Disease C.** 1983. Prevention of acquired immune deficiency syndrome (AIDS): report of inter-agency recommendations. MMWR. Morbidity and mortality weekly report **32:**101-103.
2. **Gottlieb MS, Groopman JE, Weinstein WM, Fahey JL, Detels R.** 1983. The acquired immunodeficiency syndrome. Annals of internal medicine **99:**208-220.
3. **Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A.** 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med **305:**1425-1431.
4. **Luciw PA, Potter SJ, Steimer K, Dina D, Levy JA.** 1984. Molecular cloning of AIDS-associated retrovirus. Nature **312:**760-763.
5. **Alizon M, Sonigo P, Barre-Sinoussi F, Chermann JC, Tiollais P, Montagnier L, Wain-Hobson S.** 1984. Molecular cloning of lymphadenopathy-associated virus. Nature **312:**757-760.
6. **Arya SK, Gallo RC, Hahn BH, Shaw GM, Popovic M, Salahuddin SZ, Wong-Staal F.** 1984. Homology of genome of AIDS-associated virus with genomes of human T-cell leukemia viruses. Science **225:**927-930.
7. **Shaw GM, Hahn BH, Arya SK, Groopman JE, Gallo RC, Wong-Staal F.** 1984. Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. Science **226:**1165-1171.
8. **Clavel F, Guyader M, Guetard D, Salle M, Montagnier L, Alizon M.** 1986. Molecular cloning and polymorphism of the human immune deficiency virus type 2. Nature **324:**691-695.

9. **Wain LV, Bailes E, Bibollet-Ruche F, Decker JM, Keele BF, Van Heuverswyn F, Li Y, Takehisa J, Ngole EM, Shaw GM, Peeters M, Hahn BH, Sharp PM.** 2007. Adaptation of HIV-1 to its human host. Molecular biology and evolution **24:**1853-1860.

10. **Keele BF, Van Heuverswyn F, Li Y, Bailes E, Takehisa J, Santiago ML, Bibollet-Ruche F, Chen Y, Wain LV, Liegeois F, Loul S, Ngole EM, Bienvenue Y, Delaporte E, Brookfield JF, Sharp PM, Shaw GM, Peeters M, Hahn BH.** 2006. Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science **313:**523-526.

11. **Van Heuverswyn F, Li Y, Bailes E, Neel C, Lafay B, Keele BF, Shaw KS, Takehisa J, Kraus MH, Loul S, Butel C, Liegeois F, Yangda B, Sharp PM, Mpoudi-Ngole E, Delaporte E, Hahn BH, Peeters M.** 2007. Genetic diversity and phylogeographic clustering of SIVcpzPtt in wild chimpanzees in Cameroon. Virology **368:**155-171.

12. **Jaffar S, Wilkins A, Ngom PT, Sabally S, Corrah T, Bangali JE, Rolfe M, Whittle HC.** 1997. Rate of decline of percentage CD4+ cells is faster in HIV-1 than in HIV-2 infection. J Acquir Immune Defic Syndr Hum Retrovirol **16:**327-332.

13. **Hemelaar J.** 2012. The origin and diversity of the HIV-1 pandemic. Trends Mol Med **18:**182-192.

14. **Peeters M, Courgnaud V, Abela B, Auzel P, Pourrut X, Bibollet-Ruche F, Loul S, Liegeois F, Butel C, Koulagna D, Mpoudi-Ngole E, Shaw GM, Hahn BH, Delaporte E.** 2002. Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat. Emerg Infect Dis **8:**451-457.

15. **Van Heuverswyn F, Peeters M.** 2007. The origins of HIV and implications for the global epidemic. Curr Infect Dis Rep **9:**338-346.

16. **Taylor BS, Hammer SM.** 2008. The challenge of HIV-1 subtype diversity. N Engl J Med **359:**1965-1966.

17. **Lihana RW, Ssemwanga D, Abimiku A, Ndembi N.** 2012. Update on HIV-1 diversity in Africa: a decade in review. AIDS Rev **14:**83-100.

18. **Hemelaar J, Gouws E, Ghys PD, Osmanov S, Isolation W-UNfH, Characterisation.** 2011. Global trends in molecular epidemiology of HIV-1 during 2000-2007. AIDS **25:**679-689.

19. **Araujo LA, Almeida SE.** 2013. HIV-1 diversity in the envelope glycoproteins: implications for viral entry inhibition. Viruses **5:**595-604.

20. **Abrahams MR, Anderson JA, Giorgi EE, Seoighe C, Mlisana K, Ping LH, Athreya GS, Treurnicht FK, Keele BF, Wood N, Salazar-Gonzalez JF, Bhattacharya T, Chu H, Hoffman I, Galvin S, Mapanje C, Kazembe P, Thebus R, Fiscus S, Hide W, Cohen MS, Karim SA, Haynes BF, Shaw GM, Hahn BH, Korber BT, Swanstrom R, Williamson C, Team CAIS, Center for HIVAVIC.** 2009. Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. J Virol **83:**3556-3567.

21. **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N,**

Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM. 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci U S A **105:**7552-7557.

22. **Roberts JD, Bebenek K, Kunkel TA.** 1988. The accuracy of reverse transcriptase from HIV-1. Science **242:**1171-1173.

23. **Allen TM, Altfeld M, Geer SC, Kalife ET, Moore C, O'Sullivan K M, Desouza I, Feeney ME, Eldridge RL, Maier EL, Kaufmann DE, Lahaie MP, Reyor L, Tanzi G, Johnston MN, Brander C, Draenert R, Rockstroh JK, Jessen H, Rosenberg ES, Mallal SA, Walker BD.** 2005. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. J Virol **79:**13239-13249.

24. **Brumme ZL, Brumme CJ, Heckerman D, Korber BT, Daniels M, Carlson J, Kadie C, Bhattacharya T, Chui C, Szinger J, Mo T, Hogg RS, Montaner JS, Frahm N, Brander C, Walker BD, Harrigan PR.** 2007. Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1. PLoS Pathog **3:**e94.

25. **Dong T, Zhang Y, Xu KY, Yan H, James I, Peng Y, Blais ME, Gaudieri S, Chen X, Lun W, Wu H, Qu WY, Rostron T, Li N, Mao Y, Mallal S, Xu X, McMichael A, John M, Rowland-Jones SL.** 2011. Extensive HLA-driven viral diversity following a narrow-source HIV-1 outbreak in rural China. Blood **118:**98-106.

26. **Ndung'u T, Weiss RA.** 2012. On HIV diversity. AIDS **26:**1255-1260.

27. **Louwagie J, Janssens W, Mascola J, Heyndrickx L, Hegerich P, van der Groen G, McCutchan FE, Burke DS.** 1995. Genetic diversity of the envelope glycoprotein from human immunodeficiency virus type 1 isolates of African origin. J Virol **69:**263-271.

28. **Lukashov VV, Kuiken CL, Goudsmit J.** 1995. Intrahost human immunodeficiency virus type 1 evolution is related to length of the immunocompetent period. J Virol **69:**6911-6916.

29. **Hahn BH, Shaw GM, Arya SK, Popovic M, Gallo RC, Wong-Staal F.** 1984. Molecular cloning and characterization of the HTLV-III virus associated with AIDS. Nature **312:**166-169.

30. **Cassis-Ghavami F, Curlin M, Geise R, Duerr A.** 2009. Chapter 25 - Human Immunodeficiency Virus A2 - Barrett, Edited byAlan D.T, p. 441-468. *In* Stanberry LR (ed.), Vaccines for Biodefense and Emerging and Neglected Diseases. Academic Press, London.

31. **Gentile M, Adrian T, Scheidler A, Ewald M, Dianzani F, Pauli G, Gelderblom HR.** 1994. Determination of the size of HIV using adenovirus type 2 as an internal length marker. J Virol Methods **48:**43-52.

32. **Takasaki T, Kurane I, Aihara H, Ohkawa N, Yamaguchi J.** 1997. Electron microscopic study of human immunodeficiency virus type 1 (HIV-1) core structure: two RNA strands in the core of mature and budding particles. Arch Virol **142:**375-382.

33. **Stevens W, Papathanasopolous MA.** 2009. Human Immunodeficiency Virus. *In* Mendelow BV, Ramsay M, Chetty N (ed.), Molecular Medicine for Clinicians. Wits University Press.

34. **Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312:**763-767.

35. **Kowalski M, Potz J, Basiripour L, Dorfman T, Goh WC, Terwilliger E, Dayton A, Rosen C, Haseltine W, Sodroski J.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237:**1351-1355.

36. **Ellison V, Brown PO.** 1994. A stable complex between integrase and viral DNA ends mediates human immunodeficiency virus integration in vitro. Proc Natl Acad Sci U S A **91:**7316-7320.

37. **Chow SA, Brown PO.** 1994. Juxtaposition of two viral DNA ends in a bimolecular disintegration reaction mediated by multimers of human immunodeficiency virus type 1 or murine leukemia virus integrase. J Virol **68:**7869-7878.

38. **Wang CT, Barklis E.** 1993. Assembly, processing, and infectivity of human immunodeficiency virus type 1 gag mutants. J Virol **67:**4264-4273.

39. **Loeb DD, Swanstrom R, Everitt L, Manchester M, Stamper SE, Hutchison CA, 3rd.** 1989. Complete mutagenesis of the HIV-1 protease. Nature **340:**397-400.

40. **Bohan CA, Kashanchi F, Ensoli B, Buonaguro L, Boris-Lawrie KA, Brady JN.** 1992. Analysis of Tat transactivation of human immunodeficiency virus transcription in vitro. Gene expression **2:**391-407.

41. **Fankhauser C, Izaurralde E, Adachi Y, Wingfield P, Laemmli UK.** 1991. Specific complex of human immunodeficiency virus type 1 rev and nucleolar B23

proteins: dissociation by the Rev response element. Molecular and cellular biology **11:**2567-2575.

42. **Bandres JC, Shaw AS, Ratner L.** 1995. HIV-1 Nef protein downregulation of CD4 surface expression: relevance of the lck binding domain of CD4. Virology **207:**338-341.

43. **Swann SA, Williams M, Story CM, Bobbitt KR, Fleis R, Collins KL.** 2001. HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway. Virology **282:**267-277.

44. **Cohen EA, Terwilliger EF, Jalinoos Y, Proulx J, Sodroski JG, Haseltine WA.** 1990. Identification of HIV-1 vpr product and function. J Acquir Immune Defic Syndr **3:**11-18.

45. **Kogan M, Rappaport J.** 2011. HIV-1 accessory protein Vpr: relevance in the pathogenesis of HIV and potential for therapeutic intervention. Retrovirology **8:**25.

46. **Gabuzda DH, Lawrence K, Langhoff E, Terwilliger E, Dorfman T, Haseltine WA, Sodroski J.** 1992. Role of vif in replication of human immunodeficiency virus type 1 in CD4+ T lymphocytes. J Virol **66:**6489-6495.

47. **Goncalves J, Santa-Marta M.** 2004. HIV-1 Vif and APOBEC3G: multiple roads to one goal. Retrovirology **1:**28.

48. **Reddy K, Ooms M, Letko M, Garrett N, Simon V, Ndung'u T.** 2016. Functional characterization of Vif proteins from HIV-1 infected patients with different APOBEC3G haplotypes. AIDS **30:**1723-1729.

49. **Strebel K, Klimkait T, Martin MA.** 1988. A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science **241:**1221-1223.

50. **Neil SJ, Eastman SW, Jouvenet N, Bieniasz PD.** 2006. HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. PLoS Pathog **2:**e39.

51. **Neil SJ, Zang T, Bieniasz PD.** 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature **451:**425-430.

52. **Robey WG, Safai B, Oroszlan S, Arthur LO, Gonda MA, Gallo RC, Fischinger PJ.** 1985. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. Science **228:**593-595.

53. **Zhu P, Liu J, Bess J, Jr., Chertova E, Lifson JD, Grise H, Ofek GA, Taylor KA, Roux KH.** 2006. Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature **441:**847-852.

54. **Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648-659.

55. **Pan C, Liu S, Jiang S.** 2010. HIV-1 gp41 fusion intermediate: a target for HIV therapeutics. J Formos Med Assoc **109:**94-105.

56. **Sullivan N, Sun Y, Sattentau Q, Thali M, Wu D, Denisova G, Gershoni J, Robinson J, Moore J, Sodroski J.** 1998. CD4-Induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: consequences for virus entry and neutralization. J Virol **72:**4694-4703.

57. **Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955-1958.

58. **Feng Y, Broder CC, Kennedy PE, Berger EA.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872-877.

59. **Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661-666.

60. **van Anken E, Sanders RW, Liscaljet IM, Land A, Bontjer I, Tillemans S, Nabatov AA, Paxton WA, Berkhout B, Braakman I.** 2008. Only five of 10 strictly conserved disulfide bonds are essential for folding and eight for function of the HIV-1 envelope glycoprotein. Molecular biology of the cell **19:**4298-4309.

61. **Lee JH, de Val N, Lyumkis D, Ward AB.** 2015. Model Building and Refinement of a Natively Glycosylated HIV-1 Env Protein by High-Resolution Cryoelectron Microscopy. Structure **23:**1943-1951.

62. **Aiamkitsumrit B, Dampier W, Antell G, Rivera N, Martin-Garcia J, Pirrone V, Nonnemacher MR, Wigdahl B.** 2014. Bioinformatic analysis of HIV-1 entry and pathogenesis. Curr HIV Res **12:**132-161.

63. **Hartley O, Klasse PJ, Sattentau QJ, Moore JP.** 2005. V3: HIV's switch-hitter. AIDS Res Hum Retroviruses **21:**171-189.

64. **Huang CC, Tang M, Zhang MY, Majeed S, Montabana E, Stanfield RL, Dimitrov DS, Korber B, Sodroski J, Wilson IA, Wyatt R, Kwong PD.** 2005. Structure of a V3-containing HIV-1 gp120 core. Science **310:**1025-1028.

65. **De Jong JJ, De Ronde A, Keulen W, Tersmette M, Goudsmit J.** 1992. Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support

the syncytium-inducing phenotype: analysis by single amino acid substitution. J Virol **66:**6777-6780.

66. **Hwang SS, Boyle TJ, Lyerly HK, Cullen BR.** 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science **253:**71-74.

67. **Ivanoff LA, Dubay JW, Morris JF, Roberts SJ, Gutshall L, Sternberg EJ, Hunter E, Matthews TJ, Petteway SR, Jr.** 1992. V3 loop region of the HIV-1 gp120 envelope protein is essential for virus infectivity. Virology **187:**423-432.

68. **Cho MW, Lee MK, Carney MC, Berson JF, Doms RW, Martin MA.** 1998. Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4. J Virol **72:**2509-2515.

69. **Xiao L, Owen SM, Goldman I, Lal AA, deJong JJ, Goudsmit J, Lal RB.** 1998. CCR5 coreceptor usage of non-syncytium-inducing primary HIV-1 is independent of phylogenetically distinct global HIV-1 isolates: delineation of consensus motif in the V3 domain that predicts CCR-5 usage. Virology **240:**83-92.

70. **Pollakis G, Abebe A, Kliphuis A, Chalaby MI, Bakker M, Mengistu Y, Brouwer M, Goudsmit J, Schuitemaker H, Paxton WA.** 2004. Phenotypic and genotypic comparisons of CCR5- and CXCR4-tropic human immunodeficiency virus type 1 biological clones isolated from subtype C-infected individuals. J Virol **78:**2841-2852.

71. **Coetzer M, Cilliers T, Ping LH, Swanstrom R, Morris L.** 2006. Genetic characteristics of the V3 region associated with CXCR4 usage in HIV-1 subtype C isolates. Virology **356:**95-105.

72. **Morris L, Cilliers T, Bredell H, Phoswa M, Martin DJ.** 2001. CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. AIDS Res Hum Retroviruses **17:**697-701.

73. **Raymond S, Delobel P, Mavigner M, Ferradini L, Cazabat M, Souyris C, Sandres-Saune K, Pasquier C, Marchou B, Massip P, Izopet J.** 2010. Prediction of HIV type 1 subtype C tropism by genotypic algorithms built from subtype B viruses. J Acquir Immune Defic Syndr **53:**167-175.

74. **Lin NH, Becerril C, Giguel F, Novitsky V, Moyo S, Makhema J, Essex M, Lockman S, Kuritzkes DR, Sagar M.** 2012. Env sequence determinants in CXCR4-using human immunodeficiency virus type-1 subtype C. Virology **433:**296-307.

75. **Cashin K, Gray LR, Jakobsen MR, Sterjovski J, Churchill MJ, Gorry PR.** 2013. CoRSeqV3-C: a novel HIV-1 subtype C specific V3 sequence based coreceptor usage prediction algorithm. Retrovirology **10:**24.

76. **Zhang H, Tully DC, Zhang T, Moriyama H, Thompson J, Wood C.** 2010. Molecular determinants of HIV-1 subtype C coreceptor transition from R5 to R5X4. Virology **407:**68-79.

77. **Singh A, Page T, Moore PL, Allgaier RL, Hiramen K, Coovadia HM, Walker BD, Morris L, Ndung'u T.** 2009. Functional and genetic analysis of coreceptor usage by dualtropic HIV-1 subtype C isolates. Virology **393:**56-67.

78. **Hoffman NG, Seillier-Moiseiwitsch F, Ahn J, Walker JM, Swanstrom R.** 2002. Variability in the human immunodeficiency virus type 1 gp120 Env protein linked to phenotype-associated changes in the V3 loop. J Virol **76:**3852-3864.

79. **Labrosse B, Treboute C, Brelot A, Alizon M.** 2001. Cooperation of the V1/V2 and V3 domains of human immunodeficiency virus type 1 gp120 for interaction with the CXCR4 receptor. J Virol **75:**5457-5464.

80. **Thielen A, Sichtig N, Kaiser R, Lam J, Harrigan PR, Lengauer T.** 2010. Improved prediction of HIV-1 coreceptor usage with sequence information from the second hypervariable loop of gp120. J Infect Dis **202:**1435-1443.

81. **Huang W, Eshleman SH, Toma J, Fransen S, Stawiski E, Paxinos EE, Whitcomb JM, Young AM, Donnell D, Mmiro F, Musoke P, Guay LA, Jackson JB, Parkin NT, Petropoulos CJ.** 2007. Coreceptor tropism in human immunodeficiency virus type 1 subtype D: high prevalence of CXCR4 tropism and heterogeneous composition of viral populations. J Virol **81:**7885-7893.

82. **Huang W, Toma J, Fransen S, Stawiski E, Reeves JD, Whitcomb JM, Parkin N, Petropoulos CJ.** 2008. Coreceptor tropism can be influenced by amino acid substitutions in the gp41 transmembrane subunit of human immunodeficiency virus type 1 envelope protein. J Virol **82:**5584-5593.

83. **Karageorgos L, Li P, Burrell C.** 1993. Characterization of HIV replication complexes early after cell-to-cell infection. AIDS Res Hum Retroviruses **9:**817-823.

84. **Gotte M, Li X, Wainberg MA.** 1999. HIV-1 reverse transcription: a brief overview focused on structure-function relationships among molecules involved in initiation of the reaction. Archives of biochemistry and biophysics **365:**199-210.

85. **Miller MD, Farnet CM, Bushman FD.** 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J Virol **71:**5382-5390.

86. **Farnet CM, Haseltine WA.** 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. J Virol **65:**1910-1915.

87. **Farnet CM, Bushman FD.** 1997. HIV-1 cDNA integration: requirement of HMG I(Y) protein for function of preintegration complexes in vitro. Cell **88:**483-492.

88. **Kao SY, Calman AF, Luciw PA, Peterlin BM.** 1987. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature **330:**489-493.

89. **Saltarelli MJ, Hadziyannis E, Hart CE, Harrison JV, Felber BK, Spira TJ, Pavlakis GN.** 1996. Analysis of human immunodeficiency virus type 1 mRNA splicing patterns during disease progression in peripheral blood mononuclear cells from infected individuals. AIDS Res Hum Retroviruses **12:**1443-1456.

90. **Wilk T, Gross I, Gowen BE, Rutten T, de Haas F, Welker R, Krausslich HG, Boulanger P, Fuller SD.** 2001. Organization of immature human immunodeficiency virus type 1. J Virol **75:**759-771.

91. **Zimmerman C, Klein KC, Kiser PK, Singh AR, Firestein BL, Riba SC, Lingappa JR.** 2002. Identification of a host protein essential for assembly of immature HIV-1 capsids. Nature **415:**88-92.

92. **Martin-Serrano J, Bieniasz PD.** 2003. A bipartite late-budding domain in human immunodeficiency virus type 1. J Virol **77:**12373-12377.

93. **Liao Z, Cimakasky LM, Hampton R, Nguyen DH, Hildreth JE.** 2001. Lipid rafts and HIV pathogenesis: host membrane cholesterol is required for infection by HIV type 1. AIDS Res Hum Retroviruses **17:**1009-1019.

94. **Klimkait T, Strebel K, Hoggan MD, Martin MA, Orenstein JM.** 1990. The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. J Virol **64:**621-629.

95. **Fais S, Capobianchi MR, Abbate I, Castilletti C, Gentile M, Cordiali Fei P, Ameglio F, Dianzani F.** 1995. Unidirectional budding of HIV-1 at the site of cell-to-cell contact is associated with co-polarization of intercellular adhesion molecules and HIV-1 viral matrix protein. AIDS **9:**329-335.

96. **Del Portillo A, Tripodi J, Najfeld V, Wodarz D, Levy DN, Chen BK.** 2011. Multiploid inheritance of HIV-1 during cell-to-cell infection. J Virol **85:**7169-7176.

97. **De Clercq E.** 2009. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. Int J Antimicrob Agents **33:**307-320.

98. **Kilby JM, Hopkins S, Venetta TM, DiMassimo B, Cloud GA, Lee JY, Alldredge L, Hunter E, Lambert D, Bolognesi D, Matthews T, Johnson MR, Nowak MA, Shaw GM, Saag MS.** 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nat Med **4:**1302-1307.

99. **Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, Macartney M, Mori J, Rickett G, Smith-Burchnell C, Napier C, Webster R, Armour D, Price D, Stammen B, Wood A, Perros M.** 2005. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor

CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob Agents Chemother **49:**4721-4732.

100. **Hammer SM, Katzenstein DA, Hughes MD, Gundacker H, Schooley RT, Haubrich RH, Henry WK, Lederman MM, Phair JP, Niu M, Hirsch MS, Merigan TC.** 1996. A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. AIDS Clinical Trials Group Study 175 Study Team. N Engl J Med **335:**1081-1090.

101. **Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, Eron JJ, Jr., Feinberg JE, Balfour HH, Jr., Deyton LR, Chodakewitz JA, Fischl MA.** 1997. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. N Engl J Med **337:**725-733.

102. **Larder BA, Kemp SD.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246:**1155-1158.

103. **Calza L, Danese I, Magistrelli E, Colangeli V, Manfredi R, Bon I, Re MC, Conti M, Viale P.** 2016. Dual Raltegravir-Darunavir/Ritonavir Combination in Virologically Suppressed HIV-1-Infected Patients on Antiretroviral Therapy Including a Ritonavir-Boosted Protease Inhibitor Plus Two Nucleoside/Nucleotide Reverse Transcriptase Inhibitors. HIV Clin Trials **17:**38-47.

104. **Hammer SM, Saag MS, Schechter M, Montaner JS, Schooley RT, Jacobsen DM, Thompson MA, Carpenter CC, Fischl MA, Gazzard BG, Gatell JM,**

Hirsch MS, Katzenstein DA, Richman DD, Vella S, Yeni PG, Volberding PA, International AS-USAP. 2006. Treatment for adult HIV infection: 2006 recommendations of the International AIDS Society--USA panel. Topics in HIV medicine : a publication of the International AIDS Society, USA **14:**827-843.

105. **Thompson MA, Aberg JA, Hoy JF, Telenti A, Benson C, Cahn P, Eron JJ, Gunthard HF, Hammer SM, Reiss P, Richman DD, Rizzardini G, Thomas DL, Jacobsen DM, Volberding PA.** 2012. Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel. JAMA **308:**387-402.

106. **Group ISS, Lundgren JD, Babiker AG, Gordin F, Emery S, Grund B, Sharma S, Avihingsanon A, Cooper DA, Fatkenheuer G, Llibre JM, Molina JM, Munderi P, Schechter M, Wood R, Klingman KL, Collins S, Lane HC, Phillips AN, Neaton JD.** 2015. Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. N Engl J Med **373:**795-807.

107. **Laskey SB, Siliciano RF.** 2014. A mechanistic theory to explain the efficacy of antiretroviral therapy. Nature reviews. Microbiology **12:**772-780.

108. **Coffin J, Swanstrom R.** 2013. HIV pathogenesis: dynamics and genetics of viral populations and infected cells. Cold Spring Harbor perspectives in medicine **3:**a012526.

109. **Centers for Disease C, Prevention** 2016., posting date. Opportunistic infections. [Online.]

110. **Bicanic T, Harrison TS.** 2004. Cryptococcal meningitis. Br Med Bull **72:**99-118.

111. **Kwon-Chung KJ, Bennett JE.** 1984. Epidemiologic differences between the two varieties of Cryptococcus neoformans. American journal of epidemiology **120:**123-130.

112. **McCarthy KM, Morgan J, Wannemuehler KA, Mirza SA, Gould SM, Mhlongo N, Moeng P, Maloba BR, Crewe-Brown HH, Brandt ME, Hajjeh RA.** 2006. Population-based surveillance for cryptococcosis in an antiretroviral-naive South African province with a high HIV seroprevalence. AIDS **20:**2199-2206.

113. **Chang CC, Dorasamy AA, Gosnell BI, Elliott JH, Spelman T, Omarjee S, Naranbhai V, Coovadia Y, Ndung'u T, Moosa MY, Lewin SR, French MA.** 2013. Clinical and mycological predictors of cryptococcosis-associated immune reconstitution inflammatory syndrome. AIDS **27:**2089-2099.

114. **Mwaba P, Mwansa J, Chintu C, Pobee J, Scarborough M, Portsmouth S, Zumla A.** 2001. Clinical presentation, natural history, and cumulative death rates of 230 adults with primary cryptococcal meningitis in Zambian AIDS patients treated under local conditions. Postgrad Med J **77:**769-773.

115. **Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM.** 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23:**525-530.

116. **Cilliers T, Nhlapo J, Coetzer M, Orlovic D, Ketas T, Olson WC, Moore JP, Trkola A, Morris L.** 2003. The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C. J Virol **77:**4449-4456.

117. **Idnurm A, Bahn YS, Nielsen K, Lin X, Fraser JA, Heitman J.** 2005. Deciphering the model pathogenic fungus Cryptococcus neoformans. Nature reviews. Microbiology **3:**753-764.

118. **Kwon-Chung KJ, Fraser JA, Doering TL, Wang Z, Janbon G, Idnurm A, Bahn YS.** 2014. Cryptococcus neoformans and Cryptococcus gattii, the etiologic agents of cryptococcosis. Cold Spring Harbor perspectives in medicine **4:**a019760.

119. **McClelland CM, Chang YC, Varma A, Kwon-Chung KJ.** 2004. Uniqueness of the mating system in Cryptococcus neoformans. Trends Microbiol **12:**208-212.

120. **Yan Z, Hull CM, Heitman J, Sun S, Xu J.** 2004. SXI1alpha controls uniparental mitochondrial inheritance in Cryptococcus neoformans. Curr Biol **14:**R743-744.

121. **Xu J, Ali RY, Gregory DA, Amick D, Lambert SE, Yoell HJ, Vilgalys RJ, Mitchell TG.** 2000. Uniparental mitochondrial transmission in sexual crosses in Cryptococcus neoformans. Current microbiology **40:**269-273.

122. **Chen SH, Stins MF, Huang SH, Chen YH, Kwon-Chung KJ, Chang Y, Kim KS, Suzuki K, Jong AY.** 2003. Cryptococcus neoformans induces alterations in the cytoskeleton of human brain microvascular endothelial cells. Journal of medical microbiology **52:**961-970.

123. **Alvarez M, Casadevall A.** 2006. Phagosome extrusion and host-cell survival after Cryptococcus neoformans phagocytosis by macrophages. Curr Biol **16:**2161-2165.

124. **Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, Casazza JP, Kuruppu J, Kunstman K, Wolinsky S, Grossman Z, Dybul M,**

Oxenius A, Price DA, Connors M, Koup RA. 2002. HIV preferentially infects HIV-specific CD4+ T cells. Nature **417:**95-98.

125. **Keys B, Albert J, Kovamees J, Chiodi F.** 1991. Brain-derived cells can be infected with HIV isolates derived from both blood and brain. Virology **183:**834-839.

126. **Roy S, Fitz-Gibbon L, Poulin L, Wainberg MA.** 1988. Infection of human monocytes/macrophages by HIV-1: effect on secretion of IL-1 activity. Immunology **64:**233-239.

127. **Valentin A, Albert J, Fenyo EM, Asjo B.** 1990. HIV-1 infection of normal human macrophage cultures: implication for silent infection. Virology **177:**790-794.

128. **Smed-Sorensen A, Lore K, Walther-Jallow L, Andersson J, Spetz AL.** 2004. HIV-1-infected dendritic cells up-regulate cell surface markers but fail to produce IL-12 p70 in response to CD40 ligand stimulation. Blood **104:**2810-2817.

129. **Lenardo MJ, Angleman SB, Bounkeua V, Dimas J, Duvall MG, Graubard MB, Hornung F, Selkirk MC, Speirs CK, Trageser C, Orenstein JO, Bolton DL.** 2002. Cytopathic killing of peripheral blood CD4(+) T lymphocytes by human immunodeficiency virus type 1 appears necrotic rather than apoptotic and does not require env. J Virol **76:**5082-5093.

130. **Bolton DL, Hahn BI, Park EA, Lehnhoff LL, Hornung F, Lenardo MJ.** 2002. Death of CD4(+) T-cell lines caused by human immunodeficiency virus type 1 does not depend on caspases or apoptosis. J Virol **76:**5094-5107.

131. **Bolton DL, Lenardo MJ.** 2007. Vpr cytopathicity independent of G2/M cell cycle arrest in human immunodeficiency virus type 1-infected CD4+ T cells. J Virol **81:**8878-8890.

132. **Doitsh G, Galloway NL, Geng X, Yang Z, Monroe KM, Zepeda O, Hunt PW, Hatano H, Sowinski S, Munoz-Arias I, Greene WC.** 2014. Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. Nature **505:**509-514.

133. **Holm GH, Gabuzda D.** 2005. Distinct mechanisms of CD4+ and CD8+ T-cell activation and bystander apoptosis induced by human immunodeficiency virus type 1 virions. J Virol **79:**6299-6311.

134. **Smith BA, Gartner S, Liu Y, Perelson AS, Stilianakis NI, Keele BF, Kerkering TM, Ferreira-Gonzalez A, Szakal AK, Tew JG, Burton GF.** 2001. Persistence of infectious HIV on follicular dendritic cells. J Immunol **166:**690-696.

135. **Ramratnam B, Bonhoeffer S, Binley J, Hurley A, Zhang L, Mittler JE, Markowitz M, Moore JP, Perelson AS, Ho DD.** 1999. Rapid production and clearance of HIV-1 and hepatitis C virus assessed by large volume plasma apheresis. Lancet **354:**1782-1785.

136. **Asjo B, Morfeldt-Manson L, Albert J, Biberfeld G, Karlsson A, Lidman K, Fenyo EM.** 1986. Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. Lancet **2:**660-662.

137. **Fenyo EM, Morfeldt-Manson L, Chiodi F, Lind B, von Gegerfelt A, Albert J, Olausson E, Asjo B.** 1988. Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates. J Virol **62:**4414-4419.

138. **Tersmette M, de Goede RE, Al BJ, Winkel IN, Gruters RA, Cuypers HT, Huisman HG, Miedema F.** 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. J Virol **62:**2026-2032.

139. **Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:**667-673.

140. **Bjorndal A, Deng H, Jansson M, Fiore JR, Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman DR, Fenyo EM.** 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J Virol **71:**7478-7487.

141. **Berger EA, Doms RW, Fenyo EM, Korber BT, Littman DR, Moore JP, Sattentau QJ, Schuitemaker H, Sodroski J, Weiss RA.** 1998. A new classification for HIV-1. Nature **391:**240.

142. **Edinger AL, Hoffman TL, Sharron M, Lee B, Yi Y, Choe W, Kolson DL, Mitrovic B, Zhou Y, Faulds D, Collman RG, Hesselgesser J, Horuk R, Doms RW.** 1998. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. J Virol **72:**7934-7940.

143. **Combadiere C, Salzwedel K, Smith ED, Tiffany HL, Berger EA, Murphy PM.** 1998. Identification of CX3CR1. A chemotactic receptor for the human

CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. J Biol Chem **273:**23799-23804.

144. **Sharron M, Pohlmann S, Price K, Lolis E, Tsang M, Kirchhoff F, Doms RW, Lee B.** 2000. Expression and coreceptor activity of STRL33/Bonzo on primary peripheral blood lymphocytes. Blood **96:**41-49.

145. **Neil SJ, Aasa-Chapman MM, Clapham PR, Nibbs RJ, McKnight A, Weiss RA.** 2005. The promiscuous CC chemokine receptor D6 is a functional coreceptor for primary isolates of human immunodeficiency virus type 1 (HIV-1) and HIV-2 on astrocytes. J Virol **79:**9618-9624.

146. **Nedellec R, Coetzer M, Shimizu N, Hoshino H, Polonis VR, Morris L, Martensson UE, Binley J, Overbaugh J, Mosier DE.** 2009. Virus entry via the alternative coreceptors CCR3 and FPRL1 differs by human immunodeficiency virus type 1 subtype. J Virol **83:**8353-8363.

147. **Isaacman-Beck J, Hermann EA, Yi Y, Ratcliffe SJ, Mulenga J, Allen S, Hunter E, Derdeyn CA, Collman RG.** 2009. Heterosexual transmission of human immunodeficiency virus type 1 subtype C: Macrophage tropism, alternative coreceptor use, and the molecular anatomy of CCR5 utilization. J Virol **83:**8208-8220.

148. **Coetzer M, Nedellec R, Cilliers T, Meyers T, Morris L, Mosier DE.** 2010. Extreme genetic divergence is required for coreceptor switching in HIV-1 subtype C. J Acquir Immune Defic Syndr **56:**9-15.

149. **Calado M, Matoso P, Santos-Costa Q, Espirito-Santo M, Machado J, Rosado L, Antunes F, Mansinho K, Lopes MM, Maltez F, Santos-Ferreira MO, Azevedo-Pereira JM.** 2010. Coreceptor usage by HIV-1 and HIV-2 primary

isolates: the relevance of CCR8 chemokine receptor as an alternative coreceptor. Virology **408:**174-182.

150. **Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135-1148.

151. **Jiang C, Parrish NF, Wilen CB, Li H, Chen Y, Pavlicek JW, Berg A, Lu X, Song H, Tilton JC, Pfaff JM, Henning EA, Decker JM, Moody MA, Drinker MS, Schutte R, Freel S, Tomaras GD, Nedellec R, Mosier DE, Haynes BF, Shaw GM, Hahn BH, Doms RW, Gao F.** 2011. Primary infection by a human immunodeficiency virus with atypical coreceptor tropism. J Virol **85:**10669-10681.

152. **He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, Gabuzda D.** 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature **385:**645-649.

153. **Shieh JT, Albright AV, Sharron M, Gartner S, Strizki J, Doms RW, Gonzalez-Scarano F.** 1998. Chemokine receptor utilization by human immunodeficiency virus type 1 isolates that replicate in microglia. J Virol **72:**4243-4249.

154. **Gabuzda D, He J, Ohagen A, Vallat AV.** 1998. Chemokine receptors in HIV-1 infection of the central nervous system. Seminars in immunology **10:**203-213.

155. **Agrawal L, Maxwell CR, Peters PJ, Clapham PR, Liu SM, Mackay CR, Strayer DS.** 2009. Complexity in human immunodeficiency virus type 1 (HIV-

1) co-receptor usage: roles of CCR3 and CCR5 in HIV-1 infection of monocyte-derived macrophages and brain microglia. J Gen Virol **90:**710-722.

156. **Ghorpade A, Xia MQ, Hyman BT, Persidsky Y, Nukuna A, Bock P, Che M, Limoges J, Gendelman HE, Mackay CR.** 1998. Role of the beta-chemokine receptors CCR3 and CCR5 in human immunodeficiency virus type 1 infection of monocytes and microglia. J Virol **72:**3351-3361.

157. **Gharu L, Ringe R, Bhattacharya J.** 2012. Evidence of extended alternate coreceptor usage by HIV-1 clade C envelope obtained from an Indian patient. Virus Res **163:**410-414.

158. **Cilliers T, Willey S, Sullivan WM, Patience T, Pugach P, Coetzer M, Papathanasopoulos M, Moore JP, Trkola A, Clapham P, Morris L.** 2005. Use of alternate coreceptors on primary cells by two HIV-1 isolates. Virology **339:**136-144.

159. **Cashin K, Jakobsen MR, Sterjovski J, Roche M, Ellett A, Flynn JK, Borm K, Gouillou M, Churchill MJ, Gorry PR.** 2013. Linkages between HIV-1 specificity for CCR5 or CXCR4 and in vitro usage of alternative coreceptors during progressive HIV-1 subtype C infection. Retrovirology **10:**98.

160. **Karlsson U, Antonsson L, Ljungberg B, Medstrand P, Esbjornsson J, Jansson M, Gisslen M.** 2012. Dual R3R5 tropism characterizes cerebrospinal fluid HIV-1 isolates from individuals with high cerebrospinal fluid viral load. AIDS **26:**1739-1744.

161. **Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ,**

Collman RG, Doms RW, Vassart G, Parmentier M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature **382:**722-725.

162. **Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, He T, Kang S, Ceradini D, Jin Z, Yazdanbakhsh K, Kunstman K, Erickson D, Dragon E, Landau NR, Phair J, Ho DD, Koup RA.** 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med **2:**1240-1243.

163. **Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR.** 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell **86:**367-377.

164. **de Roda Husman AM, Koot M, Cornelissen M, Keet IP, Brouwer M, Broersen SM, Bakker M, Roos MT, Prins M, de Wolf F, Coutinho RA, Miedema F, Goudsmit J, Schuitemaker H.** 1997. Association between CCR5 genotype and the clinical course of HIV-1 infection. Annals of internal medicine **127:**882-890.

165. **Scarlatti G, Tresoldi E, Bjorndal A, Fredriksson R, Colognesi C, Deng HK, Malnati MS, Plebani A, Siccardi AG, Littman DR, Fenyo EM, Lusso P.** 1997. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat Med **3:**1259-1265.

166. **Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR.** 1997. Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med **185:**621-628.

167. **Koot M, Vos AH, Keet RP, de Goede RE, Dercksen MW, Terpstra FG, Coutinho RA, Miedema F, Tersmette M.** 1992. HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. AIDS **6:**49-54.

168. **Koot M, Keet IP, Vos AH, de Goede RE, Roos MT, Coutinho RA, Miedema F, Schellekens PT, Tersmette M.** 1993. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Annals of internal medicine **118:**681-688.

169. **Cecilia D, Kulkarni SS, Tripathy SP, Gangakhedkar RR, Paranjape RS, Gadkari DA.** 2000. Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India. Virology **271:**253-258.

170. **Abebe A, Demissie D, Goudsmit J, Brouwer M, Kuiken CL, Pollakis G, Schuitemaker H, Fontanet AL, Rinke de Wit TF.** 1999. HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS. AIDS **13:**1305-1311.

171. **Jakobsen MR, Cashin K, Roche M, Sterjovski J, Ellett A, Borm K, Flynn J, Erikstrup C, Gouillou M, Gray LR, Saksena NK, Wang B, Purcell DF, Kallestrup P, Zinyama-Gutsire R, Gomo E, Ullum H, Ostergaard L, Lee B, Ramsland PA, Churchill MJ, Gorry PR.** 2013. Longitudinal Analysis of CCR5 and CXCR4 Usage in a Cohort of Antiretroviral Therapy-Naive Subjects with Progressive HIV-1 Subtype C Infection. PLoS One **8:**e65950.

172. **Lin NH, Smeaton LM, Giguel F, Novitsky V, Moyo S, Mitchell RM, Makhema J, Essex M, Lockman S, Kuritzkes DR.** 2011. Prevalence and

clinical associations of CXCR4-using HIV-1 among treatment-naive subtype C-infected women in Botswana. J Acquir Immune Defic Syndr **57:**46-50.

173. **Connell BJ, Michler K, Capovilla A, Venter WD, Stevens WS, Papathanasopoulos MA.** 2008. Emergence of X4 usage among HIV-1 subtype C: evidence for an evolving epidemic in South Africa. AIDS **22:**896-899.

174. **Brenchley JM, Hill BJ, Ambrozak DR, Price DA, Guenaga FJ, Casazza JP, Kuruppu J, Yazdani J, Migueles SA, Connors M, Roederer M, Douek DC, Koup RA.** 2004. T-cell subsets that harbor human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. J Virol **78:**1160-1168.

175. **Gartner S, Markovits P, Markovitz DM, Betts RF, Popovic M.** 1986. Virus isolation from and identification of HTLV-III/LAV-producing cells in brain tissue from a patient with AIDS. JAMA **256:**2365-2371.

176. **Gartner S, Markovits P, Markovitz DM, Kaplan MH, Gallo RC, Popovic M.** 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233:**215-219.

177. **Meltzer MS, Nakamura M, Hansen BD, Turpin JA, Kalter DC, Gendelman HE.** 1990. Macrophages as susceptible targets for HIV infection, persistent viral reservoirs in tissue, and key immunoregulatory cells that control levels of virus replication and extent of disease. AIDS Res Hum Retroviruses **6:**967-971.

178. **Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH,**

Shaw GM. 2009. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J Exp Med **206:**1273-1289.

179. **Ochsenbauer C, Edmonds TG, Ding H, Keele BF, Decker J, Salazar MG, Salazar-Gonzalez JF, Shattock R, Haynes BF, Shaw GM, Hahn BH, Kappes JC.** 2012. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. J Virol **86:**2715-2728.

180. **Arrildt KT, Joseph SB, Swanstrom R.** 2012. The HIV-1 env protein: a coat of many colors. Curr HIV/AIDS Rep **9:**52-63.

181. **Dunfee RL, Thomas ER, Gorry PR, Wang J, Taylor J, Kunstman K, Wolinsky SM, Gabuzda D.** 2006. The HIV Env variant N283 enhances macrophage tropism and is associated with brain infection and dementia. Proc Natl Acad Sci U S A **103:**15160-15165.

182. **Dunfee RL, Thomas ER, Wang J, Kunstman K, Wolinsky SM, Gabuzda D.** 2007. Loss of the N-linked glycosylation site at position 386 in the HIV envelope V4 region enhances macrophage tropism and is associated with dementia. Virology **367:**222-234.

183. **Thomas ER, Dunfee RL, Stanton J, Bogdan D, Taylor J, Kunstman K, Bell JE, Wolinsky SM, Gabuzda D.** 2007. Macrophage entry mediated by HIV Envs from brain and lymphoid tissues is determined by the capacity to use low CD4 levels and overall efficiency of fusion. Virology **360:**105-119.

184. **Sterjovski J, Churchill MJ, Ellett A, Gray LR, Roche MJ, Dunfee RL, Purcell DF, Saksena N, Wang B, Sonza S, Wesselingh SL, Karlsson I, Fenyo EM,**

Gabuzda D, Cunningham AL, Gorry PR. 2007. Asn 362 in gp120 contributes to enhanced fusogenicity by CCR5-restricted HIV-1 envelope glycoprotein variants from patients with AIDS. Retrovirology **4:**89.

185. **Rossi F, Querido B, Nimmagadda M, Cocklin S, Navas-Martin S, Martin-Garcia J.** 2008. The V1-V3 region of a brain-derived HIV-1 envelope glycoprotein determines macrophage tropism, low CD4 dependence, increased fusogenicity and altered sensitivity to entry inhibitors. Retrovirology **5:**89.

186. **Cashin K, Roche M, Sterjovski J, Ellett A, Gray LR, Cunningham AL, Ramsland PA, Churchill MJ, Gorry PR.** 2011. Alternative coreceptor requirements for efficient CCR5- and CXCR4-mediated HIV-1 entry into macrophages. J Virol **85:**10699-10709.

187. **Dunfee RL, Thomas ER, Gabuzda D.** 2009. Enhanced macrophage tropism of HIV in brain and lymphoid tissues is associated with sensitivity to the broadly neutralizing CD4 binding site antibody b12. Retrovirology **6:**69.

188. **Duenas-Decamp MJ, Peters PJ, Burton D, Clapham PR.** 2009. Determinants flanking the CD4 binding loop modulate macrophage tropism of human immunodeficiency virus type 1 R5 envelopes. J Virol **83:**2575-2583.

189. **Martin-Garcia J, Cao W, Varela-Rohena A, Plassmeyer ML, Gonzalez-Scarano F.** 2006. HIV-1 tropism for the central nervous system: Brain-derived envelope glycoproteins with lower CD4 dependence and reduced sensitivity to a fusion inhibitor. Virology **346:**169-179.

190. **Peters PJ, Sullivan WM, Duenas-Decamp MJ, Bhattacharya J, Ankghuambom C, Brown R, Luzuriaga K, Bell J, Simmonds P, Ball J, Clapham PR.** 2006. Non-macrophage-tropic human immunodeficiency virus

type 1 R5 envelopes predominate in blood, lymph nodes, and semen: implications for transmission and pathogenesis. J Virol **80:**6324-6332.

191. **Li S, Juarez J, Alali M, Dwyer D, Collman R, Cunningham A, Naif HM.** 1999. Persistent CCR5 utilization and enhanced macrophage tropism by primary blood human immunodeficiency virus type 1 isolates from advanced stages of disease and comparison to tissue-derived isolates. J Virol **73:**9741-9755.

192. **Gorry PR, Bristol G, Zack JA, Ritola K, Swanstrom R, Birch CJ, Bell JE, Bannert N, Crawford K, Wang H, Schols D, De Clercq E, Kunstman K, Wolinsky SM, Gabuzda D.** 2001. Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. J Virol **75:**10073-10089.

193. **Peters PJ, Bhattacharya J, Hibbitts S, Dittmar MT, Simmons G, Bell J, Simmonds P, Clapham PR.** 2004. Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplified from brain and lymph node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. J Virol **78:**6915-6926.

194. **Schnell G, Joseph S, Spudich S, Price RW, Swanstrom R.** 2011. HIV-1 replication in the central nervous system occurs in two distinct cell types. PLoS Pathog **7:**e1002286.

195. **Price RW, Brew B, Sidtis J, Rosenblum M, Scheck AC, Cleary P.** 1988. The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. Science **239:**586-592.

196. **Albright AV, Shieh JT, Itoh T, Lee B, Pleasure D, O'Connor MJ, Doms RW, Gonzalez-Scarano F.** 1999. Microglia express CCR5, CXCR4, and CCR3, but of these, CCR5 is the principal coreceptor for human immunodeficiency virus type 1 dementia isolates. J Virol **73:**205-213.

197. **Smit TK, Wang B, Ng T, Osborne R, Brew B, Saksena NK.** 2001. Varied tropism of HIV-1 isolates derived from different regions of adult brain cortex discriminate between patients with and without AIDS dementia complex (ADC): evidence for neurotropic HIV variants. Virology **279:**509-526.

198. **Hibbitts S, Reeves JD, Simmons G, Gray PW, Epstein LG, Schols D, de Clercq E, Wells TN, Proudfoot AE, Clapham PR.** 1999. Coreceptor ligand inhibition of fetal brain cell infection by HIV type 1. AIDS Res Hum Retroviruses **15:**989-1000.

199. **Simmons G, Reeves JD, McKnight A, Dejucq N, Hibbitts S, Power CA, Aarons E, Schols D, De Clercq E, Proudfoot AE, Clapham PR.** 1998. CXCR4 as a functional coreceptor for human immunodeficiency virus type 1 infection of primary macrophages. J Virol **72:**8453-8457.

200. **Joseph SB, Arrildt KT, Sturdevant CB, Swanstrom R.** 2015. HIV-1 target cells in the CNS. J Neurovirol **21:**276-289.

201. **Arrildt KT, LaBranche CC, Joseph SB, Dukhovlinova EN, Graham WD, Ping LH, Schnell G, Sturdevant CB, Kincer LP, Mallewa M, Heyderman RS, Rie AV, Cohen MS, Spudich S, Price RW, Montefiori DC, Swanstrom R.** 2015. Phenotypic Correlates of HIV-1 Macrophage Tropism. J Virol **89:**11294-11311.

202. **Johnston SH, Lobritz MA, Nguyen S, Lassen K, Delair S, Posta F, Bryson YJ, Arts EJ, Chou T, Lee B.** 2009. A quantitative affinity-profiling system that reveals distinct CD4/CCR5 usage patterns among human immunodeficiency virus type 1 and simian immunodeficiency virus strains. J Virol **83:**11016-11026.

203. **Joseph SB, Arrildt KT, Swanstrom AE, Schnell G, Lee B, Hoxie JA, Swanstrom R.** 2014. Quantification of entry phenotypes of macrophage-tropic HIV-1 across a wide range of CD4 densities. J Virol **88:**1858-1869.

204. **Ballabh P, Braun A, Nedergaard M.** 2004. The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiology of disease **16:**1-13.

205. **Strazza M, Pirrone V, Wigdahl B, Nonnemacher MR.** 2011. Breaking down the barrier: the effects of HIV-1 on the blood-brain barrier. Brain research **1399:**96-115.

206. **Fischer-Smith T, Croul S, Sverstiuk AE, Capini C, L'Heureux D, Regulier EG, Richardson MW, Amini S, Morgello S, Khalili K, Rappaport J.** 2001. CNS invasion by CD14+/CD16+ peripheral blood-derived monocytes in HIV dementia: perivascular accumulation and reservoir of HIV infection. J Neurovirol **7:**528-541.

207. **Fischer-Smith T, Rappaport J.** 2005. Evolving paradigms in the pathogenesis of HIV-1-associated dementia. Expert reviews in molecular medicine **7:**1-26.

208. **Xiong H, Zeng YC, Lewis T, Zheng J, Persidsky Y, Gendelman HE.** 2000. HIV-1 infected mononuclear phagocyte secretory products affect neuronal physiology leading to cellular demise: relevance for HIV-1-associated dementia. J Neurovirol **6 Suppl 1:**S14-23.

209. **Persidsky Y, Heilman D, Haorah J, Zelivyanskaya M, Persidsky R, Weber GA, Shimokawa H, Kaibuchi K, Ikezu T.** 2006. Rho-mediated regulation of tight junctions during monocyte migration across the blood-brain barrier in HIV-1 encephalitis (HIVE). Blood **107:**4770-4780.

210. **Nakamuta S, Endo H, Higashi Y, Kousaka A, Yamada H, Yano M, Kido H.** 2008. Human immunodeficiency virus type 1 gp120-mediated disruption of tight junction proteins by induction of proteasome-mediated degradation of zonula occludens-1 and -2 in human brain microvascular endothelial cells. Journal of neurovirology **14:**186-195.

211. **Andras IE, Pu H, Deli MA, Nath A, Hennig B, Toborek M.** 2003. HIV-1 Tat protein alters tight junction protein expression and distribution in cultured brain endothelial cells. J Neurosci Res **74:**255-265.

212. **Moses AV, Nelson JA.** 1994. HIV infection of human brain capillary endothelial cells--implications for AIDS dementia. Adv Neuroimmunol **4:**239-247.

213. **Epstein LG, Sharer LR, Oleske JM, Connor EM, Goudsmit J, Bagdon L, Robert-Guroff M, Koenigsberger MR.** 1986. Neurologic manifestations of human immunodeficiency virus infection in children. Pediatrics **78:**678-687.

214. **Goudsmit J, de Wolf F, Paul DA, Epstein LG, Lange JM, Krone WJ, Speelman H, Wolters EC, Van der Noordaa J, Oleske JM, et al.** 1986. Expression of human immunodeficiency virus antigen (HIV-Ag) in serum and cerebrospinal fluid during acute and chronic infection. Lancet **2:**177-180.

215. **Wiley CA, Achim CL, Christopherson C, Kidane Y, Kwok S, Masliah E, Mellors J, Radhakrishnan L, Wang G, Soontornniyomkij V.** 1999. HIV mediates a productive infection of the brain. AIDS **13:**2055-2059.

216. **Shaw GM, Harper ME, Hahn BH, Epstein LG, Gajdusek DC, Price RW, Navia BA, Petito CK, O'Hara CJ, Groopman JE, et al.** 1985. HTLV-III infection in brains of children and adults with AIDS encephalopathy. Science **227:**177-182.

217. **Reddy RT, Achim CL, Sirko DA, Tehranchi S, Kraus FG, Wong-Staal F, Wiley CA.** 1996. Sequence analysis of the V3 loop in brain and spleen of patients with HIV encephalitis. AIDS Res Hum Retroviruses **12:**477-482.

218. **Torres-Munoz J, Stockton P, Tacoronte N, Roberts B, Maronpot RR, Petito CK.** 2001. Detection of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains by laser capture microdissection. J Neuropathol Exp Neurol **60:**885-892.

219. **Petito CK.** 2004. Human immunodeficiency virus type 1 compartmentalization in the central nervous system. J Neurovirol **10 Suppl 1:**21-24.

220. **Gorry PR, Ong C, Thorpe J, Bannwarth S, Thompson KA, Gatignol A, Vesselingh SL, Purcell DF.** 2003. Astrocyte infection by HIV-1: mechanisms of restricted virus replication, and role in the pathogenesis of HIV-1-associated dementia. Curr HIV Res **1:**463-473.

221. **Jordan CA, Watkins BA, Kufta C, Dubois-Dalcq M.** 1991. Infection of brain microglial cells by human immunodeficiency virus type 1 is CD4 dependent. J Virol **65:**736-742.

222. **Burkala EJ, He J, West JT, Wood C, Petito CK.** 2005. Compartmentalization of HIV-1 in the central nervous system: role of the choroid plexus. AIDS **19:**675-684.

223. **Di Stefano M, Monno L, Fiore JR, Buccoliero G, Appice A, Perulli LM, Pastore G, Angarano G.** 1998. Neurological disorders during HIV-1 infection correlate with viral load in cerebrospinal fluid but not with virus phenotype. AIDS **12:**737-743.

224. **Ritola K, Robertson K, Fiscus SA, Hall C, Swanstrom R.** 2005. Increased human immunodeficiency virus type 1 (HIV-1) env compartmentalization in the presence of HIV-1-associated dementia. J Virol **79:**10830-10834.

225. **Abbate I, Cappiello G, Longo R, Ursitti A, Spano A, Calcaterra S, Dianzani F, Antinori A, Capobianchi MR.** 2005. Cell membrane proteins and quasispecies compartmentalization of CSF and plasma HIV-1 from aids patients with neurological disorders. Infect Genet Evol **5:**247-253.

226. **Harrington PR, Schnell G, Letendre SL, Ritola K, Robertson K, Hall C, Burch CL, Jabara CB, Moore DT, Ellis RJ, Price RW, Swanstrom R.** 2009. Cross-sectional characterization of HIV-1 env compartmentalization in cerebrospinal fluid over the full disease course. AIDS **23:**907-915.

227. **Schnell G, Price RW, Swanstrom R, Spudich S.** 2010. Compartmentalization and clonal amplification of HIV-1 variants in the cerebrospinal fluid during primary infection. J Virol **84:**2395-2407.

228. **Sturdevant CB, Dow A, Jabara CB, Joseph SB, Schnell G, Takamune N, Mallewa M, Heyderman RS, Van Rie A, Swanstrom R.** 2012. Central nervous system compartmentalization of HIV-1 subtype C variants early and late in infection in young children. PLoS Pathog **8:**e1003094.

229. **Evering TH, Kamau E, St Bernard L, Farmer CB, Kong XP, Markowitz M.** 2014. Single genome analysis reveals genetic characteristics of Neuroadaptation across HIV-1 envelope. Retrovirology **11:**65.

230. **Vazquez-Santiago F, Garcia Y, Rivera-Roman I, Noel RJ, Jr., Wojna V, Melendez LM, Rivera-Amill V.** 2015. Longitudinal Analysis of Cerebrospinal Fluid and Plasma HIV-1 Envelope Sequences Isolated From a Single Donor with HIV Asymptomatic Neurocognitive Impairment. Journal of virology & antiviral research **4**.

231. **Sturdevant CB, Joseph SB, Schnell G, Price RW, Swanstrom R, Spudich S.** 2015. Compartmentalized replication of R5 T cell-tropic HIV-1 in the central nervous system early in the course of infection. PLoS Pathog **11:**e1004720.

232. **Peeters MF, Colebunders RL, Van den Abbeele K, Nys PC, Goeman J, Colans P, Geerts Y, Van der Groen G.** 1995. Comparison of human immunodeficiency virus biological phenotypes isolated from cerebrospinal fluid and peripheral blood. J Med Virol **47:**92-96.

233. **Ellis RJ, Hsia K, Spector SA, Nelson JA, Heaton RK, Wallace MR, Abramson I, Atkinson JH, Grant I, McCutchan JA.** 1997. Cerebrospinal fluid human immunodeficiency virus type 1 RNA levels are elevated in neurocognitively impaired individuals with acquired immunodeficiency syndrome. HIV Neurobehavioral Research Center Group. Annals of neurology **42:**679-688.

234. **Churchill M, Sterjovski J, Gray L, Cowley D, Chatfield C, Learmont J, Sullivan JS, Crowe SM, Mills J, Brew BJ, Wesselingh SL, McPhee DA, Gorry PR.** 2004. Longitudinal analysis of nef/long terminal repeat-deleted HIV-1 in

blood and cerebrospinal fluid of a long-term survivor who developed HIV-associated dementia. J Infect Dis **190:**2181-2186.

235. **Pillai SK, Pond SL, Liu Y, Good BM, Strain MC, Ellis RJ, Letendre S, Smith DM, Gunthard HF, Grant I, Marcotte TD, McCutchan JA, Richman DD, Wong JK.** 2006. Genetic attributes of cerebrospinal fluid-derived HIV-1 env. Brain : a journal of neurology **129:**1872-1883.

236. **Korber BT, Kunstman KJ, Patterson BK, Furtado M, McEvilly MM, Levy R, Wolinsky SM.** 1994. Genetic differences between blood- and brain-derived viral sequences from human immunodeficiency virus type 1-infected patients: evidence of conserved elements in the V3 region of the envelope protein of brain-derived sequences. J Virol **68:**7467-7481.

237. **Haggerty S, Stevenson M.** 1991. Predominance of distinct viral genotypes in brain and lymph node compartments of HIV-1-infected individuals. Viral Immunol **4:**123-131.

238. **Arrildt KT, LaBranche CC, Joseph SB, Dukhovlinova EN, Graham WD, Ping LH, Schnell G, Sturdevant CB, Kincer LP, Mallewa M, Heyderman RS, Van Rie A, Cohen MS, Spudich S, Price RW, Montefiori DC, Swanstrom R.** 2015. Phenotypic Correlates of HIV-1 Macrophage Tropism. J Virol **89:**11294-11311.

239. **Schnell G, Spudich S, Harrington P, Price RW, Swanstrom R.** 2009. Compartmentalized human immunodeficiency virus type 1 originates from long-lived cells in some subjects with HIV-1-associated dementia. PLoS Pathog **5:**e1000395.

240. **Cecchini DM, Canizal AM, Rojas H, Arechavala A, Negroni R, Bouzas MB, Benetucci JA.** 2009. Variables that influence HIV-1 cerebrospinal fluid viral load in cryptococcal meningitis: a linear regression analysis. J Int AIDS Soc **12:**8.

241. **Brew BJ, Pemberton L, Cunningham P, Law MG.** 1997. Levels of human immunodeficiency virus type 1 RNA in cerebrospinal fluid correlate with AIDS dementia stage. J Infect Dis **175:**963-966.

242. **Morris L, Silber E, Sonnenberg P, Eintracht S, Nyoka S, Lyons SF, Saffer D, Koornhof H, Martin DJ.** 1998. High human immunodeficiency virus type 1 RNA load in the cerebrospinal fluid from patients with lymphocytic meningitis. J Infect Dis **177:**473-476.

243. **Lortholary O, Dromer F, Mathoulin-Pelissier S, Fitting C, Improvisi L, Cavaillon JM, Dupont B, French Cryptococcosis Study G.** 2001. Immune mediators in cerebrospinal fluid during cryptococcosis are influenced by meningeal involvement and human immunodeficiency virus serostatus. J Infect Dis **183:**294-302.

244. **Naranbhai V, Chang CC, Durgiah R, Omarjee S, Lim A, Moosa MY, Elliot JH, Ndung'u T, Lewin SR, French MA, Carr WH.** 2014. Compartmentalization of innate immune responses in the central nervous system during cryptococcal meningitis/HIV coinfection. AIDS **28:**657-666.

245. **Chang CC, Kangethe R, Omarjee S, Hiramen K, Gosnell B, Sojane K, Moosa MS, Lewin SR, French MA, Ndung'u T.** 2017. Relationship of Human Immunodeficiency Virus Viral Load in Cerebrospinal Fluid and Plasma in Patients Co-infected With Cryptococcal Meningitis. Open forum infectious diseases **4:**ofx032.

246. **Choi JY, Hightower GK, Wong JK, Heaton R, Woods S, Grant I, Marcotte TD, Ellis RJ, Letendre SL, Collier AC, Marra CM, Clifford DB, Gelman BB, McArthur JC, Morgello S, Simpson DM, McCutchan JA, Richman DD, Smith DM, Charter G.** 2012. Genetic features of cerebrospinal fluid-derived subtype B HIV-1 tat. J Neurovirol **18:**81-90.

247. **Eggers C, Muller O, Thordsen I, Schreiber M, Methner A.** 2013. Genetic shift of env V3 loop viral sequences in patients with HIV-associated neurocognitive disorder during antiretroviral therapy. J Neurovirol **19:**523-530.

248. **Ellis RJ, Gamst AC, Capparelli E, Spector SA, Hsia K, Wolfson T, Abramson I, Grant I, McCutchan JA.** 2000. Cerebrospinal fluid HIV RNA originates from both local CNS and systemic sources. Neurology **54:**927-936.

249. **Karlsson U, Antonsson L, Repits J, Medstrand P, Owman C, Kidd-Ljunggren K, Hagberg L, Svennerholm B, Jansson M, Gisslen M, Ljungberg B.** 2009. Mode of coreceptor use by R5 HIV type 1 correlates with disease stage: a study of paired plasma and cerebrospinal fluid isolates. AIDS Res Hum Retroviruses **25:**1297-1305.

250. **Soulie C, Tubiana R, Simon A, Lambert-Niclot S, Malet I, Canestri A, Brunet C, Murphy R, Katlama C, Calvez V, Marcelin AG.** 2009. Presence of HIV-1 R5 viruses in cerebrospinal fluid even in patients harboring R5X4/X4 viruses in plasma. J Acquir Immune Defic Syndr **51:**60-64.

251. **Falcone EL, Adegbulugbe AA, Sheikh V, Imamichi H, Dewar RL, Hammoud DA, Sereti I, Lane HC.** 2013. Cerebrospinal fluid HIV-1 compartmentalization in a patient with AIDS and acute varicella-zoster virus meningomyeloradiculitis. Clin Infect Dis **57:**e135-142.

252. **Ndung'u T, Sepako E, McLane MF, Chand F, Bedi K, Gaseitsiwe S, Doualla-Bell F, Peter T, Thior I, Moyo SM, Gilbert PB, Novitsky VA, Essex M.** 2006. HIV-1 subtype C in vitro growth and coreceptor utilization. Virology **347:**247-260.

253. **Choge I, Cilliers T, Walker P, Taylor N, Phoswa M, Meyers T, Viljoen J, Violari A, Gray G, Moore PL, Papathanosopoulos M, Morris L.** 2006. Genotypic and phenotypic characterization of viral isolates from HIV-1 subtype C-infected children with slow and rapid disease progression. AIDS Res Hum Retroviruses **22:**458-465.

254. **Smith DM, Zarate S, Shao H, Pillai SK, Letendre SL, Wong JK, Richman DD, Frost SD, Ellis RJ, Group H.** 2009. Pleocytosis is associated with disruption of HIV compartmentalization between blood and cerebral spinal fluid viral populations. Virology **385:**204-208.

255. **Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J, Anderson JA, Swanstrom R, Haynes BF, Athreya GS, Korber BT, Sharp PM, Shaw GM, Hahn BH.** 2008. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. J Virol **82:**3952-3970.

256. **Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, He HX, McLaughlin S, Shankarappa R, Margolick JB, Mullins JI.** 2003. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. J Virol **77:**13376-13388.

257. **Lengauer T, Sander O, Sierra S, Thielen A, Kaiser R.** 2007. Bioinformatics prediction of HIV coreceptor usage. Nat Biotechnol **25:**1407-1410.

258. **Cashin K, Gray LR, Harvey KL, Perez-Bercoff D, Lee GQ, Sterjovski J, Roche M, Demarest JF, Drummond F, Harrigan PR, Churchill MJ, Gorry PR.** 2015. Reliable genotypic tropism tests for the major HIV-1 subtypes. Scientific reports **5:**8543.

259. **Gupta S, Neogi U, Srinivasa H, Shet A.** 2015. Performance of genotypic tools for prediction of tropism in HIV-1 subtype C V3 loop sequences. Intervirology **58:**1-5.

260. **Raymond S, Delobel P, Mavigner M, Cazabat M, Encinas S, Souyris C, Bruel P, Sandres-Saune K, Marchou B, Massip P, Izopet J.** 2010. CXCR4-using viruses in plasma and peripheral blood mononuclear cells during primary HIV-1 infection and impact on disease progression. AIDS **24:**2305-2312.

261. **Frange P, Chaix ML, Raymond S, Galimand J, Deveau C, Meyer L, Goujard C, Rouzioux C, Izopet J, French ACOPCSG.** 2010. Low frequency of CXCR4-using viruses in patients at the time of primary non-subtype-B HIV-1 infection. J Clin Microbiol **48:**3487-3491.

262. **Mulinge M, Lemaire M, Servais JY, Rybicki A, Struck D, da Silva ES, Verhofstede C, Lie Y, Seguin-Devaux C, Schmit JC, Bercoff DP.** 2013. HIV-1 tropism determination using a phenotypic Env recombinant viral assay highlights overestimation of CXCR4-usage by genotypic prediction algorithms for CRF01_AE and CRF02_AG [corrected]. PLoS One **8:**e60566.

263. **Seager I, Travers SA, Leeson MD, Crampin AC, French N, Glynn JR, McCormack GP.** 2014. Coreceptor usage, diversity, and divergence in drug-

naive and drug-exposed individuals from Malawi, infected with HIV-1 subtype C for more than 20 years. AIDS Res Hum Retroviruses **30:**975-983.

264. **Ceresola ER, Nozza S, Sampaolo M, Pignataro AR, Saita D, Ferrarese R, Ripa M, Deng W, Mullins JI, Boeri E, Tambussi G, Toniolo A, Lazzarin A, Clementi M, Canducci F.** 2015. Performance of commonly used genotypic assays and comparison with phenotypic assays of HIV-1 coreceptor tropism in acutely HIV-1-infected patients. J Antimicrob Chemother **70:**1391-1395.

265. **Westby M, Lewis M, Whitcomb J, Youle M, Pozniak AL, James IT, Jenkins TM, Perros M, van der Ryst E.** 2006. Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. J Virol **80:**4909-4920.

266. **Saag M, Goodrich J, Fatkenheuer G, Clotet B, Clumeck N, Sullivan J, Westby M, van der Ryst E, Mayer H, Group AS.** 2009. A double-blind, placebo-controlled trial of maraviroc in treatment-experienced patients infected with non-R5 HIV-1. J Infect Dis **199:**1638-1647.

267. **Spudich SS, Huang W, Nilsson AC, Petropoulos CJ, Liegler TJ, Whitcomb JM, Price RW.** 2005. HIV-1 chemokine coreceptor utilization in paired cerebrospinal fluid and plasma samples: a survey of subjects with viremia. J Infect Dis **191:**890-898.

268. **Ndung'u T, Renjifo B, Essex M.** 2001. Construction and analysis of an infectious human Immunodeficiency virus type 1 subtype C molecular clone. J Virol **75:**4964-4972.

269. **Crous S, Shrestha RK, Travers SA.** 2012. Appraising the performance of genotyping tools in the prediction of coreceptor tropism in HIV-1 subtype C viruses. BMC Infect Dis **12:**203.

270. **Ping LH, Nelson JA, Hoffman IF, Schock J, Lamers SL, Goodman M, Vernazza P, Kazembe P, Maida M, Zimba D, Goodenow MM, Eron JJ, Jr., Fiscus SA, Cohen MS, Swanstrom R.** 1999. Characterization of V3 sequence heterogeneity in subtype C human immunodeficiency virus type 1 isolates from Malawi: underrepresentation of X4 variants. J Virol **73:**6271-6281.

Chapter 9: Appendices

The document showing approval to conduct the studies from the Biomedical Research Ethics Committee is shown:

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

27 November 2013

Professor Mahomed- Yunus Moosa **(Supervisor)**
c/o Tarryn Leslie: HPP Ethics Coordinator
Doris Duke Medical Research Institute
Nelson R. Mandela School of Medicine
University of KwaZulu-Natal

Dear Prof Moosa

PROTOCOL: Immunopathogenesis and diagnosis of cryptococcal- associated immune restoration disease in people with HIV. Dr Christina C. Chang, Department of Infectious Diseases UKZN. REF: BF053/09.

We wish to advise you that your correspondence dated 07 October 2013 requesting for approval of Mr Katlego Sojane's PhD project to be approved as a sub-study of the above-referenced project has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. Your letter dated 06 November 2013 to BREC letter dated 24 October 2013 forwarding Postgraduate approval and Research Ethics training certificate for Mr K Sojane has been noted by BREC.

This approval will be noted at the next full meeting to be held on 10 December 2013.

Yours sincerely

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics