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CHARACTERIZATION OF HIV-1 IN THE CENTRAL NERVOUS SYSTEM DURING SUPPRESSIVE THERAPY

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ABSTRACT

Combination antiretroviral therapy (cART) is effective in suppressing HIV-1 RNA levels below the lower-limit of detection of clinical assays (<20-50 copies/mL) but is not curative and more sensitive assays can detect very low levels of HIV-1 RNA in plasma even after years of suppressive therapy. If treatment is stopped HIV-1 RNA levels soon increase and immunodeficiency continues to develop. This is due to the small amount of virions present in the blood during suppressive therapy that can infect new cells and cause viral rebound if treatment is stopped. These virions are produced by latently infected resting memory CD4+ T-cells that become reactivated. In addition, other cell types may be producing infectious virions during suppressive therapy. This needs to be explored to direct efforts for the eradication of all infected cells capable of producing virions in order to cure HIV-1. Since current HIV-1 therapy is life-long, costly and not without side effects a cure would be valuable from both an individual perspective as well as from a public health perspective. In this thesis we have, using very sensitive techniques for HIV-1 RNA quantification and sequencing, studied if HIV-1 also persists in the central nervous system (CNS) during suppressive therapy.

We first analyzed paired cerebrospinal fluid (CSF) and plasma samples from elite controllers (who maintain plasma HIV-1 RNA levels at <40 copies/mL without cART) since they have been proposed to serve as a model for a functional cure. We found that, using a very sensitive assay that allows for HIV-1 RNA quantification down to less than 1 copy/mL, HIV-1 RNA could be detected at very low levels in both CSF samples and plasma samples. We then studied subjects on suppressive therapy with HIV-1 RNA levels below the lower limit of detection for clinical assays in both CSF and plasma (<40 copies/mL) and found that, using the same sensitive assay, HIV-1 RNA could be detected at very low levels in 17% of CSF samples and in 57% of plasma samples from these subjects. HIV-1 RNA could be detected in the CSF even after 10 years of suppressive therapy and the detection of CSF HIV-1 RNA was correlated to elevated levels of CSF neopterin, a marker for immune activation. We sequenced HIV-1 RNA in CSF and plasma from subjects on suppressive therapy and found a large fraction of replication incompetent hypermutants among the HIV-1 variants in CSF. In addition, we found one subject with genetically distinct variants in the CSF compared to plasma, consistent with virion production by two populations of cells, one possibly in the CNS. We did not see any signs of evolution among the sequences found in CSF during suppressive therapy. In addition, we found that subjects on suppressive therapy who had their ongoing treatment intensified by the addition of the integrase inhibitor, raltegravir, did not reduce CSF HIV-1 RNA levels or CSF immune activation.

In conclusion, HIV-1 can be detected in the CSF even after years of suppressive therapy. The detection of HIV-1 in the CSF is correlated to intrathecal immune activation. The HIV-1 found in the CSF during suppressive therapy might be produced by cells in the CNS that need to be targeted in order to cure HIV-1. Since there are no signs of viral evolution among sequences found in the CSF during suppressive therapy and the CSF HIV-1 RNA levels are not affected by treatment intensification there does not appear to be any ongoing replication in the CNS during suppressive therapy.

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II. **Dahl V**, Peterson J, Fuchs D, Gisslen M, Palmer S, Price RW
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Sequencing of the HIV-1 population in CSF during suppressive therapy
Manuscript

IV. **Dahl V**, Lee E, Peterson J, Spudich SS, Leppla I, Sinclair E, Fuchs D, Palmer S, Price RW.
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CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APOBEC	Apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like
ART	Antiretroviral therapy
ARV	Antiretroviral
cART	Combination antiretroviral therapy
CNS	Central nervous system
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ER	Endoplasmatic reticulum
GALT	Gut-associated lymphoid tissue
IN	Integrase
IQR	Interquartile range
LTR	Long terminal repeat
mRNA	Messenger RNA
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
PCR	Polymerase chain reaction
PI	Protease inhibitor
PR	Protease
PIC	Pre-integration complex
RNA	Ribonucleic acid
RT	Reverse transcriptase
RCAS	Replication-competent avian sarcoma-leukosis virus with a splice acceptor
SCA	Single-copy assay
SD	Standard deviation
SIV	Simian immunodeficiency virus
ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid

1 INTRODUCTION

1.1 THE HIV PANDEMIC

1.1.1 The origin of HIV

Human Immunodeficiency Virus (HIV) is the result of cross-species transmission of Simian Immunodeficiency Viruses (SIV) from other primates to humans. SIV belongs to a family of *lentiviruses* that can cause disease in a number of mammals including bovines, horses, sheep, felines and primates (primates can be further divided into prosimians and simians where simians contain monkeys and apes including humans). Among primates SIV can be found in over 40 different species [1], however, it is found exclusively in African monkeys and apes. This makes it likely that the introduction of *lentiviruses* in primates occurred after the split of lineages between African and Asian primates which is estimated to have occurred 6-10 million years ago [2]. It is not known when in this time-span the introduction occurred but geographically separated subspecies have been infected with the same type of SIV for at least 30 000 years proving that SIV has been circulating among African monkeys and apes for at least this long [3].

HIV can be further divided into HIV-1 and HIV-2, representing cross-species transmissions of two different types of SIV. HIV-1 is the most common type of HIV (and will be primarily discussed in this thesis) whereas HIV-2 is much rarer and those infected with HIV-2 progress more slowly to AIDS. HIV-1 is most closely related to the type of SIV (SIV_{CPZ}) that is circulating among a subtype of the common chimpanzee found in Cameroon. HIV-2 on the other hand is more closely related to the type of SIV (SIV_{SMM}) found in sooty mangabeys in West Africa. There have been several separate introductions of SIVs to humans HIV. SIV_{CPZ} appears to have been introduced to humans on at least four occasions and SIV_{SMM} on at least 8 occasions [4].

The four introductions of SIV_{CPZ} gave rise to the four groups of HIV-1 that has so far been described (M, N, O and P). Group M has spread globally and represents more than 99% of all HIV infections. Group M can be further divided into different subtypes and circulating recombinant forms that do not represent different cross-species events but appear to have evolved in humans. Subtype C is the most common subtype world-wide representing around 50% of all HIV infections whereas subtype B is the most common subtype in Europe and North America. As a result most studies (including those in this thesis) have been done on subtype B with the general caveat that the outcomes of these studies might not always be transferable to other subtypes.

The cross-species transmission event that gave rise to the HIV-1 group M pandemic is estimated to have occurred in the beginning of the 20^{th} century in West-Africa [5, 6]. It has been proposed that the transfer of SIV to humans has occurred in the context of hunting of monkeys or apes (bush meat). Transmission would then have happened during butchering of the infected animal, perhaps when the hunter or butcher cuts himself or through exposure of blood from the infected animal on mucocutaneous surfaces. For HIV-1 group M this probably happened in southern Cameroon (**Figure 1**) since this is where the most closely related type of SIV_{CPZ} is found circulating among

chimpanzees. The virus then somehow spread 700 km south to the inhabitants of Leopoldville (now Kinshasa in the Democratic Republic of Congo), at the time the largest city in the region and a hub of commerce. There HIV-1 group M started a local epidemic in the 1960s [6]. Then HIV-1 Group M was likely transferred to Haiti, maybe by Haitians returning home from work assignments in the Congo. From Haiti the epidemic then presumably spread to North America in the late 1960s and then across the world [7]. The first cases of acquired immunodeficiency syndrome (AIDS) caused by HIV-1 was described in USA in 1981 and HIV-1 was isolated in 1983 [8, 9].

Cross-species transmission of SIV has most likely occurred on many occasions but has not previously given risen to sustained human transmissions. A number of factors such as large scale injection-based treatment of various tropical diseases [10-12], urbanization [6] and increased prevalence of sexual transmitted disease [13] are factors that can have contributed to sustained transmission among humans and the spread of HIV-1 group M in West Africa making this single cross-species event the start of the pandemic.

Figure 1. West-central Africa. Google maps (www.maps.google.com).

1.1.2 The situation today

Thirty million are estimated to have died from HIV infection and 34 million worldwide were estimated to be living with the infection by the end of 2011[14]. The prevalence varies greatly between regions. The most severely affected region is Africa south of Sahara where 69% of all infected people live and 1 out of 20 adults is HIV infected. The variation between countries within this region is substantial, and for example the prevalence in South-Africa is 17% whereas the prevalence in Nigeria is 4%.

Although the number of people living with HIV is greater than ever the number of people becoming infected (2.5 million in 2011) and the number dying from AIDS-related disease (1.7 million in 2011) have been decreasing during the last years. This is partly due to the scale-up of interventions to prevent new infections and the more widespread use of combination antiretroviral therapy (cART). Since its introduction in 1995 cART has saved more than 14 million life years in low- and middle income countries making it a major achievement of modern medicine. By the end of 2011 cART reached 8 million people which for the first time was more than half (54%) of those eligible for treatment. Apart from the medical complications by HIV many of those infected are also suffering from stigma or discrimination. In conclusion, while the epidemic is slowing down and treatment has been scaled up, HIV/AIDS remains a massive global health problem.

1.1.3 HIV in Sweden

Around 10 000 people have been diagnosed with HIV (almost exclusively HIV-1) in Sweden since 1983 and around 6600 are living with HIV in Sweden today, corresponding to a prevalence of 0.06%. During 2012, 441 new infections were reported, 69% of those infected had contracted the infection outside of Sweden and 59% through heterosexual transmission [15].

1.2 HIV-1 VIROLOGY

1.2.1 Taxonomy, structure and genome

HIV-1 belongs to the *Lentivirus* genus of the *Retroviridae* family. The other known human retroviruses belong to the *Oncovirinae* genus (Human T-cell leukemia virus) and *Spumavirinae* genus (Human foamy virus). HIV-1 has an envelope and contains two copies of positive sense ssRNA molecules which are linked at the 5' end. It has a conical nucleocapsid that surrounds the viral nucleic acid and the viral enzymes reverse transcriptase (RT), protease (PR) and integrase (IN). The nucleocapsid is surrounded by an envelope which consists mainly of the host cell lipid layer with viral glycoprotein gp41 linked to the external viral protein gp120 (**Figure 2**).

The HIV-1 genome is approximately 9700 base pairs and codes for three major structural genes: group specific antigens (*gag*), polymerase (*pol*) and envelope (*env*). The Gag and Pol proteins are produced as Gag and Gag-Pol precursor polyproteins and are cleaved by PR into functional proteins. *Gag* encodes the precursor polyprotein which is further processed into p24 (capsid), p17 (matrix), p7 (nucleocapsid) and p6 (nucleocapsid). *Pol* encodes the enzymes PR, RT and IN. *Env* encodes gp120 and the transmembrane protein gp41. In addition HIV-1 has two regulatory genes and four accessory genes: *tat*, *rev*, *vif*, *vpr*, *vpu* and *nef* (**Table 1**).

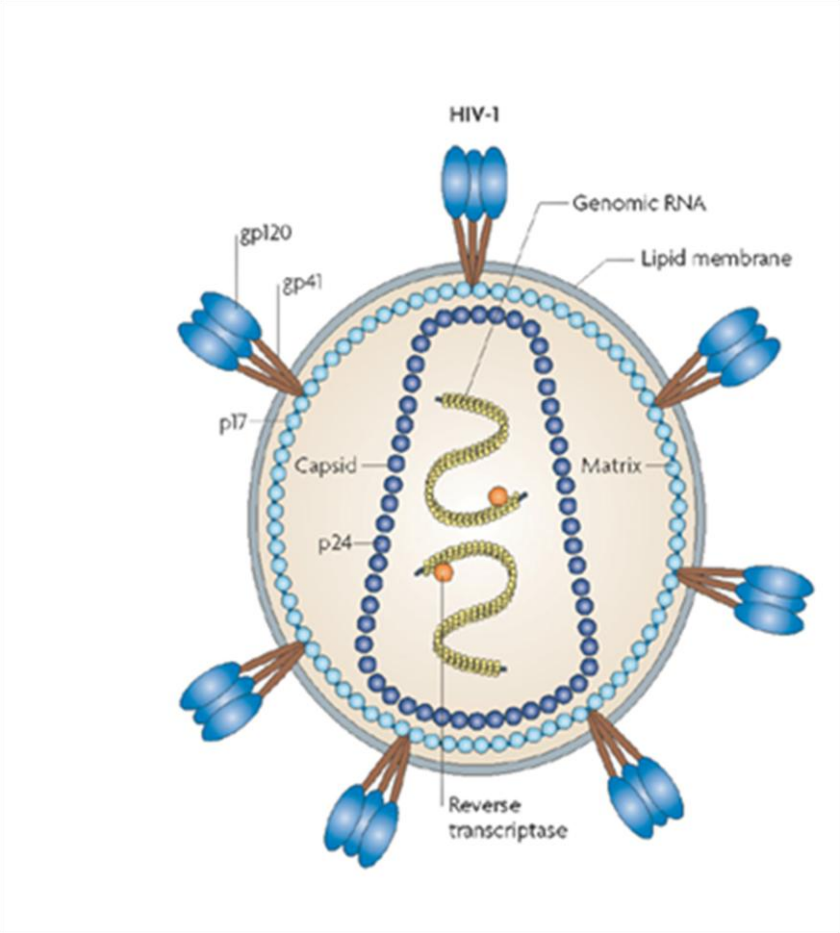

Figure 2. Schematic structure of the HIV-1 virion. Reprinted with permission from [16].

1.2.2 Replication

Replication starts by the virus attaching to the target cell and is followed by the binding of the envelope protein gp120 to its primary receptor CD4 (**Figure 3**) [17, 18]. After binding to the target cell, conformational changes occur in gp120 allowing for binding to the co-receptor CCR5 or CXCR4 [19-22]. The viral envelope then fuses with the cell and reverse transcription (conversion of the viral ssRNA by RT to ssDNA) occurs in the partially dissolved capsid [23, 24]. Only one copy of ssDNA is produced from the two copies of ssRNA [25]. The RT can switch between the two strands of ssRNA which can lead to the occurrence of a new variant if the cell had been infected by two different viral variants (this is called recombination). As ssDNA is being synthesized by RT viral RNA is degraded by a domain of the RT. The dsHIV-1 DNA binds to Vpr, IN and p17 to form a pre-integration complex (PIC). The PIC is transported to the nucleus where HIV-1 DNA is integrated into the host chromosome by IN [26]. Viral DNA is primarily inserted into transcriptionally active regions of the cellular genome [27, 28]. Some HIV-1 DNA is not integrated but instead goes through a series of circularization steps generating 2-LTR (long terminal repeat) and 1-LTR circles which prohibits further viral replication [26].

Table 1. HIV-1 regulatory and accessory genes, proteins, location and function.

Gene/Protein	Expression	In virion	Function
tat/Tat	Early	No	Tat binds to the transactivation-responsive region in the 5' end of the integrated viral DNA and enhances transcription
rev/Rev	Early	No	Rev binds to rev responsive element and facilitates the transport of unspliced or partially spliced mRNA out of the nuclesus for translation
nef/Nef	Early	Yes	Down regulates CD4 and MHC I and II of infected cells to avoid immune response
vpr/Vpr	Late	Yes	Involved in transport of pre-integration complex into the nucleus and prevents cell division
vpu/Vpu	Late	No	Induces degradation of CD4 and enhances the release of virions
vif/Vif	Late	Yes	Inhibits APOBEC thus preventing hypermutation

After integration HIV-1 can go into a latent state where it is not transcribed or, which is the exceedingly more common, replication continues by transcription of the integrated HIV-1 DNA by the host RNA polymerase II. HIV-1 RNA transcription is mediated through the binding of cellular transcription factors, most importantly NFkB to a promoter region in the 5' LTR region of the HIV-1 genome. The first transcript is a full length copy of the viral RNA. This early transcript is then spliced into mRNAs that are translated into the viral proteins Tat and Rev. Tat binds to the transactivation-responsive region which is situated downstream from the 5' LTR region where Tat enhances HIV-1 transcription thus creating a positive feed-back loop. Rev binds to the rev responsive element in the *env* region of the unspliced, or partially spliced, HIV-1 mRNA and facilitates transport of these molecules out of the nucleus for translation. During late transcription alternative splicing gives longer Gag, Gag-Pol, Env, Vif, Vpr, Vpu and full length HIV-1 RNA. All translation of viral mRNAs occurs in the cytoplasm near the endoplasmatic reticulum (ER) by the normal cellular transcription machinery. The envelope protein gp160 is processed in the ER and the Golgi complex where it is cleaved into gp41 and gp120 and glycosylated. The assembly of a new virion containing the viral the proteins Gag, Gag-Pol, Vif and Vpr and the full-length viral RNA takes place at the cellular membrane. The new virion is then released by budding, taking a part of the host cell membrane as the viral envelope. The final step in the viral replication is the maturation step where the viral enzyme PR cleaves the Gag and Gag-Pol polyproteins into matrix, capsid, nucleocapsid and p6 proteins. These proteins form the mature nucleocapsid and matrix. The viral protease also cleaves the Gag-Pol polyprotein into the viral enzymes PR, IN and RT after the viral particle has budded from the infected cell making the particle infectious [29].

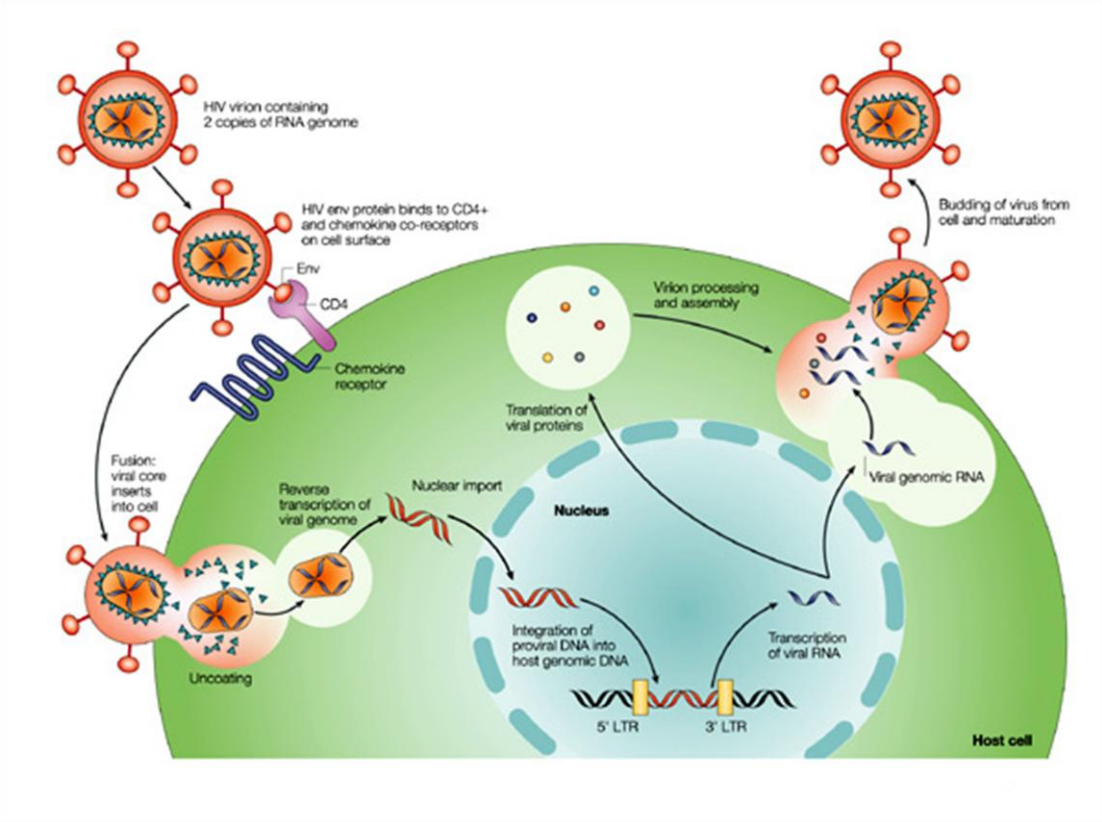

Figure 3. HIV-1 replication. Reprinted with permission from [30].

1.2.3 Genetic variation

Like all RNA viruses HIV-1 is a genetically diverse virus. This is partly due to the error rate of HIV-1 during replication (approximately one error per $3x10^{-5}$ bases) which, on average, results in 0.3 mutations in each new virion produced. These errors can be attributed to the error-prone RT that lacks proof-reading but also to the host cell RNA polymerase and G to A mutations generated by apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like (APOBEC) proteins. The error rate is comparable to what is seen in other RNA-viruses [31]. Due to the number of infected cells a great number of new virions (around 10^9) are produced every day. This means that every possible point mutation occur in an infected individual every day. Why certain mutations become fixed is partly due to classic Darwinian evolution where some mutations that give the virus an increased fitness become fixed (positive selection) whereas other mutations that decrease the fitness or render the virus replication incompetent disappear (negative selection). Some mutations that do not affect fitness will also become fixed by chance (neutral evolution) [32].

Another factor that contributes to the genetic diversity of HIV-1 is recombination. If one cell is infected by two variants of HIV-1 and these two variants happen to be assembled in the same virion then RT can switch between the RNA from these two variants during the subsequent infection of a new cell, this generates HIV-1 DNA which is a combination of the two variants. Even though cells double infected cells appear to be rare the relative importance of this phenomenon is illustrated by the fact that more than 20% of all circulating HIV-1 variants are recombinants of different subtypes [33, 34].

Point mutations and recombination in combination with the large number of virions produced each day and the long duration of infection generates an exceptional genetic diversity [31]. Amazingly the genetic diversity within a single HIV-1 infected individual is greater than the diversity of all influenza virus isolates worldwide within a year [35]. The great diversity generated during HIV-1 replication, which in the *env* (the gene with the most diversity) can reach 25-35%, has made it possible to track the spread of HIV-1 globally, between individuals and to some extent within individuals by phylogenetic analyses [7, 36, 37].

1.3 HIV INFECTION

1.3.1 Transmission and prevention

HIV-1 can be transmitted after exposure to mucosal surfaces, most commonly through vaginal or anal intercourse. Another route of infection is from an infected mother to her child, either *in utero,* during labor or through breast feeding. The virus can also spread through percutaneous inoculation, either through intravenous drug use or nosocomially by contaminated needles or blood products.

Heterosexual HIV-1 transmission is the most common form of transmission and accounts for 70% of infections world-wide despite that the risk for transmission is rather low (usually well below 1%, with a slightly higher risk of transmission from male to female compared to transmission from female to male) [38]. The risk for sexual transmission is increased by co-existing genital ulcers [39]. It is also strongly correlated to the HIV-1 RNA levels in plasma, a tenfold increase in plasma HIV-1 RNA levels results in 2.5 fold risk of transmission [40, 41]. Consequently a reduction in the HIV-1 RNA levels in plasma by cART is a very effective intervention to prevent transmission and can almost completely prevent transmission in sero-discordant couples [42, 43]. Another intervention that has been shown effective to prevent transmission is male circumcision which reduces the risk of transmission to the circumcised man by 60% [44, 45]. Proper use of condoms also greatly reduces the risk for sexual transmission.

Where available, cART, to the mother and the infant, Caesarean section and the avoidance of breast feeding can almost completely prevent mother-to-child transmission [46]. In low- and middle income countries where this is not affordable or feasible various combinations of antiretrovirals (ARVs) can at least greatly reduce the risk of transmission [47]. Single use needles and testing of blood products can reduce nosocomial transmission and needle exchange programs can reduce the rate of HIV-1 transmission among intravenous drug users (IDUs) [48].

Transmission of HIV-1 and thus establishment of infection by only a single virion appears to be the norm, occurring in 80% of the heterosexual transmission events. Infection with multiple HIV-1 variants occurs more frequently during mother-to-child transmission as well as during transmission among men who have sex with men and among intravenous drug users [49-53].

1.3.2 Cells and tissues

HIV-1 uses the CD4 receptor as its main receptor for infecting a cell and this, and the co-receptors used, determines which cells that become infected [54, 55]. The CD4 receptor is mainly expressed on the surface of CD4+ T-cells (naïve and memory) and this cell type is the primary target for HIV-1. Apart from its main receptor HIV-1 uses CCR5 or CXCR4 as co-receptors. The CCR5 expression is higher on CD4+ memory T–cells (making this subset the primary target) while naïve CD4+ T-cells express CXCR4 [56-60]. This reflected by the infection frequency of each subset, in peripheral blood the infection frequency of memory CD4+ T-cells is 0.01-1% while the infection frequency for naïve CD4+ T-cells is a tenfold lower [60-62]. Almost exclusively the infection is established by a virus using CCR5 [49, 63]. Later during infection the co-receptor usage of the virus can change so variants which use either both CXCR4 and CCR5 or CXCR4 exclusively can arise [64-67]. The appearance of viral variants that uses CXCR4 as receptors is linked to accelerating disease progression but it is unknown if it is the cause or a consequence of this.

Apart from CD4+ T-cells monocytes, macrophages, NK-cells and dendritic cells have been proposed to be infected by HIV-1 *in vivo*. There are however still uncertainties regarding this and, if infected at all, whether these cells actually can produce new virions (productive infection). According to some reports monocytes can be infected *in vivo* [68-71] while other investigators have not found infected monocytes *in vivo* despite extensive sampling [34]. Monocytes circulate for 1-3 days before they migrate into tissues and become macrophages [72]. That macrophages become infected *in vivo* is less controversial [73-77]. In addition, NK-cells can also be infected *in vivo* [78]. Dendritic cells are able to capture the virus on their cell surface and transport it to lymphatic tissue where it can infect CD4+ T-cells but it is less clear if dendritic can be infected themselves [79, 80].

The CD4+ T-cell is the primary target for HIV-1 infection and can be found in various tissues throughout the body. For practical reasons infected CD4+ T-cells found in blood have been studied the most, but they represent only 2% of the body´s lymphocytes [81]. The gut-associated lymphoid tissue (GALT) is the largest lymphoid organ in the body harboring 60% of the lymphocytes, 40% of them being CD4+ T-cells [82-85]. Massive depletion of CD4+ T-cells has been found in this compartment during early HIV-1 infection [83, 84]. Lymph nodes throughout the body also harbor CD4+ T-cells and lymph nodes have been found to contain higher concentrations of both HIV-1 RNA and HIV-1 DNA than peripheral blood [86-88].

1.3.3 Immune response

The body has two lines of defense against HIV-1 infection: the innate immune system (including restriction factors) and the adaptive immune system. Restriction factors are cellular factors which inhibit HIV-1 replication. Those that so far have been described to be protective are:

1. The APOBEC families of proteins, in particular APOBEC3G. They are expressed in virtually all human tissue [89, 90]. APOBEC3 can become integrated in the viral particle and is transferred to the target cell where it forms

a complex with the viral RT. There it deaminates cytidine residues in the negative strand viral DNA. This can result in up to 10% hypermutations of guanosine to adenosine in the positive strand sequence which renders the generated viral DNA unable to produce infectious virions [91, 92]. APOBEC is antagonized by the viral protein Vif [93-95].

2. Trim5α binds to the capsid after viral entry and accelerates capsid fragmentation and disrupts the structure of the RT [96]. Trim5α from old world monkeys effectively inhibits infection with HIV-1 and is a major reason for why old world monkey cannot be infected with HIV-1 [96].
3. Tetherin causes virions to remain trapped at the cell surface and thereby prevents the release of virions [97]. Tetherin is counteracted by the viral protein Vpu [98].

Like other ssRNA viruses HIV-1 triggers the innate immune receptors TLR7 and TLR8 which results in potent activation of dendritic cells and the release of type 1 interferons and tumor necrosis factor α. This shuts down viral replication in infected cells and activates other parts of the immune system [99, 100]. Among the cells of the innate immune system NK-cells appear to be most important for the control of HIV-1 infection. This reflected by how some variants of NK-cell receptors are correlated to a slower progression towards AIDS [101, 102].

HIV-1 infected individuals are able to mount a robust CD8+ T-cell response against HIV-1 when measured *in vitro*, yet full viral control is not achieved *in vivo* [103-105]. This could be due to that the CD4+ T-cell response (which is necessary for an effective CD8+ T-cell response) is deeply impaired during HIV-1 infection [103, 104, 106]. Certain HLA-alleles (especially HLA-B57) are linked to a slower progression of disease indicating that the CD8+ T-cell response can at least partially control infection in some individuals. [107, 108]. Initially the CD8+ T-cell response is narrow, in regards to which epitopes it targets, but it tends to broaden over time [109-112]. The virus constantly evolves to avoid the CD8+ T-cell response and escape mutations are seen within a month of infection [111].

The B-cell response to HIV-1 develops within the first weeks of infection [113]. These early antibodies do not seem to have any effect on HIV-1 RNA levels or contribute to immune pressure on the viral population [49, 113]. Even though the antibody response becomes more potent and broaden over time the B-cell response in general appears to be less associated with control of infection compared to the T-cell response [114-116]. The presence and breadth of the neutralizing antibody response is not associated to HIV-1 RNA levels and does not impact progression to AIDS [115-117].

1.3.4 Pathogenesis

HIV-1 infection is characterized by the progressive loss of CD4+ T-cells which eventually leads to immunodeficiency and death from opportunistic infections. The cause of this progressive loss of CD4+ T-cells is unclear. A recent study suggests that the integration of viral DNA triggers apoptosis in infected cells [118]. Another possible mechanism is the CD8+ T-cell mediated killing of infected cells. Indirect evidence for this is that the depletion of CD8+ T-cells in infected animal models lead to a 100-fold

increase of viremia, presumably since infected CD4+ T-cells can produce virions for a longer time if not killed by CD8+ T-cells [119, 120].

Another important feature of HIV-1 infection, apart from the loss of CD4+ T-cells, is immune activation [121]. In addition to being activated the immune system bears markers of ageing and exhaustion [122-124]. Expression of markers for immune activation are important indicators of disease progression during untreated infection [121, 125, 126]. There are several factors that can contribute to immune activation during HIV-1 infection:

1. The virus itself is recognized by the immune system.
2. Opportunistic infections such as cytomegalovirus are able to replicate during HIV-1 induced immune deficiency which could lead to immune activation [127].
3. Damage caused to the mucosal CD4+ T-cells around the gut leads to translocation of microbial products from the gut to the systemic circulation [128, 129].

The immune activation is rapidly reduced (but not entirely eliminated) when HIV-1 RNA levels are suppressed after the initiation of cART [130, 131]. In primates, such as sooty mangabeys, where SIV does not lead to immunodeficiency no immune activation or loss of CD4+ T-cells is seen [132, 133].

1.3.5 Progress of HIV-1 infection

After infection an asymptomatic period of 1-2 weeks follows when the virus replicates at the site of infection and in local lymph nodes. During this phase HIV-1 RNA is not detectable in the blood (measuring HIV-1 RNA levels is a proxy for measuring the amount of HIV-1 virions), no immune response is measurable and the infected individual remains asymptomatic [83-85, 134-137]. Then a peak in HIV-1 RNA levels follows (up to 10^7 copies of HIV-1 RNA/mL) as the infection spreads throughout the body. This is likely due to the large number of available target cells such as CD4+ T-cells in gut-associated lymphoid tissue (GALT) and peripheral lymphoid tissues and the lack of immune response against the virus. During this phase around 50% of infected subjects experience flu-like symptoms such as fever, enlarged lymph nodes and occasionally a rash [138]. After the initial depletion of a large number of target cells and the emergence of an immune response the HIV-1 RNA levels drop rapidly, by about a 100-fold, and stabilizes around a set-point. The events of early HIV-1 infection have been classified by Fiebig and colleagues based on the order of appearance of various markers for HIV-1 infection (**Figure 4**) [139].

After the viral set-point is established a period of asymptomatic infection follows. This period consists of slowly declining CD4+ T-cell levels and relatively stable HIV-1 RNA levels (**Figure 5**). Although HIV-1 RNA levels and CD4+ T-cell counts only slowly change during this time, giving the impression of this being a stable phase, this period consist of the production of a large amount of viral particles each day, massive killing of CD4+ T-cells and the body's constant struggle to replenish these cells. This period is on average 10 years, but shows considerable inter-individual variation [140].

The set-point viral HIV-1 RNA level and the degree of immune activation as well as the presence of certain HLA-alleles correlates to the time before development of AIDS [107, 108, 141]. The stage of disease can be classified either based on the WHO criteria or the CDC criteria [142, 143]. Generally AIDS is considered to have developed either when the CD4+ T-cell count falls below 200 cells/µL or by the appearance of certain opportunistic infections such as toxoplasmosis of the brain, pneumocystis jiroveci pneumonia, candidiasis of the esophagus, trachea, bronchi or lungs and Kaposi's sarcoma.

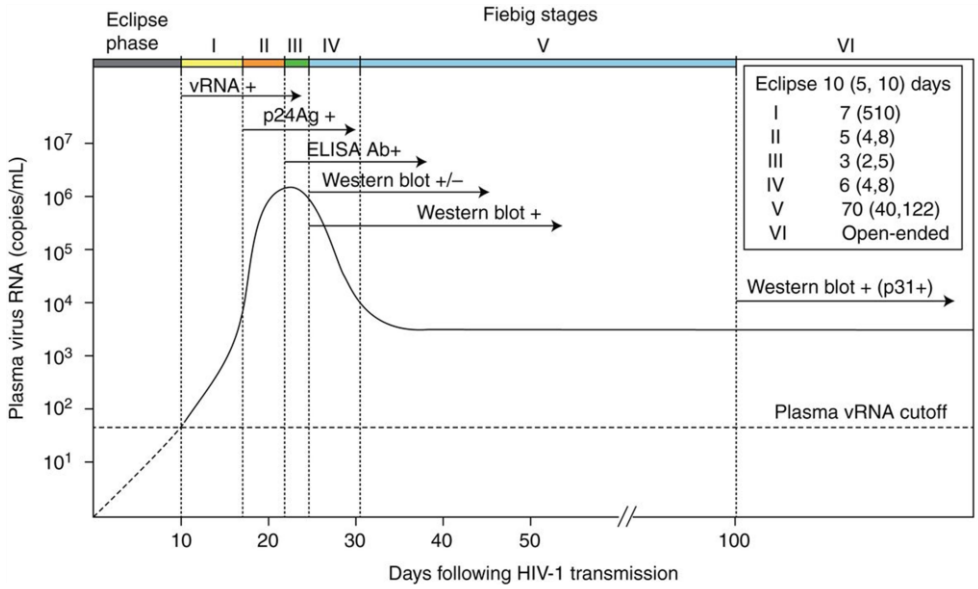

Figure 4. Laboratory staging of acute and early HIV-1 infection based on the appearance of various biological markers. The average durations and 95% confidence intervals (parentheses) of the different stages are shown in the inset. Reprinted with permission from [144].

1.3.6 Elite controllers

Elite controllers are a rare group (<1%) of HIV-1 infected individuals who, without cART, can maintain HIV-1 RNA levels in plasma below the lower-limit of detection for clinical assays (usually defined by <50 copies of HIV-1 RNA/mL) [145, 146]. Using very sensitive assays one can however detect low levels of HIV-1 RNA in plasma [147, 148]. Novel escape mutations can be found suggesting that replication occurs at a low-level [149]. Consistent with that HIV-1 RNA levels are directly correlated to disease progression most elite controllers do not progress to AIDS, although with some exceptions [150]. Replication competent virus can be isolated from most elite controllers, though in some cases elite control has occurred since the subjects had been infected with a defective virus [151, 152]. The CD8+ T cells are thought to play an important role for control and certain HLA class I alleles such as HLA-B5701, HLA-B5703 and HLA-B27 have all been associated with control. [146, 153-155]. As compared to non-controllers, freshly isolated CD8+ T cells from controllers also have a higher capacity to inhibit HIV-1 replication in infected CD4+ T cells [156]. No single factor, however, seems to be completely protective, nor is there one that is strictly required.

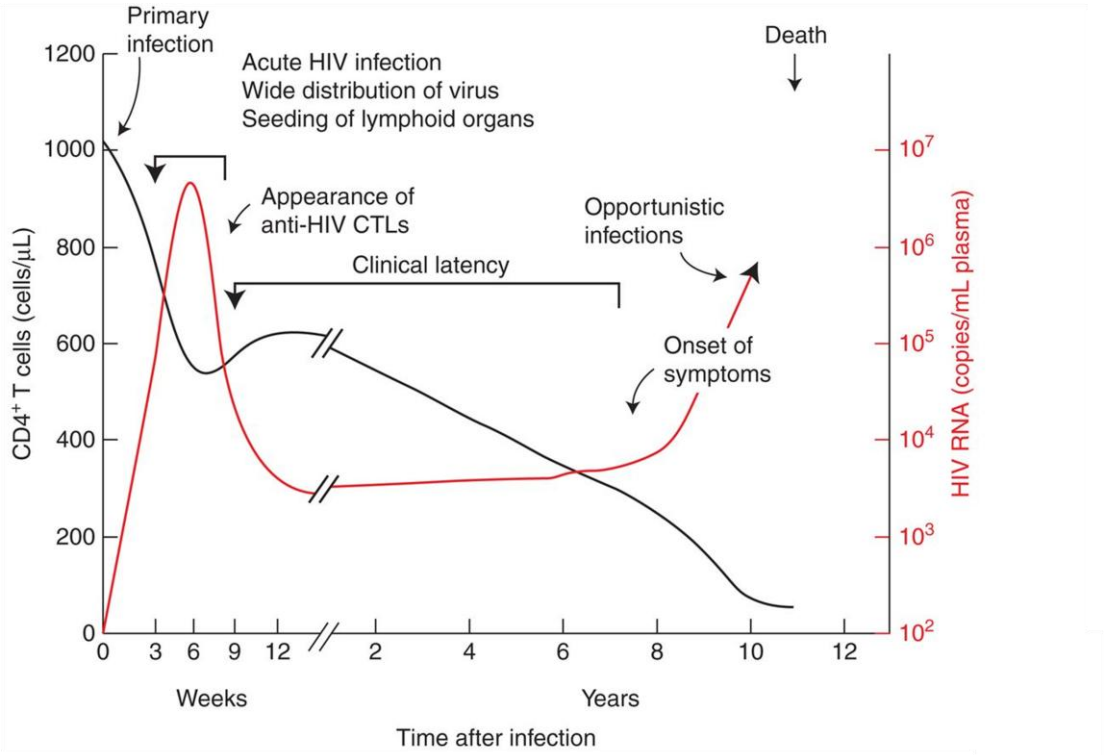

Figure 5. Time course of typical HIV-1 infection. Patterns of $CD4^+$ T cell decline and viremia vary greatly from one subject to another. Reprinted with permission from [31].

1.3.7 The central nervous system

HIV-1 enters the central nervous system (CNS) during the first weeks of infection and can be detected in the cerebrospinal fluid (CSF) throughout the subsequent course of untreated infection [157-161]. It is thought to enter the CNS by the so called "Trojan horse model", the migration of an infected cell (often suggested to be an infected monocyte) into the CNS. This model was originally proposed for visna virus, another type of *lentivirus* found in sheep [162]. Since there is no final evidence that monocytes are infected by HIV-1 *in vivo* it is more likely that CNS infection is established by the migration of an infected CD4+ T-cell into the CNS, or possibly by the transport of free virions across the blood-brain barrier.

Usually the HIV-1 RNA levels are about a 10-fold lower in CSF than in plasma, although this can vary considerably between individuals [159, 160, 163, 164]. Viral populations in the CSF and blood can follow separate evolutionary pathways and compartmentalize in some subjects [165-174]. It appears that the changes are adaptive, perhaps an adaptation to better replicate in cells in the CNS or perhaps changes that leads to an increased fitness since there is less immune response against HIV-1 in the CNS [166, 173, 175].

There has been considerable discussion about which cell types in the CNS can be infected and produce virions. Most agree that pervivascular macrophages (derived from circulating monocytes migrating into the CNS) can be infected and produce virions, this is possibly also the case with microglia (also of monocyte origin) whereas astrocytes likley can be infected but do not produce virions *in vivo* [77, 176, 177].

As in the periphery HIV-1 infection of the CNS is characterized by immune activation. This is reflected in the elevation of several markers for inflammation in the CSF such as CSF white blood cell, CSF neopterin (a marker for macrophage activation), CCL2/MCP1 and CXCL10/IP10 chemokines (which stimulate the migration of monocytes and lymphocytes into the CNS) [178]. In addition to elevated markers for immune activation markers for neuronal damage such as neurofilament light chain protein (NFL), and total tau proteins are also elevated in the CSF while amyloid precursor proteins are decreased consistent with altered neuronal meatbolism [179-182].

Table 2. Criteria for HIV associated neurocognitive disorder (HAND). Adapted from [177].

Diagnostic entity	**Cognitive performance**	**Functional status**
Normal	Normal	Normal
Asymptomatic neurocognitive impairment	Acquired impairment in at least two cognitive domains (<1 SD)	No perceived impact on daily function
Mild neurocognitive disorder	Acquired impairment in at least two cognitive domains (<1 SD)	Perceived interference with daily function to at least a mild degree (work inefficiency, reduced mental acuity)
HIV-associated dementia	Acquired impairment in at least two domains, typically in multiple domains with at least two domains with severe impairment (<2 SD)	Marked impact on daily function

Untreated HIV-1 infection is commonly associated with neurological symptoms such as attention and memory deficits, motor impairments and personality changes [183]. According to current nomenclature the neurological symptoms associated with HIV-1 should be referred to as HIV Associated Neurocognitive Disorder (HAND) and could be further classified into three subtypes depending on the degree of impairment on neuropsychological testing and functional disability (**Table 2**) [184]. The damage that HIV-1 causes to the brain is seen as diffuse brain atrophy with large ventricles, low grade inflammation with microglanodules, perivascular lymphocyte cuffing, multinucleated cells, patchy demyelination and white matter gliosis [185, 186]. Since HIV-1 does not infect neurons the neurological symptoms that can occur during HIV-1 infection must be due to indirect damage to the neurons. Two mechanisms for how this occurs have been suggested and proven to occur *in vitro* but it remains unclear which is the most important factor *in vivo* [176].

1. Viral proteins such as gp120 and Tat may be directly toxic to neurons.
2. Toxicity may be mediated by release of neurotoxic factors such as quinolonic acid, tumor necrosis factor and arachidonic acid metabolites by infected and uninfected cells responding to infection.

Untreated HIV-1 infection also predisposes to opportunistic infections in the CNS in those with more severe immune deficiency.

1.4 THERAPY

1.4.1 Drugs

Most ARVs have their effect by inhibiting the RT. The ARVs that inhibit the RT can be subdivided into nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (**Table 3**). NRTIs lack a 3' hydroxyl group which results in termination of the synthesis of viral DNA when a NRTI is incorporated [187]. NNRTIs inhibit RT through binding proximal to, but not overlapping, the active site of the RT [188, 189].

This binding changes the spatial structure of the active site and reduces RT activity [188]. Due to differences in the RT structure NRTIs inhibit HIV-2 replication whereas NNRTIs do not [188]. The third major drug class is protease inhibitors (PIs). They have their effect by inhibiting PR which cleaves Gag and Gag-Pol necessary for virion maturation. PIs are large peptide-like molecules that are quickly degraded. Therefore, ritonavir is co-administered to "boost" PI treatment by inhibiting the enzyme that degrades PIs which prolongs the activity of the PI [190, 191]. These are the major drug classes of ARVs but drugs that target other parts of the viral life cycle have also been developed. CCR5 antagonists can block binding of HIV-1 that uses CCR5 as a co-receptor [192], fusion inhibitors can block the fusion of the virion and the cell [193] and integration of HIV-1 DNA into the genome of the infected cell genome can be prevented by inhibitors of viral integrase [194, 195].

1.4.2 cART

As previously discussed, HIV-1 is a genetically diverse virus therefore variants with reduced susceptibility to an ARV can exist as minor variants before treatment is initiated or quickly evolve if replication is not completely inhibited by cART [196]. Mathematical modeling suggests that a treatment regimen should consist of a combination of ARVs where at least three mutations are required for resistance to develop to prevent the emergence of resistant variants [196]. This is consistent with clinical experience of resistance development during mono- or dual therapy, and therefore ARVs are given in combinations of at least three different drugs. According to current Swedish guidelines, first line treatment should consist of two NRTIs and one PI or one NNRTI (US guidelines also give the option of combining two NRTIs with an integrase inhibitor) [197, 198]. Swedish guidelines recommend that treatment should be initiated when the CD4+ T-cell count is between 350-500 cells/mm^3 whereas US guidelines suggests that treatment should be offered regardless of CD4+ count.

Where available cART has greatly reduced the morbidity and mortality from immunodeficiency associated with HIV-1 infection [199-202]. However cardiovascular disease, liver disease and some non-AIDS related malignancies are over-represented among HIV-1 infected individuals receiving suppressive cART [203-208]. The mechanisms behind these pathologies are not fully elucidated but they appear to some extent to be linked to the presence of low-grade inflammation during cART [209, 210]. Despite cART, some individuals do not achieve normal CD4+ T-cell counts (immune non-responders) perhaps due to fibrosis of the lymphoid tissues [211, 212].

Table 3. ARVs approved by Food and Drug Association (FDA) and European Medicines Agency (EMA).

Drug	Approved by FDA/EMA	Mechanism
NRTIs		
abacavir (ABC)	1998/1999	NRTIs lack a 3' hydroxyl group which results in termination of the viral DNA copy when a NRTI is incorporated
didanosine (ddI)	1991*	
emtricitabine (FTC)	2003/2003	
lamivudine (3TC)	1995/1996	
stavudine (d4T)	1994/1996	
tenofovir (TDF)	2001/2002	
zalcitabine (ddC)	1992	
zidovudeine (AZT)	1987/1987	
NNRTIs		
delaviridine (DLV)	1997/-	NNRTIs inhibit RT through binding proximal to, but not overlapping, the active site of the RT
efavirenz (EFV)	1998/1999	
etravirine (ETR)	2008/2008	
nevirapine (NVP)	1996/1998	
Rilpivirine	2011/2011	
Pis		
Atanzavir	2003/2004	PIs inhibit PR which cleaves Gag and Gag-Pol necessary for virion maturation.
Darunavir	2006/2008	
Fosamprenavir (fAMP)	2003/2004	
Indinavir (IDV)	1996/1996	
Lopinavir (LPV)	2000/2001	
Nelfinavir (NFV)	1997/1998	
Saquinavir (SQV)	1995/1996	
Tipranavir (TPV)	2005/2005	
Entry inhibitors		
Maraviroc (MVC)	2007/2007	Blocks binding of HIV-1 to co-receptor CCR5.
Fusion inhibitors		
Enfuvirtide (T-20)	2003/2003	Prevents fusion by binding to gp41.
Integrase inhibitors		
Raltegravir (RAL)	2007/2007	Prevents integration by binding to the complex between IN and the viral genome.

1.4.3 Resistance

Drug resistance can either be transmitted, pre-existing or develop during cART if therapy is not fully suppressive. In Sweden transmitted drug resistance is present in approximately 5% of new infections, in Europe in approximately 8% and in the US transmitted drug resistance is estimated to occur in 15% of new infections [213-215]. Resistance can also develop during cART that is not fully suppressive due to lack of adherence or due to drug interactions. Generally, resistance develops quicker for NRTIs and NNRTIs than for PIs. For NRTIs (more particular 3TC or FTC) mutations such as M184V/I and K65R changes the binding site for the NRTI and thereby prevents it from being incorporated in the elongating chain [216, 217] whereas the thymidine analogue mutations (M41L, L210W, T215Y, D67N, K70R, T215F and 219E/Q) seem to remove the incorporated NRTI (AZT and d4T) [218, 219]. Resistance against NNRTIs can occur through the emergence of single mutations (K103N and Y181C) [220, 221]. The emergence of NRTI-resistance mutations is often associated with a greater reduced fitness for the virus than NNRTI-mutations. Resistance to PIs requires the accumulation of several mutations and is therefore less likely to develop [222].

1.5 PERSISTANCE

1.5.1 Dynamics

With the assumption that cART prevents all (or almost all) infection of new cells, but does not affect the life-span of the infected cells, some conclusions about the population of infected cells can be drawn from the decay of the HIV-1 RNA level once treatment is initiated. First there is a rapid phase of decay where the level drops to 1-10% of the pre-therapy level (**Figure 6**). This phase corresponds to the decay of a population of cells with a half-life of 1-2 days, most likely activated CD4+ T-cells that where already productively infected when therapy was initiated. This is followed by a second phase of decay with a half-life of ca 2 weeks. This phase is often attributed to macrophages or possibly partially activated CD4+ T-cells, but this is uncertain [223]. Then the HIV-1 RNA level falls below the lower limit of detection for assays used in a clinical setting (<20-50 copies of HIV-1 RNA/mL) [224-226]. Using more sensitive assays it can be demonstrated that the second phase of decay is followed by a third with a half-life of 39 weeks which is followed by a plateau phase where HIV-1 RNA at a level of approximately 3 copies/mL persists even after years of suppressive therapy [148, 227, 228]. The on-therapy plasma HIV-1 RNA levels are correlated to levels of pre-therapy plasma HIV-1 RNA, however, the potency of the treatment regimen does not appear to affect the levels of persistent viremia [229].

HIV-1 virions in blood have a half-life of only minutes so the occurrence of HIV-1 RNA in blood means virions are constantly produced (methods used for detecting HIV-1 RNA measure virion-associated HIV-1 RNA) [230]. How HIV-1 virions are produced during suppressive therapy has been a question of debate. Initially it was believed that this was due to low-level replication occurring despite cART [227]. The replication would then occur in the presence of ARVs or in cells, tissues or anatomical compartments where ARVs are at suboptimal concentrations such as in the CNS.

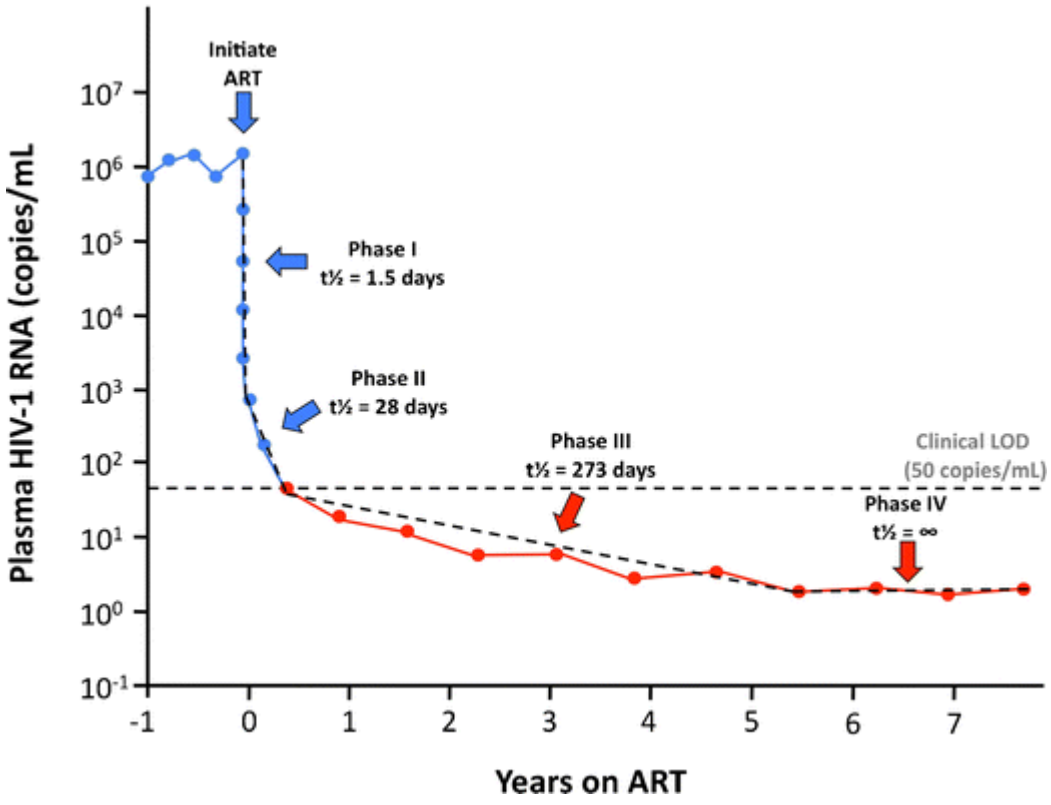

Figure 6. Decay dynamics of plasma HIV-1 RNA during cART. Upon initiation of cART, viremia decays in multiple overlapping phases, which reflects the turnover of cells infected prior to cART with different half-lives. Blue = above clinical limit of detection (LOD). Red = below clinical LOD (ie, detectable by SCA or other sensitive assays). Dotted lines = theoretical decay slopes. Reprinted with permission from [231].

Alternatively, cART inhibits all or almost all viral replication and the occurrence of HIV-1 virions during suppressive therapy is due to the production of virions from long-lived infected cells that became infected before the initiation of cART. Most studies favor the latter explanation. In summary:

- Resistance mutations usually do not emerge during suppressive cART which would likely happen if there were full cycles of replication. [232-236].
- Most studies find no substantial signs of viral evolution (a consequence of replication) during suppressive therapy [233, 236-239]. When signs of viral evolution are found it is hard to exclude the possibility that the "new variants" found during suppressive therapy were actually present pre-therapy but not found because of limited sampling or that some subjects have had short periods of non-adherence to therapy.
- The on-therapy HIV-1 RNA levels are not related to the potency of the treatment regimen [229]. This would have been expected if there was ongoing replication in some tissues or compartments since different drugs penetrate more or less well different compartments and tissues.
- Most studies which have intensified cART with and additional drug have not seen any effect on plasma HIV-1 RNA levels [240-243] . One study observed an effect on 2-LTR circles which could indicate that ongoing-replication was inhibited in the subset of subjects who were on a PI-based treatment regimen

[244]. However, it is debated if 2-LTR circles are a reliable indicator of HIV-1 replication.

- When treatment is stopped the variants that emerge do not have a close relationship to the variants that existed just before treatment was initiated. Rather it appears to be a stochastic reactivation of variants that previously have existed and have been "archived" in resting memory CD4+ T-cells [245, 246].

This does not rule out replication in other compartments during suppressive therapy. A recent study found an increase in unspliced HIV-1 RNA in the GALT during raltegravir intensification suggesting that some ongoing replication in this site was inhibited by intensifying treatment [247]. However, another study found no sign of viral evolution in cells isolated from GALT during suppressive therapy [248]. The CNS has also been proposed to be a site where HIV-1 replication can continue, this is discussed further below.

1.5.2 Cells and tissues

The cells that are capable of producing virions during suppressive therapy (often referred to as the reservoir) are of great interest. Presumably it is these cells that need to be targeted and eliminated in order to eradicate HIV-1. Phylogenetic analyses of virions in the plasma during suppressive therapy revealed that the sequences of the plasma-derived virus intermingles with the HIV-1 variants found in a reservoir of infected resting memory CD4+ T-cells. This finding suggests that the source of these plasma-derived virions is infected resting memory CD4+ T-cells [236]. These cells were then presumably infected when they were active but returned to a resting state before they died. These latently infected cells are quite rare ($1/10^6$) but remain during cART with a half-life of at least 44 months which indicates that cART will not clear HIV-1 from an infected individual within their lifetime [249-252]. Some evidence suggests that the stability of this reservoir is maintained by homeostatic proliferation of latently infected CD4+ T-cells driven by IL-7 and IL-15 [253]. Phylogenetic analyses have shown that, in some subjects, the majority of the on-therapy sequences are clonal genetically identical sequences, but these sequences are not found in circulating resting memory CD4+ T-cells [236]. The origin of these clonal sequences is unknown. It could be either a long-lived cell that continues to release virions for a long time during cART or a large population of cells infected by the same variant where individual cells are reactivated and release HIV-1 for a short while before it dies, or a progenitor cell with HIV-1 DNA integrated that divides and where the progeny produce virions for a short while before it dies. The CD34+ hematopoetic progenitor cells have been suggested to be infected with HIV-1 and to serve as a reservoir during suppressive cART possibly the source of this clonal variant [254-256]. But recent reports using very sensitive sorting techniques to ensure that the population of cells analyzed is not contaminated have not found any signs of infection of CD34+ hematopoetic progenitor cells making this less likely [62, 257]. The reservoir is established early during infection which is demonstrated by the fact that subjects who start treatment during the first months of infection nevertheless experience viral rebound if treatment is stopped [258].

1.5.3 Molecular basis of persistence

It is generally believed that HIV-1 latency is established through the infection of CD4 + T-cells that, after HIV-1 DNA is integrated, return to a resting state. Resting memory CD4+ T-cells are not very susceptible to infection [259, 260]. Several mechanisms contribute to maintaining latency on a molecular level:

- One of these mechanisms is the formation of specific nucleosomes. A nucleosome consists of DNA wrapped around histone proteins. Two nucleosomes consistently form within the 5' of the LTR and thereby regulate transcription activity since they overlap with the binding site of many key transcription factors. Maintenance of the nucleosomes is highly dependent on the state of histone aceytlation which is reflected in that histone deacetylase inhibitors (HDACi) lead to remodeling of the nucleosomes which promotes HIV-1 transcription [261, 262]. In addition, HDACi promote transcription by increasing the recruitment of RNA Pol II to HIV-1 LTR [263].
- DNA methylation at the site of HIV-1 transcription initiation is another mechanism by which HIV-1 remains latent and inhibitors of DNA methylation such as aza-Cdr can promote HIV-1 transcription [264, 265].
- The initiation of HIV-1 transcription is dependent on several cellular transcription factors, most notably NF-κB and NFAT, in resting memory cells these transcription factors are not present in the nucleus, they reside in the cytoplasm and thereby also contribute to latency [266].

1.5.4 Persistence in the CNS

Antiretroviral therapy that reduces HIV-1 RNA levels in the plasma also leads to reduced HIV-1 RNA levels in the CSF in most subjects [160, 267]. Up to ten percent of infected individuals suppressed in the blood however continue to have detectable HIV-1 RNA in CSF as measured by standard assays [268]. As CSF HIV-1 RNA levels become suppressed, markers for inflammation of the CNS such as CSF T-cell activation, CSF white blood cell count and CSF neopterin (a marker for macrophage activation) become normalized [267, 269, 270]. Even though CSF neopterin is reduced by cART it remains above what is seen in uninfected individuals, in particular it is elevated in those where CSF HIV-1 RNA is detectable by standard or sensitive assays [271, 272]. Markers for neural damage such as CSF NFL and CSF tau protein are also reduced during suppressive therapy [273]. The beneficial effects of cART on the CNS are seen in large cohort studies as a large reduction in the incidence of HIV-associated dementia in individuals receiving cART [274, 275]. An asymptomatic or mild cognitive impairment could continue to develop even during cART, possibly due to continual low grade inflammation, although this remains unresolved [271, 275, 276]. The degree of mild cognitive impairment seen in some HIV-1 infected individuals is correlated to nadir CD4+ T-cell count which might argue that the damage was done in the past [277].

The CNS is separated from the blood by the blood-brain barrier. Due to their molecular size or hydrophilicity some ARVs do not penetrate the CNS very well. This has raised the suspicion that the CNS might be a "sanctuary site" where HIV-1 can continue to replicate despite cART. Letendre and colleagues have proposed a system to estimate

how well the different ARVs penetrate into the CNS; the CNS penetration effectiveness (CPE) scoring system (**Table 4**) The efficacy of the treatment regimen is then calculated by adding the CPE-score of each drug in the treatment regimen [278]. It is not clear if treatment regimens with a higher CPE lead to a more successful suppression of CSF HIV-1 RNA levels [268, 279, 280]. Neither is it established that higher CPE scores translate into an improved neurocognitive outcome [280-282].

Table 4. CNS penetration effectiveness (CPE) scoring system adapted from [177].

Increasing CNS penetration →	**0**	**0.5**	**1**
Nucleoside reverse transcriptase inhibitors	Didanosine	Emtricitabine	Abacavir
	Tenofovir	Lamivudine	Zidovudine
	Zalcitabine	Stavudine	
	Adefovir		
Nonnucleoside reverse transcriptase inhibitors		Efavirenz	Delavirdine
			Nevirapine
Protease inhibitors	Nelfinavir	Amprenavir	Amprenavir/r
	Ritonavir	Atazanavir	Atazanavir/r
	Saquinavir	Fosamprenavir	Fosamprenavir/r
	Saquinavir/r	Indinavir	Indinavir/r
	Tipranavir/r		Lopinavir/r
Entry inhibitors	Enfuvirtide		Maraviroc
	T-1249		Vicriviroc
Integrase inhibitors		Raltegravir	
		Elvitegravir	

1.5.5 Eradication strategies

In 2009 it was reported that a man had seemingly been cured of his HIV-1 infection [283]. He was doing well on suppressive therapy when he developed acute myeloic leukemia for which he received myeloablative treatment and allogenic stem cell transplantation from an individual homozygous for a deletion in the gene that encodes for CCR5. This lead to the complete remission of his leukemia and when his cART was discontinued HIV-1 RNA levels did not increase in blood. Even after five years without therapy he did not have any detectable virus in the plasma by standard assays [284]. Using several very sensitive assays it was, however, possible to detect HIV-1 RNA in plasma and HIV-1 DNA in the rectum, although at levels much lower than seen in subjects on suppressive therapy. These very low levels of HIV-1 RNA and DNA could also possibly have been the result of contamination in the laboratory [285]. Since he remains disease free and HIV-1 RNA levels have not increased in his blood this can nevertheless be considered to be the first example of a functional cure of HIV-1 (functional meaning that not all HIV-1 has been eradicated but HIV-1 RNA levels do not increase despite the discontinuation of cART). Follow-up studies have shown that myeolablative treatment followed by autologuos stem cell transplantation is not enough to achieve a cure [286]. Myeolablative treatment with allogenic stem cell transplantation from wild type CCR5 donors without the discontinuation of cART did however lead to undetectable HIV-1 RNA and DNA in PBMCs, CD4+ T cells and plasma, in the two subjects studied [287]. Recently it was also reported at the 7th International AIDS society's conference on HIV pathogenesis, treatment and

prevention in Kuala Lumpur, Malysia, that these two individuals had now stopped cART for several weeks without experiencing an increase in HIV-1 RNA levels in their plasma. Myeloablative treatment is associated with severe side effects and risks and other options need to be explored for eradicating HIV-1. Current HIV-1 therapy is life-long, costly and not without side effects so a cure would be valuable from both an individual perspective as well as from a public health perspective.

Most HIV-1 eradication strategies are based on the hypothesis that suppressive therapy prevents infection of new cells but does not cure HIV-1 since it remains latent in long-lived cells. These cells produce low amounts of virions that cause viral rebound when treatment is stopped. Therefore, all latently infected cells capable of producing infectious virions need to be eliminated. By reactivating all the cells that are latently infected it is presumed that they would die, either from the cytopathic effect of viral replication or from the immune response directed against them. If this is done during suppressive therapy no new cells would be infected. Global T-cell activation by IL-2 and CD3 antibodies without the discontinuation of cART have been tried but were associated with severe side effects and failed to deplete the HIV-1 reservoir [288-292]. A possible explanation for why this strategy failed is that it caused clonal expansion of latently infected CD4+ T-cells of which a small fraction was not killed and were able to return to a resting state.

HDACi have been studied intensely, with the hope that they would more selectively reactivate latent HIV-1, and initially it was reported that valproic acid, a weak HDACi, lead to a modest decrease in the reservoir size [293]. Follow-up studies did however show that this was not a durable effect [294, 295]. A recent trial with the more potent HDACi, vorinostat without the discontinuation of cART, showed an increase in cell associated HIV-1 RNA in CD4 + T-cells as a marker for increased transcription but did not lead to an increase in plasma HIV-1 RNA levels measured with a sensitive assay [296]. Other studies with more potent HDACi:s are ongoing.

Screening in cell lines has found other compounds that reactivate HIV-1 without global T-cell activation. One such compound called disulfiram, which is approved for the treatment of alcoholism, reactivates HIV-1 and a clinical trial of this compound is ongoing [297, 298]. Another substance that has been shown to lead to increased levels of HIV-1 RNA in plasma during suppressive therapy as a sign of reactivation of HIV-1 is intravenously administered immunoglobulin [299].

It is uncertain if reactivation of latent HIV-1 is enough to kill the latently infected cells and eliminate the reservoir. A dual approach where a therapeutic vaccination where cytotoxic T-cells are stimulated with an HIV-1 antigen before reactivation of latent HIV-1 to ensure killing of reactivated infected cells have been shown to be more effective *in vitro* and maybe a combination of therapeutic vaccination and reactivation will also be needed *in vivo* [300].

1.5.6 Measuring the reservoir

A challenge with trials for HIV-1 eradication is how to determine if the virus has been eradicated. New compounds can first be tested *in vitro* in cell lines where HIV-1 DNA is integrated [301]. If these new compounds reactivate HIV-1 *in vitro* and are shown to be safe to use *in vivo* then their effectiveness must be tested in clinical trials. The ultimate end-point is the cessation of cART without an increase of HIV-1 RNA levels in plasma. However secondary outcome measurements are also needed since cessation of therapy is not always feasible and may be associated with risks such as the development of drug resistance. It is also necessary to estimate the relative effectiveness of compounds that are partially effective in order to choose which strategies to pursue. Several approaches have been developed to measure the size of the reservoir and the degree of reactivation. What is considered to be the gold standard is the viral outgrowth assay. In this assay resting CD4+ T-cells are, using a limiting dilution approach, co-cultured with PBMCs during global T-cell activation. HIV-1 antigen p24 can then be detected where latent HIV-1 was reactivated. Thereby, it is possible to determine the frequency of latently infected cells able to produce infectious virions [302]. Although a simplified version of this method was recently developed it is still relatively costly and time consuming [303]. A more feasible method to measure the size of the reservoir is to measure proviral HIV-1 DNA in a quantified number of resting CD4+ T-cells which can be done using various methods [304, 305]. Unfortunately these methods do not correlate well to the outhgrowth assay [306]. In general they are showing >100 fold higher infection frequencies although this relationship is highly variable. This lack of correlation between the measurement of integrated HIV-1 DNA and the outgrowth assay may be due to the fact that most latently infected cells are not able to produce infectious virions since the integrated HIV-1 DNA are hypermutants, or contain lethal insertions or deletions. In addition, the outgrowth assay correlates poorly to measurement of low levels of HIV-1 RNA in the plasma using the single-copy assay (SCA) [306]. Therefore the viral outgrowth assay is still the best assay to measure the effect of eradication efforts but since reactivation leads to transcription of HIV-1 RNA and production of virions SCA and other sensitive methods for HIV-1 RNA quantification can be valuable in measuring the degree of reactivation.

2 AIM

To characterize HIV-1 infection of the CNS during suppressive therapy.

Specific aims:

Paper I: To study how well elite controllers control HIV-1 infection of the central nervous system (CNS) by measuring HIV-1 RNA levels in the cerebrospinal fluid (CSF).

Paper II: To measure CSF and plasma HIV-1 RNA levels in subjects on suppressive therapy and to determine if the occurrence of HIV-1 in CSF during suppressive therapy is correlated to inflammation in the CNS.

Paper III: To determine if the HIV-1 populations in CSF and plasma are genetically distinct during suppressive therapy indicating that they were produced by different populations of cells.

Paper IV: To examine if the HIV-1 RNA levels in CSF during suppressive cART are reduced by treatment intensification with raltegravir.

3 MATERIALS AND METHODS

3.1 MATERIALS

In **paper I** we analyzed 28 CSF samples and 27 concurrent plasma samples from 14 subjects. The subjects had been classified as elite controllers since they, over a period of more than 12 months, had had three or more longitudinal plasma HIV-1 RNA determinations <40 copies/mL in the absence of cART. These samples were obtained from observational studies at the San Francisco General Hospital, University of California San Francisco (UCSF), San Francisco, USA.

In **paper II** we analyzed 76 pairs of CSF and plasma samples from 45 HIV-1 infected subjects with <40 copies of HIV-1 RNA/mL in both CSF and plasma. These samples were obtained from observational studies at the Sahlgrenska University Hospital, Gothenburg, Sweden and San Francisco General Hospital, UCSF, San Francisco, USA.

In **paper III** we analyzed 70 CSF and 29 plasma samples and the corresponding pre-therapy samples from 17 subjects on suppressive therapy. Twelve subjects had started treatment during chronic infection and five during early infection (a median of 61 days after the estimated day of infection). These samples were obtained from observational studies at the Sahlgrenska University Hospital, Gothenburg, Sweden and San Francisco General Hospital, UCSF, San Francisco, USA.

In **paper IV** we analyzed 63 paired CSF and plasma samples from 17 subjects. Samples were obtained from subjects enrolled in a clinical trial at San Francisco General Hospital, UCSF, San Francisco, USA. This was an open label 1:1 randomized clinical trial where subjects on suppressive cART were randomized to receive either placebo or the integrase inhibitor raltegravir for 12 weeks. Those randomized to receive placebo were offered to roll over to the raltegravir group after 12 weeks.

3.2 ETHICAL CONSIDERATIONS

All subjects participating in these studies gave written informed consent. The studies were approved by the regional ethical review board in Stockholm and Gothenburg, Sweden and by the institutional review board at UCSF, San Francisco, USA.

3.3 LABORATORY METHODS

3.3.1 Single-copy assay

In **papers I, II** and **IV** we used the single-copy assay (SCA) to measure HIV-1 RNA levels in CSF and plasma (**Figure 7**). The sensitivity of assays used in clinical practice has continuously increased as new assays have been developed. Currently the lower-limit of detection for clinical assays varies between 20-50 copies of HIV-1 RNA/mL. SCA was developed to study HIV-1 RNA concentrations in the range below this and can quantify HIV-1 RNA levels down to 0.3 copies of HIV-1 RNA/mL using a starting volume of 7 mL [307].

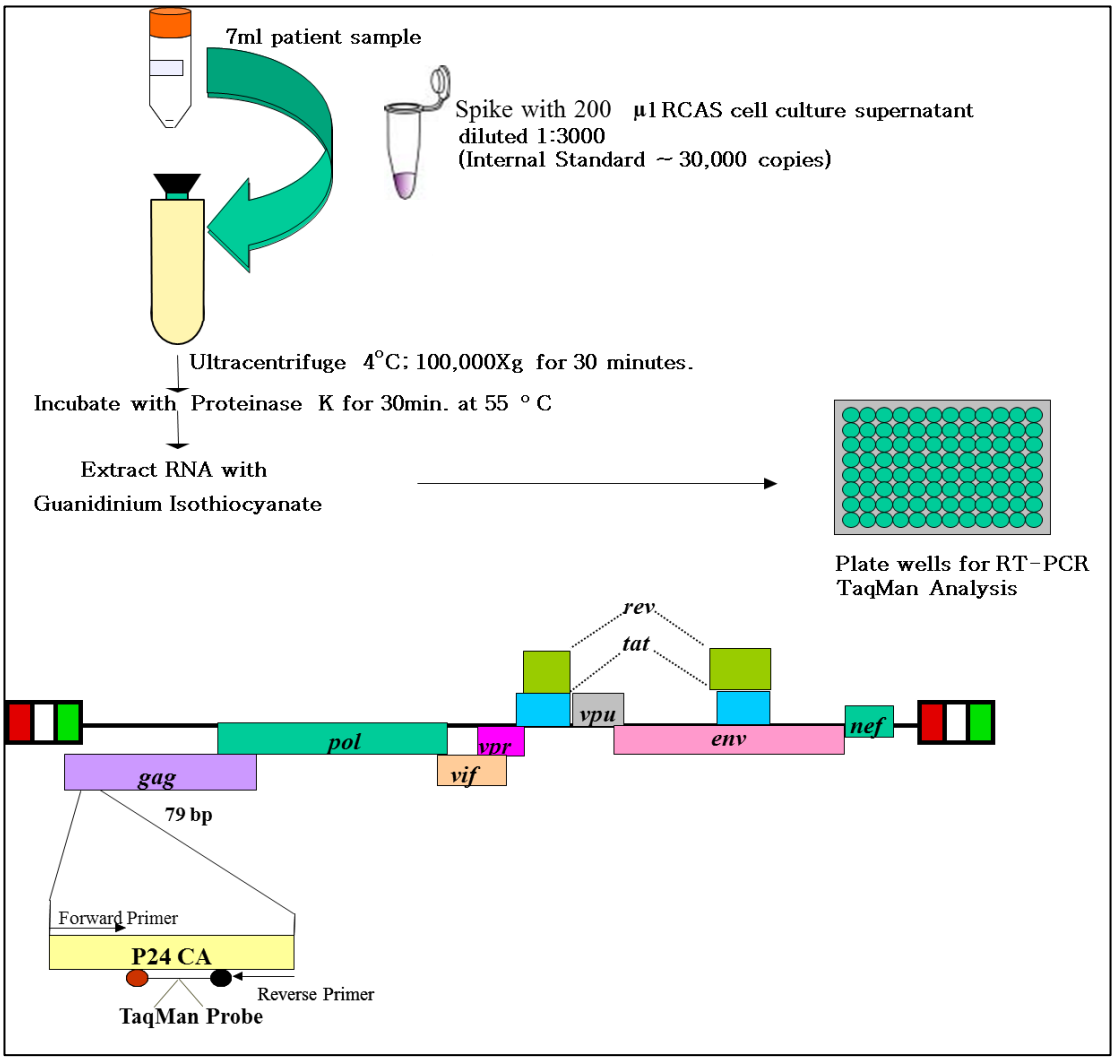

Figure 7. Single-copy assay.

Samples are initially spiked with a known quantity of replication-competent avian sarcoma leukosis virus long terminal repeat with a splice acceptor (RCAS) which is a replication competent vector derived from an avian retrovirus. This is used as internal standard to ensure that the extraction process is successful. After adding the internal standard the samples are subjected to a pre-spin for 10 minutes to remove cellular debris that might interfere with the PCR-reaction. The samples are then ultra-centrifuged at > 100 000 x g for 30 minutes to spin down the virions. The supernatant is removed and proteinase K is added to dissolve the virion. RNA is extracted with guandinium isothyocynate and glycogen. Isopropanolol is added and the lysate is centrifuged to create a pellet. The supernatant is removed and the pellet is cleaned with ethanol to remove any remaining isopropanolol since it inhibits PCR. The extracted nucleic acids were dissolved in 5 mM Tris-HCl containing dithiothretiol and an RNase inhibitor to prevent degradation. The samples are placed on a 96-well plate together with samples with known RCAS and HIV-1 concentrations to be used as standard curves. Each sample is distributed over six wells, HIV-1 RNA is quantified in triplicate, RCAS is quantified in duplicate and one well is analyzed for HIV-1 DNA by using a mix that does not contain RT during cDNA-synthesis. This setup makes it possible to analyze eight samples per plate of which two are the negative and positive controls.

The entire plate is subjected to cDNA-synthesis. After that a second mix that contains specific primers and probe for a conserved fragment of the *gag* region of HIV-1 and second set of primers and probe for the *gag* region of RCAS. The plate is then subjected to real-time PCR. Samples where less than half of the added amount of RCAS is detected, either due to poor extraction or interference with the PCR, are considered a failure and excluded from further analysis. A mean of the three wells per sample for HIV-1RNA quantification is calculated and if HIV-1 DNA is detected that amount is subtracted since measurement for HIV-1 RNA also will include HIV-1 DNA if present [307].

To be able to calculate and compare median HIV-1 RNA levels in **paper I, II** and **IV** we attributed a value for negative samples that was 0.1 below the lower limit of detection for that sample. The lower-limit of detection varied depending on the starting volume [307].

The SCA was carefully validated when developed [308]. We continuously monitored the sensitivity and precision of the assay by measuring the lower end of the standard curve for HIV-1. This was three samples that contained 3, 1 and 0.3 copies/mL respectively (**Table 5**). These standard curve-derived samples were measured in duplicate and not triplicate as clinical samples which likely contributed to a higher standard deviation (SD) than what was seen during the first validation of this assay [307].

Table 5. Precision and sensitivity of SCA.

Estimated concentration (HIV-1 RNA/mL)	**Mean measured concentration*** (HIV-1 RNA/mL, SD)	**Sensitivity** (% positive samples)
0.3	0.5 (1)	30%
0.78	*0.8 (0.7)*	
1	2 (7.8)	52%
1.56	*2.5 (2.4)*	
3	3.2 (2.9)	94%
3.125	*3.5 (1.3)*	

*Data from 94 runs of SCA where the standarad curve was further diluted down to 0.3 copies/mL. Samples containing 3, 1 and 0.3 copies/mL were then analyzed in duplicate as internal control. Data in *italics* is included as comparison and were generated during the first validation of SCA (ref) by analyzing by ten runs of analyzing a diluted sample with known HIV-1 RNA concentraion, samples were analyzed in triplicate.

3.3.2 Single-genome sequencing

In **paper III** we used single-genome sequencing (SGS) to analyze the HIV-1 populations in CSF and plasma (**Figure 8**). The advantage with SGS is that PCR re-sampling and PCR induced recombination can be avoided, and it allows for the detection of minor variants. We analyzed CSF and plasma samples obtained before therapy was initiated or just after therapy was initiated but before suppression was achieved (hereafter referred to as pre-suppression) and compared these pre-suppression sequences to sequences from samples obtained during suppressive therapy. The SGS procedure used in this thesis was developed by Palmer and co-workers [308, 309]. In conducting this assay the samples are extracted according to the same procedure

described for SCA for samples containing <50 copies of HIV-1 RNA/mL. For samples with a higher HIV-1 RNA concentrations we used a similar approach with the exception that microcentrifugation was used instead of ultra-centrifugation to concentrate virions. After extraction cDNA is generated using random primers. The samples containing cDNA are then diluted and distributed over a 96-well plate so that less than 30% of the wells are positive after nested PCR for a 1.3 kb fragment (p6 through nucleotide 1-900 of RT). Based on Poisson statistics the positive wells only contained one HIV-1 DNA molecule 80% of the time [308, 309]. Generated amplicons were then sent for sequencing.

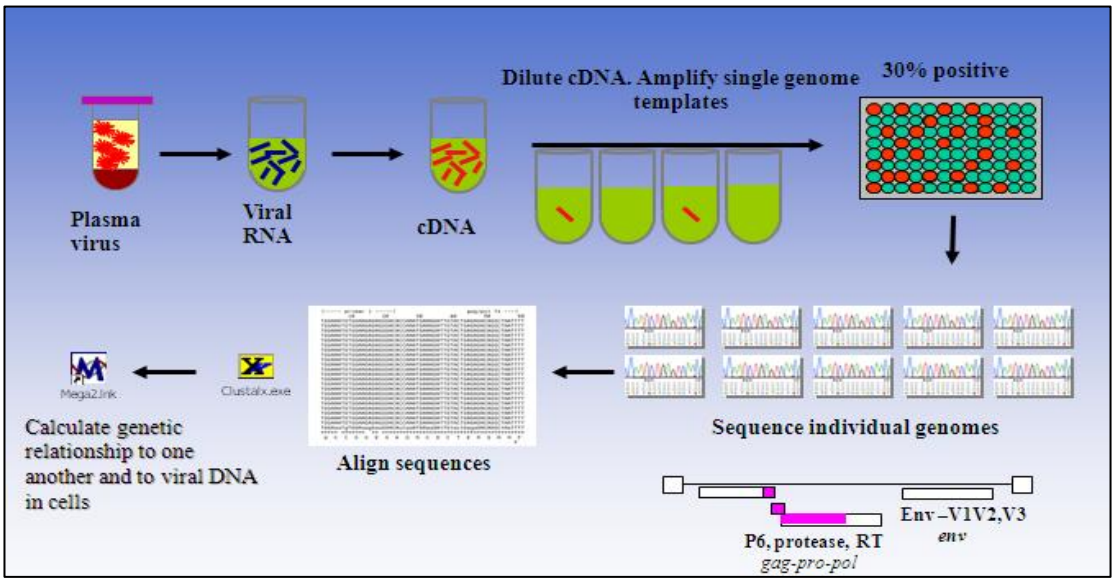

Figure 8. Single-genome sequencing. Viral RNA is extracted and converted to cDNA. The sample is then diluted and distributed over a 96 well plate until less than 30% of the wells yield a product after nested PCR to ensure that a maximum of one HIV-1 DNA molecule was present in each well. The amplified product is then sequenced and phylogenetic analyses can take place.

3.3.3 Sequences analyses

For **paper III** the raw sequence data were assembled using an in house Perl script. Alignments were constructed using MAFFT version 6.0 (http://mafft.cbrc.jp/alignment/software/). We used several strategies to ensure a high sequence quality.

1. Contigs were required to have a bidirectional sequence reads of at least 1.1 kb.
2. Contigs containing more than one single-nucleotide polymorphism were removed since this indicated amplification of more than one single cDNA template.
3. Contigs were scanned by eye and trimmed and edited when deemed necessary.

Before we continued with further analyses we constructed an alignment with all sequences and created a neighbor-joining tree to make sure that sequences from each subject clustered with each other, which would be an indication of contamination or mislabeling.

We scanned the alignments for hypermutants using the on-line tool "Hypermut" (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html) [310]. We

compared the proportion of hypermutants in CSF and plasma and then the hypermutants were removed from the alignments. Alignments were tested for recombinants using Splitstree4 (http://www.splitstree.org/) and recombinants were removed [311].

We used the Slatkin-Maddison method as implemented in HyPhy version 2.1.2 to test for compartmentalization in pre-suppression samples (http://hyphy.org/) [312]. Ten thousand permutations were performed and p-values <0.05 were considered significant evidence for compartmentalization.

To determine the relationship of pre-suppression and on-therapy CSF and plasma sequences for each subject we constructed phylogenetic trees. Maximum likelihood trees were constructed using PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) [313]. The model of evolution was selected using the Find model tool on Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov/content/index). Statistical support of the tree structures was obtained by 200 bootstrap replicates. Bootstrap values over 90% (180/200) were considered significant. Trees were rooted with a pre-suppression plasma sequence.

HIV-1 genetic diversity (average pairwise distance, APD) was calculated using the p-distance model in MEGA5.0 (www.megasoftware.net) [314].

3.3.4 Markers for immune activation

CSF white blood cell counts and differential and blood CD4+ T-cell analysis were all performed in the Sahlgrenska Hospital in Gothenburg or the San Francisco General Hospital Clinical Laboratories using standard clinical methods. Neopterin was measured in cell-free CSF by enzyme-linked immunoassay according to the manufacturer's instructions (BRAHMS Aktiengesellschaft, Henningsdorf, Germany) in Innsbruck, Austria. Normal reference values were <5.8 nmol/L in CSF [315].

3.3.5 T-Cell activation by flow cytometry

CSF and blood CD41+ and CD81+ T-cell activation were assessed by the percentage of cells in fresh specimens co-expressing surface CD38 and human leukocyte antigen (HLA)–DR or CCR5 as described previously [270, 316]. Flow cytometry data were compensated and analyzed with FlowJo (Tree Star) software version 8.8 in San Francisco, USA.

3.3.6 Neurological evaluations

Neurological performance was monitored using four brief quantitative tests (timed gait, grooved pegboard, finger tapping, and digit symbol) to obtain a simple aggregate quantitative neurological performance z score (QNPZ-4) [317, 318].

3.3.7 Statistical methods

Means, SD, median and intra-quartile range (IQR) were calculated. Variables were compared by Mann-Whitney's test, an unpaired nonparametric t-test. Proportions were compared using Fisher exact test. All p-values were 2-sided with values <0.05 considered significant. Statistical analyses were performed using Excel (Microsoft) and GraphPad Prism 6 (GraphPad). Graphs were constructed using GraphPad Prism 6 (GraphPad).

4 RESULTS AND DISCUSSION

In **paper I** we employed SCA to measure the HIV-1 RNA levels in CSF and plasma of elite controllers and found that HIV-1 RNA can be detected in the 19% of the CSF samples which was significantly less than the 54% of the plasma samples that were positive. The levels of HIV-1 RNA were also significantly lower in CSF than in plasma.

We analyzed 28 CSF samples and 27 concurrent plasma samples from 14 elite controllers. Three samples were excluded because of internal standard failure. HIV-1 RNA was detected in only 5 of 26 CSF samples compared to 14 of 26 plasma samples (P=0.02). The median levels were 0.2 (range 0.1-6) copies/mL in CSF compared to 0.8 (range 0.1-189) in plasma (P<0.0001).

A previous study, using an assay with a lower-level of detection of 2.5 copies/mL, showed that none of the eight elite controllers examined had detectable CSF HIV-1 RNA and the level of intrathecal immune activation was comparable to what is seen in those that are not infected by HIV-1 or HIV-1 infected subjects on suppressive therapy [319]. In contrast to the previous report could we detect low levels of CSF HIV-1 RNA in 5 of 14 elite controllers making our study the first, to our knowledge, to detect HIV-1 RNA in the CSF of elite controllers. That the proportion of samples where CSF HIV-1 RNA could be detected and the levels measured in the CSF are significantly lower than in plasma is similar to what we and others have found in untreated and treated HIV-1 infected subjects (**paper II, paper IV** [159, 160, 163]). Whether the infrequent and low amounts of HIV-1 in the CSF reflect production within the CNS or virion exchange between CSF and the blood is uncertain. That HIV-1 RNA could be detected in plasma of most elite controllers with sensitive methods is comparable to what other studies have shown [147]. It has been shown that HIV-1 in the blood of elite controllers can develop new immune escape mutations suggesting that source of the virions found, at least to some extent, must be ongoing replication with infection of new cells and production of virions from these cells [151].

A weakness of this study was that we did not ensure the primers and probe matched the viral strains by sequencing. In a previous report where this was done a primer or probe mismatch that was considered to be significant was found in 11% of the 62 elite controllers from where it was possible to obtain a sequence [147]. Our reported numbers could therefore underestimate the prevalence of HIV-1 RNA in CSF and plasma.

In **paper II** we employed SCA to measure the HIV-1 RNA levels in paired CSF and plasma samples from 45 subjects on suppressive therapy. We found that 17% of the CSF samples contained low levels of HIV-1 RNA, which was significantly less than the 57% of the plasma samples where HIV-1 RNA could be detected. CSF HIV-1 RNA could be detected even after 10 years of suppressive therapy. The occurrence of CSF HIV-1 RNA was correlated to elevated levels of CSF neopterin, a marker for intrathecal immune activation.

We analyzed a total of 76 pairs of samples (7 mL from each sample) from CSF and plasma from 45 subjects, including 18 with longitudinal samples. The extraction control failed for 14 of the 152 samples (8 plasma and 6 CSF); and they were excluded

from further analysis. Twelve of the 70 (17%) CSF samples were positive for HIV-1 RNA which was significantly less than the 39 of 68 (57%) plasma samples that were positive (P=<0.0001). The median CSF HIV-1 RNA levels was 0.2 copies/mL (range 0.2-3.9 IQR 0.2-0.2) compared to 0.3 copies/mL (range 0.2-15, IQR 0.2-0.9) in plasma (P=<0.0001).

The significantly lower levels of HIV-1 RNA in CSF than in plasma is similar to the relationship which is seen during untreated HIV-1 infection where the levels of CSF HIV-1 RNA is usually ten-fold lower than in plasma although this relationship varies substantially between individuals [320]. The proportion of positive samples was similar to those that were seen in **paper IV** where 14% of the CSF samples and 64% of the plasma samples were positive. Previously it has been reported that on-therapy plasma HIV-1 RNA levels are correlated to pre-therapy plasma HIV-1 RNA levels [229].We did not find any correlation between the occurrence of CSF HIV-1 RNA and the pre-therapy CSF or plasma HIV-1 RNA levels (P=0.41 and P=0.60). In addition, we did not find any correlation with between detectable plasma HIV-1 RNA and pre-therapy plasma HIV-1 RNA levels (P= 0.91). CSF HIV-1 RNA could be detected even after 10 years of suppressive therapy and plasma HIV-1 RNA up to 11 years of suppressive therapy. That HIV-1 RNA can be detected even after years of suppressive therapy is consistent with a previous report were plasma HIV-1 RNA could be detected after 7 years of suppressive therapy [228].

A previous study has demonstrated that neopterin (a marker for macrophage activation) is elevated in the CSF of HIV-1 infected subjects even after years of suppressive therapy [271]. Furthermore it has been demonstrated that subjects on suppressive therapy with "CSF-escape" (>50 copies/mL in CSF while <50 copies/mL in plasma) have elevated levels of CSF neopterin compared to those with <50 copies/mL (a median of 9.2 nmol/L vs 5.1 nmol/L). In subjects with < 50 copies/mL in both CSF and plasma those with >2 copies/mL in the CSF also have elevated levels of CSF neopterin compared to those <2 copies/mL [272]. In agreement with these earlier findings, we found that CSF neopterin levels were higher (mean 7.4 compared to 5.8 nmol/L) in time points with detectable HIV-1 RNA in CSF (P=0.03). These findings suggest that even very low levels of HIV-1 in CSF during suppressive therapy are correlated to intrathecal inflammation.

It has been suggested that the CNS is a "sanctuary site" where HIV-1 can continue to replicate during suppressive cART (plasma levels <20-50 copies/mL) since some ARVs are less effective in crossing the blood-brain barrier. The CNS penetration effectiveness (CPE) score was developed by Letendre and colleagues to calculate a rating of the estimated effect in CNS for each regimen [279, 321]. We did not find any difference in the CPE between time points with detectable CSF HIV-1 RNA to time points without detectable CSF HIV-1 RNA (P=0.59). This suggests that CPE is not a good measurement of the efficacy of a particular treatment regimen within the CNS or that there is no, or very little, ongoing replication in the CNS during therapy that is suppressive in both the CSF and plasma regardless of the CPE of the treatment regimen used.

One limitation of our study was that we did not have access to pre-therapy samples to test for primer and probe mismatch neither did we ensure this by sequencing. Our HIV-1 RNA determinations are therefore likely to be an underestimate since there was likely a primer/probe mismatch in some subjects.

In **paper III** we sequenced the HIV-1 populations in CSF and plasma during suppressive therapy. We found that a large fraction of CSF HIV-1 variants during suppressive therapy are replication-incompetent hypermutants. In one of the two subjects where we were able to obtain sequences from both CSF and plasma we found genetically distinct variants in CSF and plasma.

Seventy CSF samples with viral RNA levels <40 copies/mL were selected for analysis (median volume 8 mL). From 15 of these 70 CSF samples we generated 21 amplicons. These 15 samples were collected from 8 of the 17 subjects in the study. Next we analyzed 29 on-therapy plasma samples (median volume 7 mL, range 5-14 mL) from these 8 subjects. From the 29 on-therapy plasma samples we generated 14 amplicons. We also analyzed plasma and CSF samples collected before or immediately after therapy initiation (pre-suppression samples).

Sequence analyses revealed that 8 of 21 sequences obtained from the CSF during suppressive therapy were hypermutants compared to 0 of 13 sequences from plasma (P=0.013). Hypermutants are induced by a family of cellular cytidine deaminase referred to as the APOBEC-family and result in replication incompetent viral strains [322]. The high level of HIV-1 hypermutants in the CSF could possibly be explained by these virions being produced by a population of cells in the CNS more likely to produce hypermutants due to a high content of APOBEC. This finding should be interpreted with some caution due to the small number of subjects and due to that 6/13 of the hypermutants found in CSF came from one subject. Furthermore, we had fewer on-therapy plasma-derived sequences than CSF-derived sequences (14 versus 23).

In two of the subjects (7027 and 9058) where we generated sequences from both CSF and plasma during suppressive therapy, the on-therapy sequences from CSF and plasma did not cluster together. For subject 7027, CSF and plasma sequences from the same time point were separated by branches with significant bootstrap support (**Figure 9**). This is consistent with the hypothesis that these virions were produced by cells within the CNS and cells in the periphery (either the same or different type of cell) that had been infected with genetically distinct viral variants. But it is not conclusive evidence; it could also be explained by a population of cells in the periphery being infected with genetically distinct viruses producing virions of which some were transported into the CSF. In the subjects where we had on-therapy CSF sequences from several time points these sequences did not cluster together and in some cases they were separated by branches with significant bootstrap support. Furthermore these sequences were not found on longer branches. The lack of evolution among on-therapy CSF sequences compared to pre-suppression sequences is consistent with what other studies have shown for blood and lymphatic tissue [233, 236-239]. These findings are consistent with the hypothesis that the virions found in CSF or plasma during therapy are produced by cells which had been infected before the initiation of cART and are reactivated during cART and then produce virions for a short period of time.

This study had limitations. Notably, despite the analysis of a large number of CSF samples obtained during suppressive therapy we were unable to generate many sequences for phylogenetic analysis. This was due to the low levels of HIV-1 RNA in CSF during suppressive therapy and to the unexpected high amount of hypermutants. This, in itself is an interesting finding, unfortunately prevented an in-depth phylogenetic analysis.

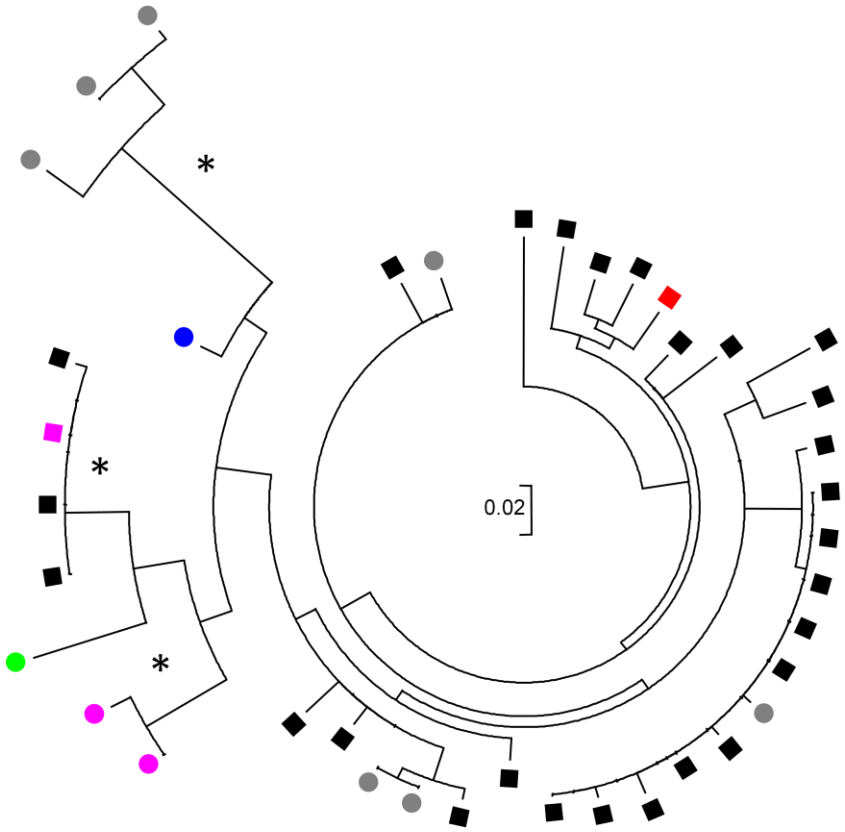

Figure 9. Maximum likliehood tree made in PhyML 3.0 under the GTR+gamma nucleotide subsitution model. Sequences from plasma are represented by squares and sequences from CSF are represented by circles. The tree was rooted with a pre-suppression plasma sequence. Pre-suppression sequences are represented by black symbols for plasma and grey symbols for CSF. Longitudinal sequences obtained during full suppression are represented by colored symbols: time point 1 red; time point 2 green; time point 3 blue, and time point 4 purple. Branch support was calculated by 200 boot-strap replicates. Branches with a boot-strap support of 180 or more are marked with a star.

In **paper IV** we found that treatment intensification with raltegravir did not affect the CSF HIV-1 levels or intrathecal immune activation in subjects whose treatment was intensified.

When the HIV-1 RNA measurements for all the CSF and plasma samples from every phase of the clinical trial were combined we found that HIV-1 RNA could be detected in 14% (8/56) CSF samples compared to 64% (32/50) of the plasma samples (P<0.001). Median for all CSF samples was 0.2 copies/mL (IQR 0.2-0.4) whereas median for plasma samples was 0.9 copies/mL (IQR 0.3-2.2 copies/mL) (P<0.0001). Both the proportion of positive samples and the median concentrations in each fluid were comparable to what was reported in **paper II**.

Treatment intensification did not reduce the proportion CSF samples where HIV-1 RNA could be detected: in the group without treatment intensification the proportion was 2 of 15 and in the group with treatment intensification it was 5 of 23 (P=0.69). Treatment intensification did not affect the level of CSF neopterin (P=0.45). The lack of effect on CSF HIV-1 RNA concentrations and CSF neopterin by treatment intensification is comparable to what was seen in another study using either enfurvitide, maraviroc or lopinavir/ritonavir to intensify treatment over a shorter period of time (8 weeks) and using a different assay for HIV-1 RNA quantification [323].

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In order to direct future attempts to eradicate HIV-1 it is important to clarify in which cells and in which parts of the body HIV-1 persists during suppressive therapy. It is also of interest to determine if there is ongoing infection of new cells during suppressive therapy since many eradication efforts are trying to achieve reactivation of all latent HIV-1 during cART with the assumption that cART will block infection of new cells. It has been suggested that the CNS could harbor latently infected cells during suppressive therapy. It has also been proposed that there is ongoing replication within the CNS during suppressive therapy since some ARVs are less effective in crossing the blood-brain barrier. In this thesis we have studied HIV-1 in the CSF of elite controllers and subjects on suppressive therapy to further characterize HIV-1 infection of the CNS. One general caveat of these studies is that they have been conducted on CSF since it is more accessible than cells from the CNS. It should however be noted that what is seen in the CSF does not necessarily always accurately reflect processes in the CNS.

Since elite controllers may be a model for a functional cure for HIV-1, we studied how they control HIV-1 infection of the CNS. We found that most elite controllers control HIV-1 infection of the CNS well with no, or very little, HIV-1 RNA present in the CSF. Contrary to previous reports, we found low levels of HIV-1 RNA in the CSF in some subjects demonstrating that cells in this compartment are infected, or can be at risk for infection since there occasionally is HIV-1 present in the CSF. Recent studies have indicated that it might be possible to induce elite control by the early initiation of cART [324, 325]. With the caveat that we studied "spontaneous elite controllers" and not "post-treatment elite controllers" the induction of elite control might be a possible strategy for a functional cure. Whether the HIV-1 RNA found in the CSF of elite controllers comes from virions produced by cells in the CNS or if it represents infected cells that have migrated to the CNS from the periphery remains unknown. Future studies should aim for sequencing the HIV-1 in CSF and plasma of elite controllers to determine the source of these virions, though this will require large volumes of plasma. It would also be of interest to study if "post-treatment elite controllers" are different than"spontaneous elite controllers".

We have also found that very low levels of HIV-1 can be detected in the CSF of subjects on suppressive therapy although at a lower frequency and lower levels than what is seen in the plasma (**paper II, paper IV**) and this is consistent with previous reports [272, 323]. We found that the occurrence of CSF HIV-1 RNA is correlated to intrathecal immune activation measured by CSF neopterin. If this CSF immune activation is shown to be correlated to the development of cognitive impairment other treatment strategies will need to be investigated. Since there does not seem to be any ongoing replication in the CNS during suppressive therapy it would not be meaningful to develop more potent ARVs, instead strategies to specifically decrease the inflammation in the CNS during cART should be explored.

It remains unclear whether the virions found in CSF during suppressive therapy are produced in the CNS. We found one example of a subject that had genetically distinct variants in CSF and plasma during suppressive therapy. This is consistent with the

production of virions by cells within the CNS that had been infected by a genetically distinct HIV-1 population compared to the population that infected cells in the periphery but it is not conclusive evidence. It could also be due to the production of virions by cells in the periphery that had been infected by genetically distinct variants. Then some virions were transported into the CSF by chance and found there giving the impression of compartmentalization. If the compartmentalization between CSF and plasma was found in other subjects it would, however, support the hypothesis of virion production in the CNS during suppressive therapy. Future studies should try to generate more sequences by analyzing more samples with larger volume to further investigate the CSF HIV-1 population on-therapy. Currently there is a loss in sensitivity for SGS when one tries to amplify longer fragments. If future techniques allows for sequencing longer fragments without losing sensitivity this should be considered in order to generate a stronger phylogenetic signal to achieve a better phylogenetic resolution. Other assays to determine the origin of virions such as characterizing cellular markers on the envelope of the virion could also be explored to further elucidate the question of the origin of HIV-1 virions found in CSF during suppressive therapy. It would also be of interest to analyze cells from the CNS of HIV-1 infected subjects on suppressive therapy who died from other causes. Then it would be possible to compare the sequences from infected cells in the CNS (if there are any) to the sequences from virions found in CSF and plasma during suppressive therapy to determine their origin.

If cells in the CNS produced virions during suppressive therapy this would mean that these cells would also need to be targeted in order to achieve a functional cure for HIV-1. The first subject to have been reported to have been functionally cured from HIV-1 did not have any HIV-1 RNA detectable in the CSF, even by single-copy assay, at follow up examinations [285]. Possibly he had had infected cells in the CNS that were eradicated during the treatment of his leukemia. When testing other compounds to eradicate HIV-1 one might need to take the CNS into account, either by designing drugs that penetrate well across the blood-brain barrier or by also administering them intrathecally, otherwise the infection might rebound from virions produced in the CNS.

We found that a large fraction of the HIV-1 found in the CSF during suppressive therapy is replication incompetent hypermutants. This finding should be interpreted with some caution since many of the hypermutants in our study were found in one subject. It could, however, be due to cells in the CNS, or cells that migrate into the CNS, contain more APOBEC than most other cells. We have also observed a lot of hypermutants in cells from the periphery in subjects on suppressive therapy (unpublished data) and this has also been reported by others [326]. Why a lot of HIV-1 variants found during suppressive therapy are hypermutants is unclear. Perhaps cells containing replication competent virus are more likely to be cleared and therefore cells containing hypermutants accumulate over time.

We did not observe any signs of evolution among CSF sequences found during suppressive therapy compared to pre-suppression sequences. This is consistent with what most studies have observed in blood and lymphatic tissue [233, 236-239]. The level of HIV-1 RNA in the CSF was not affected by treatment intensification. This is consistent with what most studies have reported for HIV-1 RNA in plasma and with the findings of one previous study on CSF [240-243, 323]. The lack of effect by treatment intensification and the absence of viral evolution do however not rule out the possibility of ongoing replication. The power to detect evolution in the CSF was limited by the number of sequences found and future studies should try to generate more sequences by analyzing more samples with larger volume. Also, new techniques that allow for

sequencing of longer fragments without loss of sensitivity would be useful. The power to detect a decrease in CSF HIV-1 RNA levels during treatment intensification is limited by the sample size, biological variation and assay variability. Perhaps larger studies would show that there is indeed a small reduction in the CSF HIV-1 RNA concentrations by treatment intensification, in all, or in a subset, of subjects. New techniques that allow for a more precise quantification of HIV-1 to minimize assay variability would also be useful for treatment intensification studies.

In conclusion, HIV-1 can be detected in the CSF even after years of suppressive therapy. The detection of HIV-1 in the CSF is correlated to intrathecal immune activation. The HIV-1 found in the CSF during suppressive therapy might be produced by cells in the CNS that need to be targeted in order to cure HIV-1. Since there are no signs of viral evolution among sequences found in the CSF during suppressive therapy and the CSF HIV-1 RNA levels are not affected by treatment intensification there does not appear to be any ongoing replication in the CNS during suppressive therapy.

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