On HIV-1 Latency and Viral Reservoirs

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ABSTRACT

HIV-1 establishes a latent infection that is inaccessible to treatment in cellular and anatomical reservoirs. This thesis concerns several problematic issues of HIV-1 persistence, including ways to measure and monitor both the virus at low viral concentrations and the depletion of the reservoir. Since the central nervous system (CNS) is a potentially important anatomical reservoir, we also explore the extent of neurological injury in HIV-1 disease.

Results from a previous study indicate that the reservoir in resting memory $CD4^+$ T-cells and levels of residual viremia was reduced through intravenous immunoglobulin (IVIG) treatment given to patients on combination antiretroviral therapy (cART). We analyzed T-cell activation markers and potential long-term effects of IVIG on residual viremia. We found no lasting effect on residual viremia, indicating that the effect of IVIG was transient. Activation markers and interleukins were not correlated to levels of residual viremia.

Correct measurements of residual viremia and of the reservoir size are crucial in HIV-1 eradication trials and may have other clinical utility. The methods employed need to be sensitive and subtype independent. We evaluated a modification of the COBAS TaqMan HIV-1 test, version 2.0 and a polymerase chain reaction (PCR) assay for total HIV-1 DNA. We achieved a sensitive quantification of plasma HIV-1 RNA that could be used to assess residual viremia. Sensitive quantification of total HIV-1 DNA in peripheral blood mononuclear cells was demonstrated and both assays were subtype independent.

Low level viremia in patients on cART, defined as a residual viral load of 20–1000 copies/ml is associated with increased risk of virologic failure. We evaluated a method used for sequencing in the case of low level viremia. The method was sensitive and also subtype independent, a feature making it useful in clinical settings where a diversity of subtypes is present.

HIV-1 establishes a chronic infection that also infiltrates the CNS and carries the risk of developing neurological symptoms. By measuring neurofilament light protein (NFL) and markers of inflammation in cerebrospinal fluid (CSF), we wished to determine the extent of neurological injury and neuropathogenesis in HIV-1 disease. We found increased CSF NFL both in patients with neurological symptoms and in neuroasymptomatic patients. Treatment decreased these levels, but treated patients still retained higher levels than controls, indicating either continued virus-related injury or an aging-like effect of HIV-1 infection.

Keywords: HIV-1, latency, intravenous immunoglobulin, residual viremia, low level viremia, ultrasensitive PCR methods, central nervous system

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SAMMANFATTNING PÅ SVENSKA

Med dagens effektiva bromsmediciner är humant immunbrist virus typ 1 (HIV-1) inte längre en dödlig sjukdom. HIV-1 etablerar dock en latent infektion i vilande minnesceller vilket gör att infektionen inte kan slås ut. Hos alla infekterade individer återfinns små virusmängder i blod trots bromsmediciner. Många studier görs nu för att försöka påverka dessa vilande minnesceller och slå ut de sista viruspartiklarna som gömmer sig för immunförsvaret. För att kunna utvärdera dessa försök krävs mycket känsliga mätmetoder. Hos vissa patienter verkar dessa små virusnivåer också ge högre risk för att utveckla mutationer som i vissa fall kan göra viruset mindre känsligt för bromsmediciner. Dessa patienter behöver monitoreras med känsliga metoder för att kunna upptäcka nya mutationer. HIV-1 är dessutom mycket mutationsbenäget och spridningen av olika subtyper i världen gör att samtliga mätmetoder behöver vara subtypsoberoende. HIV-1 etablerar också snabbt en infektion i centrala nervsystemet (CNS) vilket kan ge neurologiska symtom. CNS verkar också fungera som en fristad för viruset vilket ger konsekvenser för behandling och framtida strategier för bot. Vi behöver därför mer kunskap om vilka patienter som drabbas av neurologiska skador och hur dessa uppkommer.

Denna avhandling rör dels försök att aktivera virus i vilande minnesceller (reservoaren) samt utvärdering av mätmetoder för låga virusnivåer i blod, reservoarstorlek och genetisk typning (sekvensering) av patienter med låga virusmängder i blod. Avhandlingen innefattar också ett arbete om nervskador i olika grupper av HIV-1 infekterade patienter.

Man har tidigare visat att intravenöst immunoglobulin (IVIG) kan aktivera virus i reservoarer samt minska virusnivåer i blod hos behandlade patienter. Vi ville undersöka om immunaktivering kvarstår under längre tid samt om virusnivåer i blod är lägre hos patienter som fick IVIG. Vi kunde inte se tecken till fortsatt aktivering av immunceller och virusnivåerna i blod låg på samma nivå som före IVIG-behandling. Detta indikerar att effekten som IVIG-behandlingen hade på immunceller och HIV-1 var övergående.

Vi utvärderade två potentiellt känsliga samt subtypsoberoende metoder för polymeraskedjerreaktion (polymerase chain reaction eller PCR). PCR är ett sätt att öka mängden genetisk material i ett prov och på så sätt kan man mäta även mycket små koncentrationer av virus. Vi modifierade en kommersiell metod (COBAS Taqman HIV-1 test, version 2.0) genom tillägg av ett ultracentrifugsteg. På detta sätt ökade vi känsligheten till 3 viruspartiklar

(HIV-1 RNA) /milliliter blod jämfört med 20 HIV-RNA/milliliter blod i den ursprungliga metoden. Vi utvärderade också en PCR-metod för total mängd virus (HIV-1 DNA) i vita blodkroppar och fann en känslighet på 3 HIV-1 DNA/miljon celler. RNA-metoden kunde detektera samtliga subtyper och DNA-metoden detekterade alla subtyper utom en.

Som nämnts ovan behöver patienter under behandling med bromsmediciner monitoreras för att undvika minskad viral kontroll och risken för mutationer. Även vid låga virusnivåer i blod finns risk för mutationer. Vi utvärderade därför en kombination av metoder för sekvensering som används på Virologen, Göteborg på patienter med låga virusnivåer. I majoriteten av fallen kunde proverna sekvenseras med dessa metoder och resultaten var subtypsoberoende.

Innan effektiv behandling fanns tillgänglig drabbades många patienter av svåra neurokognitiva störningar. Dagens behandling har eliminerat dessa svåra störninger men prevalensen av mindre uttalade neurokognitiva symtom är fortsatt hög. Bakgrunden till detta är fortsatt okänd. Vi ville undersöka hur många patienter som hade tecken till pågående neurologisk skada samt om vi i så fall kunde koppla detta till någon specifik effekt inducerat av HIV-1. Vi använde oss av Neurofilament light protein (NFL) som är en biomarkör för sönderfall av nervcellernas axon. Detta sönderfall har betydelse för nervcellernas förmåga att leda nervimpulser och är delvis relaterad till normalt åldrande. Vi jämförde också nivåer av NFL med neopterin och albumin-ratio (markörer för neuroinflammation respektive blod/hjärn-barriärpermeabilitet).

Vi kunde visa att NFL var högre hos patienter utan neurologiska symtom med HIV-1 jämfört med HIV-1 negativa kontroller. Behandling minskade NFL men nivåerna var högre hos behandlade jämfört med HIV-1 negativa kontroller vilket tyder på en virusrelaterad skada alternativt en åldrande effekt inducerat av HIV-1 trots effektiv behandling. NFL korrelerade till neopterin samt till albumin-ratio vilket antyder en koppling mellan axonal skada, neuroinflammation och blod/hjärn-barriärpermeabilitet.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Tomas Mellberg*, Veronica D Gonzalez, Annica Lindkvist, Arvid Edén, Anders Sönnerborg, Johan K Sandberg, Bo Svennerholm and Magnus Gisslén. **Rebound of residual plasma viremia after initial decrease following addition of intravenous immunoglobulin to effective antiretroviral treatment of HIV**
AIDS Research and Therapy 2011, 8:21

II. Tomas Mellberg*, Jon Krabbe, Maria J Buzon, Ulrika Noborg, Magnus Lindh, Magnus Gisslén and Bo Svennerholm. **Sensitive, subtype independent HIV-1 PCR assays for assessment of residual viremia and total HIV-1 DNA**
In submission

III. Tomas Mellberg*, Jon Krabbe, Bo Svennerholm and Magnus Gisslén. **Subtype independent sequencing of low level viremia in HIV-1 infected patients, a pilot study**
In submission

IV. Jan Jessen Krut*, Tomas Mellberg*, Richard W Price, Lars Hagberg, Dietmar Fuchs, Lars Rosengren, Staffan Nilsson, Henrik Zetterberg, and Magnus Gisslén. **Biomarker evidence of axonal injury in neuroasymptomatic HIV-1 patients**
PLoS One. 2014 Feb 11;9(2):e88591

CONTENT

ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANI	Asymptomatic neurocognitive impairment
APOBEC	Apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like
BBB	Blood-brain-barrier
cART	Combination antiretroviral treatment
CCR5	Cysteine-cysteine chemokine receptor
CD4	Cluster of differentiation 4
CNS	Central nervous system
CRF	Circulating recombinant form
CSF	Cerebrospinal fluid
CXCR4	Cysteine-x-cysteine chemokine receptor
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbant assay
GALT	Gut-associated lymphoid tissue
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HDACi	Histone deacetylase inhibitors
HIV	Human immunodeficiency virus
IL-2	Interleukin 2

IL-7	Interleukin 7
IN	Integrase
IVIG	Intravenous immunoglobulin
IUPM	Infectious units per million cells
LTR	Long terminal repeat
MND	HIV-associated mild neurocognitive disorder
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NFL	Neurofilament light chain
NRTI	Nucleoside/nucleotide analog reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PIC	Pre-integration complex
PR	Protease
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
SCA	Single-copy assay
SIV	Simian immunodeficiency virus
Treg	Regulatory T-cell
URF	Unique recombinant form
WBC	White blood cell

1 INTRODUCTION

1.1 The HIV pandemic

1.1.1 Origins of HIV

On June 5 1981, the Centers for Disease Control (CDC) in the US published a report on five cases of Pneumocystis pneumonia (PCP) among previously healthy young men in Los Angeles [1]. The patients had impaired immune systems and the editorial note that accompanied the published report stated that the case histories suggested a "cellular-immune dysfunction related to a common exposure" and a "disease acquired through sexual contact". This was the first published report of what, a year later, became known as acquired immunodeficiency syndrome (AIDS) During the following years, intensive research led to the isolation of a new retrovirus later to be called Human Immunodeficiency Virus (HIV), shown to be the cause of this syndrome [2-4].

Further studies have revealed two distinct lentiviruses, HIV-1 and HIV-2, as the cause of AIDS in humans [5]. These two viruses are distinguished on the basis of their genome organization and phylogenetic (i.e., evolutionary) relationships with other primate lentiviruses. Humans are not the natural host for HIV-1 and HIV-2. Instead, these viruses have entered the human population through zoonotic, or cross-species transmission of two different types of Simian Immunodeficiency Viruses (SIV_{CPZ} and SIV_{SMM}) from other primates. HIV-1 is the most common type of HIV and comprises four different lineages, termed groups M (major or main), N (non-M, non-O), O (outlier), and P (putative). Each of these groups resulted from an independent cross-species transmission, with group M being the first to be discovered. Group M represents the pandemic form of HIV-1 and has been found in virtually every country in the world. It accounts for 99% of all HIV infections. HIV-2 has remained more geographically restricted to West Africa, with prevalence rates declining [6, 7]. HIV-2 has lower transmission rates than HIV-1 and causes a less virulent form of the disease with either no progression or slower progression to AIDS in a majority of patients [8, 9]. HIV-1 was introduced to humans via SIV_{CPZ} from common chimpanzees (Pan Troglodytes) found in Cameroon, whereas HIV-2 appears to originate from an introduction of SIV_{SMM} found in sooty mangabeys in West Africa [10]. HIV-2 will not be considered further in this thesis.

Exactly how humans acquired the ape precursors of HIV-1 is not known. However, it is considered to have occurred through cutaneous or mucous membrane exposure to infected ape blood or body fluids [11]. A diversity of primate species have been and continue to be hunted and consumed as bushmeat, and are kept as pets in west central Africa, constituting a risk for transmission [12]. The introduction of the pandemic group M to humans is estimated to have occurred around the beginning of the twentieth century in southern Cameroon [13, 14]. The virus then spread to Leopoldville (now Kinshasa in the Democratic Republic of Congo) where it became a local epidemic in the 1960s [13]. The worldwide distribution of the group M virus and its different subtypes was then facilitated by the emergence of globalisation.

1.1.2 Worldwide spread

AIDS is one of the most devastating infectious diseases ever known. It has been responsible for nearly 75 million infections. Globally, an estimated 35 million people (33.2–37.2) were living with HIV in 2013 [15] (Figure 1). The prevalence varies greatly between different regions but the pandemic continues to disproportionally affect sub-Saharan Africa, where 70% of all new infections occurred in 2013. The numbers are now declining, with 2.1 million new infections globally 2013 compared to 3.4 million in 2001. There has been a 43% decline in new HIV infections among children in 21 priority countries in Africa since 2009, partially reflecting the growing number of people benefitting from access to treatment. Still, only 36% to 40% of all persons living with HIV had obtained antiretroviral therapy in 2014 and an even smaller percentage (22% to 26%) of all children living with HIV are receiving treatment [15].

One of the most important factors in preventing the spread of HIV-1 is access to effective antiretroviral treatment. Other vital factors in restraining the pandemic include changes in sexual behaviour, such as delayed sexual debut, high levels of condom use, and reductions in multiple partners. For example, in Zimbabwe, declines in the incidence of new HIV infections were driven by shifts in behavior, notably a reduction in multiple sexual partners [16, 17]. Eliminating gender inequalities, gender-based abuse and violence while increasing the capacity of women and girls to protect themselves from HIV infection and preventing HIV-related stigma, discrimination, punitive laws, and common practices, are all important measures in fighting the pandemic. However, significant challenges remain, especially in sub-Saharan Africa. Although the pandemic is slowing down and treatment has become widely

available for more people, HIV/AIDS remains a severe global health problem.

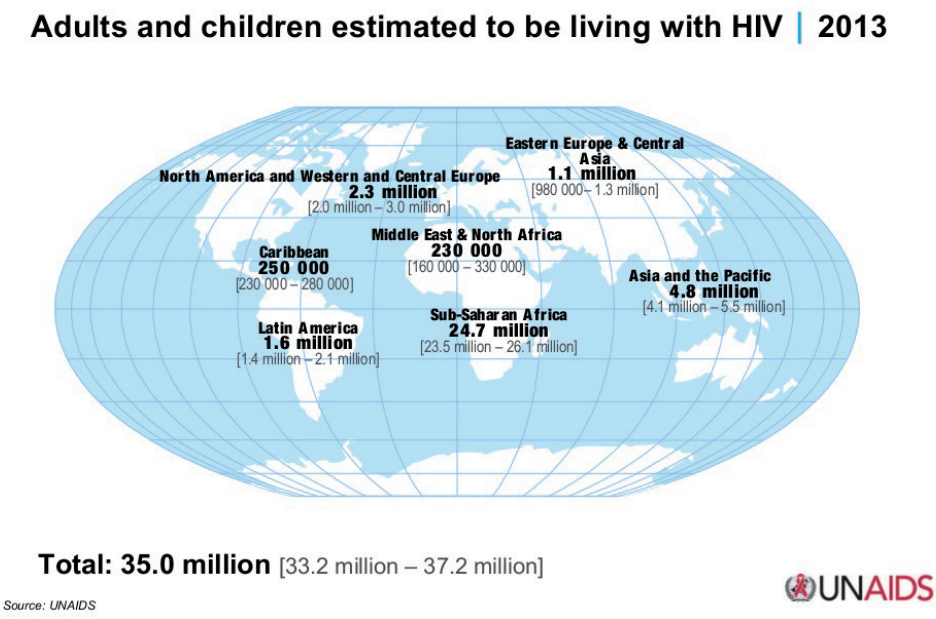

Figure 1. Estimated numbers of individuals living with HIV in 2013. Source: *UNAIDS 2014 Report on the global AIDS epidemic*

1.1.3 The situation in Sweden

The Swedish HIV-1 epidemic began in December 1979 in Stockholm with an outbreak of HIV-1B infection among men having sex with men (MSM) [18]. Since the 1990s, a growing number of incoming cases from high-prevalence countries have been the major contributing factor to the Swedish epidemic [19]. In Sweden, approximately 6600 people were living with HIV in 2014, and 400 to 500 are reported as newly-infected every year [20]. In a majority

of cases (76%) HIV was acquired abroad and the main transmission rout was heterosexual. In Sweden, the main route of transmission was sexual (72% homosexual and 26% heterosexual). HIV infection through intravenous drug abuse is rare in Sweden and has declined in numbers since 2007 [21]. The mortality rate in 2013 was below 1% [22]. The vast majority of known HIV-infected people in Sweden (94%) are under combined anti-retroviral therapy (cART) and are generally held in a virologically suppressed state [22].

1.2 HIV-1 Virology

1.2.1 Structure and genome

HIV-1 is a retrovirus belonging to the genus Lentivirus. The virus carries two copies of positive single-stranded ribonucleid acid molecules (ssRNA) associated with a nucleocapsid (NC, proteins p7 and p6). The RNA is encapsulated in a cone-shaped capsid (CA, protein p24) that constitutes the viral core. The viral enzymes reverse transcriptase (RT), protease (PR) and integrase (IN) are also packaged into the core particle (Figure 2). The capsid is surrounded by the matrix protein p17. HIV-1 has an envelope consisting of two lipid layers taken when budding from the host cell. The envelope also contains the only virus-encoding determinants on the virus surface, the envelope glycoproteins (*Envs*) gp 41 (transmembrane glycoprotein) and gp 120 (external glycoprotein) forming the HIV-1 spikes [23] (Figure 2).

The HV-1 genome contains nine open reading frames. Three major structural genes encode for *Gag*, *Pol,* and *Env* polyproteins, which after proteolyzation constitute individual proteins common to all retroviruses. *Gag* encodes the precursor polyprotein which is further processed into p24, p17, p7, and p6 proteins. *Pol* encodes the enzymes PR, RT, and IN. *Env* encodes the surface and transmembrane proteins gp 120 and gp 41. HIV-1 has two regulatory genes and four accessory genes: *tat, rev, vif, vpr, vpu,* and *nef* (Figure 2).

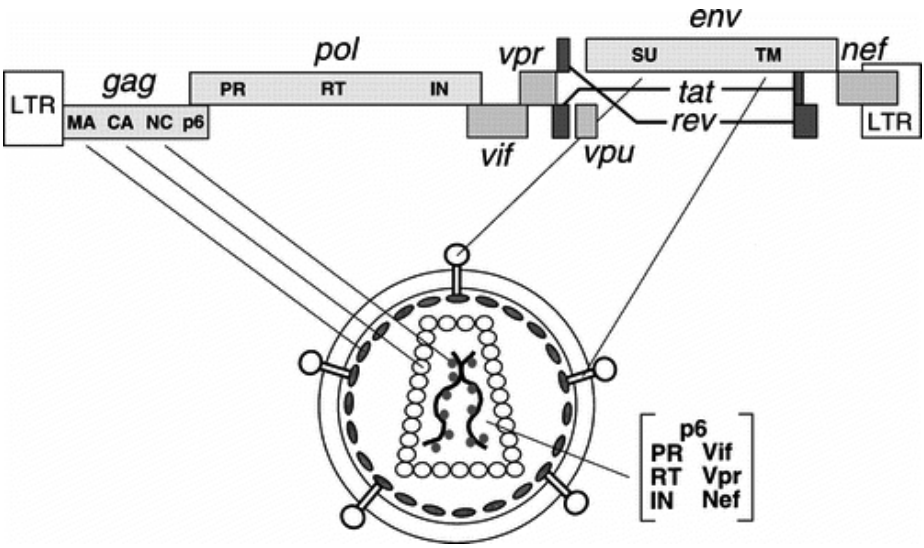

Figure 2. Schematic structure of the HIV-1 virion and genome. Adapted from *Annual Review of Biochemistry* Vol. 67: 1-25, 1998. Reprinted with permission from *Annual Review of Biochemistry®.*

1.2.2 Lifecycle

HIV entry, the first phase of the viral replication cycle, begins with the adhesion of HIV-1 *Env*, comprised of gp 120 and gp 41, to the CD4 receptor on the host cell surface. CD4 binding leads to a conformational change that allows co-receptor binding to CCR5 or CXCR4, which in turn induces membrane fusion and delivery of the viral components into the host cell cytoplasm [24, 25]. Following entry, the virus uses RT to convert ssRNA to ssDNA (reverse transcription) and subsequently a complementary DNA (cDNA) molecule is processed that is to be integrated into the host genome (Figure 3). The formation of a pre-integration complex (PIC) precedes the incorporation into the nucleus following integration of the pro-viral DNA into the host genome. Not all DNA is integrated into the host DNA. The unintegrated DNA is circularized by host DNA repair enzymes to form episomes containing two copies of the viral long terminal repeat (2-LTR circles), or undergoes recombination to form a 1-LTR circle [26]. These DNA forms represents dead ends for the virus and are considered non-

productive [27]. After integration, HIV-1 can go into a latent state where no transcription occurs. In most cases, however, replication continues by transcription of the integrated pro-virus by the host cells transcription machinery. Formation of mature viral particles is a two-step procedure: budding of the non-infectious viral particle followed by a maturation step, creating the productive virion. Particle formation requires transportation of *Gag* proteins to the plasma membrane where they associate with other cellular and viral components, producing a budding structure. These released virions are initially non-infectious particles containing a spherical layer of *Gag* polyproteins. Proteolytic cleavage at defined sites by PR then leads to the formation of the structural proteins essential for the productive virion [28] (Figure 3).

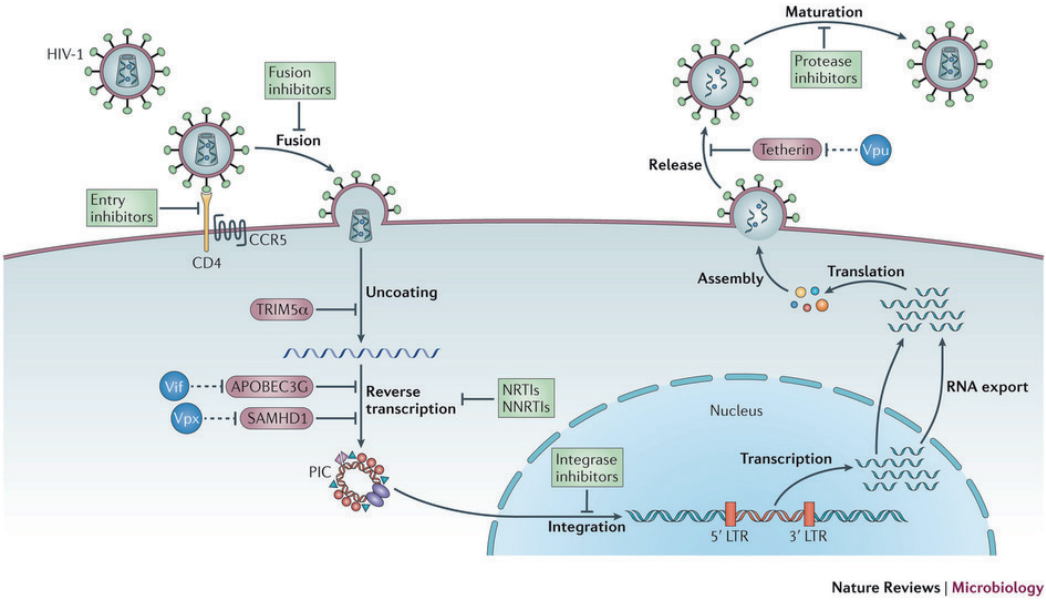

Figure 3. Lifecycle of HIV-1. Adapted from: *Nature Reviews Microbiology* 11, 877–883 (2013). Reprinted with permission from Nature Publishing Group

1.2.3 Genetic variation and evolution

HIV-1 sequences vary considerably between individuals and within a single individual. Although a small proportion of new infections represent a heterogenic viral population, most infections occur as the transmission of a single virion. This implies that diversity must take place after infection of the individual [29, 30]. In the case of HIV-1 and other retroviruses the high mutation rates are generally attributed to the very error-prone reverse transcription, but other possible sources of error include a) transcription by the host RNA polymerase II and b) hypermutation mediated by apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like G3 (APOBECG3). Whatever the cause, it is estimated that one mutation is introduced for every 1000 to 10000 nucleotides synthesized [31]. This error-rate is about average for an RNA-virus, the RT of HIV-1 is not more error-prone than RTs of other RNA-viruses. However, two other important mechanisms contribute to the extraordinary genetic diversity of HIV-1: rapid high-level virus turnover (about 10^{11} virions are produced daily and ca 10^{8} to 10^{9} cells are infected every day) and recombination. These two mechanisms, in combination with the long duration of the infection, set HIV-1 apart from most other viral infections with regard to diversity [32].

The recombination rate for HIV-1 is high compared to other viruses, retroviruses included [33]. Recombination can occur when one cell is infected by two distinct variants of HIV-1 and both RNA sequences are packaged into the same virion. During the next infection, RT can switch between the two RNAs, creating a cDNA that is a combination of the two previous variants. The viral evolution following infection is driven by immune escape in a constant positive selection. Both antibody and cytotoxic T-cell (CTL)-mediated immune selection have been shown to induce escape mutations influencing viral evolution and persistence [30, 34]. In addition to immune escape, resistance to antiretroviral treatment is an important consequence of genetic diversity that accumulates during the chronic HIV-1 infection (see below).

1.2.4 Subtypes

HIV-1 diversity has given rise to numerous subtypes and recombinants, largely as a consequence of founder effects (a single introduction followed by a rapid spread) and viral population bottlenecks. HIV-1 group M is divided into subtypes A-D, F-H, J, and K. About 20% of all circulating HIV-1 variants are recombinant forms (CRF). Unique recombinant forms exists as

well (URF). CRFs are widespread, whereas URFs are restricted to a limited number of individuals [35, 36]. Currently, there are at least 48 CRFs circulating in the world. CRFs and URFs are created after coinfection with at least two HIV-1 isolates and have genomic segments deriving from different subtypes. Whereas CRFs share the same mosaic genomic pattern and are derived from at least three different epidemiological ancestors, URFs do not [36].

The majority of people living with HIV-1 worldwide are infected with non-subtype B variants and approximately 50% of all infections globally are subtype C. However, since the spread of HIV-1 to North America and Europe was primarily attributed to subtype B, much of the research and development in the HIV field has concentrated on this subtype. However, the spread of subtypes and recombinant forms is increasing throughout the world, further underlining the importance of methods and research incorporating different subtypes [37-40]. In Swedish samples collected from 2003 to 2010, Karlsson et al. reported 41% subtype B, 19% CRF_AE, 15% subtype C, 9% subtype A, 8% CRF02_AG, and 2% subtype D. The remaining patients (6%) had a virus that was classified as other subtypes (F and G), circulating recombinant forms, unique recombinant forms, or unclassifiable [41]. Moreover, from an analysis of nearly 4000 sequences from patients diagnosed in Sweden from 1983 to 2012, Neogi et al. reported an increase of non-B subtypes in Sweden over time, compared to subtype B concluding that Sweden exhibits one of the most diverse subtype epidemics outside Africa [19].

Whether specific genotypes are associated with different phenotypic traits, such as disease progression and transmission rates has been debated. Although disease progression in HIV-1 subtype B infections is at least partially understood, infections with the dominant subtypes A, C, D, as well as CRF02_AG and CRF01_AE, which are highly prevalent in resource-poor settings have been poorly studied. Structural and regulatory differences between subtypes may influence the cellular tropism and kinetics of viral replication. Subtype C seem to have a slower progression and less pathogenic fitness compared to other group M subtypes. Subtype C uses predominantly CCR5, rarely switching to CXCR4 or dual tropism. By contrast, subtype D uses CXCR4 early in infection, which might be why individuals infected with subtype D progress to AIDS faster [42-44]. However, the overall results regarding disease progression linked to subtypes are divergent [45-49]. The notable caveat relevant to all these studies of disease progression is that confounders such as access to medical care, nutritional status, host genetic factors, and mode of viral transmission (e.g. sexual, injectable drugs, or vertical) may contribute to the divergent results. Although there are some

differences in the frequency of specific mutations when comparing subtypes, there is no compelling evidence supporting a need to consider HIV-1 subtype when selecting treatment regimens [50, 51].

1.3 HIV-1 pathogenesis

1.3.1 Transmission and course of natural infection

HIV-1 is transmitted by sexual contact across mucosal surfaces, by maternal–infant exposure and by percutaneous inoculation. The vast majority of current and new infections are transmitted sexually through the lower genital tract and the rectal mucosa. Heterosexual transmission remains the major driving force behind the pandemic, especially in southern Africa. Heterosexual infectivity rates are estimated to be fairly low in stable couples with a low prevalence of high-risk co-factors (~1 infection per 1000 contacts between infected and uninfected individuals), but the risk for sexual transmission increases considerably by co-existing factors such as genital ulcers and disease stage [52]. Transmission rates also correlate strongly with levels of plasma HIV-1 RNA. Therefore, cART is very effective in preventing transmission almost completely between discordant couples [53, 54]. Other effective measures to prevent transmission is the proper use of condoms and male circumcision [52].

Mucosal transmission is generated by infection of activated and resting $CD4^+$ T-cells in or near the epithelial layer. In most cases, establishment of infection is performed by a single virion. Initial focal replication in these cells is followed by movement of the virus to proximal lymphoid organs and then to a systemic infection [55, 56]. Following successful transmission, a typical course of infection is seen in virtually every individual who does not receive antiretroviral treatment. In the first phase, during 1 to 2 weeks, the virus replicates freely and spreads to various tissues and organs. Viremia is undetectable and there is no immune response or symptoms. The acute phase (2 to 4 weeks after infection) is characterized by high viral loads in plasma and large fractions of infected $CD4^+$ T-cells in blood and lymph nodes. This phase is often symptomatic and is accompanied by fever, pharyngitis, lymphadenopathy, and rash.

At this point during peak viremia, the immune response begins to appear, both in the form of antibodies directed against viral proteins and $CD8^+$ T-cell response against HIV-1 antigens expressed on the surface of infected cells.

At the end of the acute phase, due to some immune control and depletion of target cells, viremia declines. The next phase is characterized by a constant level of viremia (viral set-point) constituting the chronic infection and sometimes termed clinical latency. This asymptomatic period has an average duration of 10 years, but there is a large inter-individual variation [57] (Figure 4). The term clinical latency is somewhat misleading since during this period a highly active infection is going on, and virtually all patients have a gradual deterioration of their immune systems, with large numbers of $CD4^+$ T-cells infected and dying every day.

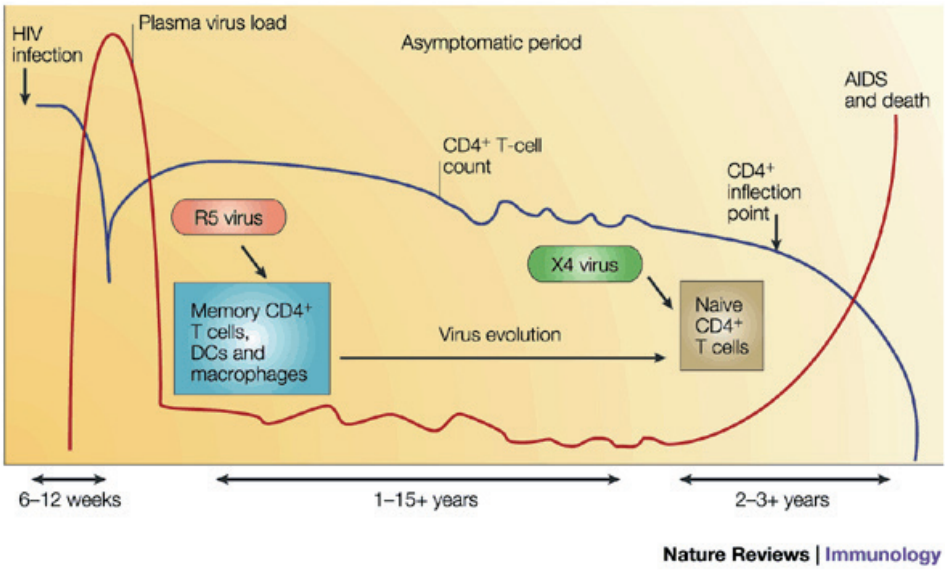

Figure 4. Course of typical HIV-1 infection. Patterns of $CD4^+$ T-cell decline and viremia vary greatly between individuals. Adapted from *Nature Reviews Immunology* 3, 343-348 (April 2003) . Reprinted with permission from Nature Publishing Group

The viral set-point or steady-state is determined by a combination of the number of available target cells, the infectivity of the virus, the number of virions produced per cell, the productive lifetime of the cell, and clearance rate of the virus. The viral level in plasma during steady-state varies greatly between individuals (< 50– >1,000,000 HIV-1 RNA copies/ml) and this set point of viremia is remarkably robust, changing little throughout the latent clinical phase [58]. The set-point viral HIV-1 RNA and the degree of immune activation correlates with the $CD4^+$ T-cell decline rate and time of progression to AIDS [59, 60]. The diagnosis of AIDS is defined by the occurrence of clinical AIDS-defining conditions, such as opportunistic infections or tumours.

1.3.2 Target cells

HIV-1 uses CD4 as the main receptor for binding to its target cells [61]. As described above, co-receptor CCR5 or CXCR4 are essential for entry into the cell. The receptor and co-receptor tropism regulates the possible target cells for the virus. The CD4 receptor is mainly expressed on the cell surfaces of naïve and memory $CD4^+$ T-cells. However, while CCR5 is largely expressed on memory $CD4^+$ T-cells, CXCR4 is the main co-receptor expressed on naïve $CD4^+$ T-cells [62]. Early in the infection, HIV-1 mainly uses CCR5 for entry into cells, making memory $CD4^+$ T-cells the main target for the virus [29, 63] (Figure 4). Later on in the disease, a co-receptor switch to CXCR4 or dual tropism can be seen in 40 to 50% of subtype B infections, an event correlated to a faster progression to AIDS [64-66].

Other cell types such as monocytes, NK-cells, and dendritic cells, have been proposed as possible HIV-1 target cells. The expression of CD4 varies in these cell types, and their role in productive infection is unclear. Some studies have reported in vivo HIV-1 replication in monocytes [67, 68], while others did not find infected monocytes during chronic infection, indicating that their frequency of infection is low, at least compared with $CD4^+$ T cells [69]. However, several studies have shown that macrophages and NK-cells can be infected in vivo [70-72]. Dentritic cells can bind to gp 120, most likely via a C-type lection such as the DC-SIGN-receptor, and thereby capture the virus and transport it to lymphatic tissues, where it can enhance infection of the target $CD4^+$ T-cells [73]. Nevertheless, whether dendritic cells can produce virions remains unclear.

1.4 Antiretroviral treatment of HIV-1

1.4.1 cART

The most significant advance in the HIV field has been the discovery of antiretroviral drugs that can suppress HIV-1 replication to undetectable levels as measured with clinical assays. To date, an array of 24 drugs approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) are available for treatment of HIV-1 infections (Table 1). These drugs are classified in six categories, depending on their mode of action: 1) nucleoside/nucleotide-analog reverse transcriptase inhibitors (NRTIs), 2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), 3) integrase inhibitors (INIs), 4) protease inhibitors (PIs), 5) fusion inhibitors, and 6) co-receptor antagonists.

The treatment of HIV-1 was revolutionized in the mid-1990s by the development of inhibitors of reverse transcription and protease, and the introduction of drug regimens that combined these modes of action to enhance the efficacy and durability of therapy (combined antiretroviral therapy or cART). The principle for effective treatment of HIV-1 infection is to combine three active drugs from at least two different classes to achieve potent inhibition of viral replication. This concept is derived from clinical experience as well as mathematical modelling of the rate of mutations required for developing resistance [32, 74]. According to current Swedish and US guidelines, first line treatment should consist of two NRTIs and one PI (or NNRTI or an integrase inhibitor). It is recommended that treatment should be offered to all patients regardless of $CD4^+$ T-cell count. [22].

The main two reasons for initiating therapy already at the onset of diagnosis are the decreased risk for transmission and HIV co-morbidity. Several studies favour early treatment to reduce the risk of both HIV-related and non-HIV-related malignancies, cardiovascular diseases and neurological diseases [75, 76]. However, there are currently no data from randomised controlled trials to support this. The risk is mainly increased in patients with low $CD4^+$ T-cell counts but morbidity and mortality is also increased in patients with $CD4^+$ T-cell counts > 350 $CD4^+$ T-cells/μl [76-80]. The risk for transmission decreases dramatically with effective antiretroviral treatment. In discordant couples where the infected individual is under full virologic suppression, HIV-1 transmission is considered extremely low, both for vaginal and anal intercourse with condom [53]. This seems to be true under the same circumstances without condom use [81].

Table 1. Approved antiretroviral drugs in Sweden 2014.

Drug	Trade name	Mechanism
NRTIs		Termination of the viral DNA copy by incorporation of a defective nucleotide
Abacavir (ABC)	Ziagen	
Didanosine (ddI)	Videx	
Emtricitabine (FTC)	Emtriva	
Lamivudine (3TC)	Epivir	
Stavudine (d4T)	Zerit	
Tenofovir (TDF)	Viread	
Zidovudine (AZT, ZDV)	Retrovir	
NNRTIs		Inhibition of RT by binding to it's active site
Efavirenz (EFV)	Stocrin, EFV TEVA	
Nevirapine (NVP)	Viramune	
Etravirine (ETR)	Intelence	
Rilpivirin (RPV)	Edurant	
PIs		Inhibiton of PR resulting in non-mature virions
Atazanavir (ATV)	Reyataz	
Darunavir (DRV)	Prezista	
Fosamprenavir (fAPV)	Telzir	
Indinavir (IDV)	Crixavan	
Lopinavir (LPV)	Kaletra	
Nelfinavir (NFV)	Viracept	
Saquinavir (SQV)	Invirase	
Tipranavir (TPV)	Aptivus	
Ritonavir (RPV)	Norvir	
Integrase inhibitors (INI)		Prevents integration of viral DNA by binding to the complex between IN and viral genome
Raltegravir (RAL)	Isentress	
Dolutegravir (DTG)	Tivicay	
Elvitegravir (EVG)	Vitekta	
Fusion inhibitors (FI)		Prevents fusion by binding to gp41
Enfuvirtid (T-20)	Fuzeon	
CCR5-antagonists		Blocks binding of HIV-1 to co-receptor CCR5
Maraviroc (MVC)	Celsentri	

1.4.2 Resistance and low level viremia

Due to the enormous intrinsic ability of HIV-1 to exhibit genetic diversity, virus variants with reduced susceptibility to some of the antiretroviral drugs may preexist in the viral quasispecies before initiating therapy [32]. Drug resistance can also be transmitted with a prevalence of 5 to 15% in the case of new infections (5% in Sweden, 8% in Europe, and 15% in the US) [41, 82, 83]. Furthermore, the effect of therapy can be impaired by non-adherence or poor drug tolerance. Drug interactions among antiretroviral agents or other medications may also decrease optimal drug levels. Each of these can lead to virologic failure and the development of drug resistance associated mutations (DRAMs).

Current guidelines state that the goal of therapy is virologic suppression below the limit of detection with clinical assays (< 20 to 50 copies/ml, depending on the assay used). In Sweden, virologic failure is defined as a repeatedly measured viral load > 150 HIV-1 RNA copies/ml for a period > 6 months. Importantly, the vast majority of treated patients have plasma HIV-1 RNA < 50 copies/ml [22]. According to US guidelines, virologic failure is defined as the inability to achieve or maintain suppression of viral replication to an HIV-1 RNA level < 200 copies/ml [84]. Below this level, patients with stable cART and no signs of further virologic progression may not be counselled to switch treatment. However, in some cases, a HIV-1 RNA level just above 50 copies/ml may reflect an incipient virological failure and drug resistance testing can also be important at these levels of viremia. Drug-resistant testing by sequencing of the viral coding regions of reverse transcriptase (RT), protease (PR), and in some cases integrase (IN) is a valuable tool for treatment strategies during virologic failure. In general, resistance testing is not routinely recommended in patients with a viral load < 500 to 1000 HIV-1 RNA copies/ml, primarily due to a lack of commercially available and reliable assays for HIV-1 RNA levels in this range [84]. It may also be argued that sequencing at these levels of viremia may not be representative of the actual viral population and so could be misleading. In recent years, however, there has been an increasing interest in these lower levels of viremia, and efforts have been made to enhance the assays to also allow sequencing below 500 to 1000 HIV-1 RNA copies/ml.

Low level viremia is a term used in research and is usually defined as a consistent detectable viral load in the range between the detection limit of the assay (20 to 50 HIV-1 RNA copies/ml) and 1000 HIV-1 RNA copies/ml. As reflected by the current guidelines, many patients experience virologic failure when their viral load reaches 150 to 200 HIV-1 RNA copies/ml and above. Many groups have reported an increased risk for both virologic failure and

the development of DRAMs during low level viremia [85-92]. When viral loads remain at these levels (especially between 50 and 200 copies/ml) the best management and clinical consequences remain unknown. Recent works by Laprise et al. and Doyle et al. has demonstrated the association between these lower segments of low level viremia and virologic failure [93, 94]. Even a detectable viral load, less than 50 HIV-1 RNA copies/ml was correlated to a higher risk for virologic rebound to > 400 HIV-1 RNA copies/ml [94]. Such levels of viremia (< 50 HIV-1 RNA copies/ml) are often called very low levels of viremia. The risk for virologic rebound to HIV-1 RNA > 50 copies/ml in patients with very low levels of viremia has also been described elsewhere [95, 96]. Those findings are controversial and other studies point to the contrary [97-99]. All these studies have methodological problems since there is a significant widening of the coefficients of variation at the lower limits of the quantitative range in the methods used in these studies [100]. The clinical significance of very low levels of viremia also remains unclear since little or no support for the development of DRAMs at these viral levels has been reported [95, 99, 101]. Many patients with a rebound > 200 HIV-1 RNA copies/ml also resuppressed to levels < 50 HIV-1 RNA [95]. The question of viral evolution during suppressive therapy with viral loads < 50 HIV-1 RNA copies/ml is further discussed in Subchapter 1.5.3.

Risk factors for low level viremia vary between studies, but adherence, higher pre-cART plasma viral load, and lower $CD4^+$ T-cell counts have all been proposed [94, 96, 102]. Treatment regimens may also influence the risk for low level viremia [96, 103], but such results may be correlated to adherence and so called channeling bias (patients with a more severe disease may receive PI treatment to a larger extent) rather than favourable mechanisms of a specific regime. Both the level of viremia and the time period at higher levels of viremia seem to be important determinants for virologic failure, as is the development of DRAMs during low level viremia [93, 94, 96, 104, 105]. With higher viral loads, it may be assumed that immune responses are increased thereby providing a larger number of target cells for the virus, and in turn increasing the rate of viral evolution and the risk for DRAMs. A higher degree of HIV-1 specific responses by cytotoxic T-cells and consistent increased markers of activation in patients with low level viremia was reported by Karlsson et al. [87]. A higher degree of immune activation was also observed in patients with intermittent detectable viremia (blips). However, no signs of virologic failure or the development of DRAMs were seen in this group [87]. This is also supported by others who report no association between blips and virologic failure or DRAMs [101, 106].

1.5 HIV-1 persistence during therapy

1.5.1 Latency

Viral latency is defined as a state of reversibly non-productive infection of individual cells. For HIV-1, the term latency was initially used to describe the asymptomatic period during natural infection during which a viral set-point is established. As assays for more sensitive quantification of HIV-1 RNA were developed, it became clear that a massive viral and cellular turnover is produced during this stage of infection and that HIV-1 also had the ability to establish a latent infection in individual T-cells. The induction and maintenance of a latent reservoir requires long-lived HIV-1 susceptible cells that do not become activated and die as a result of viral cytopathic effects or host immune responses. Resting $CD4^+$ T-cells were soon proposed as candidates. In 1995 Chun et al. showed that replication-competent virus could be rescued by cellular activation of resting $CD4^+$ T-cells [107]. These cells are rare (about $1/10^6$ $CD4^+$ T-cells) and HIV-1 is mainly found in the memory subset of resting $CD4^+$ T-cells [108, 109]. Shortly after cART was introduced, three groups simultaneously showed that latently infected cells persisted in patients under suppressive treatment [110-112]. For the HIV-field, this has led to a specific definition of the term latency and reservoirs. It is currently proposed that one define a HIV-1 reservoir as an infected cell population that allows persistence of replication-competent HIV-1 in patients on optimal cART regimens over a period of years [113]. That the resting memory $CD4^+$ T-cell phenotype is a major HIV-1 reservoir during cART is supported by the finding that during therapy sequences of virions intermingle with the sequences of virions from resting memory $CD4^+$ T-cells [114]. Since HIV-1 does not efficiently establish productive infection in resting $CD4^+$ T-cells, it is thought that those cells are infected in their activated state when transitioning back to a resting memory state, resulting in a stably integrated but transcriptionally silent provirus in a long-lived memory $CD4^+$ T-cell [115, 116]. This state is maintained by a specific transcriptional environment in resting $CD4^+$ T-cells, including inhibition of host transcriptional factors and epigenetic modifications in the cells [113]. HIV-1 latency could therefore be seen as an accidental consequence of the virus tropism for activated $CD4^+$ T-cells. As discussed below, the decay rate of this pool is extremely slow and eradication of this reservoir with cART alone is now considered impossible during a human lifetime [117, 118]. In a study by Chomont et al., it is suggested that the stability of this reservoir is maintained by low level homeostatic proliferation in the pool of resting memory $CD4^+$ T-cells [108]. Evidence for homeostatic proliferation of those cells comes from another more recent study, reporting clonal expansion of T-cells carrying mutant viruses with a defect that would preclude replication [119] A specific type of

memory T-cell termed T-memory stem cell (T_{SCM}), representing approximately 2 to 4% of the total T-lymphocyte population, has been identified. These cells seem to be extremely durable and have the capacity to differentiate themselves into more mature central memory, effector memory, and effector T-cells while maintaining their own pool size by homeostatic renewal [120]. In a longitudinal study of patients on cART spanning for more than 10 years of treatment, the HIV-1 DNA found in these cells remained largely stable compared to other $CD4^+$ T-cell types [121]. It has, therefore, been proposed that these cells play an important role in maintaining the reservoir during cART. The reservoir in resting memory $CD4^+$ T-cells is established early in infection, as reflected by the rebound of archival pre-therapy viruses at treatment interruption, as well as the size of the reservoir being correlated to the timepoint of treatment initiation [119, 122-125]. Furthermore, higher HIV-1 DNA levels, as markers of a larger reservoir, predict faster rebound after treatment interruption [126].

1.5.2 Cells, compartments, and sanctuary sites

Since the description of resting $CD4^+$ T-cells as a reservoir for HIV-1, several cell types and anatomical sites have been proposed as additional reservoirs. Evidence for reservoirs other than resting $CD4^+$ T-cells comes from extensive analysis of residual viremia. Although many sequences of plasma viruses in most patients on cART are identical to those found in resting $CD4^+$ T-cells, in some patients a majority of the sequences seem to be derived from oligoclonal populations called predominant plasma clones (PPC). These clones differ from the proviruses in circulating $CD4^+$ T-cells and contribute to the residual viremia in those patients [114]. Different cells have been proposed as cellular reservoirs in addition to resting $CD4^+$ T-cells. However, few are able to meet the definition of a cell that allows persistence of replication-competent HIV-1 for long periods of time in the presence of optimal cART (see the definition of HIV-1 latency above). Macrophages have been proposed by several groups, but most studies on macrophages in different tissues were performed in the pre-cART era [127-129]. The infection is typically productive in these cells, and evidence is still lacking for a truly latent infection in macrophages after an extensive period of effective cART. The question of whether progenitor cells in the monocyte-macrophage lineage or hematopoietic stem cells may be infected in vivo is controversial. This issue was highlighted in 2010 when Carter et al. showed that HIV-1 can infect hematopoietic progenitor cells and establish a latent infection [130]. However, a more recent study contradicts those results and the question remains unresolved [131].

The term compartment is defined as a site that has a limited exchange of viral genetic information with other sites of infection. Thus, in vivo virologic compartments are cell types or tissues among which there is a restricted virus flow. In comparison, virologic reservoirs are cell types or tissues in which there is a relative restriction of replication [132]. When a specific anatomical compartment is poorly penetrated by antiretroviral drugs, thereby allowing persistent viral replication, it is termed a sanctuary site [113]. The largest lymphoid organ in the body is the gut-associated lymphoid tissue (GALT) and a majority of the cells harbored by GALT are lymphocytes. During untreated infection, most of HIV-1 replication takes place here [133]. GALT has been proposed as an anatomical compartment for HIV-1 during cART, based on higher frequencies of cells carrying HIV-1 DNA in GALT as compared to plasma in patients receiving effective cART [134, 135]. Whether GALT represents a sanctuary site with ongoing rounds of viral replication remains an open question. The genitourinary tract has also been proposed as a potential compartment and sanctuary site for HIV-1 [136-138]. This was recently challenged by Awery et al. in a study showing that, although the total concentrations of efavirenz was higher in plasma compared to seminal fluid, the free proportion of the drug was equally distributed between these two anatomical sites [139].

There is strong evidence for HIV-1 compartmentalization of the central nervous system (CNS) and concern that this compartment also works as a sanctuary site for the virus. Many studies have reported distinct viral variants in the CNS as compared to blood [140-143] as well as local viral evolution in the CNS during cART [144]. Independent replication in the CNS in patients with progressive CNS symptoms despite otherwise effective cART (so-called symptomatic cerebrospinal fluid [CSF] escape) has also been observed [145, 146], indicating that the CNS compartment can serve as a sanctuary site for the virus in selected cases. Furthermore, it has been shown that some patients exhibit CSF HIV-1 RNA levels > 50 copies/ml although plasma HIV-1 RNA levels remain < 50 copies/ml [147]. Despite these results, the clinical and neuropathological consequences of asymptomatic HIV-1 CSF escape remain unclear. During untreated infection, HIV-1 in the brain is mainly found in microglial cells and macrophages and these cells are also thought to be the cellular sources of sustained HIV-1 replication in the CNS due to their long-lived profiles [148]. In untreated HIV-1 infection, a gradually increasing compartmentalization occurs over the course of infection [149]. After treatment initiation, the decay rate of HIV-1 compartmentalized viral variants is similar to plasma viral decay rates. However, in neurologically impaired individuals, CNS-derived viral variants decay at a slower rate, indicating other cellular sources in these individuals [150]. There is a variation in CSF concentrations compared to plasma for different drugs, and an index for the

rate of drug penetration into the CNS has been developed [151]. The clinical use of such an index has been debated due to some caveats. First, measuring drug concentrations in the CNS is hampered by the same issues as in the genitourinary tract (see above). Antiretroviral drugs are substrates for a variety of transporters that could potentially affect drug levels [152]. As an example, Dahl et al. found no difference in this index between timepoints with detectable CSF HIV-1 RNA and to timepoints without detectable CSF HIV-1 RNA [153]. Second, penetration indexes are based on pharmacokinetic data rather than pharmacodynamic considerations, and cofactors such as intracellular metabolism may influence the effectiveness of drugs in the CNS [154]. Furthermore, despite lower drug concentrations in the CNS and a slower viral decay rate during treatment, there is still an overall effectiveness of a wide variety of drug regimens in suppressing CSF HIV-1 RNA, as well as in preventing or improving neurocognitive problems seen in untreated HIV-1 infection [154-157]. HIV-1 infection in the CNS is further discussed in Chapter 1.8.

1.5.3 Residual viremia

Once antiretroviral treatment is initiated, plasma viral loads decrease as cART prevents all (or almost all) infection of new target cells. Since free plasma virus has a very short half-life [158], viral decay in plasma during treatment with cART is primarily affected by the life span of previously infected cells. Several phases of viral decay during treatment have therefore been identified. First, a rapid exponential decay with a half-life of 1 to 2 days is seen, corresponding mainly to the infection of activated $CD4^+$ T-cells [159]. This is followed by a gradual phase 2 decay with a half-life of 2 to 3 weeks [158, 160]. The cells corresponding to this phase of decay are not known; however, based on the expected life span of these cells, macrophages has been proposed. Although most patients achieve viral suppression < 50 HIV-1 RNA copies/ml during this second phase of viral decay, a key observation during cART is HIV-1 persistence [110-112, 161]. Using more sensitive methods of quantification, a third phase of decay with a half-life of 39 to 63 weeks (9 to 15 months) has been identified, followed by a fourth, extremely stable phase in which no further decay has been demonstrated [162, 163] (Figure 5). After the third phase of decay, a stable viral set-point around 1 to 3 HIV-1 RNA copies/ml seems to establish itself in most patients on cART [161-163]. This level of cryptic viremia during cART has been termed residual viremia. The half-life of the cells corresponding to the third phase of decay matches the described characteristics of resting memory $CD4^+$ T-cells. A very stable proportion of these cells may also constitute the fourth phase, corresponding to cells with a possible rate of division equal to their death rate. To some extent the residual viral set-point is correlated to pre-

therapy plasma viral levels but not to treatment regimen [162]. Levels of pre-treatment total HIV-1 DNA have also been correlated to levels of residual viremia [164]. Both of these correlating variables reflect the establishment of infection in long-lived cells early in infection, which is also confirmed by the non-evolutionary nature of viruses captured from resting $CD4^+$ T-cells in patients on cART [111, 112, 165]. A more recent study highlights adherence as the only clear predictor of levels of residual viremia [102]. In this study, no clear association between residual viremia and either the duration of virologic suppression, $CD4^+$ T-cell count, or cART regimen was significantly associated with levels of residual viremia.

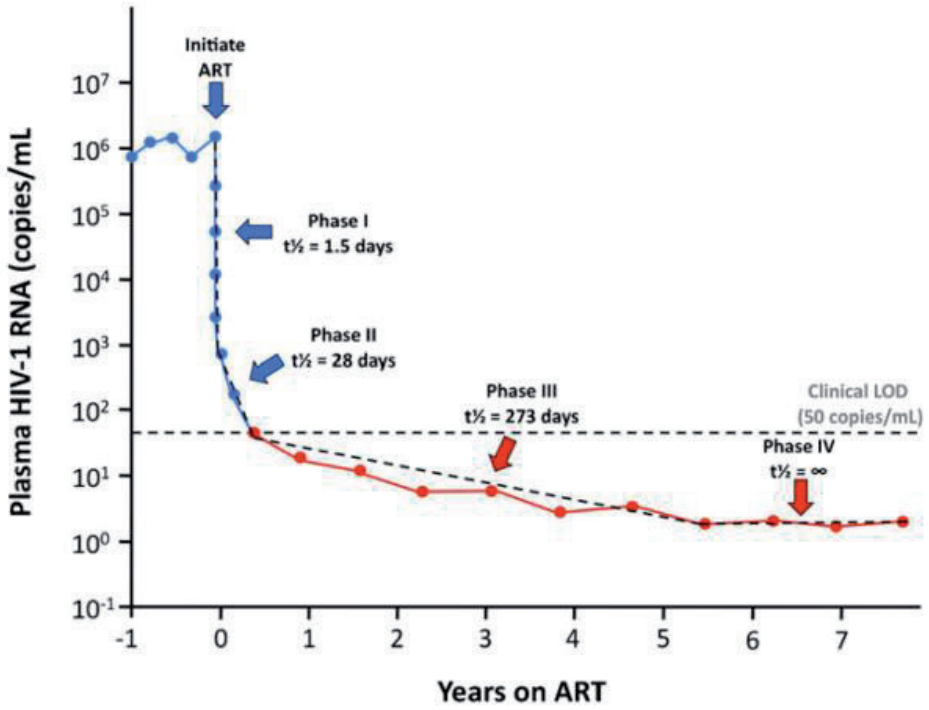

Figure 5. Decay dynamics of plasma HIV-1 RNA during cART treatment. Upon initiation of cART, viremia decays in multiple overlapping phases, which reflects the turnover of cells infected prior to cART with different half-lives. Blue = above clinical limit of detection (LOD). Red = below clinical LOD (detectable by ultrasensitive PCR assays). Dotted lines = theoretical decay slopes. Adapted from: *Curr HIV/AIDS Reports* 2012; 9:91-100. Reprinted with permission from Springer Publishing.

The source of viremia and how HIV-1 virions are produced during therapy has been debated. In principle, residual viremia could result from a low degree of ongoing viral replication, the release of virus from latently infected cells that have become activated, or the release of virus from other stable reservoirs. When residual viremia was first confirmed, it was assumed that ongoing cycles of replication were possible despite cART [161]. Due to the nature of HIV-1, this theory implies a few important assumptions. First, this scenario raises the possibility of viral evolution and the emergence of drug resistant viral strains during residual viremia. Viral evolution has been reported [166, 167], but in most studies to date, no solid evidence for viral evolution during cART has been found [106, 111, 114, 119, 168]. Furthermore, although some data indicates a risk for virologic rebound to HIV-1 RNA plasma levels > 50 copies/ml in patients with detectable plasma viral loads < 50 HIV-1 RNA copies/ml (see above) [94-96], the development of DRAMs is generally not seen in patients with stable low residual viremia [99, 101, 106, 114, 165, 166, 169]. This is also consistent with the finding that rebound viremia in patients interrupting antiretroviral therapy has demonstrated no evidence of ongoing genetic variability during suppression [122]. When signs of viral evolution are found, it is often hard to exclude the possibility that these viral variants did not exist before therapy but were not found because of limited sampling. PCR errors and the lack of longitudinal sampling are other significant factors [99]. The issue of adherence is also essential and can never be fully elucidated in these studies. Furthermore, in many studies reporting viral evolution and resistance mutations, there is an uncertainty regarding the phase of decay patients were in: they may not have reached the residual viral set-point. Another assumption in the case of ongoing replication during cART is the possibility of affecting residual viremia by intensified cART with an additional drug. Most studies of intensified cART with additional drugs found no effect on HIV-1 RNA levels [169-173]. Buzon et al. reported elevated levels of episomal HIV-1 DNA (2-LTR circles) in patients who were given raltegravir, which could indicate ongoing replication [174]. This finding is controversial and no effect on residual viremia was seen in the study. In the confirmatory studies that followed, conflicting results regarding 2-LTR circles were reported [170, 175].

The current data regarding the source of residual viremia is in general consistent with the release of archival viruses from stable reservoirs. Although multiple reservoirs may exist, replication-competent HIV-1 can be isolated from resting $CD4^+$ T-cells in plasma of all patients on cART, regardless of the duration of treatment. This makes this reservoir the best established barrier to HIV-1 eradication. However, some of the residual viremia may be due to ongoing rounds of replication, perhaps in

compartments not effectively penetrated during cART treatment, such as GALT or the CNS (discussed further in Subchapters 1.5.2 and 1.8.1). In studies where ongoing replication could be suspected due to increased 2-LTR circles in the presence of raltegravir, this was mainly seen in patients with a protease inhibitor (PI) treatment. However, whether these effects were the result of poor drug penetration in specific tissues remains to be clarified [173, 175]. The use of 2-LTR circles as reflective of viral replication has also been questioned by the stability of these DNA forms, at least in vitro [176] (see also Chapter 1.7). Under optimal circumstances, cART blocks replication of virus to such an extent that resistant viruses that do arise seem unable to establish a foothold and expand within the population [99]. The key issues regarding drug resistance appear to be primarily related to toxicity and adherence, and so the main focus should be on appropriate monitoring of patients [102].

1.6 Curing the infection

1.6.1 Eradication strategies

With the discovery of HIV-1 persistence in resting memory $CD4^+$ T-cells it became clear that cART alone would not eradicate the infection. Several different strategies to purge the reservoir have been pursued but most of them have failed to have any proven sustained effect on the latent reservoir. There is, however, one case of potential cure that has been described and has generated renewed optimism in the field. This case ("the Berlin patient") involved an adult HIV-1 infected patient who received a hematopoietic stem cell transplantation (HSCT) for acute lymphatic leukemia [177]. The donor was carefully selected to be homozygous for a 32-base-pair deletion in the HIV-1 co-receptor CCR5. The patient had been doing well on cART with undetectable HIV-1 RNA levels in plasma. Prior to transplantation, the patient was subjected to chemotherapy, antithymocyte globulin treatment, and whole-body irradiation. cART was discontinued at the time of transplantation but no viral rebound was seen. Extensive analysis of different tissues did not find consistent evidence for persistent HIV-1 infection 5 years after transplantation with discontinuation of cART [178]. Several factors apart from the homozygous CCR5 deletion in the transplanted cells, were probably responsible for eliminating the HIV-1 reservoir, including graft-versus-host reaction as well as lympholytic and immunosuppressive interventions [177]. Similar trials involving HSCT in HIV-1 infected patients have attempted to purge the reservoir by transplanting cells from individuals with wildtype CCR5 to HIV-1 infected patients heterozygous for the 32-base-pair deletion in CCR5. Although a considerably long-term suppression of

viral replication in the absence of cART was seen, both patients rebounded with high viral loads [179]. Persons who are homozygous for a 32-base-pair deletion in CCR5 are almost completely protected from acquiring HIV infection [180, 181]. Heterozygous individuals are infectible, yet the course of infection tends to be less aggressive [180, 182]. It is unclear whether the heterozygous deletion of CCR5 played a role in the outcomes of these HSCT cases.

The second case of an HIV-1 cure ("the Mississippi baby") was thought to have been achieved in a perinatally infected baby who was given cART within 30 hours of delivery. cART was discontinued after 18 months of treatment and viremia remained undetected for more than 1 year [183]. Unfortunately, viremia returned after more than 2 years after discontinuation of cART [184]. This suggests that latently infected cells may be dormant for long periods of time. However, this case provides strong in vivo evidence to support the early establishment of a latent reservoir after infection and emphasizes the importance of early therapy initiation.

In recent years, latency-reversing agents to upregulate HIV-1 gene expression have attracted much attention. By reactivating all the cells that are latently infected, it is presumed that they will die, either from the cytopathic effects of viral replication, or from immune responses directed against them. In the presence of cART, no new rounds of infection should appear, thus eliminating the infection. Early versions of this approach explored the effects of global T-cell activation by recombinant human interleukin-2 and CD3 antibodies [185-187]. However, global T-cell activation resulted in unacceptable toxic side effects, and no depletion of the reservoir was achieved. Instead, other more selective strategies of reversing latency have been studied. Currently, histone deacetylase inhibitors (HDACi) are the most prominent latency-reversing agents being tested in human clinical trials. In 1996, Van Lint et al. showed that histones positioned at specific sites in the HIV-1 LTR can inhibit HIV-1 gene expression [188]. It was also shown in several studies that histone deacetylation was important for HIV-1 latency and that HDACi could reverse latency [188-190]. The use of HDACi as a latency-reversing agent is attractive because HDACi have been used for treatment of different malignancies, and several drugs containing them already exist on the market [191-193]. Generally, HDACi are well tolerated, with the most common acute toxicities being fatigue, gastrointestinal, and transient cytopenias. The first HIV-1 clinical trial with valproic acid, a weak HDACi, reported a modest decrease of reservoir size but in follow-up studies no sustained effect was seen [194-196]. A more potent HDACi, vorinostat, showed increased cell-associated HIV-1 RNA, indicating increased HIV-1 transcription, but failed to show any increase in residual viremia [197]. A

follow-up multiple-dose study did not show a sustained increase in HIV-1 gene expression or any effect on residual viremia [198]. In a comparative study evaluating the potency of different HDACi, panobinostat was found to be the most potent stimulator of HIV-1 expression from latently-infected cell lines [199]. Cyclic dosing of this drug is currently being tested in clinical trials and preliminary data show increases in cell-associated unspliced HIV-1 RNA, as well as increased detection of plasma HIV-1 RNA in patients treated with panobinostat [200]. Further studies will reveal if this HDACi is potent enough to show a sustained diminishing effect on the HIV-1 reservoir.

1.6.2 IVIG

Intravenous immunoglobulin (IVIG) is a therapeutic preparation of normal human polyclonal IgG obtained from pooled plasma taken from thousands of healthy blood donors. It was initially introduced as replacement therapy in primary and secondary immune deficiencies [201] but is now widely used in a number of autoimmune and inflammatory diseases [202-206]. IVIG has multiple modes of action that are thought to act synergistically. The different modes of action involve the modulation of expression and function of Fc receptors on target cells; modulation of dendritic cell; and T and B-cell activation, differentiation, and their effector functions [207, 208]. IVIG has also been shown to modulate the production of cytokines causing antiinflammatory effects in vivo [209]. In the pre-cART era, IVIG was used for infection prophylaxis in pediatric HIV infections and has been used for replacement therapy in pediatric HIV-infected patients on cART with combined hypogammaglobulinemia [210-212]. IVIG therapy is relatively safe. Adverse reactions to IVIG occur in 5 to 10% of all patients and are usually mild. Rare although serious side effects including anaphylactic reactions, occur in patients with IgA deficiency, which is associated with the development of anti-IgA antibodies that react with IgA in the IVIG preparation. Other rare side effects are thromboembolic events occurring in patients with a high pre-therapy risk of thrombosis [201]. Two trials, one of them presented in this thesis (Paper I), have been performed to explore the potential of IVIG as a latency-reversing agent [213, 214].

1.7 Measuring the reservoir and monitoring the infection

1.7.1 Methods to measure the reservoir

Approaches for targeting the latent reservoir are being developed based on an understanding of how the reservoir is measured. Since the major reservoir for

HIV-1 during suppressive treatment is considered to be resting memory $CD4^+$ T-cells (see above), most techniques for measuring the reservoir are directed at this cellular subset. Although these cells are distributed in both plasma and lymphatic tissues and have possible compartment issues [134], there is a cross-infection between these two sites [119]. For convenience, studies are usually performed on cells found in blood. Currently, the infectious units per million cells (IUPM) assay is considered the most sensitive and precise technique for measuring viral reservoirs. This assay is based on co-culture and activation of highly purified resting $CD4^+$ T-cells from the patient together with peripheral blood mononuclear cells (PBMCs) from a HIV-negative donor [215]. Using a limiting dilution in the activation step of this procedure, it is possible to estimate the replication-competent proportion of the viral reservoir. This assay has its drawbacks in being costly and time consuming, as well as having a high variance–a feature making it difficult to measure small differences in replication-competent viruses with accuracy. Furthermore, a recent report indicates that this assay underestimates the actual replication-competent viral reservoir [216].

An alternative way of estimating the viral reservoir is by quantitative polymerase chain reaction (qPCR) of HIV-1 DNA, and many assays for this have been developed. Estimating a reservoir by qPCR has the advantage of being less labour intensive than IUPM and may also offer a more stable technique for monitoring viral reservoirs in patients. Latent infection can occur in either a pre-integration or a post-integration state. Pre-integration is considered a labile state and is represented by a linear DNA form with an estimated half-life of 1 to 5 days [217]. Some of the viral DNA is not integrated to reach a stable post-integration state and forms episomal DNA forms (2-LTR and 1-LTR circles) that are not integrated into the host cell genome [27]. These circular DNA forms are mainly produced during active replication. Some studies propose a more stable profile of these DNA forms, at least in vitro [176] or in vivo during treatment with the integration inhibitor raltegravir [218] but there is also in vivo evidence for instability of these forms during cART [219]. The stability of these unintegrated DNA forms has consequences for the measurement of the viral reservoir by qPCR. Since linear unintegrated HIV-1 DNA has a short half-life in vitro and circular DNA is considered to be mainly produced during active replication, one could reason that most of the HIV-1 DNA will be integrated if replication is halted by cART for a long period of time. Measures of total and pro viral HIV-1 DNA have, therefore, been proposed as interchangeable surrogates for reservoir size [217]. Since measurement of total DNA is easier and less laborious to perform, this technique would be favourable for monitoring viral reservoirs. Total HIV-1 DNA has been shown to be correlated to integrated HIV-1 DNA in patients on suppressive cART [220], a

finding also confirmed by our group (Figure 9). Other studies have suggested that measurement of integrated pro-viral HIV-1 DNA may be important in estimating reservoir size since some patients have been shown to have an access of unintegrated HIV-1 DNA even during cART [221]. This would agree with the findings of Murray et al. [218]. Perhaps a combination of these assays is important for estimation of the viral reservoir [220]. A major problem with all qPCR-assays is their inability to differ replication-competent viruses from non-viable genetic information incorporated into the host cell genome. In a structured analysis of different assays to measure the viral reservoir, Eriksson et al. showed that there was a high variability in the relationship between different qPCR methods and IUPM. In general the former showed > 100-fold higher infection frequencies than IUPM [222]. The true reservoir appears to lie somewhere between measures by IUPM that underestimate the reservoir and measurements of total or integrated HIV-1 DNA that overestimate the reservoir. Nevertheless, qPCR measurements of plasma HIV-1 RNA have lately been shown to reflect the replication-competent reservoir in resting $CD4^+$ T-cells, and it has been proposed that very sensitive measurements of residual HIV-1 RNA viremia may estimate the actual HIV-1 reservoir [194, 223]. This is also consistent with a study by Josefsson et al. showing that sequences of resting $CD4^+$ T-cells and residual viremia intermingle [69].

1.7.2 Measuring residual viremia

Correct and robust measurements of residual viremia are crucial in evaluating HIV-1 eradication strategies. In recent years it has also been proposed that, at least in some patients, it may be clinically relevant to monitor viremia even < 50 HIV-1 RNA copies/ml [94, 96]. The single copy assay (SCA), a quantitative technique that can detect less than 1 copy of HIV-1 RNA/ml, has been widely used in recent years [153, 173, 224, 225]. However, it is labour intensive and it is currently limited to quantification of subtype B strains [226]. As discussed in Subchapter 1.2.4, there is a need for subtype-independent assays, since many research and clinical sites must deal with a diversity of subtypes, and the spread of different subtypes throughout world is increasing. For measurement of HIV-1 viremia in a reliable and automated way, the commercial qPCR kits available today offer a quality control and performance that most in-house methods lack. With a particular kit, uniform performance on a day-to-day basis can be expected. The commercial kits also provide quantification of most HIV-1 group M subtypes. However, these assays are not sensitive enough to measure residual viremia and they have a high variability in samples with an HIV-1 RNA copy number less than 20 to 50 copies/ml depending on the assay [227, 228]. At very low copy numbers (< 20 copies per reaction tube in the assay), the random variation due to

sampling error (Poisson's error law) becomes significant [229]. Stochastic influences can be reduced by increasing the sample volume, followed by ultracentrifugation to enrich the material. This modification has been done in commercial assays [105, 230] as well as in in-house assays [161, 226] in order to study residual viremia. Havlir et al. used the Roche Amplicor Ultrasensitive assay to reach a 2.5 HIV-1 RNA copy/ml sensitivity, whereas Yukl et al. used the Abbot RealTime assay and managed to quantify HIV-1 RNA at a single-copy/ml level [105, 230]. At present, the commercial assays that are mainly used to monitor plasma viral load in patients are the Abbot RealTime assay and the COBAS Taqman HIV-1 test version 2.0. Both are based on real time PCR, which have replaced many of the older PCR assays. When compared using clinical samples (without any assay modifications), these assays show equivalent performance on higher viral loads, but substantial variation at lower viral loads, making them hard to compare when used in different studies [228]. The same method must be used in longitudinal monitoring of patients on cART. The older version of COBAS Taqman HIV-1 test (version 1.5) underestimated viral loads in some HIV-1 group M non-B strains [231, 232]. To address this problem, Roche developed an assay with a dual-target strategy, incorporating both *gag* and LTR regions. It showed improved efficiency and corrected previous underestimated results [228, 233]. The COBAS Taqman HIV-1 test, version 2.0, seems to have higher detectability rates than the Abbot RealTime assay [228] and has also lowered the limit of quantification to 20 copies/ml, compared to 40 copies/ml in the older version. At present, no group has reported any attempt to modify the COBAS Taqman HIV-1 test, version 2.0 and no reports on subtypes have been incorporated into modifications of either the Amplicor test or the Abbot RealTime assay. As part of this thesis, our group has performed the first modification of the COBAS Taqman HIV-1 test, version 2.0 to allow quantification of residual viremia and also incorporate clinical performance on different HIV-1 subtypes.

1.8 HIV-1 and the central nervous system

1.8.1 Neuropathogenesis

Before the cART era, neurological manifestations of HIV-1 infection was common and in many cases severe. Without treatment, HIV-1 causes subacute encephalitis in approximately 20% to 30% of the patients [234]. This state was initially recognized as AIDS dementia complex (ADC), now termed HIV-associated dementia (HAD). With the introduction of cART, the incidence of HAD has been markedly reduced in developed countries, and even severely impaired patients have improved after treatment initiation

[235-237]. Even so, despite virological suppression, neurological symptoms continue to manifest themselves in HIV-1 infected patients. These neurological effects are now grouped under a broader term: HIV-associated neurocognitve disorders (HAND). They comprise a spectrum of HIV-related neurological impairments including asymptomatic neurological impairment (ANI), mild neurocognitive disease (MND), and fully-manifest disease (HAD). The prevalence of HAND differs widely between studies ranging from 18% to 69% of all patients on cART [238-240]. These diverging results could be explained by the fact that prevalence of HAND in the cART era is almost exclusively driven by the milder forms of HAND (ANI and MND). ANI and MND diagnosis are based on neuropsychometric testing abnormalities with or without functional impairment in activities of daily living. When compared to the best available population controls, it appears that approximately half of the HIV population have lower levels of performance than predicted. However, the lack of proper matched norms, knowledge of prior performance, and other confounding effects leave the diagnosis of ANI and its clinical utility controversial, and an overestimation of the actual neurological effects in the HIV-infected population could be suspected [241, 242]. It is, however, evident that a large proportion of cART-treated HIV-1 infected patients suffer from neurological symptoms, and intensive research has been directed towards the mechanisms of viral persistence and pathogenesis in the CNS. This is important since milder forms of HAND are associated with decreased quality of life, poor treatment adherence, and higher mortality risk [243-245].

HIV-1 establishes an infection in the CNS very soon after transmission. Virus can be found in the CSF [246, 247] and in brain tissues [248] during primary infection. Exactly how the virus enters the CNS through the blood brain barrier (BBB) is not fully understood. It is generally believed that viral entry occurs through BBB crossing of virus-carrying monocytes and $CD4^+$ T-cells and subsequently, local cells within the CNS are infected [249-251]. Compartmentalization of HIV-1 in the CNS is further discussed in Subchapter 1.5.2. The damage that HIV-1 does to the brain is thought to be mediated by neurotoxic molecules or by oxidative stress caused by neuroinflammation. Neurotoxic molecules may be produced directly by the virus or as a result of immune activation. For example, HIV-1-transactivating protein (Tat) have the ability to disrupt the integrity of the BBB [252, 253] and has been shown to be the target of $CD4^+$ T-cell autophagy [254]. Gp 120 is also known to cause neurotoxicity, at least in vitro [250]. Indirect neurotoxicity is suggested to be mediated through the reaction of macrophages and microglia to the chronic infection [255]. Associated neurotoxic factors include quinolinic acid, tumor necrosis factor, platelet activating factor, and arachidonic acid metabolites [250]. Inflammation and

immune activation can be detected immediately after viral entry into the CNS [247, 256]. Markers of intrathecal immunoactivation is gradually increased in untreated primary infection [256] and it has been shown that up to 10% of all individuals develop neurological symptoms during this early stage of infection. Early CNS injury, measured by levels of CSF neurofilament light protein (markers of axonal disruption) and proton-magnetic resonance spectroscopy (MRS)-based metabolites has also been correlated to markers of neuroinflammation in neuroasymptomatic patients during primary infection [257]. According to these studies, it seems likely that neuronal injury and dysfunction already occurs during this stage of disease.

cART dramatically decreases the level of immune activation in blood and CSF as well as reducing neuronal damage [258-261]. Even so, several studies have shown that a low-grade neuroinflammation persists despite suppressive cART [262-265]. However, it is not known whether this low-grade immune activation entails a risk for the progression to HAND. HIV-1 associated neurocognitive disorders are also associated with nadir $CD4^+$ T-cell counts [239]. A higher degree of prior immunosuppression (reflected by a lower $CD4^+$ nadir) might establish a "neurological legacy" whose consequences may persist. Whether CNS damage in treated HIV-1 infections is associated with ongoing neuronal disruption or by residual brain damage prior to treatment initiation is still unclear, but both these mechanisms may be of significance. Effective antiretroviral treatment benefits the brain via multiple mechanisms, including immune recovery, reduced immune activation, and viral suppression – both systemically and in the CNS. Early treatment therefore, may reduce the risk of developing HIV-1 related neurocognitive disorders.

1.8.2 Biomarkers of CNS infection in HIV-1 disease

As discussed above, the formal diagnosis of HAND relies on neuropsychometric testing against normative controls, a procedure with many confounding factors. These tests do not distinguish between ongoing brain injury from residual brain damage and do not help to describe HIV-1 neuropathogenesis. Furthermore, brain tissue is obviously not accessible for sampling in living HIV-1 infected individuals. The main pathogenic components of HIV-1 infection in the CNS can be measured by biomarkers of the CSF, an approach that has gained clinical interest in the past decade. Three components are needed to estimate the systemic effects of HIV-1 in the CNS: viral, immune-inflammatory, and neural (CNS injury).

Nearly all untreated HIV-1 infected patients have measurable levels of CSF HIV-1 RNA that limits the diagnostic value of this biomarker. However, characterization of the viruses in the CSF is important, especially in patients on cART with neurosymptomatic viral escape [145, 146]. Using the new quantitative PCR assays, HIV-1 RNA is also an important biomarker to assess disease progression and treatment effects. With very sensitive assays (SCA), HIV-1 RNA can also be found in neuroasymptomatic patients on cART at levels that seem to be associated with intrathecal immune activation measured by neopterin [153, 156]

Biomarkers of immune and inflammation responses include soluble markers such as cytokines, chemokines, markers of cell activation and BBB dysfunction [266]. Cell-based phenotypic markers of activation are not appropriate for estimation of CNS inflammatory responses because of the scarcity of such cells in the CSF. Neopterin, a soluble metabolite of pteridine, has been shown to be a cardinal marker of intrathecal immune activation in HIV-1 infection. This biomarker is produced by cells of the monocyte-macrophage lineage and most likely also by astrocytes in response to stimulation by interferon gamma and reflects activation of these cells [267, 268]. Neopterin is closely linked to intrathecal immune activation and participates in several biological pathways involving macrophages and oxidative stress [269, 270]. Neopterin is also simultaneously released with neurotoxic substances, such as quinolinic acid after induction by HIV-1 regulatory proteins *tat* and *nef,* reflecting the possible use of this biomarker also for these neurotoxic processes [271]. In untreated HIV-1 infected patients, CSF neopterin is almost always increased and levels are correlated to disease progression with higher neopterin levels with decreasing $CD4^+$ T-cell counts [270]. Patients with HAD exhibits particularly high neopterin levels. After initiation of cART, neopterin decreases but despite suppression of CSF HIV-1 RNA < 50 copies/ml, mild elevations of neopterin can be seen in many patients, indicating a low-level persistent intrathecal immune activation [263, 265, 270].

Several neural biomarkers to assess CNS damage have been studied. At present, the most promising seem to be the light subunit of neurofilament protein (NFL). This is a major structural component of myelinated axons determining axon calibre and is important to maintain effective nerve conduction [272]. Levels of NFL is normally correlated to age and can be measured in CSF. Pathological levels reflects axonal disruption which can be seen in many neurological conditions, such as neurodegenerative diseases, relapsing multiple sclerosis, extrapyramidal symptoms, and cerebral infarction [273-275]. NFL has also been studied in HIV-1 disease and it has been shown that NFL is substantially increased in patients with HAD.

Increased levels has also been shown in some untreated, neurologically asymptomatic patients, mainly in those with low $CD4^+$ T-cell counts, indicating subclinical brain injury [276]. Those findings were confirmed in Paper IV in this thesis [258]. NFL may also predict progression to HAD in untreated patients [277]. Furthermore, normalization of NFL has been shown both in patients with and without HAD after initiation of cART [259]. NFL has the advantage of reflecting ongoing CNS injury and with the new, more sensitive assay, NFL can now be measured in most normal subjects allowing the detection of smaller increases in pathological states which is further elaborated in Paper IV of this thesis [258, 278].

2 AIMS

To expand the knowledge of methods for monitoring HIV-1 during treatment; potential strategies to eradicate the infection and neurological damage due to the chronic infection. The specific aims were:

I. To evaluate potential long-term effects of intravenous immunoglobulin on residual viremia when given to patients on cART. To analyze the relationship between residual viremia and T-cell activation markers.

II. To enhance the sensitivity of the COBAS Taqman HIV-1 test, version 2.0, to enable subtype-independent assessment of residual viremia, and to evaluate a sensitive method of measuring total HIV-1 DNA in PBMC.

III. To evaluate a potentially sensitive and subtype-independent method of sequencing HIV-1 low level viremia.

IV. To examine axonal injury and neuropathogenesis in HIV-1 patients by measuring the light subunit of neurofilament protein (NFL) and markers of neuroinflammation in CSF.

3 MATERIALS AND METHODS

3.1 Patients

3.1.1 Paper I

Nine HIV-1 infected patients were followed at the Department of Infectious Diseases, Sahlgrenska University Hospital, Gothenburg, Sweden. They had all been previously taken part in a pilot study of the potential effects of IVIG treatment [213]. All patients had been on cART for more than two years and were virologically suppressed (HIV-1 RNA < 50 copies/ml) for at least 1.5 years prior to IVIG therapy. All patients remained virologically suppressed at the time of the follow-up study. A control group of fourteen HIV-1 infected patients fulfilling the same inclusion criteria as the treated group was added in the follow-up study.

3.1.2 Paper II

Archived samples from four HIV-1 infected patients were used to assess sensitivity and reproducibility in both the modified version of the COBAS Taqman HIV-1 test, version 2.0, and the in-house real time PCR for total HIV-1 DNA. Samples from these patients were selected for their high viral loads in plasma in order to perform dilution series. (See methods section for more detailed information on these patients.)

Both methods were tested in a correlation study performed on samples archived from nineteen HIV-1 infected patients. All patients had been on cART for more than 1 year and were virologically suppressed (< 50 HIV-1 RNA copies/ml for a median of 54 months). All patients but two had undetectable HIV-1 RNA (measured by the COBAS Taqman HIV-1 test, version 2.0). All clinical samples were collected by the Department of Infectious Diseases, at Sahlgrenska University Hospital, Gothenburg, Sweden.

3.1.3 Paper III

We collected sequenced samples longitudinally from thirty-two HIV-1 infected patients on cART. All samples were collected by the Department of Clinical Virology, Sahlgrenska University Hospital, Gothenburg, Sweden. For each sample, a specific request for sequencing had been made the patient's clinician. Inclusion in the study was based on the need for ultra-

centrifugation and the use of nested PCR because of low HIV-1 RNA copy numbers (< 1000 copies/ml).

3.1.4 Paper IV

A longitudinal research project on HIV-1 infection in the CNS was undertaken at the Department of Infectious diseases, Sahlgrenska University Hospital, Gothenburg, Sweden in 1985. Since then, voluntary patients willing to undergo lumbar punctures for research purposes have been sampled yearly in both CSF and plasma. Additionally, lumbar and venous punctures are performed at the beginning and three months after initiation or cessation of cART therapy. Similar protocols have been performed at the Department of Neurology, University of California, San Francisco, California, USA. In two studies, one cross-sectional and the other a longitudinal cohort design, we collected archived samples and data from HIV-1 infected patients at the two aforementioned centers. The cross-sectional study included 252 patients divided into 6 subgroups. Four groups consisted of untreated patients without overt neurological disease who were subdivided according to levels of blood $CD4^+$ T-cells into < 50 $CD4^+$ T-cells per ml (n = 42), 50 to 199 cells per ml (n = 49), 200 to 349 cells per ml (n = 52), and > 350 (n = 57) cells per ml; one group of HAD patients (n = 14); and one group on suppressive cART with plasma HIV-RNA < 50 copies/ml for at least one year (n = 85). Forty-six subjects were included in both untreated and treated groups sampled at different points in time. We also included 204 healthy HIV-negative controls. The longitudinal cohort study included 78 neuroasymptomatic patients who were treatment-naive or had been off treatment for > 6 months. CSF samples were analyzed before and after treatment initiation, with a median time of 15 weeks.

3.2 Laboratory methods

3.2.1 Ultrasensitive HIV-1 RNA PCR methods

In Paper I, we measured residual viremia with a modified version of the Roche Amplicor Monitor Test (version 1.5, Roche Diagnostic Systems, Hoffman-La Roche, Basel, Switzerland). Increased sensitivity was achieved through the same technique of ultra-centrifugation prior to PCR, as presented in Paper II below.

In Paper II, we used the COBAS AmpliPrep/COBAS Taqman HIV-1 test, version 2.0, for measurements of plasma HIV-1 RNA. This is a nucleic acid amplification test for the quantification of HIV-1 RNA in human plasma. Specimen preparation is automated using the COBAS AmpliPrep Instrument

and amplification and detection is automated using the COBAS TaqMan Analyzer. The method employs a dual target approach, with primers and probes directed at both LTR and *gag* genes. HIV-1 RNA is quantified using a non-infectious armored RNA construct (Quantitation Standard or QS) that contains HIV sequences with identical primer binding sites as the HIV-1 target RNA and a unique probe binding region that allows HIV-1 QS amplicon to be distinguished from HIV-1 target amplicon. The HIV-1 QS is added to each specimen at a known copy number and is carried through the specimen preparation, reverse transcription, PCR amplification, and detection steps of cleaved dual-labeled oligonucleotide detection probes. The COBAS TaqMan Analyzer calculates the HIV-1 RNA concentration in the test specimens by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen and control. To enrich the material prior to PCR and thus creating a more sensitive assay, the samples were ultra-centrifuged at 50,000 x g at 4°C for 60 minutes. The supernatant was removed and HIV-1 seronegative plasma was added to each sample to resuspend the pellet. The suspension was vortexed and transferred to PCR tubes, then stored at –70°C. PCR was conducted in accordance with the manufacturer's protocols.

3.2.2 Methods to measure total and integrated HIV-1 DNA

Quantification of total HIV-1 DNA was performed using a real time PCR in a single-step procedure. Extraction of total HIV-1 DNA was done using a DNA Blood Extraction Kit from Qiagen. For each extraction 10 to 15 million PBMCs were used. Primers and probes were designed to target the LTR region of HIV-1. Quantification was determined in reference to a standard curve generated by amplification of plasmid pCR2.1, harboring an LTR-LTR junction and two copies of the human CCR5 gene. This plasmid showed excellent performance with a linearity of 0.99 for both LTR and CCR5 and slopes -3.37 for LTR and -3.34 for CCR5. The amplification efficiency could be calculated to 98% for LTR and 99.3% for CCR5.

Integrated HIV-1 DNA was quantified using a two-step PCR procedure. Extraction of HIV-1 DNA was performed in the same way as for total HIV-1 DNA. HIV-1 is integrated near to the up-stream or down-stream of the human *alu*-repeat segment. Therefore, a chronically infected cell line harboring HIV-1 DNA integrated genomes was used as the quantification standard. The first PCR is an end-point PCR in eight cycles with primers designed to target the region *alu-gag*, thus also incorporating the LTR-region. As a negative control (background), a separate reaction with primers targeting only *gag* was included in the first PCR-step. This was followed by a second nested real time PCR for 45 cycles with the same primers and probes

as used for the PCR for total HIV-1 DNA (LTR quantification). The result was compared to the *gag*-only background, and the amount of integrated HIV-1 DNA in the sample was calculated in reference to the amount of CCR5 and integrated LTR in the standard curve generated from the chronically infected cell-line.

3.2.3 Sequencing

Two methods of sequencing low level viremia in HIV-1 patients have been used at the Department of Clinical Virology in Gothenburg. The methods differ in the first amplification step, where either an in-house protocol or the ViroSeq® HIV-1 Genotyping System by Abbot has been used. The same in-house nesting protocol was applied regardless of the initial PCR. Large plasma volumes (preferably 10 ml, but such volume was not always available) were concentrated by ultra-centrifugation at 50000 x g at 4°C for 60 minutes. The pellet was resuspended in 500 µl of the supernatant to concentrate the sample approximately 20-fold. The suspension was vortexed and transferred to PCR tubes, and then stored at –70°C until analysis. In the in-house protocol for the first amplification step, PR and RT amplification were performed in separate reactions as opposed to the Viroseq® PCR amplifying both coding regions in one reaction (Figures 6 and 7). The nesting PCR reaction was performed, targeting RT and PR coding regions in separate reactions (Figures 6 and 7). (Details regarding extraction procedures, volumes and PCR conditions are described in the method section in Paper III.) Nested products, detected by gel electrophoresis and UV illumination, were purified with the QIAquick PCR Purification kit (QIAGEN, Germany), according to the manufacturer's protocol. The purified products were cycle-sequenced in unilateral reaction mixtures with the nesting primers and the Big Dye Terminator Kit, version 1.1 (Applied Biosystems, Foster City, CA, USA), as recommended by the manufacturer. Sequencing products were analyzed on the Avanti 3130 XL genetic analyzer (Applied Biosystems). Sequence proofreading, alignment, and comparative sequence analysis were performed using Sequencher, version 4.9 (Gene Codes Corp, Ann Arbor, MI, USA).

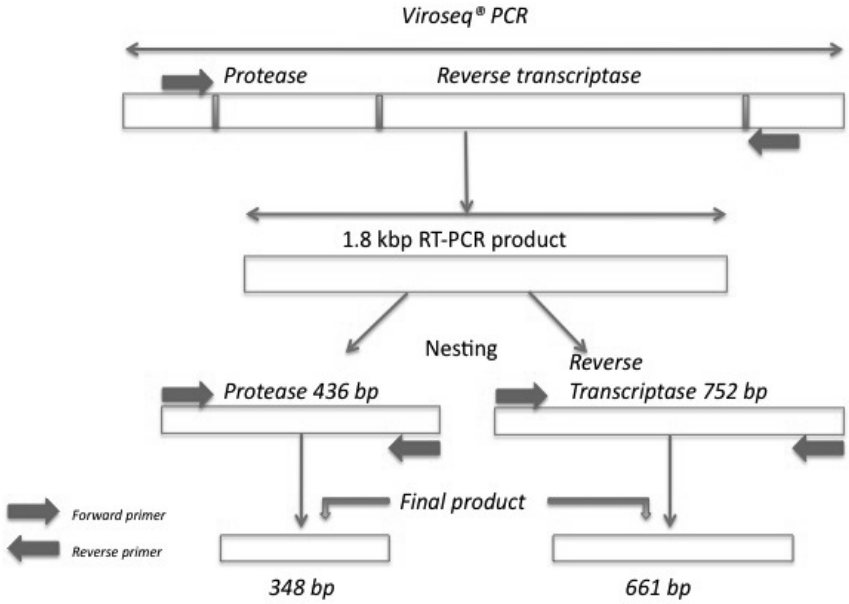

Figure 6. Initial PCR using Viroseq® HIV-1 Genotyping System (Abbot) followed by an in-house nested PCR.

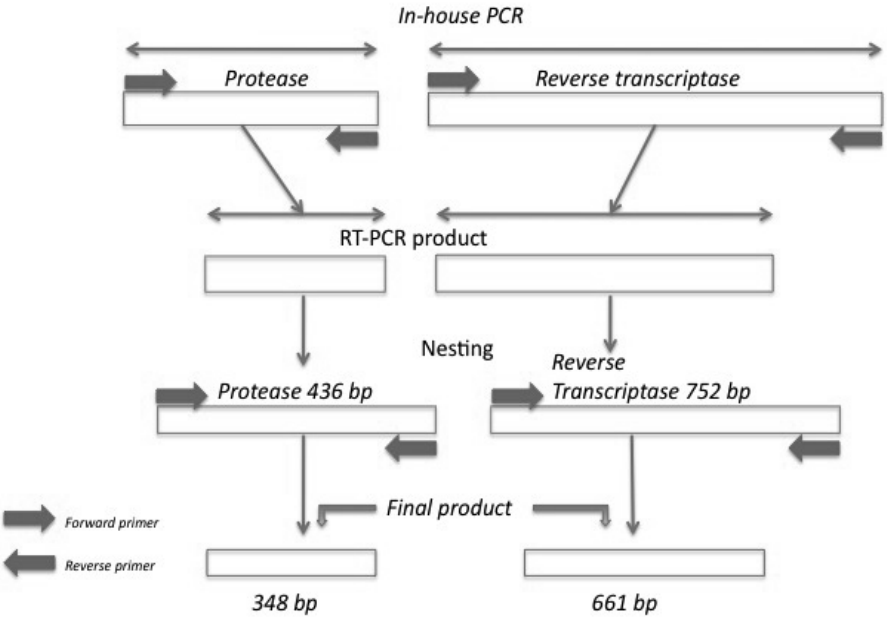

Figure 7. Initial in-house PCR followed by in-house nested PCR.

3.2.4 T-cell characterization, activation markers and cytokines

In Paper I, flow cytometry was used for phenotypic analysis of lymphocytes and measurement of T-lymphocyte activation markers CD38 and HLA-DR. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). HIV-1 responses by $CD8^+$ T-cells were measured by means of a specific HIV-*gag* p55 peptide pool from JPT Peptide Technologies, Berlin, Germany. CMV, EBV, and Flu (CEF) control peptide pool, as well as Staphylococcal Enterotoxin B (SEB) (SIGMA-Aldrich Logistic GmbH, Schnelldorf,

Germany), were added as positive controls. Plasma samples from all patients were analyzed for the presence of IL-2 and IL-7 cytokines on a Luminex 100™ System (Luminex Corp, Austin, TX, USA). (Details regarding these analyses are presented in Paper I.)

3.2.5 CSF biomarkers

In Paper IV, CSF NFL protein was measured in the Neurochemistry Laboratory at the University of Gothenburg using a novel, sensitive sandwich ELISA method (NF-lightH ELISA kit), as described by the manufacturer (UmanDiagnostics AB, Umeå, Sweden). The lower limit of quantification was 50 ng/l and the intra- and inter-assay coefficients of variation were 4.1% to 13%. Neopterin was analyzed in CSF using a commercially available immunoassay (BRAHMS, Berlin, Germany) with an upper normal reference value of 5.8 nmol/l [270].

4 RESULTS

4.1 Paper I

In an earlier study, our group investigated the effect on the latent reservoir of intravenous immunoglobulin (IVIG), given in addition to suppressive cART [213]. This concept originated from the observation of a HIV-1 infected patient with Guillan-Barré syndrome who was given IVIG during cART treatment. In the course of IVIG treatment, a temporary plasma HIV-1 RNA increase was observed, and later, when cART was discontinued, the patient remained virologically suppressed for several months [279]. In the proof-of-concept study that followed, several effects of IVIG treatment during suppressed HIV-1 infection were revealed. As observed in the Guillan-Barré patient, an initial transitory low level increase in plasma HIV-1 RNA was seen during IVIG treatment. These viruses seem to originate from the resting memory $CD4^+$ T-cell compartment. A reduction in reservoir size as measured by IUPM, was observed in a majority of the patients. At 8 to 12 weeks after IVIG treatment, only one patient exhibited detectable plasma HIV-1 RNA, compared to five patients at base-line (Figure 8). IVIG also resulted in increased levels of serum interleukin 7 (IL-7) in the patients responding to treatment and a consistent increase of $CD25^+$ $CD127^-$ regulatory T-cells (Tregs) was seen in all patients after IVIG treatment [213].

The main purpose of the follow-up study presented here was to investigate potential long-term effects on residual viremia in patients treated with IVIG. We also wanted to study the levels of Tregs in these patients and the relationship between residual viremia and T-cell activation markers in both IVIG-treated patients and controls. Fourteen HIV-1 infected patients on suppressive cART were included as controls. We found that six out of nine IVIG treated patients had detectable levels of residual viremia at follow-up (48 to 104 weeks after IVIG treatment) and showed a rebound of residual viremia to pre-treatment levels (Figure 8). We did not detect any significant difference in the Treg ($CD4^+$ $CD25^+$ $CD127^-$ $FoxP3^+$) proportion of peripheral blood $CD4^+$ T-cells between IVIG treated patients and controls. We found no correlation between residual viremia and T-cell activation markers or specific HIV-1 responses in any of the groups. In an ad-hoc analysis, we found that levels of residual viremia were correlated to $CD4^+$ T-cell counts in both groups indicating that a higher $CD4^+$ T-cell population may harbor additional quantities of virus. Our results indicate that the effect of IVIG treatment on residual viremia was transient. Although we did not

estimate reservoir size with IUPM in this follow-up study, we assume, based on the previous findings, that the effect on reservoir size is reflected by the levels of residual viremia. Therefore, we suggest that the effect on reservoir size was also transient. A correlation between HIV-1 RNA and $CD4^+$ T-cell count might indicate that patients with a higher $CD4^+$ T-cell count harbor a larger residual pool of latently infected $CD4^+$ T-cells.

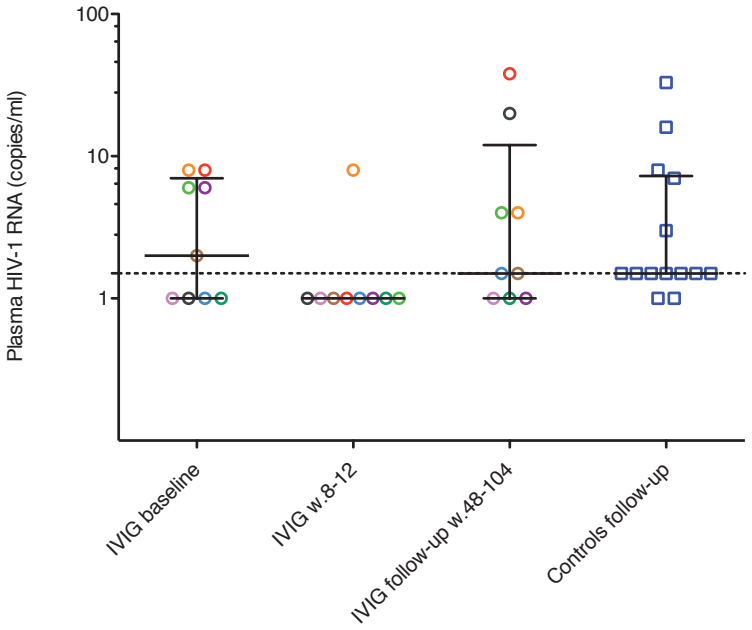

Figure 8. The effect of IVIG on residual viremia. At 8 to 12 weeks after IVIG administration, only 1 out of 9 patients exhibited detectable plasma HIV-1 RNA. At follow-up 6 out of 9 patients had detectable plasma HIV-1 RNA and no difference was seen compared to controls included at follow-up. Detection limit marked by grid line.

4.2 Paper II

The most sensitive published assay to measure residual viremia is the single-copy assay (SCA) developed by Palmer et al. [226]. This method is limited to subtype B viral clades, which limits its use since a majority of HIV-1 infections worldwide are non-B isolates. We, therefore, evaluated a modification of the COBAS Ampliprep/COBAS Taqman HIV-1 test, version 2.0 (AS-CT2), with the hypothesis that this would enable subtype-independent estimation of residual viremia. We achieved a sensitive quantification of plasma HIV-1 RNA down to 3 copies/ml and concluded that this assay could be used to assess residual viremia (Table 2). We tested the assay on subtypes B, C, D, F, and CRF_AE and all subtypes were detected by this ultrasensitive assay. We also evaluated a sensitive assay for quantification of total HIV-1 DNA in PBMC and found good reproducibility and a limit of quantification at 3 copies/million PBMC. This assay quantified all subtypes but CRF_AE, probably reflecting primer mismatch. We studied the relationship between total HIV-1 DNA in PBMC and residual viremia and found no significant correlation in this study. In an additional study, integrated HIV-1 DNA was quantified and a significant correlation was seen between integrated and total HIV-1 DNA (result not reported in Paper II) (Figure 9). We believe that the plasma HIV-1 RNA assay and the assay for quantification of total HIV-1 DNA are suitable for clinical use and for research in settings where a variety of HIV-1 subtypes are present.

Table 2. Comparison of The COBAS Ampliprep/COBAS Taqman HIV-1 test, version 2.0 (AS-CT2) with an ultra-centrifugation step prior to the standard protocol (Ultrasensitive AS-CT2) in two separate dilution series (Panel 1 and Panel 2). Input HIV-1 RNA copies/ml are theoretical concentrations calculated from a dilution series originated from a plasma sample quantified using AS-CT2. All samples were run in quadruplicate.

Panel 1	AS-CT2			Ultrasensitive AS-CT2		
Input HIV-1 RNA copies/ml	Mean HIV-1 RNA copies/ml	SD	Samples detected (%)	Mean HIV-1 RNA copies/ml	SD	Samples detected (%)
2683	3283	684	100	1675	307	100
537	639	49	100	442	205	100
107	118	23	100	69	22	75
26.8	-	-	100	26	15	100
5.4	-	-	75	5.7	0.4	75
2.7	-	-	50	-	-	100

Panel 2	AS-CT2			Ultrasensitive AS-CT2		
Input HIV-1 RNA copies/ml	Mean HIV-1 RNA copies/ml	SD	Samples detected (%)	Mean HIV-1 RNA copies/ml	SD	Samples detected (%)
27	91	75	100	93	23	100
9	36.5	11	100	31	8.3	100
3	-	-	25	8.7	4.7	75
1	-	-	-	2.5	0.6	75

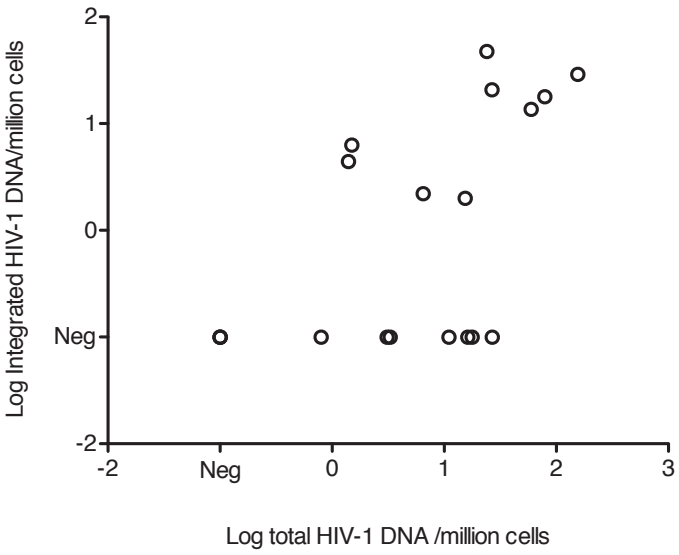

Figure 9. Correlation between total and integrated HIV-1 DNA/million PBMCs. Neg = negative. Spearman r = 0.57, p = 0.01.

4.3 Paper III

Clinical assays for amplification and sequencing offer the ability to amplify and sequence different HIV-1 subtypes. However, at low levels of viremia their sensitivity is inadequate. Validated results are usually not guaranteed below 1000 HIV-1 RNA copies/ml. Therefore, an in-house protocol for enrichment of genomic material by ultra-centrifugation and a nested PCR has been developed at the Department of Clinical Virology, Gothenburg. In a longitudinal study, we investigated the success rate of amplification and sequencing at low level viremia in 32 patient samples. In each case, a specific request for sequencing was made by the patient's clinician. Inclusion was

based on the need for ultra-centrifugation because of low RNA copy numbers (< 1000 copies/ml).

For the initial PCR step, both the ViroSeq® HIV-1 Genotyping System by Abbot and an in-house protocol was used, the two methods being in part complementary to one another. The same in-house nested PCR was used regardless of the initial PCR. Eight previously known subtypes were represented; A, B, C, D, F, G, CRF_AE, and CRF_AG. The success rate of amplification of both PR and RT coding regions in the same sample was 100% in samples with a viral load above 100 copies/ml. Below 100 copies/ml we managed to amplify both regions in 7/13 (54%) of the samples. Amplification of either PR or RT in a sample was successful in 30 out of 32 samples (94%), with a median plasma viral load of 119 copies/ml (range < 20 to 966 copies/ml). The assays were able to amplify and sequence either PR or RT in all but one subtype A specimen. We observed DRAMs in 12 patient samples. However, the study was not designed to evaluate de novo resistance mutations, but only to show the possibility of sequencing coding regions at these levels of viremia. We concluded that the use of an ultra-centrifugation step and a nested in-house PCR increased sensitivity and enabled subtype-independent amplification and sequencing of HIV-1 low level viremia.

4.4 Paper IV

The mechanisms behind neurological impairment in HIV-1 disease are not fully understood. Diagnosing milder forms of HIV-1 neurocognitive impairment is also problematic since the diagnosis only relies on neuropsychological testing. We wanted to examine axonal injury in HIV-1 patients in a more precise way by measuring the light subunit of NFL in CSF with a novel, sensitive method (see Chapter 1.8). In order to further study HIV-1 neuropathogenesis, we evaluated the relationship between this biomarker and markers of intrathecal immunoactivation (neopterin) and BBB integrity (CSF/plasma albumin ratio) as well as the association with the level of systemic and CNS viral replication as measured by levels of plasma and CSF HIV-1 RNA, and systemic immunosuppression as reflected in blood $CD4^+$ T-cell counts. In a cross-sectional study, we included both HAD patients and neuroasymptomatic patients in different $CD4^+$ T-cell strata and compared these groups with HIV-1 negative controls. In a longitudinal study we compared levels of CSF NFL in neuroasymptomatic patients before and after cART initiation. We found that 13/14 of the HAD patients had elevated CSF NFL concentrations. Additionally, elevated CSF NFL concentrations were found in a substantial number of the untreated neuroasymptomatic subjects, particularly those with lower blood $CD4^+$ T-cell counts. The

association between disease progression and CSF NFL was also shown by a strong correlation of CSF NFL with the blood $CD4^+$ T-cell counts in neuroasymptomatic untreated patients.

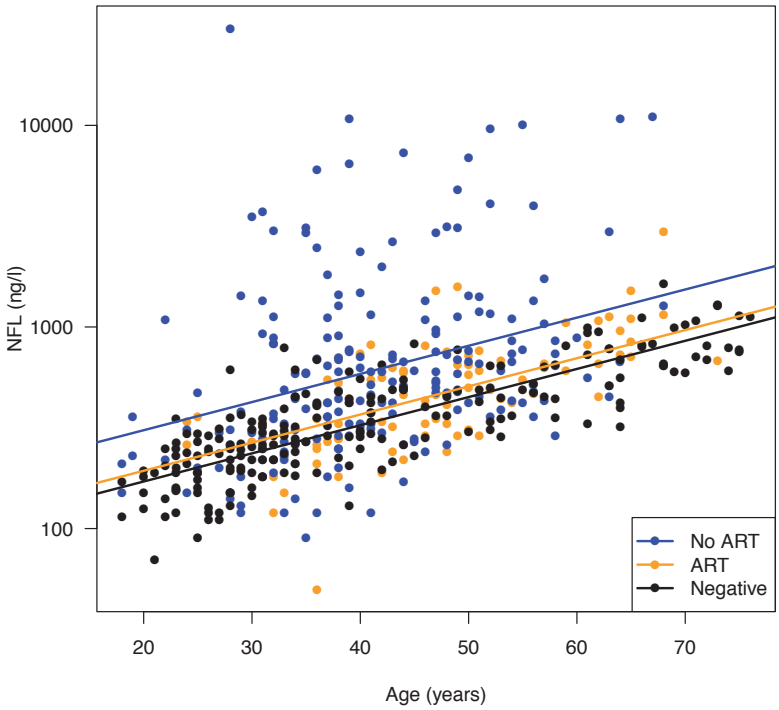

Figure 10. CSF NFL related to age and treatment effect. Concentrations of CSF NFL in neuroasymptomatic untreated HIV-1 infected subjects (No ART) were equivalent to those of HIV-1 negative subjects (Negative) who were 18.5 years older ($p < 0.001$). CSF NFL concentrations in the treated group (ART) were equivalent to those of HIV-1 negative subjects who were 3.9 years older ($p < 0.01$).

When analyzing age as a covariate (since CSF NFL normally is correlated to age), we found that the CSF NFL in untreated neuroasymptomatic patients was equivalent to subjects 18.5 years older than the HIV-1 negative controls. Even HIV-1 infected patients on suppressive cART exhibited an increased CSF NFL, compared to HIV-1 negative controls, corresponding to 3.9 years of "axonal aging" (Figure 10). In a multivariate analysis, we found that apart from age, levels of CSF NFL were correlated to CSF neopterin and CSF

white blood cell (WBC) counts in untreated patients. CSF neopterin was not independently associated with CSF NFL in the treated group. However, CSF albumin ratio was an independent predictor for CSF NFL in both treated and untreated HIV-1 infected patients. Pre cART plasma viral load was also found to be an independent predictor of CSF NFL levels in the treated group. In general, treatment decreased levels of CSF NFL in neuroasymptomatic patients when initiating cART. A significant decrease of CSF NFL levels was only seen in the proportion of patients who exhibited elevated levels before treatment initiation. We concluded that CSF NFL has a value as an independent biomarker of neurological damage in HIV-1 disease. Our data indicate the presence of subclinical neurological damage in a large proportion of untreated patients. Even virologically-suppressed patients exhibited higher levels of CSF NFL than HIV-1 negative controls, suggesting ongoing low grade injury or an aging effect of HIV-1 infection that is not fully reversed by treatment. The most important influencing factors in addition to age were $CD4^+$ T-cell counts, CSF neopterin levels, CSF WBC counts, and CSF/plasma albumin ratio, reflecting the importance of systemic disease progression, immune activation and BBB dysfunction in HIV neuropathogenesis.

5 DISCUSSION

Although effective treatment is available for a growing number of infected individuals, HIV-1 is the cause of morbidity and death in millions of people worldwide. With treatment, a majority of patients manage to control HIV-1 replication and avoid progression towards AIDS. However, the chronic infection induced by HIV-1 continues to cause co-morbidities and stigma in many patients. The latent reservoir established by HIV-1 in resting memory $CD4^+$ T-cells and the presence of anatomical reservoirs, such as the CNS, have become some of the most challenging obstacles in HIV research.

This thesis seeks to provide additional information on several issues regarding the latent reservoir and ways to monitor infection during cART.

No one has yet succeeded in demonstrating long-term effects on the latent reservoir through the use of latency-reversing agents. In order to understand how to reverse HIV-1 latency, we need reliable and sensitive methods to measure the reservoir. We also need to be able to monitor the virus at low levels, both to evaluate eradication trials, and to manage treatment strategies in the case of low-level replication with a potential risk for viral rebound. The worldwide diversity of HIV-1 subtypes is another challenge that needs to be addressed with the use of subtype independent methods. Finally, HIV-1 infection within the CNS constitutes a major problem in individuals on suppressive cART as well. We need to obtain more knowledge on neuropathogenesis and the extent to which neurological damage is present during different stages of disease. To accomplish this, more objective ways to estimate neuronal degradation in HIV-1 infected patients would be helpful.

Purging the latent reservoir

The latent reservoir is established very early in infection. Even treatment initiation within a few hours of transmission has not been sufficient to eradicate the infection [183, 184]. Several strategies other than early treatment have been pursued in attempts to purge the reservoir. Among those, latency-reversing agents such as HDACi have gained interest in recent years. Although an effect on latent HIV-1 gene expression has been shown in a majority of trials, a concern is that very little effect has been seen on levels of residual viremia [194, 195, 197, 198, 200]. Residual viremia is generally thought to emanate from stable reservoirs that release viruses into the blood. Furthermore, several studies have linked residual viremia to the viruses that persists in resting memory $CD4^+$ T-cells [69, 194, 213, 223]. When activating

viral gene expression in these cells, some effect on residual viremia would be expected, at least if the activation was potent enough.

We have previously reported that IVIG could serve as an activator of HIV-1 replication. Lindkvist et al. showed indications of a decreased reservoir size correlating to a temporary increase in residual viremia when high dosage IVIG was given to patients with suppressive cART [213]. A reduction in residual viremia was seen 8 to 12 weeks after IVIG administration. In a previous trial, IVIG was administered to HIV infected children on cART with the hope of decreasing viral loads. In this study, IVIG infusion was not associated with a reduction in HIV viral load in any patient. On the contrary, in most patients a transient increase in plasma viremia was observed during IVIG treatment, which further indicates that IVIG seems to stimulate HIV expression [280]. In Paper I, we found that residual viremia was detected in 6 out of 9 IVIG treated patients at approximately 1 year post treatment, compared to detectable viremia in a single patient at 8 to 12 weeks after IVIG was given. We concluded that the effect on residual viremia in the previous study was transient. In line with the argumentation above, we reasoned that this was probably true of the size of the reservoir as well.

Exactly how IVIG would activate HIV-1 gene expression in latently-infected cells is not easily explained. Expression of Fc gamma receptors on T-cells is rare [281], and therefore the mechanism might be indirect, perhaps mediated by cytokines. In the first IVIG study, an increase of interleukin-7 (IL-7) was seen in all patients who decreased their reservoirs during IVIG treatment. It has been shown that IL-7 can activate virus expression and reduce the latent HIV-1 reservoir in a mouse model [282]. IL-7 has also been shown to induce proviral reactivation from resting $CD4^+$ T-cells isolated from HIV-1 infected patients on cART [283]. Furthermore, transient increases in plasma HIV-1 RNA levels and inducement of T-cell cycle entry has been demonstrated when administrating recombinant IL-7 to HIV-1 infected patients on cART [284]. These data suggests IL-7 as a possible mediator of HIV-1 activation during IVIG treatment. A consistent increase of Tregs was also seen in the study by Lindkvist et al. Tregs are important in modulating chronic inflammatory responses [285], and elevated levels of Tregs have been reported in elite controllers (individuals capable of controlling viral load without cART) [286]. It is possible that IVIG has a modulating effect on the immune activation seen in HIV-1 infection. However, no differences in Tregs or in IL-7 were seen as compared to controls in Paper I, indicating that these effects also were transiently induced by IVIG treatment.

This study had some limitations. It would have been favourable if also IUPM had been available for use in measuring the reservoir in the follow-up study.

However, this was not possible at the time and so we used residual viremia as an indirect measurement of the reservoir size. Even though a set-point of residual viremia is thought to establish itself in all patients on suppressive cART [162, 163], some fluctuations in the levels of HIV-1 RNA could be expected. There might also be a large variance in the PCR measurements at these plasma viral levels. Therefore, repeated measurements of plasma HIV-1 RNA, both before and after IVIG administration, might have been useful in determining a more precise individual level of residual viremia.

As mentioned above, efficient reversal of latency should lead to transient increases in plasma HIV-1 RNA and changes in residual viremia. These changes could be measured with ultrasensitive PCR methods such as the SCA [226]. However, this method is labor intensive and currently limited to subtype B strains. In Paper II therefore, we modified the commercially available COBAS Taqman HIV-1 test, version 2.0. By adding an ultra-centrifugation step we enriched the genomic material and hypothesized increased assay sensitivity with sustained subtype independence. We obtained an approximately 10-fold increased sensitivity, quantifying HIV-1 RNA down to 3 copies/ml plasma, and all subtypes tested were detectable.

The variation in quantification with PCR is dependent on factors such as laboratory handling, primers/probes specificity, and efficacy of the PCR, including the RT-step for quantification of RNA. In general, a large variance is seen in PCR measurements at low levels of viremia as reflected by large standard deviations and a high signal-to-noise ratio near the limit of detection [287]. When the starting samples are very low, as in assessment of levels of residual viremia, the smallest error in the initial PCR cycles will have great impact on the result since the PCR-reaction is exponential. Interpretation of PCR results at these levels of viremia is also problematic because of stochastic influences. We decreased these problems by increasing the sample volumes, followed by ultra-centrifugation for enrichment of the material. We did not attempt to concentrate virus from more than a maximum 10 ml of plasma, but a larger volume might have lowered the limit of quantification in the assay further. The need for large volumes is also reflected in the HIV-1 DNA assay where it is important to have at least 10 x 10^6 PBMCs before extraction of DNA from cells.

A considerably large variation could be seen when comparing the two HIV-1 RNA PCR sensitivity panels in Paper II (approximately 0.5 log) (Table 2). Even when modifying the assay, this variation could be expected due to the issues mentioned above (intrinsic characteristics of PCR methods and stochastic influences at such low viral concentrations). Therefore, studies of residual viremia in eradication trials using PCR methods available today

might not be precise enough to prove an effect on the latent reservoir. On the other hand, truly effective latency-reversing treatment with a curing potential might result in transient increases in viremia detectable also with standard clinical assays [116, 288]. As a result, the appropriate place for ultrasensitive HIV-1 RNA PCR assays may be in monitoring patients after the eradication trial. For monitoring patients in larger interventions of this kind in the future, subtype independence of the assays would be of utmost importance. The assay presented in Paper II may, therefore, be suitable for close monitoring of residual viremia after eradication trials. The method is easy to perform and is sensitive enough to detect low level viremia before reseeding of the reservoir occurs.

In order to study a potential activation or depletion of the HIV-1 reservoir, proper methods to measure HIV-1 replication and reservoir size are needed. IUPM is considered a good method for estimating reservoir size in resting memory $CD4^+$ T-cells since it takes into account the problem of measuring replication-incompetent viruses [215]. With this assay, only viruses capable of replicating themselves are measured. However, the method is expensive, time-consuming, and requires large volumes of blood (> 100 ml). Furthermore, the dynamic range of the assay is limited to the low frequencies of latently-infected cells. With any substantial reduction in the size of the reservoir, latently-infected cells would no longer be measurable using this assay without an even larger input of cells [116, 215]. The IUPM method also seems to underestimate the true HIV-1 reservoir [216].

PCR methods have been proposed as efficient and reproducible methods of estimating reservoir size. These methods fail to distinguish between replication-competent viruses from incomplete HIV-1 DNA that cannot produce virions, and therefore they overestimate the productive HIV-1 reservoir [222]. However, PCR measurements are less costly and not as labour-intensive to perform as compared to IUPM. Both measurements of total and integrated HIV-1 DNA have been used in several studies to estimate reservoir size [109, 135, 174, 178, 217, 220, 289]. Measurements of total and integrated HIV-1 DNA have also been proposed as interchangeable surrogates for reservoir size in long-term virologically-suppressed individuals [217]. A correlation between total HIV-1 DNA and integrated HIV-1 DNA has previously been described [220] and also confirmed by our group (Figure 9). In Paper II, we evaluated a realtime PCR for quantification of total HIV-1 DNA and demonstrated a sensitivity ranging down to 3 HIV-1 DNA copies/million PBMCs. We were able to quantify all but one subtype (CRF_AE) and concluded that this assay may be useful in research settings where a diversity of subtypes is present.

Subtype independent monitoring of patients on cART

As the PCR assays for monitoring patients on cART have become more sensitive, many patients are found to have measurable levels of HIV-1 RNA in plasma. The best management of this low level viremia is not clear and there is a concern that lower levels of plasma HIV-1 RNA might increase the risk for virological rebound and the development of DRAMs [85, 86, 88, 90, 91]. An increased risk for virological rebound to > 50 HIV-1 RNA copies/ml in patients with a detectable viral load by clinical assays (detection limit 40 to 50 HIV-1 RNA copies/ml) has been described [94, 95]. Even a viremia > 3 copies/ml has been linked to an increased risk for virological failure, compared to patients with undetectable residual viremia measured with an ultrasensitive assay [96]. When interpreting these results one must bear in mind that low level genotyping has some analytical issues, especially in levels < 50 HIV-1 RNA copies/ml because sequence analysis must be obtained from a very limited number of RNA molecules. This present obvious problems with sampling making it hard to draw distinct conclusions as to whether mutations found are new mutations or rather represent older ones not detected in earlier sampling. Although these results are controversial and contradicted by some [97, 98], they highlight the importance of sensitive assays for sequencing at these lower levels of viremia.

Sweden exhibits one of the most diverse HIV-1 epidemics outside Africa [19, 41]. Moreover, the diversity of HIV-1 subtypes is increasing worldwide [19, 37-40]. Subtype independent sequencing assays are, therefore, vital. In Paper III, we presented sequencing results for patients infected with different subtypes who exhibited low level viremia. An ultra-centrifugation step followed by an in-house nesting PCR resulted in subtype independent amplification of both PR and RT coding regions in all samples with a plasma viral load > 100 HIV-1 RNA copies/ml and in a majority of samples below this level. This was a pilot-study and no tests on the reproducibility of the assay were performed. Sequencing at these low viral loads requires extensive amplification of RNA, increasing the risk for PCR-generated errors and contamination of the viral sequences obtained. A possible concern is also that the nested PCR approaches to low-copy sequencing will increase the number of ambiguities in the sequences [290]. Therefore, before this in-house technique can be applied in a broader clinical context, reproducibility tests would be needed. However, the use of this assay show that subtype independent sequencing in low level viremia could be achieved by the use of an ultra-centrifugation step and nested PCR.

Neurological damage in HIV-1 disease

HIV-1 compartmentalization in the CNS has raised concern that this compartment also acts as a sanctuary site for the virus [140-144]. This potential reservoir will, therefore, also need to be considered in order to eradicate the virus. Gama et al. recently highlighted this in a study. They reported that treatment with a potential latency-reversing agent, PKC activator ingenol-3-hexanoate (Ing-B), in SIV-infected macaques led to a decrease of the reservoir in resting memory $CD4^+$ T-cells. However, this treatment also activated viral genomes in the brain, resulting in severe CNS disease in one of the primates [291]. Further insights in HIV-1 neuropathogenesis and CNS accessibility for different therapies are of great importance when developing strategies for a cure.

With the introduction of cART in the mid 1990s, the severe neurological symptoms previously seen in HIV-1 infected patients declined [235-237]. However, milder forms of neurocognitive impairment persist in the cART era and even patients who are virologically suppressed exhibit neurological symptoms. The pathogenesis behind these findings is not clear. HIV-1 can be found in the CSF in virtually all untreated patients early in infection. Signs of intrathecal immunoactivation and ongoing neurological damage have been reported during primary infection [256, 257]. It may be that the neurological effects seen in a subset of patients on suppressive cART reflect residual brain damage acquired before treatment initiation. Markers of intrathecal inflammation as measured by neopterin are elevated in untreated patients but drops significantly when cART is begun [259, 265]. However, even during suppressive cART, intrathecal immune activation remains elevated in a majority of patients [262, 264, 265]. Whether intrathecal immune activation during cART is linked to neuropathogenesis in HIV-1 infection is unclear, but the low-level residual elevation of neopterin in treated patients suggests that suppressive cART does not always restore the normal CNS immunological environment. In Paper IV, we did not find neopterin to be a predictor of CSF NFL in neuroasymptomatic treated patients. However, CSF albumin ratio was an independent predictor for CSF NFL in both treated and untreated HIV-1 infected subjects, reflecting a compromised BBB with increased permeability of serum proteins and other substances into the brain. BBB dysfunction is a common finding in HAD and our data suggest that an increased permeability across the BBB might be linked to neuropathogenesis in both untreated and treated HIV-1 infected individuals [292, 293].

If some of the neurological effects seen during cART could be explained by an ongoing neuronal disruption, it might have future consequences for treatment. CSF NFL has been shown to be a sensitive biomarker for axonal

disruption, and with a new assay CSF NFL can now be measured in most normal subjects, allowing the detection of smaller increases in pathological states. In Paper IV, we found that CSF NFL levels were elevated not only in patients with HAD and in those with progressive disease (< 200 $CD4^+$ T-cells / µl), but in many neuroasymptomatic patients with higher $CD4^+$ T-cell counts as well. CSF NFL was higher in untreated subjects regardless of age, and was equivalent to 18.5 years older HIV-1 negative controls. This elevation was largely reversible with cART, and a majority of patients with elevated CSF NFL decreased their levels when treatment was initiated. Nevertheless, levels of CSF NFL in the treated and virologically suppressed group remained significantly higher than those levels in the HIV-1-negative population, a difference equivalent to 3.9 additional years of life in the treated HIV-1 infected group.

The clinical impact of the generally mild elevations of CSF NFL that we found in Paper IV is unclear. However, a previous study showed that increased CSF NFL was found to be predictive of HAD development in neuroasymptomatic untreated patients as compared to $CD4^+$ T-cell count matched controls in a case-control study [277]. This indicates that untreated asymptomatic patients with elevated CSF NFL might suffer ongoing subclinical neurodegeneration that could give rise to neurological effects in the future. Furthermore, our results indicate a higher degree of axonal disruption in a subset of HIV-1 infected individuals, despite being on a suppressive cART regimen. In these patients, a discrete neuroinflammation and a compromised BBB with increased permeability might be linked to a mild but continuously ongoing axonal degradation. These findings might also indicate that some viruses are not accessible to treatment and that the CNS constitutes a sanctuary site for the infection.

The findings in Paper IV support the use of CSF NFL as a biomarker of CNS damage at different stages of disease. To further evaluate this biomarker, future studies will need to incorporate neuropsychometric testing. This would also help in understanding the prognostic value of CSF NFL in HIV-1 disease.

6 CONCLUSIONS

The use of IVIG as a latency-reversing agent in HIV-1 disease did not result in a measurable long-lasting effect on residual viremia or on T-cell activation. However, this treatment is one of few that has shown some effect on levels of residual viremia. The potency of this agent to reverse HIV-1 latency need to be assessed in a larger placebo-controlled study, perhaps with cyclic dosing.

By modification of a commercial assay, we achieved a sensitive quantification of plasma HIV-1 RNA that could be used to assess residual viremia. Evaluation of a realtime PCR for quantification of total HIV-1 DNA in PBMC showed a sensitivity of 3 copies/million cells. The assays were subtype independent, making them useful for research and clinical application in settings where a variety of HIV-1 subtypes is present.

Subtype independent sequencing of HIV-1 low level viremia was achieved through the use of ultra-centrifugation and nested PCR. This technique can be applied to monitor patients when an increased risk for viral rebound and subsequent viral failure is suspected.

Increased CSF NFL indicates ongoing axonal injury in many neuroasymptomatic patients. Although treatment decreases CSF NFL, treated patients retain higher levels than controls, indicating either continued virus-related injury or an aging-like effect of HIV-1 infection. CSF NFL correlates with neopterin and albumin ratio, suggesting an association between axonal injury, neuroinflammation, and BBB permeability. CSF NFL appears to be a sensitive biomarker of subclinical and clinical brain injury in HIV-1 and warrants further assessment for broader clinical use.

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