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ANTIRETROVIRAL TREATMENT OF HIV-1 IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

HIV-1 invades the central nervous system (CNS) early in the infectious course. It establishes a chronic progressive infection, and triggers an intrathecal immune response. If left untreated, a majority of patients will develop neurological complications, caused by opportunistic pathogens or HIV-1 itself. The most devastating manifestation of HIV-1 in the CNS is AIDS dementia complex (ADC), a subcortical dementia, occuring in about 20% of untreated patients. The incidence of neurological complications has decreased dramatically since the introduction of antiretroviral drugs. In order for these drugs to act in the CNS, they must penetrate the blood-brain barrier (BBB) into the cerebrospional fluid (CSF). It is, therefore, important to determine which agents have this capacity, and what impact they have on HIV-1 CNS infection.

We analysed CSF concentrations of three protease inhibitors (PIs): lopinavir co-formulated with a low dose of ritonavir, and saquinavir in combination with nelfinavir. Lopinavir was detectable in 15/15 samples. The concentrations achieved were probably high enough for antiviral activity in the CSF, generally exceeding severalfold the concentration needed to inhibit viral replication by 50% (IC_{50}). The concentrations of saquinavir were very low, and only detectable in 7/15 CSF samples. Nelfinavir was detectable in 9/15 CSF samples, with concentrations in the range of the IC_{50}. Antiretroviral treatment (ART) containing lopinavir/ritonavir or saquinavir/nelfinavir significantly reduced plasma and CSF viral loads, as well as intrathecal cell-mediated immunoactivation, measured as decreasing levels of CSF neopterin and β2-microglobulin.

HIV-1 has the capacity of establishing viral latency in resting memory CD4+ T-cells, making the virus impossible to eradicate with ART alone. Even in patients on effective ART, a low-level viral replication in plasma can be detected. This probably originates from latently infected cells. To determine whether there is a similar low-level viral replication in CSF, we used an HIV-1 RNA quantification assay with a detection limit of 2 copies/mL in 13 neurologically asymptomatic individuals on effective ART. All patients had CSF viral loads < 2 copies/mL. In plasma, 5/13 patients had levels ranging from 2.3 to 8.2 copies/mL. This makes it unlikely that the CSF in neurologically asymptomatic individuals acts as a viral reservoir in which HIV-1 can replicate independently from the periphery.

CSF neopterin levels remain abnormal in many patients, despite a long period on successful ART. We retrospectively evaluated what influence various levels of CSF HIV-1 RNA, different antiretroviral regimens, and different levels of plasma viral load have on CSF neopterin levels in patients on effective ART. We found that patients with the lowest CSF viral loads (< 2.5 copies/mL) also had the lowest CSF neopterin concentrations. Subjects treated with PI- or non-nucleoside analogue-based regimens had CSF neopterin in the same range. Plasma HIV-1 RNA levels did not affect CSF neopterin levels. These findings indicate that the low-grade persistent intrathecal immunoactivation observed in treated patients is mainly driven by residual viral replication within the CNS. The more the antiretroviral regimen suppresses viral replication in the CNS, the less the intrathecal immunoactivation.

Key words: HIV-1, cerebrospinal fluid, antiretroviral treatment, lopinavir, saquinavir, nelfinavir, neopterin, beta-2-microglobulin, HIV-1 RNA, IgG index, blood-brain barrier, albumin ratio, ultra-ultra sensitive PCR

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. Yilmaz A, Ståhle L, Hagberg L, Svennerholm B, Fuchs D, Gisslén M. **Cerebrospinal fluid and plasma HIV-1 RNA levels and lopinavir concentrations following lopinavir/ritonavir regimen**. *Scand J Infect Dis* 2004, 36:823–828.

II. Yilmaz A, Fuchs D, Hagberg L, Nillroth U, Ståhle L, Svensson JO, Gisslén M. **Cerebrospinal fluid HIV-1 RNA, intrathecal immunoactivation, and drug concentrations after treatment with a combination of saquinavir, nelfinavir, and two nucleoside analogues: the M61022 study**. *BMC Infectious Diseases* 2006, 6:63.

III. Yilmaz A, Svennerholm B, Hagberg L, Gisslén M. **Cerebrospinal fluid viral loads reach less than 2 copies/mL in HIV-1-infected patients with effective antiretroviral therapy**. *Antiviral Therapy* 2006, 11:833–837.

IV. Yilmaz A, Price RW, Spudich S, Fuchs D, Hagberg L, Gisslén M. **Persistent intrathecal immune activation in HIV-1-infected individuals on antiretroviral therapy**. *Submitted*.

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ABBREVIATIONS

ADC	AIDS Dementia Complex
AIDS	Acquired Immune Deficiency Syndrome
ART	antiretroviral treatment
BBB	blood-brain barrier
BID	bis in die (twice a day, Latin)
β2M	beta-2-microglobulin
CCR5	cysteine-cysteine chemokine receptor
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention
CNS	central nervous system
CSF	cerebrospinal fluid
CXCR4	cysteine-x-cysteine chemokine receptor
DNA	deoxyribonucleic acid
env	envelope
FI	fusion inhibitor
gag	group antigen gene
gp 120	glycoprotein 120
HAART	highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HPLC	high performance liquid chromatography
IC_{50}	drug concentration needed to inhibit 50% of viral replication
IgG	Immunoglobulin G
IQR	interquartile range
LOD	limit of detection
MCP-1	Monocyte Chemoattractant/chemotactic Protein 1
nef	negative factor
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
PCR	polymerase chain reaction
PI	protease inhibitor
pol	polymerase
rev	regulator of virion protein expression
RIA	radio-immuno assay
RNA	ribonucleic acid
RT	reverse transcriptase
SIV	Simian Immunodeficiency Virus
tat	transactivator
TDM	therapeutic drug monitoring
TNF-α	Tumor Necrosis Factor-alfa
vif	viral infectivity factor
vpr	viral protein R
vpu	viral protein U
UV	ultraviolet
WBC	white blood cell

INTRODUCTION

A great deal has occurred since 1981, when the first cases of acquired immune deficiency syndrome (AIDS) were discovered in previously healthy, young, homosexual men in the United States [3]. The causative agent of this syndrome, human immunodeficiency virus type 1 (HIV-1) [4], has spread at a ferocious rate, creating one of the greatest epidemics the world has ever seen. In 2006, there were an estimated 4.3 million newly-infected people with HIV and 2.9 million AIDS-related deaths [5]. Sub-Saharan Africa constitutes the worst affected region: it contains 63% of the world's 39.5 million HIV-infected adults and children (Figure 1) [5]. The vast majority of people living with HIV do not know that they are infected, and for those who are aware of their condition, stigma and discrimination related to HIV remain major issues throughout the world. Despite global efforts, including increased access to effective treatment and prevention programmes, the epidemic continues to outpace attempts to diminish it [6].
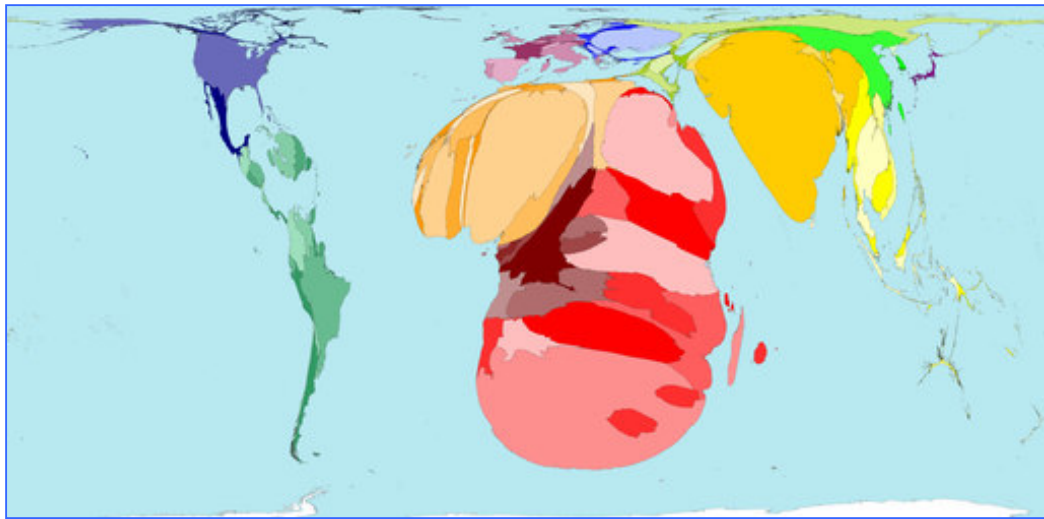

Figure 1. A map of the world in which the size of each country is proportional to the number of people ages 15 to 49 living with HIV/AIDS. (Copyright 2006 SASI Group, University of Sheffield, and Mark Newman, University of Michigan.) <www.worldmapper.org>

The origin of HIV-1 is the simian immunodeficiency virus (SIV), whose natural host is the chimpanzee *Pan troglodytes troglodytes* [7]. There are three distinct groups of HIV-1: major (M), novel (N), and outliers (O) [8]. Only group M strains are of epidemic importance, and they can be further divided into 9 subtypes (A, B, C, D, F, G, H, J, and K); an increasing number of circulating recombinant forms (CRFs); and several unique recombinant forms (URFs) [8, 9]. Subtype B is the most prevalent subtype in Europe and North America. HIV-2 is another retrovirus that can cause AIDS in humans.

Compared to HIV-1, it is less contagious, and it takes longer for HIV-2-infected individuals to develop AIDS [10]. Apart from their diverging clinical features, they also differ in origin and epidemiology. HIV-2 is mostly endemic to West Africa, and is more related to SIV from the sooty mangabey monkey than it is to HIV-1 (hereafter referred to as HIV) [10, 11].

HIV can be transmitted through sexual contact, intravenous drug use, infected blood products, or from mother to child (vertical transmission). Heterosexual contact is the principal route of transmission worldwide, and is the driving force behind the epidemic in sub-Saharan Africa, whereas intravenous drug use contributes substantially to the spread of the disease in Eastern Europe. The mother-to-child transmission can be almost completely eradicated with antiretroviral treatment (ART). No child has been born with HIV in Sweden since 1999 [12]. The HIV prevalence in Sweden remains low, although in recent years condom use has decreased and the incidence of Chlamydia infection has increased, especially in adolescents [13], making it theoretically possible that the number of HIV cases may rise as well. There are currently about 4000 people living with HIV in Sweden, 390 of whom were diagnosed in 2006 [13].

HIV is a retrovirus belonging to the lentivirus genus. The HIV genome consists of three major genes (gag, pol, env) that encode structural proteins, and three viral enzymes: reverse transcriptase (RT), protease, and integrase. There are also six minor genes, coding for regulatory (tat, rev) and accessory (nef, vif, vpr, vpu) proteins. All these proteins have important roles in the viral replicative cycle and viral infectivity [14].
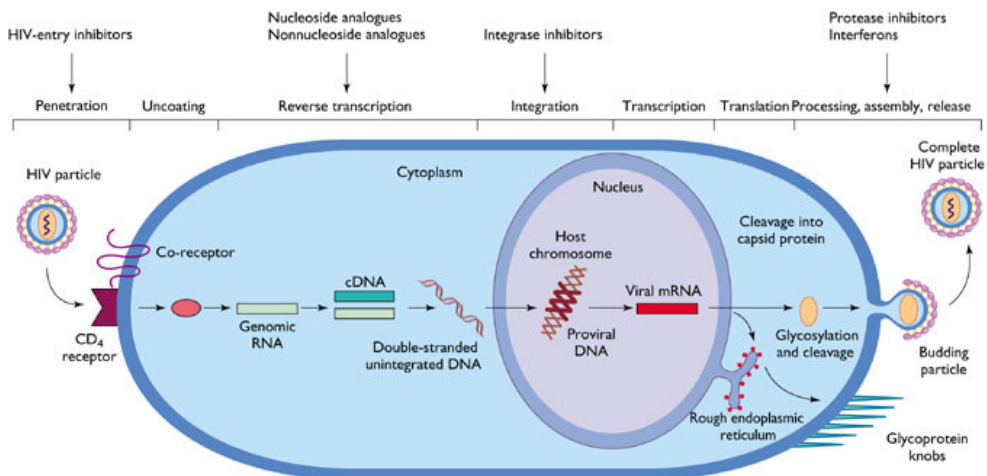

Figure 2. The replicative cycle of HIV and targets for antiretroviral therapy. <www.images.md>

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The first step in the HIV lifecycle involves binding of the viral surface protein glycoprotein 120 (gp120) to the cluster of differentiation (CD) 4 receptor, expressed on the surface of T-lymphocytes, monocytes, macrophages, microglia, and dendritic cells [15]. For successful fusion, additional binding to a β-chemokine co-receptor, mainly CCR5 (R5 strains) or CXCR4 (X4 or T-tropic strains, since they prefer to replicate in T-lymphocytes), is required [16]. R5 strains (also known as M-tropic strains, since they mainly replicate in macrophages) are characteristically found in the early stages of infection. In time, the tropism broadens, allowing the virus to infect a wider repertoire of cells. CCR5 is primarily expressed by memory T-cells, and CXCR4 by naive T-cells [17]. In later stages, about 50% of individuals switch from R5 to R4 tropism. This is associated with a sharp decline in the number of CD4+ T-lymphcocytes (subsequently simplified as CD4 cells) and accelerated disease progression [18]. After cell entry, the viral RNA is reversely transcribed to a DNA intermediate (unintegrated provirus) that, in the nucleus, becomes integrated with the host genome (integrated provirus). Like herpes viruses, HIV has the capacity of establishing latency. There are two different kinds of latency. If HIV enters the CD4 cell in a resting phase (G_0) of the cell cycle, this will lead to preintegration latency, with unintegrated HIV DNA in the cytoplasm. This DNA is unstable and will be decomposed in a few days unless the cell becomes activated. The second type, the postintegration latency, is more stable, leading to integrated provirus in the host genome [19]. HIV from latently infected cells constitutes only about 1% of the total viral population in plasma [20].
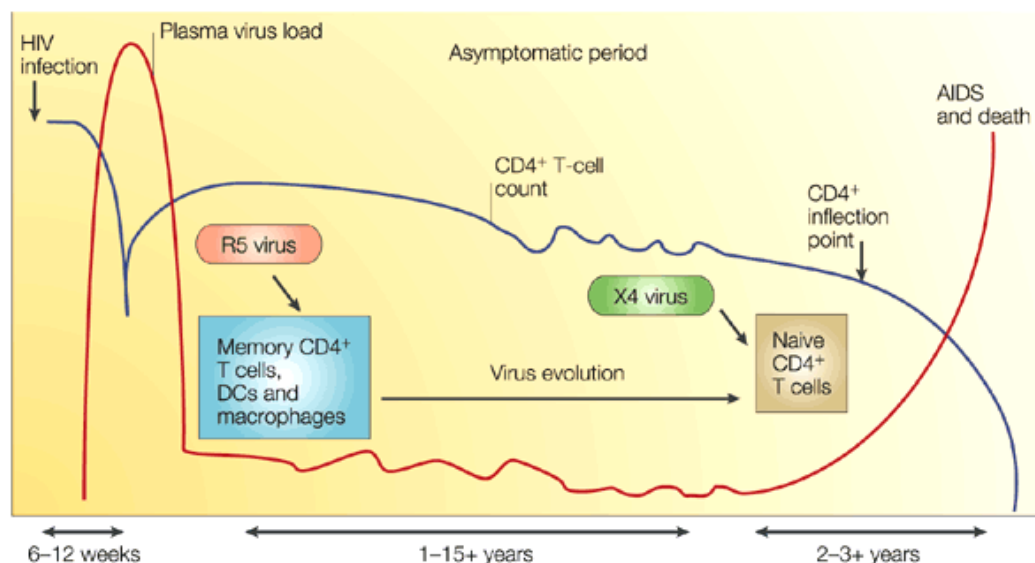

Figure 3. Schematic diagram of the natural course of an untreated HIV-1 infection, showing virus load (red line), and CD4+ T-cell count (blue line). DC, dendritic cell. (Reprinted by permission of Macmillan Publishers Ltd., *Nat Rev Immunol*, Copyright 2003, reference [1].)

Approximately 14 days after transmission, the majority of infected individuals develop a symptomatic primary HIV infection with fever, fatigue, sore throat, rash, and headache [21]. During this initial stage of infection there is a marked increase in plasma HIV RNA, and a fall in the CD4 cell count [22]. The plasma viremia subsides alongside the clinical symptoms after a few weeks, reaching a fixed set point that has shown inself to be predictive of the rate of disease progression. During this clinically quiescent phase, virus particles are produced at an enormous rate, and large amounts of CD4 cells are destroyed and replenished daily [23, 24]. Over time, there is a gradual loss of CD4 cells (Figure 3), making the individual increasingly susceptible to severe infections and tumors. The time span for AIDS to evolve can vary from less than one to more than 20 years [24]. In 1993, the Centers for Disease Control and Prevention (CDC) designed a classification system for HIV-infected individuals based on clinical symptoms and CD4 cell levels (Table 1).

Table 1. 1993 revised classification system for HIV infection and expanded AIDS surveillance case definition for adults and adolescents $\geq$ 13 years of age, and AIDS-defining conditions.

CD4+ T-cell count (x 10^6/l)	Clinical categories		
	Asymptomatic, primary HIV, or PGL* (A)	Symptomatic, not (A) or (C) conditions (B)	AIDS-defining condition (C)
> 500	A1	B1	C1
200–499	A2	B2	C2
> 200	A3	B3	C3

* Persistent generalised lymphadenopathy. The shaded area indicates categories defined as AIDS in Europe. In North America, A3 and B3 are also classified as AIDS.

AIDS-defining conditions (category C)

Candidiasis of bronchi, trachea, or lungs

Candidiasis, esophageal

Cervical cancer, invasive

Coccidioiomycosis, disseminated or extrapulmonary

Cryptococcosis, extrapulmonary

Cryptosporidiosis, chronic intestinal (> 1 month's duration)

Cytomegalovirus disease (other than liver, spleen, or lymph nodes)

Cytomegalovirus retinitis (with loss of vision)

Encephalopathy, HIV-related

Herpes simplex: chronic ulcer(s) (> 1 month's duration)

Herpes simplex: bronchitis, pneumonitis, or esophagitis

Histoplasmosis, disseminated or extrapulmonary

Isosporiasis, chronic intestinal (< 1 month's duration)

Kaposi's sarcoma

Lymphoma, Burkitt's (or equivalent term)

Lymphoma, immunoblastic (or equivalent term)

Lymphoma (primary) of brain

Mycobacterium avium complex or *M. kansasii*, disseminated or extrapulmonary

Mycobacterium tuberculosis, any site (pulmonary or extrapulmonary)

Mycobacterium, other or unidentified species, disseminated or extrapulmonary

Pneumocystis jiroveci pneumonia

Pneumonia, recurrent

Progressive multifocal leukoencephalopathy

Salmonella septicemia, recurrent

Toxoplasmosis of the brain

Wasting syndrome due to HIV

Treatment of HIV-infection

The first antiretroviral drug, zidovudine, was introduced in 1987. It was soon followed by didanosine and zalcitabine. Monotherapy with one of these three nucleoside reverse transcriptase inhibitors (NRTIs) was the only treatment available for HIV-infected individuals for many years. The NRTIs exert their effect by competitive inhibition of the HIV RT. In 1996, the first protease inhibitor (PI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) were registered for clinical use. The PIs target the enzyme protease and thereby inhibit the cleavage of newly-produced HIV polypeptides. The NNRTIs act by inhibiting the RT in a non-competitive way. Enfuvirtide is so far the only registered fusion inhibitor (FI), belonging to a class of drugs called entry inhibitors. Integrase inhibitors and CCR5 antagonists, the latter being the first antiretroviral drug directed against a human target, are available in expanded access programmes, and will probably be registered within the near future (Table 2).

The introduction of combination ART in 1996 led to a dramatic change for many HIV-infected individuals in the developed world, namely, extended survival, fewer opportunistic infections, and improved quality of life. For a while there was even great optimism that HIV could be completely eradicated with a sufficiently prolonged course of highly active antiretroviral therapy (HAART, most often defined as at least three drugs from two different drug classes). However, after finding that HIV also infects long-lived cells like microglia, and establishes latency in resting memory CD4 cells, these hopes were abandoned [25-27]. Treatment is nowadays expected to be life-long.

Recommendations about when to initiate treatment have changed over the years. In the early days it was thought best to "hit hard and early"; but since treatment is not without side effects, the prevailing strategy of late has been to wait for as long as possible before introducing ART. In the most recent Swedish guidelines, published in 2007 [28], the pendulum appears to be swinging the other way. Commencement of therapy is now recommended in individuals with symptomatic infection, AIDS, or before the CD4 cell count drops below 250 cells/μL. This is because HIV-infected individuals have an increased risk of developing non-HIV related diseases and malignancies as well, e.g., anal carcinoma, Hodgkin's lymphoma, and lung cancer [29]. Treatment is initiated with 2 NRTIs plus either a PI or an NNRTI.

The fist line of therapy is the one with the highest probability of achieving success. The virological goal is a decrease of at least 2 $\log_{10}$ in plasma viral load after 4 weeks of treatment, and a viral load < 50 copies/mL within 3 to 4 months. A crucial factor for successful therapy is the patient's willingness and ability to carry out the treatment prescribed. Suboptimal drug concentrations in conjunction with the high replication rate of HIV and the

high error rate of the RT (1 nucleotide/10,000 nucleotides) increases the risk of developing resistance. Poor adherence is the most common cause of suboptimal drug levels, but pharmacokinetic factors can also contribute. Determination of drug concentrations, so-called therapeutic drug monitoring (TDM), for PIs and NNRTIs can be performed in cases of deviant pharmacokinetics (pregnancy, liver or kidney disease, or drug-drug interactions). TDM may sometimes be prompted by pronounced side-effects, the absence of treatment response when dose adjustment is possible, or routinely 2 to 4 weeks after initiation of treatment for drugs where the optimal drug exposure has been defined. Resistance is present when the virus has reduced susceptibility to a particular drug. Some agents only require one mutation to develop resistance (drugs with a low genetic barrier), e.g., lamivudin and the NNRTIs; whereas other drugs have high genetic barriers, e.g., most PIs. Resistance can be determined via genotypic or phenotypic testing (the former being used in Sweden).

Much interest has lately been focused on microbicides. They have been developed to offer women an opportunity to protect themselves against HIV and other sexually transmitted infections without necessitating their partner's involvement. This is especially needed in the developing world, where women may not be in a position to refrain from sex or able to negotiate condom use. Microbicides contain different active substances and are intended for local application in the vagina (gel, creme, or ring), but none have yet been approved. Male circumcision is advocated by some as another way to decrease transmission of HIV [30].

An important side-effect of ART is lipoatrophy of the face, extremities, and the gluteal region. The mechanism behind this is thought to be mitochondria toxicity, mainly caused by NRTI, with increased risk when using stavudine or zidovudine. Mitochondria toxicity can also lead to lactate acidosis, a condition with a high mortality rate [31]. The most common adverse effects for PIs are metabolic disturbances, including hyperlipidemia, insulin resistance and, in the long run, increased risk for cardiovascular disease [32]. The NNRTI efavirenz can cause psychiatric symptoms and rashes, whereas nevirapine may provoke more serious skin reactions. The FI enfuvirtid, which is administred subcutaneously, has as its most common side-effect a reaction at the injection site.

The biggest problem with antiretroviral treatment in the developing world is clearly its absence. The vast majority of HIV-infected individuals worldwide do not have access to these drugs. WHO (the World Health Organisation), UNAIDS (the joint United Nations programme on HIV/AIDS), and UNICEF (the United Nations Children's Fund) have launched major scale-up programmes with the goal of increasing global access to treatment. It has been estimated that by the end of 2006 only 28% of the people living with HIV in low- and middle-income countries were receiving treatment, out of approximately 7.1 million in need [5].

Table 2. Antiretroviral drugs currently used in Sweden.

Class	Group	Generic name	Trade name	Abbreviation	Year of approval
Reverse transcriptase inhibitors					
	Nucleoside analogues (NRTI)				
		abacavir	Ziagen	abc	1999
		didanosine	Videx	ddI	1992
		emtricitabine	Emtriva	ftc	2003
		lamivudine	Epivir	3TC	
		stavudine	Zerit	d4T	1996
		tenofovir*	Viread	tdf	2002
		zidovudine	Retrovir	zdv	1987
	Non-nucleoside analogues (NNRTI)				
		efavirenz	Stocrin	efv	1999
		nevirapine	Viramune	nvp	1998
		etravirin**	—	TMC-125	2007[†]
Protease inhibitors (PI)					
		atazanavir	Reyataz	atv	2002
		darunavir	Prezista	drv, TMC-114	2007
		fosamprenavir	Telzir	f-apv	2004
		indinavir	Crixivan	idv	1996
		lopinavir/rtv	Kaletra	lpv	2001
		nelfinavir	Viracept	nfv	1998
		saquinavir/tablet	Invirase	sqv	2005
		saquinavir/soft gel	Fortovase	sqv-sg	2000
		ritonavir***	Norvir	rtv	1996
		tipranavir	Aptivus	tpv	2005
Integrase inhibitors					
		raltegravir**	Insentress	MK-0518	Jan 2008[†]
Entry inhibitors					
	Fusion inhibitor (FI)				
		enfuvirtid	Fuzeon	T-20	2003
	CCR5 antagonists				
		maraviroc	Celsentri	—	2007[†]

* nucleotide analogue; ** available on licence; *** only used for boosting other PIs

[†] preliminary approval

Neurological manifestations of HIV

Before the introduction of ART, neurological manifestations commonly appeared during the course of HIV in at least 70% of infected individuals [33]. A wide range of neurological disorders are associated with HIV (Table 3). They affect both the central and peripheral nervous system [34]. Neurological symptoms can sometimes appear during primary infection as meningitis, meningoencephalitis, or peripheral neuropathy. Overall, these conditions are rare, but as immune suppression progresses and the CD4 cell count drops, the frequency of AIDS-related neurological complications rises sharply. During these later stages of disease, both opportunistic infections and AIDS dementia complex (ADC), caused by HIV itself, can develop [34, 35]. Among these opportunistic infections, cerebral toxoplasmosis, progressive multifocal leucoencephalopathy (PML), and primary central nervous system (CNS) lymphoma are characterised as focal CNS diseases, often presenting with focal hemispheral dysfunction (i.e., hemiparesis, aphasia, apraxia, or hemisensory impairment) [36]. Causes of non-focal CNS conditions include cytomegaloviral (CMV) encephalitis, metabolic and toxic encephalopathies, and ADC.

Table 3. HIV-associated neurological complications.

Opportunistic infections	Primary viral HIV syndromes
Cerebral toxoplasmosis	AIDS dementia complex
Cryptococcal meningitis	Vacuolar myopathy
Progressive multifocal leukoencephalopathy	Aseptic meningitis
Cytomegaloviral encephalitis	
Other opportunistic infections	**Other**
	Peripheral neuropathy
Tumors	Myopathy
Primary central nervous system lymphoma	Guillain-Barré syndrome
	Cerebrovascular complications

ADC, also called HIV-associated dementia, is characterised by a constellation of cognitive, motor, and behavioral symptoms [37, 38]. The diagnosis of ADC is based on clinical determinations and the exclusion of other diseases by cerebrospinal fluid (CSF) analysis and radio imaging of the brain [39]. The severity of symptoms varies, with deterioration increasing over time. The initial presentation of ADC may include difficulties in concentrating and slowing of mental functions, gradually affecting work or activities of daily life. Motor symptoms develop somewhat later and include slowing of rapid movements of limbs and eyes, and hyperreflexia. The rate at which ADC progresses exhibits large interpatient variability [40]. The Memorial Sloan-Kettering Scale used worldwide to stage ADC consists of 6 degrees ranging from 0 to 4 (0, 0.5, 1, 2, 3, and 4), where 0 represents normal mental and motor functioning, and 4 is an end-stage dementia with a near vegetative state resulting in a) virtual or complete mutism, and b) paraparesis with urinary and fecal incontinence [37].

In the pre-HAART era, about 20% of untreated HIV-infected individuals developed ADC [41, 42]. Nowadays ADC is rare and almost exclusively seen either in patients without ART, or those undergoing treatment but with insufficient virological suppression—for example, because of poor adherence or multiple drug resistance. The majority of patients with ADC placed on ART experience improvement of their symptoms [43, 44]. Paradoxically, the prevalence of ADC in the developed world has increased as a consequence of the prolonged lifespan of HIV-infected individuals [45]. Even though ADC is uncommon in treated patients, neurological symptoms still appear in individuals on HAART whose immune systems remain intact [44]. Such symptoms are often more subtle than in patients with ADC, and are sometimes referred to as HIV-associated mild neurocognitive disorder (MND) or asymptomatic neurocognitive impairment (ANI) [46].

HIV and the central nervous system

Lentiviruses such as HIV exert pathogenic effects in both the immune system and the nervous system. Viral invasion of the CNS occurs very early, often during primary infection [21]. HIV presumably gains access to the CNS via infected monocytes trafficking across the blood-brain barrier (BBB) that once inside the CNS differentiate into perivascular macrophages (the "Trojan horse" theory) [47, 48]. Other proposed mechanisms of CNS entry are passage of cell-free virions via transcytosis through brain microvascular endothelial cells, by direct infection of brain microvascular endothelial cells, or by means of infected lymphocytes [49-51].

Neuropathogenesis of HIV

Once HIV has entered the CNS, it will persist there for a long time—most probably life-long. Virus production mainly takes place in macrophages and microglial cells [52], the only cells in the brain expressing CD4, and with CCR5 as their most common co-receptor [53, 54]. There is no correlation between the number of infected macrophages or microglia and the severity of symptoms in ADC, and it has even been shown that some individuals with ADC seem to lack HIV-producing cells [55]. Endothelial cells do not express the receptors commonly used by HIV, and there is no evidence that this cell type becomes productively infected [56]. Astrocytes have been thought to harbour HIV in a restrictive fashion. The mechanism of viral entry into astrocytes is unclear because they have neither detectable levels of CD4 nor the main HIV co-receptors on their surfaces [57]. Oligodendrocyte infection has not been demonstrated *in vivo* [58]. The neurons are not infected by HIV, but there is still significant neuronal cell death in patients with ADC. This finding indicates that other mechanisms must be involved in the neuropathogenesis of HIV. In fact, a large number of viral as well as host cellular factors have been associated with HIV-induced neurodegeneration (Figure 4).

Infected macrophages and microglia produce HIV and thereby release viral proteins such as gp 120, tat, and vpr. They all display toxic activity against neurons and/or astrocytes *in vitro*, although their effects *in vivo* are not fully established [59-63]. In addition to these viral products, the activated cells also secrete cellular products known to have neurotoxic effects, such as cytokines, quinolonic and arachidonic acid, platelet activating factor (PAF), and nitric oxide. The cytokines tumor necrosis factor-alfa (TNF-α) and interleukin 1β (IL-1 β) can activate uninfected cells and promote the recruitment of activated T-cells from the periphery [48, 64]. The activation of astrocytes leads to astrocytosis and to increased permeability of the BBB, enhancing the leakage of cells and serum products into the CNS. By increasing the release of Ca^{2+} and glutamate, and by decreasing the glutamate uptake, the extracellular concentrations of glutamate and other neurotoxins increase, resulting in neuronal death. Balancing these deletorius effects, the activated macrophages/microglia also have the capacity to release neuroprotective products: β-chemokines, CX3C-chemokine ligand 1 (CX3CL1), and growth factors can promote neuron survival by regulating the Ca^{2+} homeostasis in neurons, by stimualting anti-apoptotic signaling pathways, and by decreasing neurotoxicity caused by gp 120 [59, 65]. TNF-α has also been described as having neuroprotective properties that prevent the accumulation of Ca^{2+} in neurons [66].
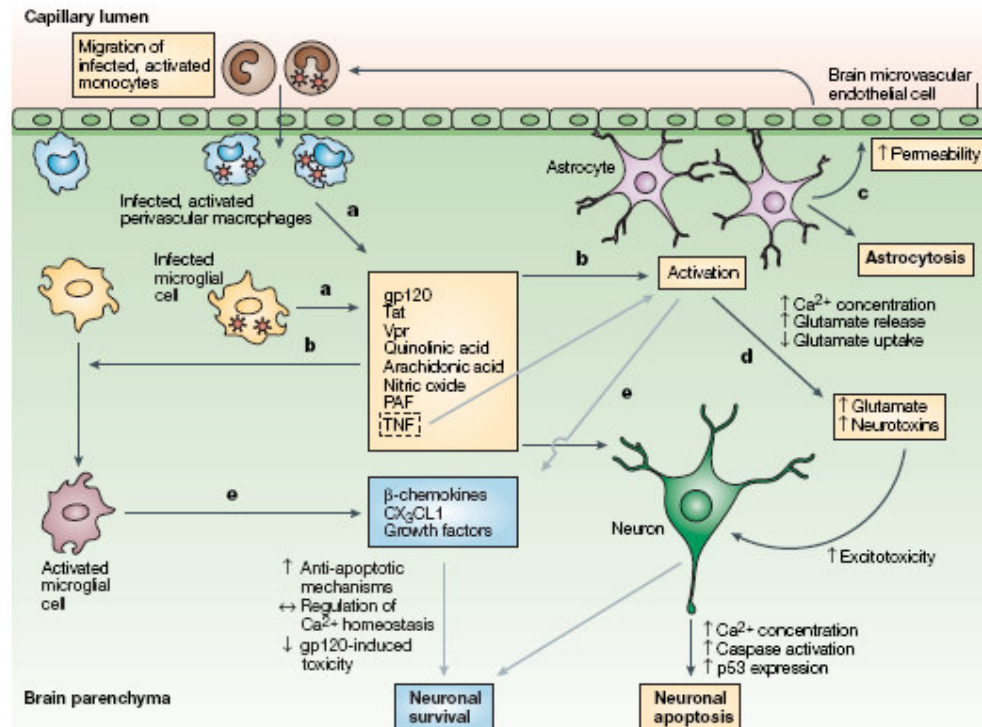
Figure 4. Mechanisms of neurodegeneration and neuroprotection in HIV-infection: a) Infected macrophages and microglia release viral proteins and cytokines with neurotoxic effects; b) The cellular products also activate microglia and/or macrophages and astrocytes; c) Activated astrocytes can modify the permeability of the blood-brain barrier; d) Astrocytes are also involved in increasing the extracellular concentrations of Ca^{2+} and glutamate, which are toxic to the neurons; e) Grey arrows indicate neuroprotective pathways. (Reprinted by permission of Macmillan Publishers Ltd., *Nat Rev Immunol*, Copyright 2005, reference [2].)

The most striking neuropathological feature of HIV encephalitis (the pathological correlate to ADC) is the presence of multinucleated giant cells formed by gp 120-mediated fusion of macrophages and microglia [53]. Additional characteristics include myelin pallor, monocyte infiltration, astrogliosis, loss of neurons, and activated microglial cells [53, 67]. The basal ganglia, subthalamic nucleus, and substantia nigra constitute some of the most heavily affected parts of the brain [67].

Cerebrospinal fluid analysis

CSF is produced by the choroid plexus at a rate of 350 µL/min, and the total quantity (140 mL) is replaced 4 to 5 times a day. The presence of HIV and its effects on brain tissue are difficult to examine directly: the closest one can get are postmortem studies of human brains, animal models, and CSF analysis. The advantages of using CSF are that it is easily accessible in living subjects via lumbar puncture and it reflects events in brain tissue, since the CSF is connected to the perivascular space of the brain. Two disadvantages limiting its use may be that some individuals are reluctant to undergo lumbar punctures and because, although the CSF stands in a very close physiological relationship to the brain, it is in some ways still another compartment.

Detection and quantification of HIV

In the early days, virus culture was the only method available for the detection of HIV in CSF, with the highest recovery rate during primary infection and the later stages of disease [68]. Nucleic acid amplification by polymerase chain reaction (PCR) is at present one of the most commonly used methods to detect and quantify HIV viral load in various body fluids. HIV RNA determinations in plasma can be used as a prognostic marker in untreated subjects or, most significantly, to estimate treatment efficacy in individuals undergoing treatment [69]. The diagnostic and prognostic importance of HIV RNA levels in CSF is less clear. Almost all untreated HIV-infected individuals have detectable CSF HIV RNA, with widely varying levels [70]. Despite this broad range, CSF HIV RNA levels are often lower than plasma levels [71]. In general, higher levels are found during primary infection, opportunistic infections, and ADC [72-75]. Individuals with severe dementia have higher HIV RNA levels than those with milder symptoms [72, 73, 75].

Among patients with opportunistic CNS infections, high levels of CSF HIV RNA can be found during cryptococcosis or tuberculous meningitis, i.e., conditions with increased inflammatory meningeal infiltrates [72, 76]. The elevated CSF viral load in these situations is thought to originate from CSF lymphocytes, since a correlation between these two parameters has been observed; although both pathogens have also been shown *in vitro* to enhance HIV replication [77, 78].

In the early stages of infection, most of the CSF viral load emanates from short-lived CD4 cells trafficking into the CNS from the periphery. In contrast to this transitory infection, there is a more autonomous infection that occurs in later stages, with viral production predominantly originating from long-

lived macrophages and microglia inside the CNS [70, 79, 80]. Antiretroviral therapy, especially when several agents are used in combination, has proven to be very efficient in reducing CSF HIV RNA levels [81-87]. HIV-infected patients with neurocognitive impairment starting ART have a reduction of the CSF viral load in parallel with an improvement of neuropsychological symptoms [88-90].

Markers of inflammation

During healthy conditions, the CSF is an acellular fluid with few white blood cells (WBC). In HIV-infected individuals, CSF pleocytosis (WBC > 4 x 10^6/L) is a frequent finding [91, 92], appearing more often during early, asymptomatic stages than late, advanced stages [93, 94]. Pleocytosis usually resolves as a response to ART [94, 95]. In the early stages, the amount of pleocytosis has been shown to correlate with the CSF viral load [96], but it is not fully understood which is, as it were, the chicken and which is the egg.

Neopterin is a low molecular compound (253 Daltons, Da), predominantly produced by macrophages and microglial cells after stimulation with interferon-gamma (IFN-γ). Serum and urine concentrations of neopterin have been used as a marker of cell-mediated immune activation in HIV infection since the beginning of the epidemic, and levels have been found to convey prognostic information [97, 98]. Neopterin is a very sensitive marker of cellular immune activation, with increased levels already evident at the end of an incubation period. Because of this characteristic, serum neopterin levels are used in Austria in screening blood and organ donors [99]. Elevated CSF levels of neopterin are found throughout the course of HIV infection, its highest values occuring in patients with ADC and opportunistic CNS infections [100, 101]. In patients with ADC, levels of CSF neopterin have also been shown to correlate with the severity of the dementia [72]. After commencement of ART, CSF neopterin decreases markedly, but remains slightly abnormal in a large number of patients, despite several years of receiving ART [102, 103]. Even patients with systemic virological failure exhibit a marked reduction of CSF neopterin concentrations [104].

β2-microglobulin (~ 17 kDa) is a part of the major histocompatibility complex (MHC) class I molecule, expressed on the surface of all nucleated cells (except neurons). Its levels in body fluids increase during inflammatory conditions or lymphoproliferative malignancies, due to increased cell turnover. As for neopterin, CSF levels of β2M are higher in individuals with neurological symptoms, especially ADC, and tend to correlate with ADC stages [105, 106]. Until quantification of HIV RNA became available, β2M was considered the best CSF marker of ADC [105]. Lately, the predictive value of β2M has come under discussion. It does not seem that β2M is as

sensitive as neopterin in reflecting the cellular immune response. Firstly, levels of β2M decrease rapidly after commencement of therapy and remain normal in all patients after 2 years of treatment [102]. Secondly, in a recent study, CSF β2M concentrations failed to predict neuropsychiatric deterioration in individuals receiving HAART [107].

HIV also induces intrathecal antibody formation during the entire course of HIV infection. This can be measured as an increased immunoglobulin G (IgG) index or by detecting specific oligoclonal bands [91, 93, 108, 109].

The above-mentioned markers are the ones used in this study, and are therefore more thoroughly described. But there are numerous other markers of inflammation that have been analysed in the CSF of HIV-infected individuals. Among the cytokines, TNF-α is one of the most extensively studied. CSF levels of TNF-α have been shown to correlate with stage of infection, neurological symptoms, CSF HIV RNA levels, and levels of other cytokines, e.g., IL-2 [110-112]. One difficulty in measuring TNF-α is that it is rapidly cleared from CSF [113].

Chemokines are small proteins that mediate migration of uninfected or HIV-infected leukocytes through the BBB to areas of infection. Monocyte chemotactic protein-1 (MCP-1) plays an important part in recruiting monocytes and macrophages [114]. Several studies have indicated that MCP-1 could be a reliable marker of macrophage and microglial activation. CSF levels of MCP-1 are usually elevated in patients with ADC, and low in neurologically asymptomatic HIV-infected individuals [115].

Interferon-γ-inducible protein 10 (IP-10 or CXCL10) can be produced by T-cells, macrophages, astrocytes, and endothelial cells [116, 117]. It attracts activated T-cells, natural killer cells, and monocytes from the periphery [116, 117]. CSF concentrations of IP-10 closely correlate to CSF mononuclear cell counts and CSF HIV RNA levels [95]. However, no associations have been found between CSF IP-10 levels and stage of HIV-infection or neurological symptoms [95].

Several more inflammatory markers have been evaluated in HIV-infected individuals: adhesion molecules, RANTES (regulated upon activation, normal T-cell expressed and secreted), MIP-1α (macrophage inflammatory protein 1α), and fractalkine (also called CX3CL1)—to mention only a few, although they will not be discussed further here.

Markers of blood-brain barrier permeability

The BBB (between blood and brain interstitial fluid) and the blood-CSF barrier (between blood and CSF) are semipermeable barriers surrounding the CNS. They serve as gatekeepers to the brain, preventing most molecules from entering the CNS, thereby maintaining a stable environment for the brain. The principal route of entry for most molecules into the CNS is via the BBB because the BBB has a much larger surface area than the choroid plexus [118]. The BBB differs from other capillaries in the body in a number of ways. It is formed by endothelial cells that are fused together by tight junctions. These cells lack intercellular pores, they have a paucity of pinocytosis, and they possess a large mitochondrial content to fuel the transport pumps situated in the BBB. The endothelial cells and pericytes are enclosed by a basement membrane and almost completely surrounded by astrocyte foot processes (Figure 5). An easy way to assess the BBB permeability is by using the CSF/plasma albumin ratio: since the liver is the only organ synthesizing albumin, any albumin found in CSF must have come from the periphery. BBB impairment can occur during any period of HIV infection, but is more common in advanced disease, ranging from 5% to 22% in individuals without neurocognitive disease, to about 50% in patients with AIDS, and affecting virtually all patients with ADC [108, 119-121].
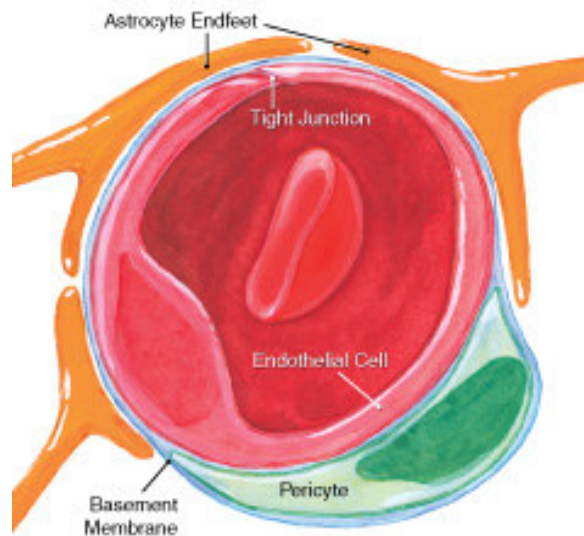

Figure 5. The blood-brain barrier. (Reprinted by permission of R&D systems.) <www.RnDSystems.co.uk>

Antiretroviral drugs and the CNS

For an antiretroviral drug to effectively inhibit viral replication in the CNS, it must be able to penetrate the BBB. The CNS-penetrating capacity of a drug depends on a number of factors: molecular size, lipophilicity, degree of ionisation and plasma protein binding, and whether or not the drug is a substrate for transmembrane transporters. Host characteristics like cerebral blood flow and degree of local inflammation may also be contributory factors [122]. The easiest way to determine whether a drug is capable of entering into the CNS is by measuring drug concentrations in CSF. It has not been clearly established what constitutes "good" penetration, but the drug concentration should preferably exceed that needed to inhibit 50% of viral replication (IC_{50}) by severalfold [118]. The inhibitory quotient (IQ) is a measure introduced in an attempt to combine the achieved drug concentration with the susceptibility of the virus to that particular drug, and is calculated as plasma trough concentration/IC_{50} [123].

Among the NRTIs, zidovudine, stavudine, lamivudine, and abacavir are well known to penetrate the BBB, but didanosine and zalcitabine are not [82, 124-129]. Tenofovir has been shown to penetrate into CSF, at least in guinea pigs [130]. The NNRTIs efavirenz and nevirapine reach CSF concentrations exceeding their IQs [131, 132]. In general, the PIs are large molecules, and extensively bound to plasma proteins, such as α-1-acid glycoprotein and albumin. Additionally, they are to varying degrees substrates for P-glycoprotein, a transport pump situated in the BBB, making them more or less susceptible to active efflux. Indinavir is the only PI able to achieve therapuetic concentrations in CSF in the abscence of ritonavir, while adding ritonavir leads to significantly increased CSF concentrations [133]. Previous studies have failed to detect nelfinavir, saquinavir, lopinavir, or ritonavir in CSF [134-137]. The more recently licensed PIs amprenavir and atazanavir have been shown to have low and very low CSF/plasma ratios, respectively [138, 139]. The FI enfuvirtide penetrates into CSF poorly, which is not surprising considering its pharmacological properties [140].

A number of studies have evaluated changes in CSF viral load in response to ART. Monotherapy with zidovudine reduces CSF HIV RNA levels [81]. This is also true for dual-NRTI treatment with lamivudine plus zidovudine or stavudine [82]. HAART leads to a rapid decline of the CSF viral load in most neurologically asymptomatic individuals, whereas some studies indicate that the response in CSF is slower [80, 86, 94, 141]. This slower treatment response may be due to the fact that the virus in CSF has it origin in long-lived cells in the CNS. Such slower viral kinetics are predominantly observed in patients with ADC and/or low CD4 cell counts, lending support to the previous hypothesis [80, 88].

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AIMS

The overall aim was to attain greater understanding of the pharmacokinetics of different antiretroviral drugs in CSF and their effect on HIV infection in the CNS, as well as to learn more about HIV neuropathogenesis. The specific aims were to:

- analyse CSF concentrations of some PIs and study their impact on CSF viral load, intrathecal immune activation, and BBB function (Papers I and II);

- investigate whether CSF can act as a viral reservoir in HIV-infected individuals undergoing effective treatment (Paper III); and

- gain more understanding of the factors associated with persistent intrathecal immune activation in HIV-infected individuals on antiretroviral therapy (Paper IV).

PATIENTS

The Department of Infectious Diseases at the Sahlgrenska University Hospital/Östra has a unique bank of stored CSF and blood samples from HIV-infected individuals. This longitudinal research project on HIV infection in the CNS was launched in 1985 and continues to enroll new subjects. Individuals willing to participate undergo annual sampling of CSF and blood. Lumbar punctures are also performed prior to and 3 months after initiation or cessation of therapy. Up to date, 1103 lumbar punctures have been performed on 324 persons. This thesis includes a total of 353 HIV-infected patients, 235 from Göteborg, and 118 from the Department of Neurology, University of California, San Francisco General Hospital, San Francisco, CA, USA (Figure 6). Participants were included after they had given their informed consent, and the study was approved by the research ethics commitee at each site.
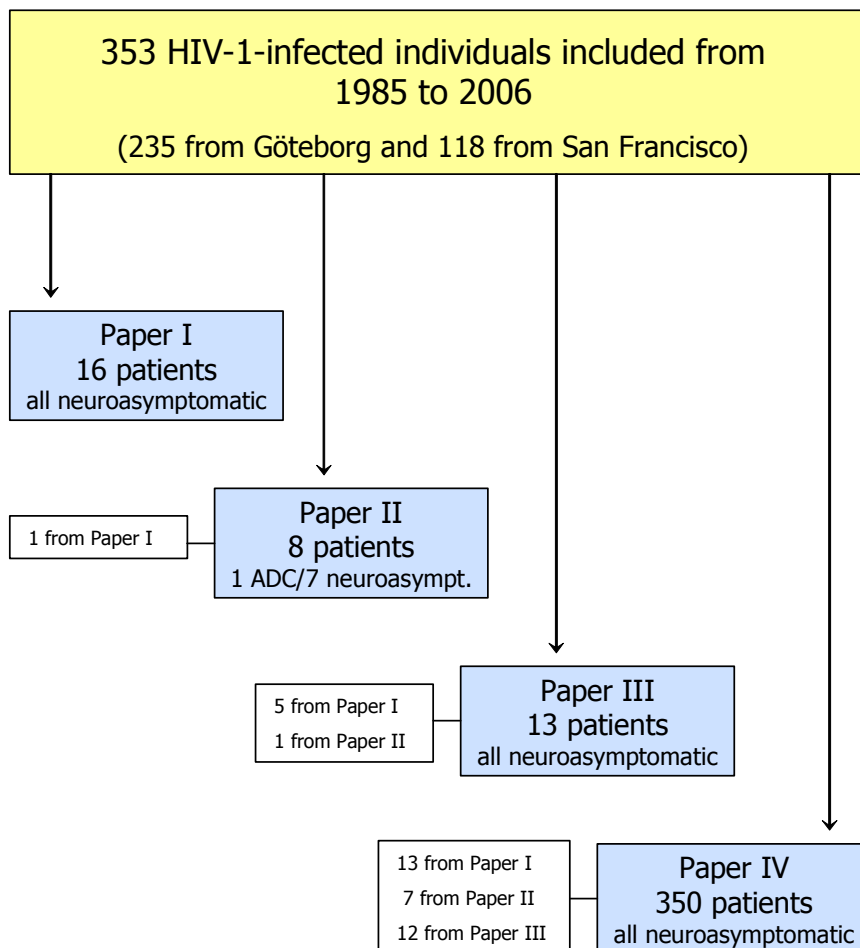

Figure 6. The number and distribution of participants in Papers I–IV.

Paper I

A total of 16 HIV-infected individuals were enrolled in this study. Nine out of 16 were antiretroviral-naïve, 3 had been off treatment for at least 3 months, and 4 were already on a lopinavir/r (ritonavir)-based regimen. The subjects received lopinavir/r 400/100mg BID, and all but one received 2 NRTIs. Characteristics of the 12 subjects initiating lopinavir/r-based ART are shown in Table 4. Lopinavir concentrations were measured in a total of 13 patients (9 who started lopinavir/r containing ART, and 4 already on treatment with lopinavir/r). Two patients had two different plasma and CSF samples analysed, making 15 measurements in all for determination of plasma unbound (P_{ub}) and CSF total (CSF_{tot}) concentrations. For plasma total (P_{tot}) concentrations, two values were missing, resulting in a total of 13 samples. Lumbar punctures were performed at baseline and after approximately 3 and 12 months of treatment.

Table 4. Characteristics of participants in Papers I and II initiating treatment with lopinavir/r or saquinavir/nelfinavir.

	Paper I	Paper II
	n = 12	n = 8
Female/Male	3/9	4/4
Age	38 (18–53)	39 (27–59)
CD4 cell count	110 (6–490)	200 (20–310)
CDC stage A/B/C	4/2/6	4/3/1
NRTIs	zdv + 3TC (n = 6), d4T + 3TC (n = 4)	zdv + 3TC (n = 7)
	d4T + ddI (n = 1), abc + ddI (n = 1)	zdv + ddI (n = 1)
AIDS defining events	Candida esophagitis (n = 2)	ADC (n = 1)
	Atypical mycobacterial infection (n = 2)	
	Pneumocystis jirovecii pneumonia	
	Kaposi´s sarcoma	
Comments	1 patient developed cerebral toxoplasmosis at week 12, others neuroasymptomatic	1 with ADC at start of study, 7 neurologically asymptomatic

CDC: Center for Disease Control and prevention; ADC: AIDS Dementia Complex; NRTI: nucleoside analogues. Age and CD4 cell count are given in median (range).

Paper II

Eight treatment-naïve HIV-infected individuals commencing ART with saquinavir-sgc 1200 mg BID and nelfinavir 1250 mg BID were recruited for the study. Patient characteristics are given in Table 4. A total of 37 samples (5 from each of 6 patients, 4 from 1, and 3 from another) for analysis of P_{tot} were obtained. The P_{ub} and CSF_{tot} concentrations were analysed in 15 samples (twice in 7 patients and once in 1).

Paper III

Thirteen HIV-infected subjects on ART without neurocognitive symptoms, whose plasma viral loads had been < 20 copies/mL for more than 12 months, were included. Six patients were asymptomatic (CDC A), 3 were classified as symptomatic but did not meet the criteria for AIDS (CDC B), and 4 had an AIDS-defining diagnosis (CDC C). Six patients each were undergoing treatment with NRTIs + PI or NRTIs + NNRTI, and one patient received a NRTI-sparing regimen with efavirenz + atazanavir/r.

Paper IV

In this retrospective study we included patients chronically infected with HIV, but with no neurological symptoms, who had received stable ART $\geq$ 6 months. One hundred and fifty-seven individuals from the cohorts in Göteborg and San Francisco matched these criteria. Among them, 134 were on HAART, 11 on a single NRTI, and 12 on two NRTIs. A control group of 193 individuals who were either antiretroviral naïve or had been off ART for $\geq$ 6 months were also included. Subjects were further divided into different subgroups according to the parameter to be evaluated (Figure 7).
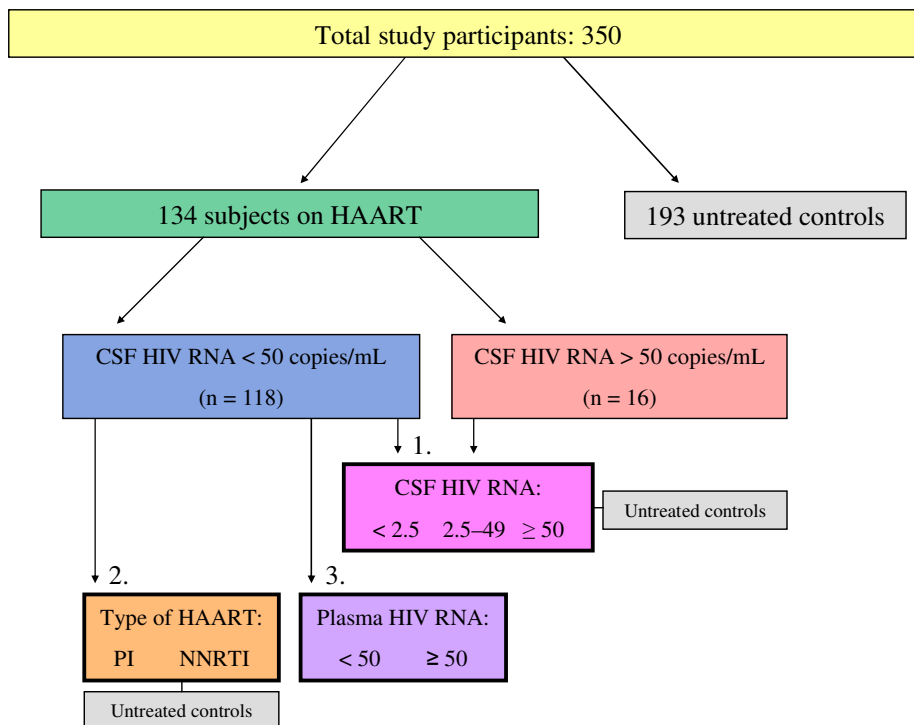

Figure 7. A flow chart for the group formations in Paper IV. Subjects on HAART (green box) were initially divided into groups based on their CSF HIV RNA levels (pink box): < 2.5, 2.5–49, and $\geq$ 50 copies/mL (Step 1). The 118 subjects on HAART with CSF viral loads < 50 copies/mL (blue box) were further analysed according to HAART-regimen (orange box): PI- or NNRTI-based (Step 2), or according to plasma HIV RNA (purple box): < 50 or $\geq$ 50 copies/mL (Step 3). Grey boxes indicate untreated controls.

METHODS

All lumbar punctures were performed in a standardised manner, early in the morning, and prior to the intake of antiretroviral drugs. A total of approximately 13 mL CSF was routinely collected. The initial part of each sample was used to determine cell count, protein, and β2M, and the last mL was used for HIV RNA quantification. The remaining CSF was centrifuged and aliquots were stored at –70°C for future analysis. All CSF samples with a red blood cell count > 30 x 10^6/L were excluded. In Paper III, another 10 mL of CSF was obtained for HIV RNA quantification using the ultra-ultra sensitive technique. Patients underwent phlebotomy at the same time, and blood samples were treated in the same way as CSF.

HIV RNA quantification

Quantification of HIV RNA in cell-free plasma and CSF was performed with the Roche Amplicor Monitor Test (version 1.5, Roche Diagnostic Systems, Hoffman-La Roche, Basel, Switzerland). The assay has a dynamic range down to 50 copies/mL (1.70 $\log_{10}$ copies/mL), and a lower detection limit of 20 copies/mL (1.20 $\log_{10}$ copies/mL). In order to yield a detection limit of 2.0 copies/mL (Paper III), 8.25 mL of the samples (where available) were heavily centrifuged at 180,000 G and 4°C for 30 minutes in Beckmans Preparative Ultracentrifuge L7-55 prior to quantification. In Paper IV, some of the patients had their plasma and CSF analysed by a slightly different ultra-ultra sensitive assay with a detection limit of 2.5 copies/mL [142]. To avoid the inter-assay variation, all samples for each patient were analysed on the same occasion. HIV RNA levels were transformed to $\log_{10}$ copies/mL before statistical analysis.

Markers of immune activation and BBB permeability

Neopterin was analysed by a radio-immuno assay (RIA) from Henningtest Neopterin, BRAHMS, Berlin, Germany. The normal reference value for serum was ≤ 8.8 nmol/L in serum in all studies where neopterin was used [143]. The CSF neopterin normal reference values were ≤ 5.3 nmol/L in Paper I [143], and ≤ 4.3 nmol/L for studies reported in Papers II and IV [144]. The reference value was changed because a greater quantity of reference material had been analysed. The neopterin concentrations for participants from San Francisco in Paper IV were measured with an enzyme-linked immunosorbent assay (ELISA). The values between these two assays are interchangable [145], and the same normal reference values were utilised.

Analyses of β2M were included in Papers I–III. In the case of all patients in Paper II, and in 6 out of 12 subjects in Paper I, β2M was measured by ELISA [146], with normal reference values ≤ 2.4 mg/L for serum and ≤ 2.2 mg/L for CSF. The laboratory later switched to performing the analysis by nephelometry. The remaining 6 subjects from Paper I had their β2M concentrations analysed by nephelometry (3 with Dade Behring and 3 with Beckman Coulter). In Paper III, the Dade Behring nephelometry was used in 10 patients, and the Beckman Coulter nephelometry in 3 patients. The methods were tested against each other, and the reference values for each method were determined at the laboratory where the study was performed.

Quantitative determinations of albumin and IgG were carried out by nephelometry (Behring Nephelometer Analyser, Behringwerke, AG, Marburg, Germany). The BBB permeability was assessed using the albumin ratio, defined as [CSF albumin (mg/L)/serum albumin (g/L)] [147], with reference values < 6.8 for persons under age 45 and < 10.2 for those 45 or older [148]. Inthrathecal IgG production was determined by the IgG index, calculated as [CSF IgG (mg/L)/serum IgG (g/L)]/albumin ratio, the reference value being < 0.63, independent of age [148].

Analysis of antiretroviral drug concentrations

The concentrations of lopinavir, saquinavir, and nelfinavir were determined by high performance liquid chromatography (HPLC), isocratic reversed phase, with an Ace 3 C18 3 µm, 50 x 3 mm column (Scantec), and ultraviolet (UV) detection. Analysis of P_{tot} were performed by precipitating samples, centrifugation, and injection of the supernatant onto the HPLC column. For determination of P_{ub}, plasma was initially centrifuged in Amicon Centrifree cartridges to obtain ultrafiltrates. Plasma samples were subsequently treated in the same manner as CSF samples: they underwent water-water extraction, and were then run on the HPLC column. The levels of detection (LODs) for the different assays are given in Table 5.

Table 5. Levels of detection for HPLC assays
used in Papers I and II.

	Level of detection	
	P_{tot} (µM)	P_{ub} and CSF $_{tot}$ (nM)
Lopinavir	0.50	20.0
Saquinavir	0.08	2.5
Nelfinavir	0.13	2.0

P_{tot} = plasma total concentration
P_{ub} = plasma unbound concentration

Other analysis

The peripheral CD4 cell count was measured by direct immuofluorescence in a flow cytometer (FACS, Becton Dickinson, Mountain View, CA, USA). The CSF cell counts were immediately analysed in a Fuch-Rosenthal chamber.

Statistical analysis

In all papers, non-parametric methods were used for group descriptives: median and range or interquartile range (IQR). Evaluation of pre- and on-treatment samples in Papers I and II was performed by Wilcoxon's signed rank test. Spearman's rank correlation coefficient or product moment correlation analysis was used to evaluate correlations. For comparisons between two independent groups in Paper IV, the Mann-Whitney U-test was used, and in the case of more than two independent groups, the Kruskal-Wallis test.

RESULTS

The impact of combination antiretroviral therapy containing lopinavir/r or saquinavir/nelfinavir on CSF viral load, intrathecal immune activation, and BBB integrity (Papers I and II)

The effects of the ART combinations used in Papers I and II on viral load, neopterin, and β2M in CSF and blood are illustrated in Figures 8 and 9. The first follow-up for patients on lopinavir/r-based HAART took place after a median (range) of 3.0 (2.6–6.0) months, and the second at the end of 12.1 (6.0–16.5) months.
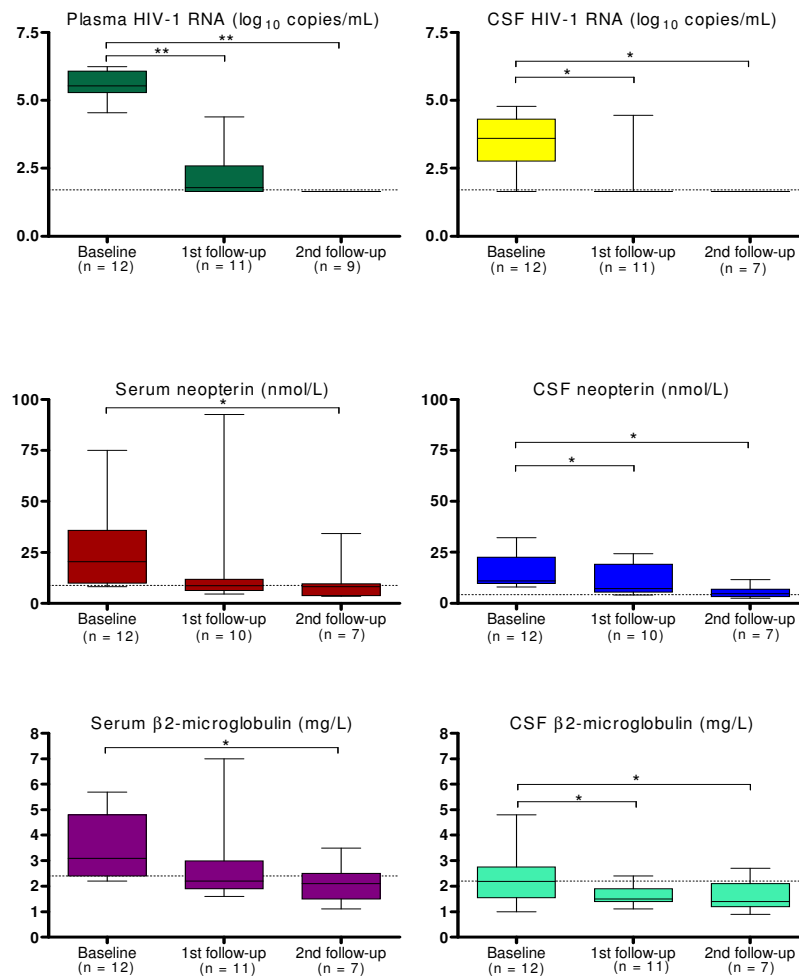

Figure 8. The effect of lopinavir/ritonavir containing ART on viral load, neopterin, and B2-microglobulin in blood and CSF. The dotted lines indicate levels of detection or normal reference values. The horizontal bar inside each box shows the median value and interquartile range (25th to 75th percentile) within the box. Short bars outside each box show range. * = $p < 0.05$; ** = $p < 0.01$.

In both CSF and blood, HIV RNA, neopterin, and β2M decreased significantly after initiation of therapy. The viral loads in CSF and plasma were < 50 copies/mL for all 7 individuals on a lopinavir/r-based regimen who continued until the second on-treatment follow-up, and for 5 out of 7 of the individuals on a saquinavir/nelfinavir-based regimen. Throughout the study periods, CSF levels of β2M were within reference values in a larger number of patients than CSF levels of neopterin. The CSF monocytic cell count determined in Paper II also decreased considerably in response to treatment, but the albumin ratio and IgG index remained unchanged. The CD4 cell count increased significantly in both studies.
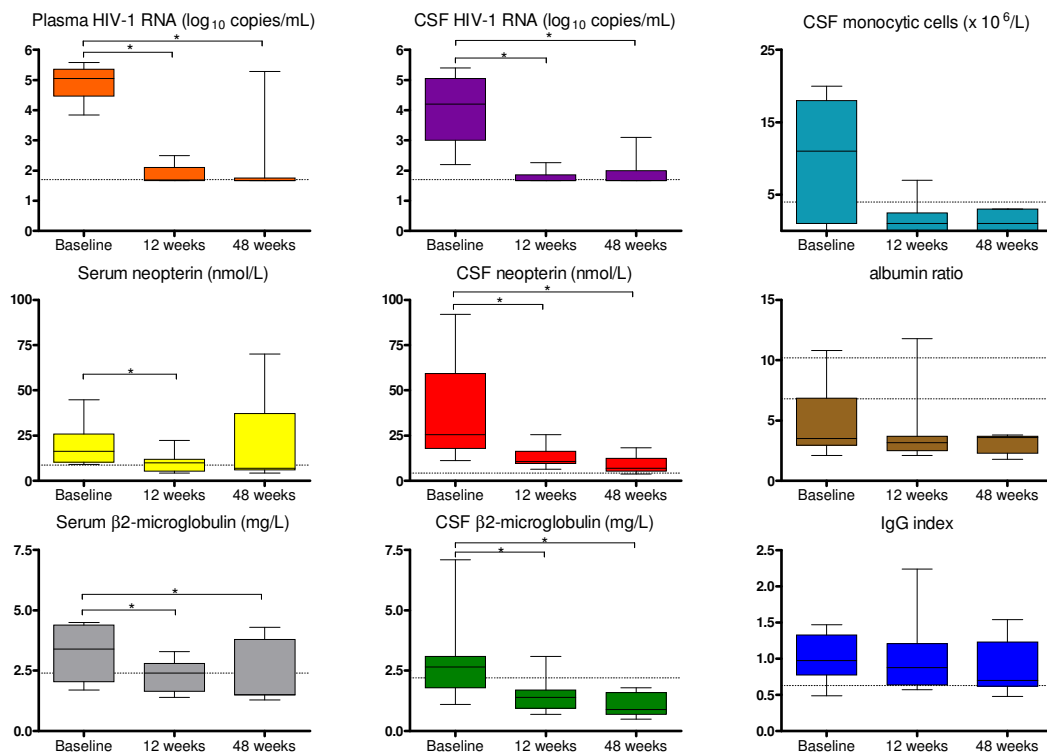

Figure 9. The effect of saquinavir/nelfinavir containing ART on viral load, neopterin, β2-microglobulin in blood and CSF, CSF monocytic cell count, albumin ratio, and IgG index. There were 8 subjects at baseline and at the first on-treatment follow-up, and 7 at the second on-treatment follow-up. The dotted lines indicate levels of detection or normal reference values. The horizontal bar inside each box shows the median value and interquartile range (25th to 75th percentile) within the box. Short bars outside each box show range. $* = p < 0.05$.

CSF and plasma concentrations of lopinavir, saquinavir, and nelfinavir (Papers I and II)

The individual P_{ub} and CSF_{tot} concentrations for lopinavir, saquinavir, and nelfinavir for all study participants in Papers I and II are shown in Table 6. Lopinavir was detectable in 15 out of 15 CSF samples, saquinavir in 7 out of 15, and nelfinavir in 9 out of 15.

Table 6. The plasma unbound (P_{ub}) and CSF total (CSF_{tot}) concentrations for each patient in Papers I and II.

	Lopinavir		Saquinavir		Nelfinavir		
H post-dose	P_{ub} (nM)	CSF_{tot} (nM)	P_{ub} (nM)	CSF_{tot} (nM)	P_{ub} (nM)	CSF_{tot} (nM)	H post-dose
10.5	103	37	0	0	0	0	0.3
12.3	39	24	56	5	12	7	9.8
10.0	21	28	96	9	31	19	7.0
12.0	–	25	25	7	24	17	12.0
2.0	35	23	0	3	0	0	–
12.0	86	115	0	0	15	16	13.3
11.0	45	30	0	0	0	0	11.5
9.0	44	48	0	0	14	3	9.0
–	–	23	0	0	9	2	14.0
11.0	40	118	0	3	20	13	11.0
–	63	35	0	0	0	0	–
11.0	55	22	0	0	5	0	11.5
6.0	36	28	0	0	3	0	12.0
11.5	39	51	25	5	15	16	9.3
–	88	24	16	6	10	23	6.0

Post-dose hours for lopinavir on far left, for saquinavir and nelfinavir on far right. Values under detection limit of assays listed as 0.

CSF HIV RNA levels determined by ultra-ultra sensitive PCR assay (Paper III)

All 13 subjects had CSF HIV RNA < 2.0 copies/mL. Of the 5 patients who did not achieve < 2.0 copies/mL plasma, viral replication ranged between 2.3 and 8.2 copies/mL. Measures of CSF inflammation were mostly normal: one patient had a WBC count just above the normal reference value (5 WBC x 10^6 cells/L), and three had minimally elevated CSF β2M concentrations. Albumin ratio was elevated in only one individual, while IgG index was elevated in 6 out of 13 subjects.

CSF neopterin levels in individuals on stable antiretroviral therapy (Paper IV)

1. HIV RNA and neopterin levels for total study population

Individuals on HAART and two NRTIs had lower CSF HIV RNA and neopterin levels than those on one NRTI ($p < 0.01$) or the untreated controls. Plasma HIV RNA and neopterin levels were significantly lower in subjects on HAART compared to the three other groups; serum neopterin was lower for subjects on two NRTIs compared to one NRTI ($p < 0.05$) (Table 7). None of the parameters differed significantly between individuals on one NRTI and the untreated controls.

Table 7. Results for all subjects included in the study in Paper IV, listed according to treatment regimen.

	Antiretroviral treatment			
	HAART	2 NRTIs	1 NRTI	Untreated controls
Number of subjects	134	11	12	193
CSF neopterin (nmol/L)	6.7 (5.1–9.5)	8.5 (6.4–12.4)	15.0 (12.8–23.5)	16.0 (10.0–26.9)
Serum neopterin (nmol/L)	7.9 (5.6–12.7)	13.3 (9.1–15.2)	19.7 (13.8–25.8)	16.1 (9.8–24.4)
CSF HIV RNA (log10 copies/mL)	1.3 (1.3–1.3)	1.4 (1.3–2.2)	3.5 (3.1–3.6)	3.4 (2.4–4.1)
P HIV RNA (log_{10} copies/mL)	1.3 (1.3–2.4)	3.6 (2.5–4.5)	4.3 (3.9–4.9)	4.3 (3.6–4.9)

Values given in median (interquartile range). HAART: highly active antiretroviral therapy; NRTI: nucleoside analogue.

2. HIV RNA and neopterin levels for individuals on HAART, grouped according to CSF HIV RNA levels

Among individuals on HAART, patients with CSF HIV RNA < 2.5 copies/mL had significantly lower CSF neopterin and plasma HIV RNA levels than patients with 2.5–49.0 CSF HIV RNA copies/mL. Duration of treatment did not differ significantly between the groups with different CSF HIV RNA levels (Table 8). The untreated controls had CSF neopterin in the same range as their CSF HIV RNA matched group undergoing treatment.

Table 8. Results for subjects on HAART and untreated controls, grouped according to CSF HIV RNA levels.

	CSF HIV RNA (copies/mL)			
	< 2.5	2.5–49.0	≥ 50	≥ 50 untreated
Number of subjects	35	13	16	16
CSF neopterin (nmol/L)	6.1 (5.1–9.1)	8.3 (7.2–13.5)	9.9 (7.7–13.5)	9.6 (7.3–14.7)
Serum neopterin (nmol/L)	8.9 (5.9–13.9)	7.9 (5.0–12.2)	12.4 (5.8–14.6)	12.1 (8.6–18.5)
P HIV RNA (log_{10} copies/mL)	0.4 (0.4–0.8)	2.6 (1.6–3.5)	4.1 (2.7–4.7)	3.3 (2.3–4.4)
No. of patients on HAART with:				
- PI	15	10	10	–
- NNRTI	19	3	3	–
- 3 NRTIs	1	0	3	–
Duration of ART in years	2.9 (1.2–4.0)	2.2 (1.0–3.5)	1.1 (0.6–4.0)	–

Values given in median (interquartile range). HAART: highly active antiretroviral therapy; PI: protease inhibitor; NRTI: nucleoside analogue; NNRTI: non-nucleoside analogue. CSF neopterin and plasma HIV RNA levels differed significantly between treated groups ($p < 0.01$ and $p < 0.001$) by the Kruskal-Wallis test; serum neopterin did not.

3. HIV RNA and neopterin levels for individuals on HAART having CSF viral loads < 50 copies/mL according to HAART regimen

The CSF neopterin and plasma HIV RNA levels did not differ significantly between individuals on a PI- or NNRTI-based regimen. Nor were there any statistically significant differences in these parameters when analysing individual PIs or NNRTIs, although for some PIs there were too few subjects to draw statistical conclusions (Table 9).

Table 9. Results for subjects on PI- or NNRTI- based HAART and untreated controls having CSF viral loads < 50 copies/mL.

	HAART based on:		
	PI	NNRTI	Untreated controls
Number of subjects	70	43	20
CSF neopterin (nmol/L)	6.1 (4.9–8.3)	7.6 (4.9–9.5)	11.1 (8.2–15.7)
Serum neopterin (nmol/L)	7.2 (5.3–11.9)	8.6 (6.1–13.3)	11.5 (8.1–22.8)
P HIV RNA (log_{10} copies/mL)	1.3 (1.3–1.8)	1.3 (1.3–1.3)	3.2 (2.1–3.8)
Duration of treatment in years	1.9 (1.0–3.2)	2.9 (1.1–4.0)	–
PI or NNRTI used	lopinavir/r (n = 22)	efavirenz (n = 24)	
	indinavir (n = 11)	nevirapine (n = 19)	
	atazanavir/r (n = 9)		
	nelfinavir (n = 8)		
	saquinavir/nfv (n = 8)		
	saquinavir/r (n = 5)		
	indinavir/r (n = 5)		
	saquinavir (n = 2)		

Values given in median (interquartile range). HAART: highly active antiretroviral therapy; PI: protease inhibitor; NRTI: nucleoside analogue; NNRTI: non-nucleoside analogue.

4. HIV RNA and neopterin levels for individuals on HAART having CSF viral loads < 50 copies/mL, according to level of plasma HIV RNA

The plasma viral load, whether < 50 or ≥ 50, had no significant impact on CSF and serum neopterin in subjects on HAART with suppressed viral replication in CSF (Table 10).

Table 10. Results for subjects on HAART having CSF HIV RNA < 50 copies/mL, according to plasma HIV RNA level.

	Plasma HIV RNA (copies/mL)		
	< 50	≥ 50	p-value
Number of subjects	92	26	
CSF neopterin (nmol/L)	6.2 (4.9–9.1)	7.0 (5.4–11.5)	n.s.
Serum neopterin (nmol/L)	7.6 (5.6–11.7)	7.9 (5.8–12.6)	n.s.
P HIV RNA ($\log_{10}$ copies/mL)	1.3 (1.3–1.3)	3.1 (2.4–3.7)	< 0.001
Duration of treatment (years)	2.0 (1.0–3.5)	2.3 (1.0–3.0)	n.s.

Values given in median (interquartile range); n.s.: non-significant.

DISCUSSION

Shortly after the first cases of AIDS were described in 1981, it became clear that the causative agent, HIV, was a virus that not only was destructive to the immune system, but also had the capability of entering and causing harm to the CNS. HIV was first isolated from the CSF, brain, and spinal cord of patients with AIDS and neurological disorders in 1985 [149-151]. It was later evident that the virus can be detected by PCR in the CSF of virtually all HIV-infected individuals, beginning with the primary infection and continuing throughout the entire infectious course [152]. Despite the fact that viral entry into the CNS occurs as a very early event, it usually takes several years for HIV-infected individuals to develop CNS manifestations. ADC is the most ravaging neurological complication, affecting a considerable proportion of untreated patients. For many years, the CNS was thought of as a distinct and difficult to treat compartment of the body. In theory, this may be attributed to the CNS being surrounded by protective barriers, inside of which the primary targets for HIV are cells that have a slow turnover. The HIV-infection in CSF is, in fact, often "compartmentalised" in the sense that viral strains in CSF and blood, although related, differ to varying degrees, depending on the stage of systemic infection and the presence of ADC [79, 153, 154]. Further, many antiretroviral drugs penetrate the BBB poorly, creating an environment that might favor the selection of resistant virus populations [155-157].

Concerns that the CNS would act as a sanctuary site, difficult to reach by antiretroviral drugs, and therefore allowing HIV to replicate independently from the periphery, have not been borne out in practice. Firstly, the incidence of ADC has been dramatically reduced since the introduction of ART [158, 159]. A declining incidence of ADC was observed even when zidovudine was used as a monotherapy in the late 1980s [41]. Secondly, studies of the viral burden in CSF and plasma have demonstrated that HIV is generally well suppressed in CSF in the context of effective, systemic ART [84, 85, 88].

The CNS-penetrating capacities of PIs have not been as extensively studied as have those of NRTIs and NNRTIs. Although PIs are rather lipophilic, they are large molecules, with a high degree of binding to plasma proteins (with the exception of indinavir). These characteristics, together with the fact that PIs are substrates for P-glycoprotein, and hence susceptible to active efflux, makes it more difficult for them to traverse the BBB [160, 161]. The CSF and plasma concentrations of ritonavir-boosted lopinavir are analysed in Paper I, and those of saquinavir and nelfinavir used in combination in Paper II. CSF lopinavir concentrations exceeded the median IC_{50} of 3.0 nM severalfold in all 15 samples [162]. These results have been confirmed in two additional studies, where CSF levels of lopinavir were found in the same

range [163, 164]. Neither saquinavir nor nelfinavir had previously been detected in CSF [134, 135], probably because the methodology in those studies had higher limits of detection than the HPLC we employed. The CSF concentrations of saquinavir were only detectable in 7 out of 15 samples and did not exceed the IC_{50} for wild-type virus [165]. Nelfinavir, on the other hand, exhibited CSF concentrations in the range of the IC_{50} in 9 out of 15 samples where it could be detected [165]. In another study published in 2006, CSF nelfinavir concentrations were found at the same levels in 19 patients on combination therapy with nelfinavir and 2 NRTIs [166].

There are certain considerations to be taken into account when analysing CSF concentrations of antiretroviral drugs. It is difficult to establish the total CSF drug exposure by only one concentration determination. The ideal would be to obtain several CSF and plasma concentrations over the duration of the dosing interval, making it possible to create an area under the concentration-time curve (AUC) for each compartment, then calculate the AUC_{CSF}/AUC_{plasma} ratio. This ratio reflects the percentage of steady-state plasma concentrations achieved in CSF. However, the method is time-consuming, expensive, and inconvenient for patients, so it is seldom performed [167].
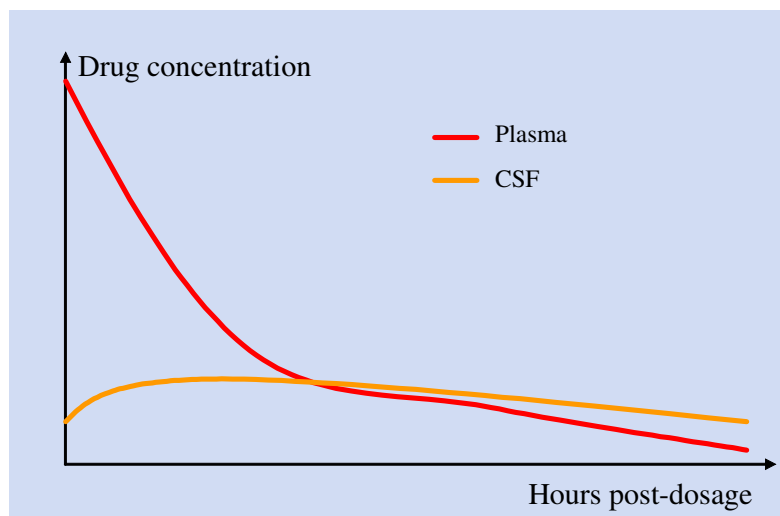

Figure 10. An example of a concentration-time curve in cerebrospinal fluid (CSF) and plasma after administration of a drug intravenously. The CSF concentration rises later than in plasma, and can even exceed the plasma concentration by the end of the dosing interval.

One alternative method is to simultaneously analyse CSF and plasma antiretroviral drug concentrations at fixed post-dosage time intervals. In doing so, one must be aware of the divergent drug distribution properties in each compartment. The plasma concentration often increases and decreases rapidly, exhibiting large variations in concentration over time. The maximum CSF concentration is reached later than that in plasma, and the concentration variations are smaller, so that at the end of the dosing interval the concentrations in CSF can exceed the ones in plasma (Figure 10).

Another complicating factor when interpreting CSF and plasma concentrations is the binding of drugs to proteins. We were only able to determine the total CSF concentrations (i.e., bound plus unbound drug) of lopinavir, saquinavir, and nelfinavir. The unbound and pharmacologically active fraction in CSF would most likely be below the detection limit of our method. It would appear that only one study has been published in which unbound CSF concentrations of an antiretroviral drug (indinavir) have been determined [168]. The binding of antiretrovirals to plasma proteins has been extensively studied. Most PIs are highly bound to α-1-acid glycoprotein and albumin. The CSF levels of proteins are much lower than in plasma, but the CSF protein content still exceeds the concentrations achieved by antiretroviral drugs, so that binding in the CSF should be taken into consideration. This is particularly important when comparing the CSF drug concentration with the IC_{50} value for that drug. In plasma, the IC_{50} or the IQ serve as indicators for viral susceptibility for a particular drug. The IC_{50} and IQ can vary considerably, depending on which cell-line or virus strain has been used, how much human serum or purified proteins the cultures contain, and what quantification method has been used [165]. The situation is even more complicated for drugs that are intracellularly metabolised, such as the NRTIs. A protein-adjusted IC_{50} or IQ would be the optimal alternative, but is most often unattainable.

How do drug concentrations in CSF translate into drug concentrations in brain tissue? Measuring drug exposure in the CNS is a challenging task. In order to thoroughly estimate CNS drug exposure, biological fluids or tissue samples from different parts of the CNS must be obtained. For obvious reasons, this is difficult to accomplish in living subjects, although such studies have been performed on animals. Alternatively, clinical studies have used antiretroviral drug concentrations in the CSF as a measure of CNS drug exposure. However, it is not known how accurately CSF concentrations of antiretroviral drugs reflect drug exposure in the brain parenchyma [169]. In general, during a steady-state infusion of a drug in the absence of transport mechanisms, a concentration gradient will arise, so that the unbound plasma drug concentration will exceed the concentration in brain extracellular fluid, and this concentration will be greater than that in the CSF [170, 171]. This is, however, not the case in real life, where consideration has to be taken of a

number of transport mechanisms. The NRTIs zidovudine and stavudine achieve CSF concentrations in the same range, but animal models show better uptake into brain tissue for zidovudine [172]. Another example of the disparity between CSF and brain tissue concentrations is the antifungal drug amphotericin, which is undetectable in CSF, but achieves good penetration into the brain parenchyma [173].

It is also important to keep in mind that NRTIs, NNRTIs, and PIs exert their effect intracellularly, and that extracellular concentrations of a drug do not always correlate with intracellular concentrations. This is the case for NRTIs that require triphosphorylation by cellular kinases to become active, and is one reason why concentrations of NRTIs are not routinely analysed in TDM programmes [174]. For NNRTIs and PIs, a good correlation has been found between extra- and intracellular concentrations [175].

We found that a combination ART containing either lopinavir/r or saquinavir/nelfinavir significantly reduced CSF HIV RNA levels. However, it is not possible to draw far-reaching conclusions about the efficacy of lopinavir/r or saquinavir/nelfinavir in reducing the CSF viral load from our two studies, since all patients were on concomitant treatment with 2 NRTIs. A dual NRTI regimen has previously been demonstrated to substantially decrease the CSF viral load in HIV-infected individuals [83]. A recent monotherapy study of lopinavir/r that included CSF analysis showed that 10 out of 11 subjects had CSF HIV RNA < 50 copies/mL after 24 weeks of treatment [164]. Nelfinavir and saquinavir/r, on the other hand, have failed to suppress the CSF viral load when used as single agents [136, 176].

An issue that is currently being heavily debated concerns the number of CNS-penetrating antiretroviral drugs needed to substantially reduce the viral load, or to improve neurocognitive impairment. Direct comparisons between studies that have been conducted in this area are difficult to make because definitions of CNS penetration and study outcomes vary. While some researchers have demonstrated a correlation between the number of penetrating drugs and CNS effectiveness [177, 178], others have not [132]. The monotherapy study with lopinavir/r cited earlier [164] indicates that control of viral replication in the CSF may be achieved by using one potent antiretroviral drug, at least in the short run. Time will tell if such patients run the future risk of developing virological failure with resistance, and possibly neurological complications as well. Some individuals are on treatment with NRTI-sparing regimens, i.e., antiretroviral combinations without NRTIs, because of side-effects or resistance. Only one patient on such a regimen appears in our study in Paper III, where we used an ultra-ultra sensitive PCR. This patient had CSF HIV RNA < 2 copies/mL, which suggests that virological control in CSF is possible to achieve with this treatment regimen. Although this represents a single case, it is important that all aspects of

combinations other than the ones recommended as the first line of therapy be investigated, since these are used in an increasing number of HIV-infected individuals. Monotherapy is not recommended in treatment guidelines nowadays, although it is currently being studied in several clinical trials, both for the PIs, lopinavir/r and darunavir/r, and for the integrase inhibitor raltegravir (MK-0518). These protocols also serve as a golden opportunity to study the pharmacokinetics and effects of a single drug in the CSF.

Many studies on viral kinetics in CSF have been performed on neurologically asymptomatic individuals who are initiating treatment. The situation for patients with ADC is quite different. In the vast majority of cases, the CSF viral load in patients with late stage disease (and neurological complications) is produced by long-lived macrophages and microglia residing in the CNS [52, 80]. This is evidenced by the slower rate of decay of HIV RNA in CSF than in plasma in patients with ADC [80, 88, 94]. When initiating treatment in an individual with neurological symptoms, it would seem of particular importance to choose a regimen that penetrates well into the CNS, since the virus is causing harm locally.

The CNS, the male genital tract, and latently infected cells, such as CD4 memory cells and naive CD4 cells, have all been implicated as viral reservoirs [25-27, 141, 179]. Several studies have demonstrated a low-grade viremia in individuals on effective HAART [142, 180, 181]. This probably arises from continuous or intermittent activation of latently infected cells, or from other stable cellular or anatomical reservoirs.

In Paper III we used an HIV RNA quantification assay with a detection limit of 2.0 copies/mL in order to determine whether low-level viral replication was present in the CSF of subjects on stable and effective HAART. All 13 neurologically asymptomatic individuals had CSF viral loads < 2.0 copies/mL, compared to 8 out of 13 in plasma. This result is in agreement with several other studies [104, 166, 180, 181]. In 19 patients receiving nelfinavir and 2 NRTIs for at least 18 months, 12 had CSF viral loads < 3.0 copies/mL [166], compared to 6 out of 19 in plasma. In a cohort of HIV-infected individuals in San Francisco undergoing successful therapy (defined as stable combination ART for at least 3 months), 34 out of 47 (72%) had CSF HIV RNA < 2.0 copies/mL [104]. One explanation for the discrepancy between the American study and ours, besides the shorter duration of ART in the former, could be that they analysed CSF viral loads using ultra-ultra sensitive PCR when the viral load was < 400 copies/mL. However, all these studies indicate that the CNS is not as difficult to treat as was once believed. Perhaps this is because the viral load in CSF is generally lower than in plasma in the case of neurologically asymptomatic individuals [71], or that macrophages and microglia are not activated in the same manner as lymphocytes. It is also possible that antiretroviral drugs have different effects

on these two cell-types. *In vitro*, NRTIs exhibit greater activity against HIV in macrophages than in replicating cells such as activated lymphocytes [182]. This is because the NRTIs are competitive inhibitors of HIV RT. The antiretroviral activity of NRTIs depends on the intracellular concentration of their triphosporylated metabolite and the concentrations of natural 2´deoxy-nucleoside triphosphates (dNTPs), the building blocks of the growing DNA chain. The intracellular pool of dNTPs is much smaller in macrophages than in active lymphocytes, leading to a more pronounced effect [182]. The NNRTIs are non-competitive inhibitors of HIV RT, so their antiviral activity should not be affected by the dNTP pool—something that has been confirmed *in vitro* [182]. The PIs are equally active in macrophages and lymphocytes; however, higher concentrations are required to inhibit replication in the former, at least *in vitro* [183].

The CSF viral load alone does not suffice for either diagnosing ADC, identifying patients at risk of developing neurological disease, or evaluating treatment effects in the CNS. Even though CSF HIV RNA levels have been shown to correlate with severity of dementia in patients with ADC [72], there are many HIV-infected individuals that have high CSF viral loads for years without developing neurological symptoms. There will probably never be just one marker for the diagnosis and evaluation of HIV-associated neurological complications. This is more likely to be achieved using a combination of markers reflecting different aspects of HIV neuropathogenesis. We have used neopterin and β2M as markers of cell-mediated immune activation in this thesis. Both of these well-delineated markers decreased significantly after initiation of HAART containing lopinavir/r or saquinavir/nelfinavir. As mentioned in the introduction, neopterin is currently considered a more sensitive marker of macrophage/microglial activation than β2M. The studies in Papers I and II, where a larger proportion of patients had concentrations of neopterin that were greater than β2M in CSF, confirm this. Two other studies have demonstrated elevated CSF neopterin levels in 45% and 81% of their subjects after 2 and 4 years of treatment, respectively [102, 103]. Patients with herpes simplex virus type 1 (HSV-1) encephalitis displayed a similar reduction of CSF neopterin [184], whereas reduction was much faster after viral meningitis [185]. Persisting CSF neopterin levels might be associated with an ongoing low-grade viral replication within the brain, a more difficult to control immune response in the CNS, or possibly autoimmune phenomena.

The gradual breakdown of the BBB is a key event in the neuropathogenesis of HIV, and is therefore more common in later stages of the disease [108]. The BBB deterioration is mediated by activated cells in the CNS and the viral and cellular products they secrete, leading to the entry of activated monocytes and serum products into the CNS [186]. BBB dysfunction was rarely encountered in our studies, which merely included neuroasymptomatic

subjects. The only patient in Paper II with an elevated albumin ratio was a male with ADC. It was unfortunately not possible to evaluate the long term effect of ART on his albumin ratio since he had a virological failure on the occasion of the first on-treatment follow-up, most probably the result of poor adherence. It is difficult to assess the effects of ART on albumin ratio in neurologically asymptomatic individuals, who usually have values within the normal range [121]. However, in patients with ADC, BBB integrity has been shown to improve after initiation of treatment [187].

The IgG index did not change significantly in response to ART (Paper II). Many patients treated for as long as 4 years with effective HAART show signs of ongoing intrathecal immune activation, with elevated IgG indices [103]. Only a small proportion of the secreted antibodies are HIV-specific [188], which might indicate a role for autoimmune mechanisms in the neuropathogenesis of HIV. Autoantibodies against neuron-associated gangliosides, cerebellar soluble lectin, and myelin basic proteins have been found in the CSF of HIV-infected individuals [189-191]. The mechanisms behind persisting CSF neopterin levels and intrathecal IgG production are probably similar.

Even though we know that many HIV-infected individuals have signs of ongoing intrathecal humoral and cell-mediated immune activation, we do not know what the clinical implications of this are. Might such processes be harmful to the brain, or is it possible they have a beneficial effect? It has been suggested that low-level viral replication in blood could have positive effects by continuously stimulating the immune system [192]. On the other hand, it is difficult to imagine the positive effects of having an active immune system enclosed in a protected, immunologically distinct compartment such as the CNS. Chronic inflammation and immune stimulation is regarded as harmful in the long run in many diseases, such as arteriosclerosis, ulcerus colitis, and chronic infectious and non-infectious hepatitis. If this is also the case with HIV CNS-infection, one clinical implication of our study would be to choose treatment regimens that consistently result in CSF viral loads less than 2.5 copies/mL.

Almost all neopterin detected in CSF is produced intrathecally [193]. One step in understanding the clinical importance of persisting intrathecal immune activation is to learn more about the mechanisms that stimulate it. Perhaps it is not so surprising that we found no difference in CSF neopterin levels for individuals with CSF viral loads < 50 copies/mL on either PI- or NNRTI-based HAART (Paper IV). Nor were there any differences in subjects on HAART and CSF viral loads < 50 copies/mL whose plasma viral loads varied. It is striking that, when subjects on stable HAART were grouped according to levels of CSF HIV RNA, those with the lowest CSF viral loads also had the lowest CSF neopterin levels. This indicates that intrathecal viral

production plays a role in triggering the cellular immune response within the CNS. By suppressing the CSF viral load to a maximum, a further decrease in CSF neopterin levels may be obtained. Despite this, only 6 out of 35 individuals (17%) with CSF viral loads < 2.5 copies/mL had CSF neopterin levels within the normal range; this was not true of any individuals with higher CSF viral loads. These findings give some support to the existence of continuous low-level viral replication in brain tissue during HAART, a replication that has not been possible to detect, even with ultra-ultra sensitive PCR.

CSF analysis of HIV-infected individuals is primarily employed in research projects, but not in clinical praxis. Even though there are rare case reports about so-called CNS escape [194], i.e., disproportionately high CSF HIV RNA levels in treated patients, most studies have demonstrated that HAART effectively suppresses the CSF viral load. This is even true in the case of individuals with systemic virological failure [104]. If future studies were to indicate that low-level intrathecal viral replication or persistent immune activation is harmful, the situation would be different. Regular CSF analysis would then probably become a routine part of patient monitoring, as might the use of ultra-ultra sensitive quantification assays. A significantly cheaper alternative approach might be monitoring CSF neopterin levels. If the results of our study are confirmed by other researchers, one might assume that the CSF viral load in a patient with normal CSF neopterin levels is most likely to be less than 2.5 copies/mL.

The normal reference value for CSF neopterin ($\leq$ 4.3 nmol/L) is based on studies of 71 controls. Among these, 24 were healthy volunteers [143]. The remaining 47 individuals had their CSF analysed because they complained of headache or vertigo. Infections and other diseases were excluded at the time, and CSF albumin and cell count were normal [144]. However, these controls may have had some undiagnosed condition causing increased intrathecal immune activation. If anything, this might have led to an overestimation of the reference value, rather than an underestimation. Concentrations of neopterin in CSF increase with age. The controls used for determination of the normal reference value in this thesis were between 18 and 76 years of age, and the patients were in almost the same range (18 to 79).

Despite the attention given to characterising and studying HIV-associated neurological complications over the last two decades, many unanswered questions remain. For example, it is still a mystery why some patients develop neurological impairment while others do not, even when a considerable amount of virus is present in the CSF. HAART has had a profound impact on the incidence of ADC, but the HAART era is not without neurological complications. Several studies indicate that the clinical presentation of HIV-induced CNS injury has changed, with the appearance of milder forms of

HIV-related brain disease and a shift in symptomatology [46]. We currently lack objective, laboratory-based methods for diagnosing and assessing these conditions. The future situation may be even more difficult, as HIV-infected individuals live longer and therefore run the risk of being affected by disorders such as Alzheimer's disease and vascular dementia.

Future studies need to focus on finding diagnostic tools for HIV-related neurocognitive disease, while continuing to investigate the CNS-penetrating capacities of various antiretroviral drugs. Studies of CCR5 antagonists and their effectiveness in penetrating the CNS will be particularly useful, since viruses using the CCR5 co-receptor are predominant in CSF, even in patients with ADC [195]. It is also important to follow HIV-infected individuals longitudinally with regard to the long-term effects of hosting ongoing viral replication and immune activation in the CNS, so that one may evaluate different treatment strategies. Since 1996, ART has had a tremendous impact on reducing the morbidity and mortality of HIV, but such therapy remains, in essence, a clinical experiment on a grand scale, whose long-term treatment effects and side-effects, as well as its influence on low-grade viral activity, is unknown.

CONCLUSIONS

- HAART containing lopinavir/r or saquinavir/nelfinavir effectively reduces the CSF viral load and intrathecal immune activation in HIV-infected individuals commencing treatment.

- The CSF concentrations of lopinavir/r exceed the IC_{50} severalfold and are probably sufficient for antiviral activity, even when lopinavir/r is used on its own, whereas the CSF concentrations of saquinavir and nelfinavir are not.

- Fewer HIV-infected individuals on stable, effective HAART have detectable virus in their CSF than they do in plasma, as analysed by ultra-ultra sensitive quantification assays, rendering it unlikely that the CSF of neurologically asymptomatic subjects acts as a reservoir in which HIV can replicate independently from the periphery.

- CSF HIV RNA levels are an important driving force behind persistent intrathecal immune activation. By maximal suppression of viral replication, it is possible to reduce the CSF neopterin levels to normal values. Despite this, 29 out of 35 subjects (83%) in our study with CSF HIV RNA < 2.5 copies/mL still had elevated CSF neopterin levels.

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SVENSK SAMMANFATTNING

HIV är ett virus som infekterar och förstör celler i immunsystemet, framförallt CD4 + T-hjälpar celler. Så småningom leder detta till en immunbrist, känd som AIDS. Mindre känt är att HIV även kort efter smittotillfället tar sig in i det centrala nervsystemet (CNS) och infekterar makrofager och mikroglia, celler som är viktiga för immunförsvaret i CNS. Närvaron av HIV i CNS stimulerar immunsystemet lokalt med cellaktivering och antikroppsbildning som följd. Kring mitten av 80-talet, när det inte fanns några läkemedel mot HIV, utvecklade ca 20% av HIV-infekterade individer en demens som var direkt relaterad till HIV. Förutom denna demens kan HIV-infekterade individer drabbas av infektioner som vanligen inte angriper personer med välfungerande immunsystem.

Nuförtiden har vi en hel arsenal med anti-HIV-läkemedel och det är inte tillnärmelsevis lika många patienter som drabbas av den fruktade AIDS-demensen eller andra infektioner i CNS. För att ett läkemedel ska kunna utöva sin effekt i CNS måste det passera blod-hjärn barriären (ett membran som skyddar hjärnan från potentiellt farliga substanser). Det är därför viktigt att ta reda på vilka anti-HIV-läkemedel som kan passera blod-hjärn barriären in till likvor (vätskan som omger hjärnan och ryggraden), och vilken effekt de har på HIV-infektionen i hjärnan. Individer som påbörjar en kombinationsbehandling mot HIV sänker effektivt virusmängden i likvor och minskar även graden av immunaktivering.

Vi har mätt likvor-koncentrationerna av tre olika anti-HIV-läkemedel (lopinavir, saquinavir och nelfinavir) i två skilda studier. Vi fann att lopinavir-koncentrationerna var tillräckligt höga för att hindra viruset från att föröka sig. Nivåerna av saquinavir i likvor kunde bara mätas hos mindre än hälften av patienterna i den andra studien. Resultatet för nelfinavir var något bättre, men troligen är koncentrationerna av dessa båda läkemedel för låga för att på egen hand klara av att hämma virusförökningen.

HIV har förmågan att infektera vilande minnes CD4 celler och "gömma" sig i värdcellens DNA, vilket resulterar i att viruset inte går att behandla bort med de läkemedel som är tillgängliga idag. Till och med patienter som behandlats med en effektiv kombinationsbehandling i flera års tid har en låggradig virusförökning i blodet, som bara kan mätas med mycket känsliga metoder. Dessa få virus-kopior härstammar troligtvis från de vilande minnes-CD4 cellerna. Vi undersökte 13 patienter och fann att ingen av dem hade en liknande låggradig virusreplikation i likvor, men att 5/13 hade det i blodet. Detta tyder på att likvor inte är så svårbehandlat som man tidigare trodde, då man spekulerade i att virus i likvor skulle kunna föröka sig fritt i frånvaron av tillräckliga läkemedelsnivåer och därmed kunna ge upphov till resistent virus som kunde sprida sig till resten av kroppen.

Neopterin är ett välstuderat ämne som används som markör för att bedöma graden av aktivering hos makrofager och mikroglia (de celler som främst infekteras av HIV i CNS). Nivåerna av neopterin i likvor stiger vid ökad inflammatorisk aktivitet och sjunker när den minskar, till exempel efter insatt behandling. Trots flera års behandling har många HIV-infekterade individer kvar förhöjda värden av neopterin i likvor. För att ta reda på vad som ligger bakom denna aktivering av immunsystemet undersökte vi om typ av kombinationsbehandling eller virusmängd i blod och/eller likvor hade någon inverkan på neopterin-nivåerna i likvor. Det enda som föll ut var virusmängden i likvor, på så sätt att ju lägre virusnivåer i likvor vi fann desto lägre var neopterin-nivåerna. Detta tyder på att virusförökningen är en viktig pådrivare av immunaktiveringen, och om man kan pressa ner den minskar även graden av immunaktivering i CNS.

TÜRKÇE ÖZET

HIV virüsü bağışıklık sistemindeki hücreleri ve özellikle CD4 yardımcı T-hücrelerini enfekte ederek yok eden bir virüstür. Bu enfeksiyon ileri dönemde AIDS (kazanılmış immün yetersizlik sendromu) diye bilinen bağışıklık sisteminin yetersizliği ile sonuçlanır. HIV´in daha az bilinen bir yanı da bulaşmasından kısa bir süre sonra merkezi sinir sistemine (MSS) girerek, bağışıklık sistemindeki makrofaj ve mikroglia hücrelerini enfekte etmesidir. Virüsün MSS'de bulunması bağışıklık sistemini faaliyete geçirerek, bölgesel bağışıklık sistemine ait bu hücrelerin aktifleşmesine ve antikor olarak bilinen bağışıklık sistemi tarafından yabancı yapılara karşı geliştirilen proteinlerin üretimine yol açar. 1980'li yılların ortalarında, HIV'e karşı ilaç geliştirilmeden önce, bu virüs ile enfekte olmuş kişilerin % 20'sinde HIV ile ilişkili olarak demans adı verilen bellek bozukluğu görülmekteydi. Günümüzde HIV' e karşı etkin ilaçlarla tedavi daha önce korkulan AIDS hastalığına yakalanma riskini azaltmıştır.

Bir ilacın MSS'nde etkili olabilmesi için beyni koruyan ve zararlı olabilecek maddelerin geçmesine mani olan kan-beyin engelini aşması gerekir. Bu nedenle hangi HIV ilaçlarının kan-beyin engelini aşarak beyin omurilik sıvısına geçebildiği ve MSS'nde HIV enfeksiyonuna karşı etkilerini tespit etmek oldukça önemlidir.
Araştırmalarımızda üç ayrı HIV ilacının (lopinavir, saquinavir ve nelfinavir) beyin omurilik sıvısındaki seviyesi değerlendirilmiştir. Çalışmalarımızın sonuçları lopinavirin beyin omurilik sıvısında virüsün üremesine engel olabilecek seviyeye ulaşabildiğini, ancak saquinavirin beyin omurilik sıvısındaki seviyesinin hastaların yarısından azında ölçülebildiğini tespit ettik. Nelfinavir seviyesinin ise saquinavir seviyesine oranla daha yüksek olduğunu, ancak tek başına kullanıldığında virüsün beyin omurilik sıvısında üremesine engel olabilecek seviyeye ulaşamayacağını gözlemledik.

HIV'in dinlenmekte olan CD4 bellek T-hücrelerinin DNA'sında gizlenebilmesi, bu virüsün mevcut ilaçlarla tedavisini zorlaştırır. Birkaç yıl etkili ilaç tedavisi görmüş hastaların kanlarında bile, düşük derecede virüs üreme oranı görülebilmektedir. Daha önce, beyin omurilik sıvısında yeterli seviyede ilaç bulunmaması halinde, virüsün serbestçe üreyerek, ilaçlara dayanıklı hale gelebildiği ve vücudun diğer kısımlarına da yayılabileceği düşünülüyordu. Çalışmalarımızda incelediğimiz 13 hastanın beyin omurilik sıvısında benzer derecede düşük virüs üreme oranı gözlenmedi, ancak bu 13 hastadan 5'inin kanında virüs tespit edildi. Bu, virüsün beyin omurilik sıvısında üremesinin önlenebildiğini göstermektedir.

Neopterin makrofaj ve mikroglia hücrelerindeki faaliyet derecesini tespitte kullanılır. Örneğin, başlanan bir tedavinin sonucu olarak, beyin omurilik sıvısındaki neopterin seviyesi, iltihabi faaliyetler arttığında artar, azaldığında ise düşer. Yıllarca süren tedaviye rağmen, HIV ile enfekte birçok hastanın beyin omurilik sıvısında hala yüksek seviyede neopterin bulunmaktadır. Çalışmalarımızda bağışıklık sistemindeki bu faaliyetin sebebini araştırmak üzere seçilen karma ilaç tedavisinin veya kandaki virüs miktarının, beyin omurilik sıvısındaki neopterin seviyesine nasıl bir etkisi olduğunu inceledik ve beyin omurilik sıvısında virüs miktari düşük olan hastalarda neopterin seviyesinin de düşük olduğunu tespit ettik. Bu da gösteriyor ki beyin omurilik sıvısında virüs üremesi bağışıklık sistemindeki faaliyeti önemli ölçüde etkilemektedir ve virüsün üremesinin önlenmesi ile bu faaliyet de azalabilmektedir.