On Cerebrospinal Fluid Biomarkers of HIV-1 Infection

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“We have killed each other with our ignorance, our prejudice, and our silence. We may take refuge in our stereotypes, but we cannot hide there long, because HIV asks only one thing of those it attacks. Are you human?” —Mary Fisher 1992

ABSTRACT

HIV invades the central nervous system (CNS) shortly after transmission and is present throughout the course of infection, causing immune activation and neuroinflammation. If left untreated, more than 20% of patients with late-stage HIV/AIDS develop HIV-associated dementia (HAD). With combined antiretroviral treatment (cART), HAD is rare, but mild neurocognitive deficits are commonly noted and have been termed HIV-associated neurocognitive disorders (HAND). The diagnosis of HAND relies solely on neuropsychological testing, which might overestimate the prevalence of HAND. Analysis of biomarkers could enhance diagnostic precision. With an aging HIV-infected population, methods to distinguish HAND from other dementias, especially Alzheimer's disease (AD), will increase in importance.

This thesis evaluates biomarkers related to neuronal injury (neurofilament light chain protein [NFL] and total tau [t-tau]); immune activation (neopterin); and altered metabolism (soluble amyloid precursor protein α and β [sAPP], beta-amyloid$_{1-42}$ [Aβ_{1-42}], and phosphorylated tau [p-tau]) in cerebrospinal fluid (CSF) of HIV patients with and without cognitive deficits. For the purposes of differential diagnosis, AD patients and HIV-negative subjects with CNS infections were included.

HAD patients exhibited a biomarker pattern with normal to low Aβ_{1-42}, decreased sAPPs, normal p-tau, and increased t-tau, thus differentiating HAD from AD, neuroasymptomatic (NA) HIV-infected patients, and controls. Although CSF p-tau occurs physiologically with aging, p-tau levels were normal or decreased in HIV. HIV-related opportunistic infections (OI) and CNS infections in HIV-negatives were similar to HAD, indicating that neuroinflammation might induce a pathologic processing of amyloid that is separate from the metabolism in AD. Amyloid and tau metabolites could be useful biomarkers to distinguish HAD from AD.

CSF NFL was highest in HAD patients, but NA patients, both with and without cART, also exhibited increases in NFL. This indicates ongoing axonal disruption at all stages of HIV, including some patients on cART. Most likely this is due to HIV-induced axonal disruption. CSF NFL levels increased in younger HIV-infected patients as compared to controls.

Keywords: HIV-associated neurocognitive disorders, HIV-1, amyloid protein, tau protein, neurofilament protein, biomarker, central nervous system, cerebrospinal fluid.

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SAMMANFATTNING PÅ SVENSKA

Humant immunbrist virus typ 1 (HIV) har sedan det första beskrivna fallet 1981 utvecklats till en världsomspännande pandemi, med uppskattningsvis 36 miljoner smittade personer världen över. Obehandlad fortlöper sjukdomen utan sjukdomssymtom under många år, samtidigt som HIV infekterar och förstör CD4-positiva T-hjälparceller, vita blodkroppar centrala för ett fungerande immunförsvar. Slutstadiet är Acquired Immunodeficiency Syndrome (AIDS), då svår och slutligen dödlig sjuklighet orsakad av infektioner och cancerformer yttrar sig på grund av den förvärvade immunbristen. En välkänd komplikation till långt gången HIV/AIDS är också HIV-associerad demens (HAD), en demensform som primärt drabbar den vita substansen i hjärnan. Det är väl belagt att HAD uppkommer på grund av en direkt, HIV-associerad, aktivering av immunsystemet samt inflammatorisk skada på nervceller i centrala nervsystemet (CNS). Kliniskt yttrar sig HAD som ett komplex av nedsatt kognitiv förmåga, beteendeförändringar och motoriska svårigheter.

Nu när bromsmediciner (cART) finns tillängliga är HAD en ovanlig företeelse. Ett spektrum av lindriga kognitiva störningar hos såväl obehandlade som cART-behandlade patienter är dock vanligt förekommande. Dessa sammanfattas tillsammans med HAD i begreppet HIV-associated neurocognitive disorders (HAND). HAND diagnosticeras idag enbart med neuropsykologisk testning och det finns invändningar mot att förekomsten av HAND sannolikt överskattas.

Denna avhandling fokuserar på att närmare kartlägga proteiner som signalerar dels nervskada, dels aktivering av immunsystemet och slutligen förändrad metabolism i cerebrospinalvätska (CSF). Tre övergripande frågeställningar går som en röd tråd genom avhandlingen: 1) Kan biomarkörer användas för att skärpa diagnostiken av HAND? 2) Har HIV-patienter ett annat mönster vid analys av biomarkörer jämfört med friska personer eller patienter med andra sjukdomar? 3) Kan analys av biomarkörer hjälpa oss att närmare förstå de effekter som HIV utövar i CNS vid HAND?

I delarbete I undersöktes nedbrytningsprodukter av amyloid prekursor protein och olika former av tauprotein i CSF. Vi jämförde nivåer mellan patientgrupper med HAD, HIV-positiva utan neurologiska symtom, HIV-infekterade med opportunistiska infektioner i CNS (OI) och Alzheimers sjukdom (AD). Vi fann att HAD uppvisade en annorlunda proteinprofil jämfört med AD och föreslår att amyloid och tauproteiner verkar vara lovande komplement till neuropsykologisk testning för såväl diagnos av HAD som för att skilja AD från HAD. Patienter med OI gick inte att

skilja från HAD genom analys av amyloid och tauproteiner. Sannolikt orsakades de snarlika förändringarna av inflammation i CNS, men det gick inte att utesluta en separat effekt av HIV.

Då det i delarbete I var oklart om förändringarna amyloid och taunivåer var en effekt specifikt relaterad till HIV, eller om det var en generell effekt orsakad av inflammation i CNS, valde vi i delarbete II att undersöka samma proteiner på HIV-negativa personer med infektioner i CNS. Vi fann ett snarlikt mönster som vid HAD och OI. Sannolikt orsakas förändringarna i proteinnivåer av inflammation och aktivering av immunförsvaret i CNS.

Mildare former av HAND är vanligt även hos välbehandlade HIV-patienter. Vi valde därför att i delarbete III undersöka pågående nervskador i CNS genom analys av neurofilament light chain protein (NFL), en markör för axonalt sönderfall i CSF. Vi fann att patienter med HAD såväl som HIV-infekterade utan neurologiska symtom hade förhöjda nivåer. Trots att bromsmediciner verkar minska nervskadan, reflekterat i NFL, hade även patienter med cART förhöjda nivåer jämfört med friska kontroller. Denna studie visar att en låggradig och pågående nervskada sannolikt sker hos HIV-patienter och att HIV eventuellt orsakar ett för tidigt åldrande av hjärnan. Vidare är NFL i CSF tydligt förhöjt vid HAD och skulle kunna utgöra ett komplement till neuropsykologisk testning.

I delarbete IV undersökte vi fosforylerat tau (p-tau) i CSF från patientgrupper med HAD och HIV-infektion utan kognitiva störningar. Nivåer av P-tau i CSF stiger med åldern i hela befolkningen. Vår hypotes var att p-tau eventuellt ökar i tidigare åldrar vid HIV-infektion som tecken på för tidigt åldrande av hjärnan. P-tau skiljde sig inte hos HIV-patienterna jämfört med friska kontroller och man kan inte med hjälp av p-tau diagnosticera nervskador i CNS vid HIV.

LIST OF PAPERS

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

I. Gisslén M, Krut J, Andreasson U, Blennow K, Cinque P, Brew BJ, Spudich S, Hagberg L, Rosengren L, Price RW, Zetterberg H. **Amyloid and tau cerebrospinal fluid biomarkers in HIV infection**.
BMC Neurology. 2009; 9:63.

II. Krut JJ, Zetterberg H, Blennow K, Cinque P, Hagberg L, Price RW, Studahl M, Gisslén M. **Cerebrospinal fluid Alzheimer's biomarker profiles in CNS infections**.
J Neur. 2013; 260:620-626.

III. Krut JJ, Mellberg T, Price RW, Hagberg L, Fuchs D, Rosengren L, Nilsson S, Zetterberg H, Gisslén M. **Biomarker Evidence of Axonal Injury in Neuroasymptomatic HIV-1 Patients**.
PLoS One. 2014 Feb 11;9(2):e88591

IV. Krut JJ, Price RW, Zetterberg H, Fuchs D, Hagberg L, Yilmaz A, Cinque P, Nilsson S, Gisslén M. **No support for premature CNS aging in HIV-1 when measured by cerebrospinal fluid hyperphosphorylated tau (p-tau)**.
In submission

CONTENT

ABBREVIATIONS

AD	Alzheimer's Dementia
ADC	AIDS Dementia Complex
AICD	APP Intracellular Domain
AIDS	Acquired Immunodeficiency Syndrome
ANI	Asymptomatic Neurocognitive Impairment
APP	Amyloid Precursor Protein
$A\beta_{1-42}$	Beta-amyloid protein
BBB	Blood-Brain Barrier
cART	Combined Antiretroviral Therapy
CDC	Centers for Disease Control
CHARTER	CNS HIV Antiretroviral Therapy Effects Research
CMV	Cytomegalovirus
CNS	Central Nervous System
CRF	Circulating Recombinant HIV-1 Forms of Group M virus
CSF	Cerebrospinal Fluid
CTF	C-terminal Fragment
CVD	Cardiovascular Disease
ELISA	Enzyme-linked Immunosorbent Assay
Env	Envelope

Gag	Group specific antigen
HAD	HIV-associated Dementia
HAND	HIV-associated Neurocognitive Disorder
HIV	Human Immunodeficiency Virus
HSV-1	Herpes Simplex Virus type 1
INI	Integrase Inhibitor
IQR	Interquartile Range
IVDU	Intravenous Drug User
LAV	Lymphadenopathy Associated Virus
LP	Lumbar Puncture
MND	Minor Neurocognitive Disorder
MSM	Men who have sex with men
NA	Neuroasymptomatic
NFL	Neurofilament Light Chain Protein
NNRTI	Non-nucleoside/nucleotide Reverse Transcriptase Inhibitor
NP	Neuropsychological Testing
NRTI	Nucleoside Reverse Transcriptase Inhibitor
OI	HIV-related Opportunistic Infection
P-tau	Phosphorylated tau protein
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
PML	Progressive Multifocal Leukoencephalopathy

Pol	Polymerase
ROC	Receiver-operator Characteristics
sAPP	Soluble Amyloid Precursor Protein
SIV	Simian Immunodeficiency Virus
T-tau	Total tau protein
Tat	Transactivator of transcription
TNF	Tumor Necrosis Factor
URF	Unique Recombinant HIV-1 Forms of Group M virus
WBC	White Blood Cell

1 INTRODUCTION

The story of HIV/AIDS is multifaceted. On one side, it is a story of fear. Initially, there was anxiety as the pandemic grew to enormous proportions while politicians, scientists, and people affected by the virus watched helplessly. But even after 1996, when the first effective combination antiretroviral treatment (cART) was launched and up until present day, it is obvious that social stigmata and prejudice are to a great extent central elements in the daily lives of those who are HIV positive. Some groups, such as men having sex with men (MSM) with HIV, are faced with a double stigma and face even greater discrimination and exclusion from society in many parts of the world.

It is also a story about global inequalities. An overwhelming majority of HIV-infected people live in countries with limited access to proper healthcare and education, poor economic resources and gender inequalities—all factors facilitating the progression to AIDS and the spread of the disease.

On the other side, the story of HIV is one of the greatest successes in modern medicine. With the collaboration of the scientific community, the medical industry and both governmental and non-governmental organizations, HIV/AIDS has gone from a disease leading to certain death, to a treatable condition with normal life expectancy and little risk of developing symptomatic HIV.

1.1 History

In 1981, several scientific reports described unusual cases of severe illness among previously healthy, homosexual males in the U.S. The men all exhibited clinical signs of immune deficiency with rare opportunistic infections such as Pneumocystis Carinii (Jirovecii) pneumonia, mucosal candidiasis, malignancies (Kaposi sarcoma), and severe lymphopenia [1-3]. Initially, it was believed that only gay men were affected by the disease, but cases soon emerged among hemophiliacs, intravenous drug users, and heterosexuals, leading to the suspicion that the immune deficiency seen was

caused by a both sexually transmitted and blood-borne infectious agent [4,5]. Initially, the nomenclature was rather confusing, but by 1983 the acronym AIDS (acquired immune deficiency syndrome) had been accepted.

However, AIDS was not confined to the US. Reports of AIDS-like symptoms emerged in increasing numbers from around the world, and sub-Saharan Africa later proved to be the epicenter of the pandemic.

In 1983, Barré-Sinoussi and Montagnier isolated a T-lymphotrophic retrovirus from a patient with symptoms of AIDS, and they named it LAV (lymphadenopathy associated virus). It was an effort for which they received the 2008 Nobel Prize in medicine. In the following year, Gallo et al. stated that AIDS is caused by the HTLV-III virus [6,7], which proved to be the same virus as LAV. Consensus was reached in 1986, and the name was changed to HIV (human immunodeficiency virus).

The year 1987 marked a change in paradigm, when the nucleoside analogue (NRTI) AZT was introduced as the first effective drug against HIV/AIDS [8]. The jubilation was short-lived, however, as AIDS patients within months or years developed resistance to AZT monotherapy and progressed to severe disease and death. In the following years, several new NRTIs (ddI, ddC, d4T, and 3TC) were developed, but it was not until 1996, when the first protease inhibitor (PI), saquinavir, soon followed by indinavir, were introduced and used in combination with two other drugs, usually two NRTI, that sustainable suppression of HIV through cART was possible. The principle of cART remains the same to this day, but drugs with other modes of action (Non-nucleoside inhibitors (NNRTI), integrase inhibitors, fusion inhibitors, and CCR-5 inhibitors), less toxicity, and increased simplicity of use are now available.

The origin of HIV was long disputed, but in the late 1990s it was concluded that HIV-1 was derived from simian immunodeficiency virus (SIV) found in chimpanzees (*Pan Troglodytes troglodytes*) [9]. HIV-2 showed great similarities with the type of SIV found in sooty mangabey monkeys (*Cercocebus atys*) [10].

Phylogenetical mapping of SIV from feces of wild chimpanzees in West Central Africa showed great similarities with two groups of HIV: pandemic (M-type) and N-type [11]. By using the same technique, the first human case of HIV group M has been estimated to have occured between 1910 and 1930 in Kinshasa [12-14]. It is not fully established how transmission from monkeys to humans took place, but the dominant theory is by contact with blood during hunting and slaughtering [15].

1.2 Demographics

According to estimates from UNAIDS, about 35 million people were living with HIV globally in 2013. This number is increasing year by year, mainly due to greater access to cART and rising long-term survival. An estimated 24.7 million HIV-infected (70%) lived in low- and middle-income countries in sub-Saharan Africa (Figure 1.). Roughly 37% had access to cART. Worldwide, heterosexual transmission is by far the most common route, but in Western Europe and North America, MSM accounted for the largest proportion of those newly-infected. An estimated 13% of all intravenous drug users worldwide are also infected with HIV, and are an important reason for increasing numbers of HIV-infected people in parts of Asia and Eastern Europe.

There has been an obvious decrease in those newly infected (approximately 2.1 millions in 2013 compared to 3.4 millions in 2001). This is in part due to increased use of cART, since viral suppression greatly reduces the risk of transmission; but it is also due to preventive measures such as condom use, male circumcision, avoiding mother-to-child transmission, education, and empowerment of women [16].

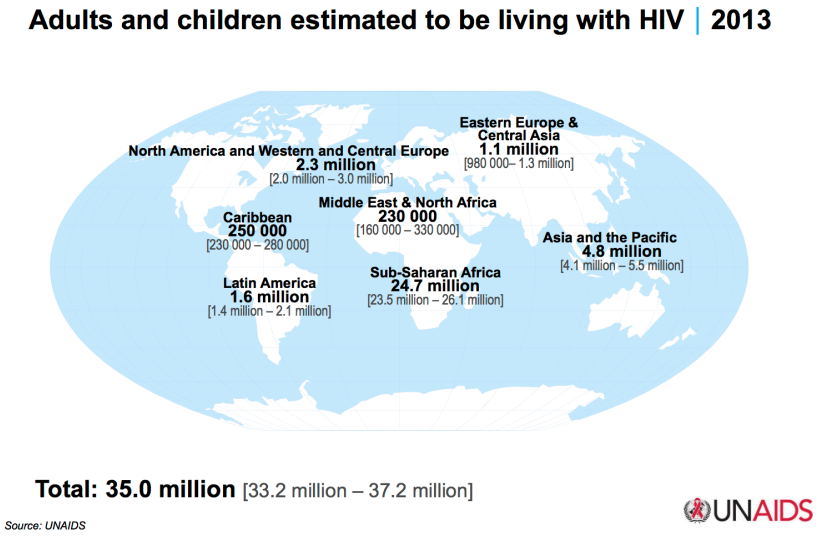

Figure 1. Estimated numbers of HIV-infected people in 2013. Source: UNAIDS 2014 Report on the global AIDS epidemic

Since the post-infection progression to AIDS is typically slow, ranging from less than one to more than 20 years, a large number of unknown HIV-positive people exist throughout the world [17]. Due to the social stigma still surrounding HIV, disclosure to sexual partners and insufficient testing are major concerns in controlling the pandemic.

According to Folkhälsomyndigheten, the Swedish equivalent to Center for Disease Control, a total of 6400 people were verified HIV-positive in Sweden 2013. This equates to an HIV-prevalence in the population of 0.06%. There were 461 HIV-infections newly diagnosed. Out of these, 353 had been infected abroad, mainly through heterosexual contact. Of the 86 people infected in Sweden in 2013, a majority were MSM (62 cases). Judging by the 10-year trend, HIV is at a stable level of newly-infected cases in Sweden. Most Swedish patients have been well managed by suppressive cART, but 37

patients presented with AIDS, mainly due to previously unknown HIV. [18]. Those numbers are similar as compared to InfCare, a database used by all HIV clinics in Sweden. In October 2015, 6919 patients were registered as HIV-positive. Out of those, 94.7% were treated with suppressive cART (defined as HIV-RNA <50 copies/mL plasma).

1.3 Molecular properties of HIV-1

HIV belongs to the family *Retroviridiae*, genus *Lentivirus*. Two distinct types of HIV viruses exist in man: HIV-1 and HIV-2. Whereas HIV-2 is mainly confined to West Central Africa, HIV-1 accounts for the pandemic spread of the virus. HIV-1 (hereafter HIV) is divided phylogenetically into four groups: M (major), N (novel), O (outliers), and P (putative) [19-21]. The M-group is responsible for the pandemic spread and is subdivided into genetic subgroups/clades (A–K); several mosaic combinations of two viruses; circulating recombinant forms (CRF); and unique recombinant forms (URF). Worldwide, subtype C is predominant, but there are clear regional differences. Western Europe, North and South America are mainly affected by subtype B, while Southeast Asia exhibits CRFs as the predominant subtypes [22].

Structurally, the HIV virion has a diameter of 100nm. At the surface, it consists of a lipoprotein membrane "studded" with glycoproteins (gp120 and gp41). These are important for adhesion to CD4-positive (CD4+) white blood cells (WBC). Attached to the inside of the membrane is a matrix protein (p17). A central capsid, rich in the core antigen p24, surrounds two single-stranded RNA copies and the three main enzymes: reverse transcriptase, integrase and protease (Figure 2).

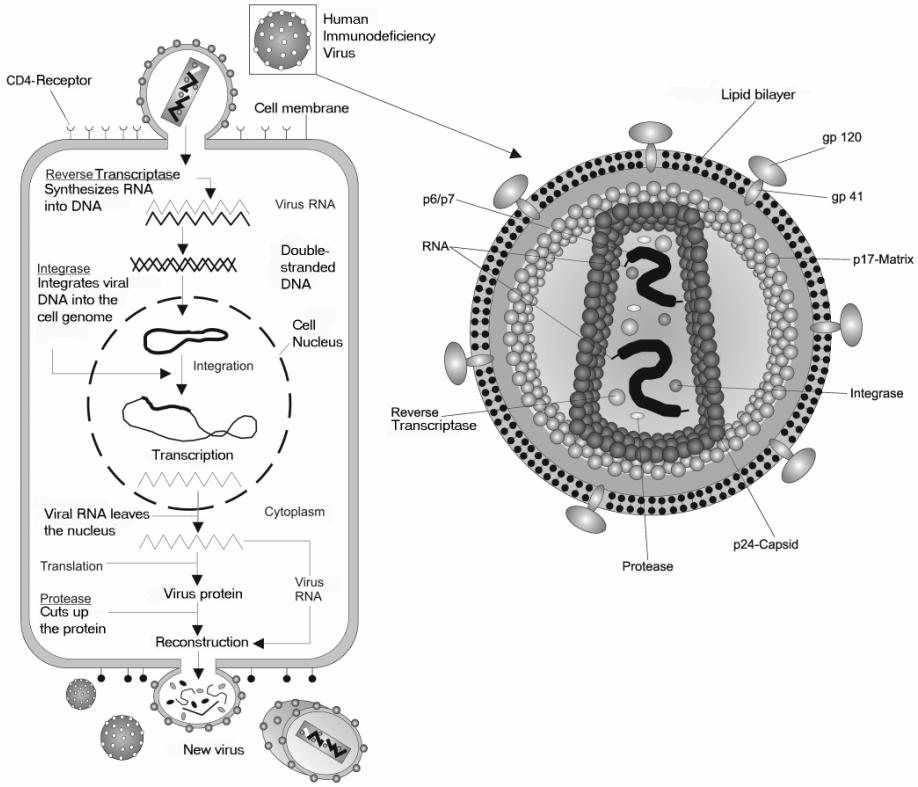

Figure 2. HIV replication within CD4+ cells (left). Structure of the HIV-1 virion (right). Used under the GNU Free Documentation License

The genome of HIV-1 consists of a number of important genes. Group-specific antigen (gag) encodes for structural proteins p6, p7, p17, and p24, which are building blocks for the capsid and matrix. DNA polymerase (pol) encodes for the reverse transcriptase, protease, and integrase enzymes. The envelope (env) gene encodes for the membrane proteins gp41 and gp120. In addition, a number of regulatory genes exist.

1.4 HIV-1 replication

HIV replicates in CD4+ WBC, mainly T-lymphocytes crucial to proper functioning of the adaptive immune response. The protein gp120 in the virion membrane binds to the CD4 glycoprotein of the WBC. This process exposes a domain at gp120 that binds into the different co-receptors CCR5 or CXCR4 of the WBC and simultaneously exposes gp41 in the virion membrane. CCR5 is mainly expressed by monocytes, macrophages, dendritic cells, and activated CD4+ T-lymphocytes. Viruses that prefer adhesion to CCR5 are referred to as M-tropic. CXCR4 is mainly expressed by T-lymphocytes and virus attaching to them is referred to as T-tropic [23,24].

The binding of virus to WBC cause a fusion of membranes through the action of gp41, and the contents of the viral capsid is injected into the WBC. In the cell cytoplasm, reverse transcriptase converts the single-stranded RNA into proviral DNA in the cell cytoplasm.

The viral DNA is then integrated into the host DNA via the action of integrase. After integration, two different paths are possible. In the first path, the cell is activated and starts to synthesize new HIV-RNA (messenger RNA) and protein precursors. The precursors are cleaved by HIV-protease into a number of proteins (reverse transcriptase, integrase etc.) and packaged together with two single-stranded HIV-RNA into a new virion that buds off from the host cell (Figure 2).

In the second path, the HIV-infected CD4+ cells progress to a latent or dormant phase. The virus invades active WBCs followed by integration of provirus in the host genome, so-called post-integration latency. These cells then progress to a latent state, thereby maintaining a stable viral reservoir over the course of infection. Important reservoirs are found in peripheral compartments (monocytes/macrophages, latent T-cells), and account for approximately 1% of the total number of infected CD4-cells [25]. If the CD4+ cells are already in a resting state at the time of infection, the provirus does not integrate in the host genome, so-called pre-integration latency. These cells are normally short-lived. In the central nervous system (CNS), microglia is likely functioning as a viral reservoir.

1.4.1 Mutations

Reverse transcriptase, translating single-stranded HIV-RNA to proviral DNA, lacks proofreading mechanisms and is error prone. In combination with the abundant production rate of new virus particles, the amount of mutated quasispecies is high. It is estimated that five to ten mutations per genome are introduced every replication cycle, or 10^{10} new mutations eacg day [26]. Some of these are as virulent as wild-type virus. In a treatment-naïve individual, several quasispecies of the virus exist simultaneously. cART aims to suppress the virus levels to a minimum, thereby reducing the risk of mutation and selection of drug-resistant strains. Resistances to all drug-classes are described, although the genetic barrier is higher for some drugs (PIs for example).

1.5 Pathogenesis

1.5.1 HIV-1 pathogenesis in the peripheral compartments

For a schematic presentation of HIV-pathogenesis, see Figure 3.

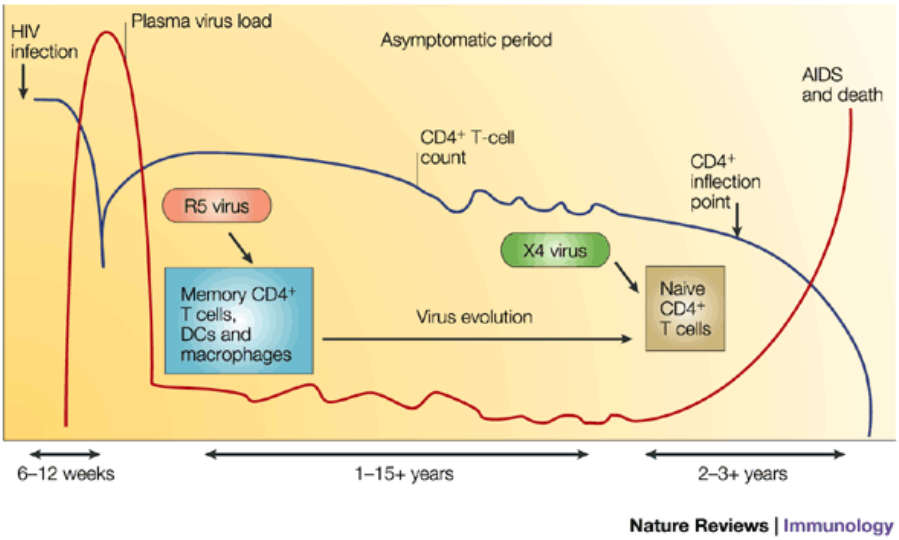

Figure 3. Schematic diagram showing the course of HIV-1 infection. Used with permission from Nature publishing groups

Upon entering the body, HIV-1 rapidly attaches to WBCs expressing CD4-receptor as explained above. During the first couple of weeks post-transmission, an asymptomatic phase occurs characterized by rapid increase in HIV-RNA and decrease in CD4+ WBC. The virus is distributed throughout the body, including the CNS, during the early course of infection, establishing reservoirs of memory cells. Some but not all patients enter an acute, symptomatic phase after 2 to 4 weeks post-transmission (primary HIV). Clinically this phase is characterized by mononucleosis-like symptoms (fever, myalgia, lymphadenopathy, pharyngitis, and rash). The viral load is at high levels and a further decline in CD4+ T-cells is noted.

After some days or weeks, the immune system regains partial control and a sharp decrease in the viral load is noted. This is largely an asymptomatic phase, but a continuous HIV replication and concurrent depletion of CD4+ T-cells occur. In median, HIV patients remain clinically asymptomatic for 10 years, although high levels of viremia, fast depletion of CD4+ T-cells, and negative host factors could speed up the process and reduce the interval to a couple of years. When levels of CD4+ T-cells drop below $350*10^6$ cells/ml blood, discrete signs of T-cell-mediated immune deficiency such as Herpes zoster and oral candidiasis usually appear. When the CD4 count falls below $200*10^6$ cells/ml blood, severe opportunistic infections or HIV-associated tumors become apparent, thereby fulfilling the criteria for Acquired Immune Deficiency (AIDS). (Table 1.)

CD4 cell-count categories	Clinical categories		
	A: Asymptomatic, acute HIV, or persistent generalized lymphadenopathy	*B: Symptomatic conditions, not A or C**	*C: AIDS indicator conditions***
> 500 cells/mL	A1	B1	C1
200-499 cells/mL	A2	B2	C2
< 200 cells/mL	A3	B3	C3
A3, B1 and C1-C3 are defined as AIDS in USA; C1-C3 are AIDS-defining in Europe			

*B: Symptomatic conditions	**AIDS-indicator conditions
• **Oropharyngeal candidiasis** • **Bacillary angiomatosis** • **Persistent vulvovaginal candidiasis** • **Pelvic inflammatory disease** • **Cervical dysplasia (moderate/severe) cervical carcinoma in situ** • **Oral hairy leukoplakia** • **Herpes zoster involving two or more episodes or at least one dermatome**	• Bacterial pneumonia, recurrent (two or more episodes in one year) • Candidiasis of bronchi, trachea, or lungs • Esophageal candidiasis • Biopsy-confirmed invasive cervical carcinoma • Coccidioidomycosis, disseminated or extrapulmonary • Cryptococcosis, extrapulmonary • Cryptosporidiosis, chronic intestinal • Cytomegalovirus disease (other than liver, spleen, or nodes)

*B: Symptomatic conditions (continued)	**AIDS-indicator conditions (continued)
• **Idiopathic thrombocytopenic purpura** • **Constitutional symptoms such as fever (> 38.5°C) or diarrhea lasting > 1 month** • **Peripheral neuropathy**	• Encephalopathy, HIV-related • Herpes simplex: chronic ulcers (> 1 month in duration), or bronchitis, pneumonitis, or esophagitis • Histoplasmosis, disseminated or extrapulmonary • Isosporiasis, chronic intestinal (> 1 month in duration) • Kaposi's sarcoma • Lymphoma, Burkitt, immunoblastic, or primary central nervous system • Mycobacterium avium complex (MAC) or Mycobacterium kansaii, disseminated or extrapulmonary • Mycobacterium tuberculosis, pulmonary or extrapulmonary • Mycobacterium, other species or unidentified species, disseminated or extrapulmonary • Pneumocystis jiroveci pneumonia (PCP) • PML • Salmonella septicemia, recurrent • Toxoplasmosis of brain • Wasting syndrome caused by HIV

Table 1. 1993 CDC Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults. Listed are AIDS-defining criteria based on CD4 T cell count and AIDS-defining conditions, as well as "indicator" conditions related to HIV-induced immunosuppression.

1.5.2 HIV-1 pathogenesis in the central nervous system

The CNS is surrounded by the blood-brain barrier (BBB), a selectively permeable membrane separating the systemic blood circulation and the capillaries in the CNS. The barrier is constituted of astroglia, endothelial cells, perivascular macrophages, pericytes, and a basal lamina, forming tight junctions [27]. Water and small, lipophilic substances diffuse passively across the BBB, but the main route for transporting larger, hydrophilic compounds occur due to selective active transport. This functions as an effective protection for the CNS by limiting the import of pathogens and toxic

substances, but can also be an issue in terms of achieving therapeutic concentrations of drugs in the CNS [28,29].

HIV enters the CNS early in the course of infection and persists throughout the chronic stage [30]. The exact mechanisms for CNS entry are not yet fully understood, but HIV is most likely transported across the BBB via infected blood monocytes [31]. Others suggest that lymphocytes might harbor virus that is transported into the CNS, infecting macrophages [32]. It may also be that free virions are also being transported across the BBB.

Cells exposing the CD4-receptor, in common with the CCR5 co-receptor, are infected in the CNS. The main productive cell reservoirs for HIV are perivascular macrophages and microglia. Astrocytes, without expression of CD4 or CCR5 can also harbor the virus [33]. Due to the discriminatory properties of the BBB, a separate phylogenetical evolution of HIV can occur in the CNS as compared to the rest of the body, so-called compartmentalization [34,35].

HIV does not productively infect neurons. However, studies show that HIV-antigen, proviral DNA and gene sequences can be found in neurons [36,37]. The pathological values of these findings are disputed. It has been well established that an intrathecal immune activation and neuronal injury is induced by the chronic low-level viremia in the CNS, as visible by an array of cerebrospinal fluid (CSF) biomarkers [38-41]. Clinically, there is clear overrepresentation of cognitive and motor disorders in HIV-patients. These range from asymptomatic cognitive impairments to overt dementia, and are summarized under the term HIV-associated neurocognitive disorders (HAND), further explained below [42].

Neuronal degeneration in HIV infection is complex. Viral proteins, such as gp120, tat and vpr exert neurocytotoxic effects in vitro [43,44]. Furthermore, HIV-associated proteins stimulate macrophages and microglia to produce neurotoxic compounds, mainly excitatory amino acidsn quinolonic acid [33,45] and, other cytokinesm and chemokines, thereby inducing an array of mediators and effectors in the inflammatory response.

1.6 HIV-1 related morbidity

1.6.1 Opportunistic infections and malignancies

With the subsequent depletion of CD4+ T-lymphocytes, commonly at a CD4-count of around $200*10^6$ cells/ml, a number of severe opportunistic infections (OI) and malignancies related to T-cell deficiency begin to appear. In the developed world, AIDS is diagnosed by a combination of lowest CD4-count (nadir) and clinical symptoms of OIs as stated by the CDC [46] (Table 1). In resource-poor settings, where it is seldom possible to measure CD4, a clinical staging algorithm developed by WHO and based solely on clinical features is used [47].

1.6.2 Non-AIDS defining morbidity and mortality

In addition to morbidity exerted by opportunistic infections and malignancies related to T-cell deficiency, HIV patients are at increased risk for a number of other conditions. In general, inflammation related to high viral load and low CD4 is present, but patients on suppressive cART are at greater risk of this than the general population.

Apart from the AIDS-defining malignancies outlined in Table 1, the incidence of other malignancies, such as malignant melanoma, malignant lymphomas, anal-, oropharyngeal-, lung-, and liver cancer is increased in HIV patients both on and off cART [48]. It is debatable if anal cancer should be considered a non-AIDS-defining disease or an AIDS-defining criterion. It is almost exclusively associated with human papilloma virus and is most common among MSM. Its pathogenesis shares great similarities with cervical cancer in women, which is an AIDS-defining criterion.

Cardiovascular disease (CVD) is significantly higher in HIV patients. Although risk factors for CVD such as dyslipidemia and smoking are common in the HIV population, an independent effect of HIV-related

inflammation is noted. Low CD4 is also a predictor for the development of CVD [49,50].

Co-infection of HIV with hepatitis B or C leads to the rapid development of end-stage liver disease (cirrhosis and hepatocellular cancer) [51]. Some nucleoside/nucleotide analogues as one of the components in cART have a dual effect against HIV replication as well as Hepatitis B and C, and therefore treatment with cART decreases the rate of progression to end-stage liver disease [52].

HIV-related nephropathy is relatively common among the black population. A clear connection between high viral loads and nephropathy is seen, and cART effectively hinders further progression of renal decline [53]. Lastly, HIV is responsible for an array of neuronal damages, both in the CNS and in peripheral nerves, as explained in detail below.

1.7 Combined antiretroviral treatment (cART)

The discovery of PIs in 1996, and combination with NRTI marked the start of the cART era. HIV, which previously led to AIDS and certain death, became a manageable condition with a near normal life expectancy.

The goal of suppressive cART is to decrease plasma viral load to an undetectable level (< 20 copies of HIV-RNA/ml in Sweden). When the viral load burden decreases, the number of CD4+ T-cells increases. There are two main reasons to treat HIV-patients with cART: 1) to stop disease progression and concomitant morbidity/mortality and 2) to decrease the risk of HIV transmission.

According to the 2014 guidelines issued by the Swedish Reference Group for Antiretroviral Therapy, all patients diagnosed with HIV, regardless of CD4-

status, are offered to start on cART. This is in accordance with U.S. guidelines. Up until recently, evidence of beneficial effects on morbidity when treating HIV-patients above 350 CD4+ T-cells/μL was scarce. However, the START-study, published in 2015, provide sound evidence that immediate treatment initiation regardless of CD4 T-cell levels is superior compared to deferred treatment regarding both AIDS-related and non-AIDS-related morbidity [54]. In Sweden, first-line cART consists of two NRTIs plus ritonavir-boosted PI, two NRTIs plus one NNRTI, or two NRTI plus one integrase inhibitor (INI) [55]. Significant resistance mutations need to be taken into consideration when deciding on which treatment to use, and so resistance testing is routinely performed before initiation of cART. Co-morbidities, the vast amount of interactions between cART and other medicines, the presence of the HLA-B*5701 allele (due to severe allergic reactions to abacavir), and side effects are also important factors when choosing a regimen. Currently, there are six separate groups of antiretrovirals approved for clinical use, see Table 2. The mechanisms of action for the different antiretroviral groups are summarized in Figure 4.

Generic name	*Trade name*
Nucleoside analogues (NRTI)	
• Abacavir (ABC)	• Ziagen
• Didanosine (ddI)	• Videx
• Emtricitabine (FTC)	• Emtriva
• Lamivudine (3TC)	• Epivir
• Stavudine (d4T)	• Zerit
• Tenofovir (TDF)	• Viread
• Zidovudine (AZT, ZDV)	• Retrovir
Combined formulations	
• ABC+3TC	• Kivexa
• TDF+FTC	• Truvada
• AZT+3TC	• Combivir
• AZT+3TC+ABC	• Trizivir
•	
Non-nucleoside RT inhibitors (NNRTI)	
• Efavirenz (EFV)	• Stocrin
• Nevirapin (NVP)	• Viramune
• Etavirin (ETR)	• Intelence
• Rilpivirin (RPV)	• Edurant
Combined formulations	
• EFC+TDF+FTC	• Atripla
• RPV+TDF+FTC	• Eviplera

Protease inhibitors (PI)	
• Atazanavir (ATV)	• Reyataz
• Darunavir (DRV)	• Prezista
• Fosamprenavir (fAPV)	• Telzir
• Indinavir (IDV)	• Crixivan
• Saquinavir (SQV)	• Invirase
• Ritonavir (RTV)	• Norvir
• Tiprinavir (TPV)	• Aptivus
Combined formulations	
• Lopinavir+RTV (LPV+RTV)	• Kaletra
Integrase inhibitors (INI)	
• Raltegravir (RAL)	• Isentress
• Dolutegravir (DTG)	• Tivicay
• Elvitegravir (EVG)	• Vitekta
Combined formulations	
• DTG+ABC+3TC	• Triumeq
• EVG (+cobicistat)+TDF+FTC	• Stribild
Fusion inhibitors (FI)	
• Enfuvirtid (T-20)	• Fuzeon
CCR5-inhibitors	
• Maraviroc (MVC)	• Celsentri

Table 2. Antiretroviral drugs available for clinical use in Sweden, 2014. Adapted from the Swedish Reference Group for Antiviral Therapy (RAV).

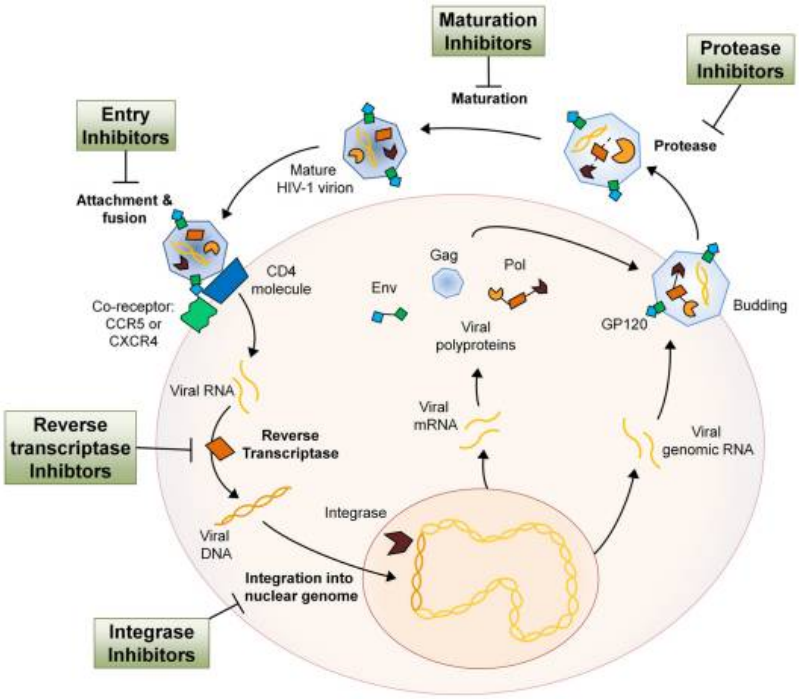

*Figure 4. Mechanism of action for different antiretroviral classes. Adapted from Smith, R. L., et al. (2012). "Premature and accelerated aging: HIV or HAART?" Front Genet **3**: 328, under the terms of the Creative Commons Attribution License.*

1.8 Neurocognitive disorders in HIV

1.8.1 HIV-associated neurocognitive disorder (HAND)

Before the cART-era, progressive neurocognitive disorder with memory difficulties, psychomotor slowing, and decreased cognition was observed in approximately 20% of AIDS patients. The cognitive symptoms were frequently associated with behavioral changes (dysphoria, apathy, and psychosis) and motor disabilities such as ataxia, decreased fine motor skills, and tremor. This complex of symptoms was initially termed AIDS dementia complex (ADC) and found to evolve due to a neuroinflammatory effect of HIV in the CNS [56-58]. Overt dementia is rare after the introduction of cART

(and subsequent suppression of HIV in CNS). However, discrete neurocognitive and motor impairments are still commonly noted among treated patients treated for HIV-infections. New terminology was decided upon, where three levels of impairment were distinguished; asymptomatic neurocognitive impairment (ANI), minor neurocognitive disorder (MND), and HIV-associated dementia (HAD). Collectively, these groups have been summarized as HAND. The criteria for HAND were revised in 2007 (Frascati criteria) and are still used as the gold standard today [59,60].

1.8.2 Diagnosis of HAND

Where possible, HAND is diagnosed using elaborate neuropsychological (NP) testing. Seven domains are assessed: verbal/language; attention/working memory; abstraction/executive; memory (learning, recall); speed of information processing; and sensory, perceptual, and motor skills. In settings where access to NP testing is limited, simplified surveys, for example a cog-state exam, are used. Confounders, such as HIV-related opportunistic infections, substance abuse, and other neurocognitive impairments/dementias or states of confusion/delirium need to be excluded in the analysis.

For a diagnosis of ANI, a decreased function of > 1 SD, compared to a demographically adjusted HIV-negative group, needs to be observed in at least two cognitive domains of at least five tested. Furthermore, the decreases measured should not interfere with everyday functioning. MND is defined by the same test-criteria as ANI above, but the cognitive deficits do interfere to a mild extent with activities of living, either as reported by the patient or by others in close contact with the patient. HAD is defined as a decrease in at least two cognitive domains > 2SD compared to demographically corrected means. Moreover, there is an obvious decline in everyday cognitive functioning with regard to ability to work, general competence in managing a home, and social skills, all as noted clinically [59].

1.8.3 HAND in the cART era

Ever since cART became widely available, the clinical spectrum of HAND has changed. Suppressive treatment decreases HIV-induced neuroinflammation. Although somewhat controversial, decreased inflammation is possibly limiting neuronal decline as well. HAD is nowadays

a rare occurrence among treated patients, with a reported prevalence of between 2 to 4% in several studies. However, a large proportion of patients (37-74%) are still diagnosed with milder forms of HAND when using standardized NP testing. A majority of these are diagnosed as ANI, with no apparent clinical deficits [60-62]. Questions have been raised as to whether NP testing alone might lead to a large overestimation, and it has been suggested that other methods such as biomarker analysis and radiological means be included in the diagnostic armamentarium to increase diagnostic precision [63]. Another drawback of NP-testing is that it doesn't discriminate ongoing from residual brain damage; an important factor when it comes to the HIV infected survivors of the pre-cART era. With an aging HIV-positive population under treatment, good methods for differentiating HAND from other types of cognitive impairments and dementias will become increasingly important.

1.8.4 Alzheimer's dementia as a model

Alzheimer's disease (AD) is a good example of a neurocognitive disease where a multifaceted approach of diagnostic methods is used in clinical practice to achieve a conclusive diagnosis. As in other types of dementia, NP testing is an important part in determining the type and severity of cognitive deficits. Pathologic metabolism of amyloid and tau proteins are hallmark signs of AD, and a distinct biomarker pattern of beta$_{1-42}$ amyloid (Aβ_{1-42}), total tau (t-tau) and hyperphosphorylated tau (p-tau) can be readily measured in CSF. Furthermore, amyloid deposition in senile plaques and neurofibrillary tangles made of phosphorylated tau can be visualized in the brain on radiological examination.

Several biomarkers have proven to be promising candidates in diagnosing HAND as well as for distinguishing it from other types of dementia, but none of them are presently in clinical use. The biomarkers related to HAND are described in detail below.

1.8.5 Confounders of HAND

Apart from other types of dementia, a number of co-morbidities and lifestyle factors affecting cognitive performance are especially present in the HIV-infected population. When doing NP testing, all confounders need to be accounted for and excluded for proper diagnosis of HAND.

Opportunistic infections and malignancies such as cryptococcal meningitis, mycobacterial infection, progressive multifocal leukoencephalopathy (PML), toxoplasmosis, and lymphoma in the CNS can mimic HAND. Chronic, non-AIDS-related CNS infections, such as tertiary syphilis, but also acute infections (viral encephalitis, bacterial meningitis), need to be accounted for in the analysis. Psychiatric disorders such as depression and psychosis are also important confounders.

In parts of the world, the prevalence of recreational use of narcotics, both intravenously (IVDU) and orally/nasally is increased in HIV-infected people. The use of narcotics can lead to acute episodes of confusion, delirium, or manifest psychosis. The effects of long-term use include progressive psychomotor decline in the case of certain drugs such as metamphetamine, where a neurocytotoxic effect is exerted by the drug itself [64].

The long-term effects of cART regarding cognitive decline are not completely known. In vitro evidence suggests that some NRTIs, NNRTIs and PIs cause mitochondrial and neuronal toxicity at therapeutic concentrations [65].

1.9 Biomarkers in Cerebrospinal fluid

According to the National Institutes of Health (NIH), a biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [66]. The use of biomarkers is widespread in clinical practice, ranging from standard laboratory tests like C-reactive protein to markers for specific diseases like Alzheimer's dementia and cancers.

Due to the practical difficulties and potential dangers involved in acquiring brain biopsies, the analysis of CSF serves as a "mirror" for pathological processes in the CNS. Although not a perfect method of assessing pathology

in brain tissue, CSF-analysis is considered sensitive enough to assess low-level changes of biomarkers correlated to disease. Presented below are a number of biomarkers relevant to HIV research that appears to be the most promising ones available. A complete list is beyond the scope of the present thesis.

1.9.1 Clinical procedures

CSF is collected by lumbar puncture (LP). A cannula is inserted in the interstitium of lumbar vertebrae two and three or three and four. The patient is preferably lying down on one side, curled up in a fetal position. It is also possible to perform LP with a patient sitting, but measuring CSF pressure is then ruled out. CSF is manually collected through the cannula into a number of tubes for whatever diagnostic analysis desired.

In the HIV-research setting at the Department of Infectious Diseases in Gothenburg, all patients who consent to participate under the parameters of the research protocols receive an LP before treatment initiation and at least annually as part of the clinical follow-up. LP is performed early in the morning with the patient instructed to abstain from breakfast and cART before the procedure. Approximately 24 ml of CSF is removed and analyzed together with blood samples in accordance with Table 3. A sample of the CSF is also stored in a -70°C freezer for future use in research.

	Cerebrospinal fluid	Blood
All patients	• Cell count (poly, mono, erythrocytes) • Albumin ratio (CSF:blood) • IgG analysis • IgG bands • β_2 microglobulin • Neurofilament light chain (NFL) • Quantitative HIV-RNA • CSF saved at -70°C	• Hemoglobin, WBC with differential count, platelets • Plasma glucose • ALT, ALP, bilirubin, • Creatinin, phosphate • Cholesterol, HDL, LDL, triglycerides • CD4, CD8, CD4/CD8-ratio • Quantitative HIV-RNA • Isolation of HIV (stored for future analysis) • Extra blood for storage at −70°C 1.
Untreated patients	• Isolation of HIV	• HLA-B5701 type (first visit) • Isolation of HIV
Treated patients	• Extra CSF for storage at −70°C	• Extra blood for storage at −70°C

Table 3. CSF and blood tests performed annually on consenting HIV-infected patients at the Department of Infectious Diseases, Sahlgrenska University Hospital, Gothenburg, Sweden.

1.9.2 Neurofilament light chain protein (NFL)

NFL is the light subunit of neurofilament protein and is a major constituent of myelinated axons in the CNS. The main functions of NFL are to maintain axonal caliber and to facilitate nerve conduction [67]. Although a slight physiological increase in NFL levels occur with aging, large increases reflect pathological axonal disruption, mainly at a subcortical level. Notably, NFL seems to clear from the CSF rather quickly after the disruption of axons stops; NFL is therefore considered a marker for ongoing axonal injury. A number of neurological diseases exhibit increased CSF NFL, among them HAD, amyotrophic lateral sclerosis, and cerebral infarction [68-71]. Even more significantly, NFL increases in neuroasymptomatic HIV-infected patients, both on and off treatment, suggesting a smoldering HIV-induced neuroinflammation and a clinically "quiet" axonal degeneration [41,72].

1.9.3 Total tau (t-tau) and hyperphosphorylated tau (p-tau) proteins

T-tau protein is a constituent of mainly non-myelinated cortical axons in the CNS, where it promotes microtubule stability and transport of organelles [73]. As with NFL, increases in CSF t-tau are considered a reflection of axonal damage. Several studies have shown high levels of t-tau among HIV-infected patients with HAD and opportunistic CNS infections, but sensitivity seems to be lower for t-tau than NFL in HIV settings [39,72-74].

P-tau protein is a result of hyperphosphorylation of t-tau, causing decreased stability of the axons due to detachment of tau from the microtubules [75]. Increased levels of CSF p-tau are noted physiologically in the developing brain, but are considered a pathological finding in the adult brain [76]. Whereas increased t-tau reflects axonal disruption, high levels of p-tau represent a pathological process that is not yet fully understood. In HAND, CSF p-tau is generally not increased.

A number of diseases collectively characterized as tauopathies, of which AD is the most studied, consistently exhibit increased levels of both t-tau and p-tau. For the purposes of differential diagnosis between HAND and Alzheimer's dementia in an aging HIV-positive population, tau proteins might serve as a useful tool [39,77].

1.9.4 Amyloid metabolites

Amyloid precursor protein (APP) is a transmembrane protein with widespread expression in human cells. Its main functions are thought to be acting as a receptor, and promoting extra- to intracellular signaling, neural plasticity and cell development [78]. APP is cleaved enzymatically by secretases, and two different metabolic pathways are possible. In the non-amyloidogenic pathway, α-secretase digests APP into soluble APPα (sAPPα) and a C-terminal fragment (α-CTF). Further cleavage of α-CTF occurs by the action of γ-secretase and results in release of the APP intracellular domain (AICD) and a p3 fragment. There is no evidence for

neurocytotoxic effects of the products from the non-amyloidogenic pathway.

In the amyloidogenic pathway, APP is cleaved by the enzyme β-secretase instead, resulting in sAPPβ and β-CTF. The C-terminal fragment is subsequently cleaved by γ-secretase into $A\beta_{1-40}$ and $A\beta_{1-42,}$ and also releases AICD [79] (Figure 5).

Amyloid metabolites in conjunction with tau proteins are central biomarkers in diagnosing AD. β-amyloid$_{1-42}$ ($A\beta_{1-42}$) is the most studied amyloid metabolite in AD and is a main constituent of amyloid plaques in AD. A significant decrease of $A\beta_{1-42}$ is commonly noted in CSF analysis of AD patients, most likely due to sequestration in the plaques mentioned. Plaque formation is not a common finding in HAND, and CSF $A\beta_{1-42}$ is at variable levels, ranging from normal to low, in patients with HAND regardless of severity.

Other amyloid metabolites have proven to be useful as biomarkers in the diagnosis of both AD and other neurological diseases. sAPPα and -β diffuse into CSF and can be readily measured. HAD exhibits decreased concentrations of sAPPs, especially the β-form, compared to controls in several papers. This is in contrast to AD, where the soluble forms of APP are generally normal or increased [39,72].

Measurement of secretases is also an attractive option when attempting to map the amyloid pathogenesis in neurological disease.

1.9.5 CSF Neopterin

Neopterin is a monocyte/macrophage-derived marker of intrathecal immune activation. Upon inflammatory stimulation by primarily interferon gamma of the monocyte/macrophage, guanosine triphosphate is metabolized to form neopterin [80]. CSF neopterin has proven to be a sensitive biomarker signalling HIV-induced immune activation in the CNS. All HIV-infected subjects exhibit increased levels of CSF neopterin, but a gradual increase is seen with

the severity of immune status and development of HAD. Moreover, neopterin can be used to measure the treatment effect in the CNS, as a decline in neopterin levels follows the treatment effect [40].

1.9.6 Blood-brain barrier integrity

HIV produces inflammation of the BBB, both by inflammatory and toxic compounds released by the virus and from inflammatory cytokines and chemokines. In effect, the BBB becomes more permeable, and an increased exchange of substances occurs between the blood and the CNS [81,82]. From a biomarker perspective, the ratio of CSF and blood albumin indicates BBB dysfunction. That measurement is always available since albumin is routinely analyzed when performing LP.

1.9.7 CSF HIV-RNA

CSF HIV-RNA is a good biomarker for the severity and progress of CNS symptoms. Although proper suppression of HIV-RNA in blood is readily achieved by modern cART, a low-level viremia in CNS is commonly found. HIV induces both immune activation and neuronal damage in the CNS, and a clear association between levels CSF HIV-RNA, neopterin and NFL exist [40,68].

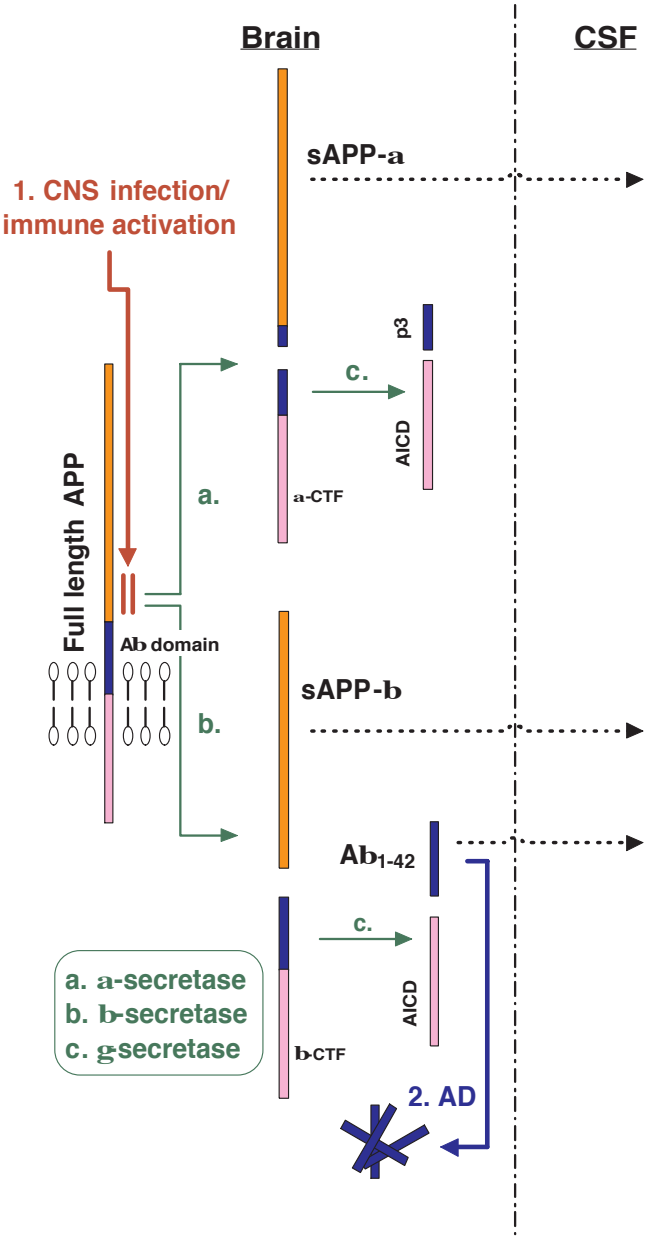

*Figure 5. Schematic presentation of amyloid precursor protein (APP) metabolism. Adapted from Gisslen, M., et al. (2009). "Amyloid and tau cerebrospinal fluid biomarkers in HIV infection." BMC Neurol **9**: 63.*

2 AIMS

The overall aim was to assess CSF biomarkers of neuronal injury (NFL and t-tau), immune activation (neopterin), and pathological metabolism (p-tau, sAPPα and -β, $A\beta_{1-42}$) in HIV patients with or without neurocognitive deficits as a means of gaining a greater understanding of the pathogenesis in HIV-associated neurocognitive disorders. The specific aims for each paper were:

I. To evaluate CSF biomarkers of amyloid- and tau protein metabolism as a potential diagnostic tool complementing neuropsychological testing in diagnosis of HAD/ADC. To determine whether amyloid and tau could be used as biomarkers in differential diagnostics to distinguish HAD from AD and HIV-related CNS OIs.

II. To determine whether the altered CSF biomarker pattern of amyloid and tau metabolites in OIs is due to an HIV effect, or if the alterations in each biomarker is also induced by CNS infections in HIV-negative individuals.

III. To survey axonal damage in HIV-patients with and without neurocognitive impairment by measuring low-level changes in CSF NFL and other biomarkers of immune activation and inflammation.

IV. To examine whether CSF p-tau is at higher levels in HIV-infected patients compared to HIV-negative people of the same age, indicating premature or accelerated aging.

3 PATIENTS AND METHODS

3.1 Patients

Beginning in 1985, a large biobank of CSF, frozen at -70°C, has been built up at the Department of Infectious Diseases, Gothenburg for research purposes. As of 3rd of November, 2015, 1919 CSF samples are stored from at total of 514 HIV-infected individuals HIV-infected patients, who volunteer to participate under the oversight of ethics boards and research protocols, are given lumbar punctures before treatment initiation and at yearly intervals thereafter. Similar protocols have been used at collaborative centers in San Francisco and Milan. Papers I to IV all used a retrospective cross-sectional design based on stored and frozen CSF in these biobanks. In Paper III, a longitudinal cohort design was used to assess the effect on NFL before and after treatment initiation with cART.

3.1.1 Paper I

A total of 150 patients were subdivided into an HIV-positive (n = 86) and an HIV-negative group (n = 64). The HIV-infected patients were further stratified into three subgroups: ADC (the old term for HAD) (n=21), neuroasymptomatics (n = 40), and CNS OI (n = 25). The OIs consisted of cytomegalovirus-encephalitis (n = 4), cryptococcal meningitis (n = 6), cerebral toxoplasmosis (n =6), and PML (n = 9). The HIV-negative patients were subdivided into three groups; AD (n = 21), healthy young controls (*cont y*), age-matched to the HIV-infected population (n = 22); and an older group of healthy controls (*cont o*) age-matched to the patients with AD (n = 21). The HIV-infected patients were recruited from the Department of Infectious Diseases, Sahlgrenska University Hospital in Gothenburg, Sweden; the Clinic of Infectious Diseases, San Rafaele hospital in Milan, Italy; and the Department of Neurology, San Francisco General Hospital, USA. All patients with HIV were either naïve to cART or had not received treatment for at least six months prior to analysis. The AD patients and controls were recruited from the Department of Neurochemistry, Sahlgrenska University Hospital, Gothenburg, Sweden, in collaboration with Karolinska University Hospital, Stockholm, Sweden. The HIV-negative controls were recruited as volunteers, consisting mainly of relatives to patients with AD.

3.1.2 Paper II

A total of 119 patients were included, subdivided into four main groups: HIV-negative individuals with CNS infections (n = 35), HAD (n = 21), AD (n = 21), and healthy controls (n = 42). Those with CNS infections were further divided into herpes simplex-1 encephalitis (HSV-1) (n = 10), bacterial meningitis (n = 12) and lyme neuroborreliosis (n = 13). The bacterial meningitis group was further categorized by different bacterial agents: S. pneumoniae (n = 6), H. influenzae (n = 2), N. meningitidis (n = 1) and E. coli (n = 1). In two patients, no bacterial agents were found, but clinical presentation and remaining laboratory analyses excluded other causes. The patients with CNS infections were recruited from the Department of Infectious Diseases, Sahlgrenska University Hospital, Gothenburg, Sweden, between the years 1997 and 2008. The HAD patients and healthy controls were made up of the same population used in paper I. In the case of HAD this was due to difficulties in recruiting new patients because of the good coverage by suppressive cART. AD patients were in part previously those used in another study [83].

3.1.3 Paper III

Paper III is divided into one retrospective cross-sectional part and a second longitudinal cohort part. The cross-sectional part included an HIV-positive group (n = 252) divided into six subgroups: Four groups of neuroasymptomatic (NA) patients without treatment, stratified according to levels of CD4+ T-lymphocytes; patients with HAD; patients on suppressive cART (plasma HIV-RNA <50 copies/mL for at least one year); and 204 HIV-negative, healthy individuals as controls. Forty-six patients were included in both the treated-suppressed and the NA groups, but individual samples were used in the different groups, with a median time of 5.9 years between samples.

The longitudinal part of the study included 78 patients. At baseline, all were neuroasymptomatic and they were either treatment naïve or had been off cART for > 6 months. After initiation of treatment, CSF analyses were performed at a median time of 15 weeks after treatment initiation. The patients were recruited from the previously mentioned Departments of Infectious Diseases, Gothenburg and Neurology, San Francisco. See Table 4.

Groups	Number	Median age (years)
Cross-sectional study		
NA CD4 < 50	42	39
NA CD4 50-199	49	39
NA CD4 200-349	52	40
NA CD4 > 350	57	42
HAD	14	47
cART	85	47
HIV negative	204	36
Longitudinal cohort study		
All subjects	78	40
Normal CSF NFL	52	40
Elevated CSF NFL	26	42

Table 4. Paper III. Subject characteristics. NA = Neuroasymptomatic; HAD = HIV-associated dementia; cART = suppressive combined antiretroviral treatment.

3.1.4 Paper IV

A total of 225 HIV-infected patients comprised the study, divided into three groups: HAD (n = 31), suppressive cART (n = 49), and NA (n = 145). Seventy-nine healthy HIV-negative volunteers were used as controls. All samples were collected between the years 1986 and 2014 and stored at -70°C until analysis. Samples came from the Departments of Infectious Diseases, Gothenburg, Neurology, San Fransisco and Infectious diseases, Milan.

3.2 Methods

3.2.1 Neurocognitive evaluation

All HIV-infected patients were evaluated clinically for neurological and psychiatric diseases at follow-ups. If no obvious signs or symptoms were noted by the investigator or reported by the patient, they were defined as NA. Only on clinical suspicion of neurocognitive decline was NP testing performed. In recent years, assessment of cognitive function by cog-state evaluation was performed on all patients who agreed to participate.

We defined HAD using the CDC and American Academy of Neurology Task Force criteria in all the papers presented here [84,85]. Staging of HAD severity was done according to the Memorial Sloan Kettering Scale [86].

AD was diagnosed in accordance with the criteria outlined by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [87].

3.2.2 Laboratory analyses

Sandwich ELISA was used to analyze amyloid metabolites, t-tau, p-tau, and NFL. The principle of sandwich ELISA is summarized in Figure 6. $A\beta_{1-42}$, t-tau, and p-tau, phosphorylated at threonine 181, were analyzed according to procedures previously described [88-90]. sAPPα and -β were analyzed using the MSD© sAPPα/sAPPβ multiplex assay as described by the manufacturer (Meso Scale Discovery, Gaithersburg MD, USA). NFL was evaluated using the commercial NF-light© ELISA kit as described by the manufacturer (UmanDiagnostics AB, Umeå, Sweden). Neopterin was measured using a commercially available immunoassay (BRAHMS, Berlin, Germany).

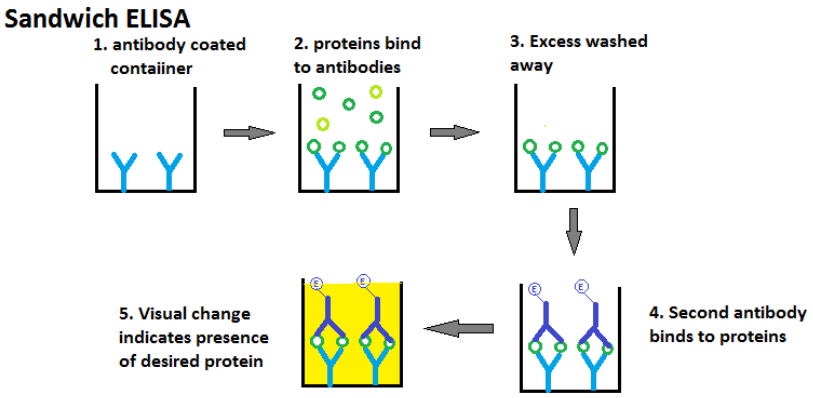

Figure 6. Simplified outline of sandwich ELISA method. 1) Capture antibodies of known quantity coat plate, binding to protein/antigen if present (for example NFL). 2) An Enzyme-linked detecting antibody added, binding to protein/antigen. 3) Enzymatic activation by substrate causes color change in the presence of specific protein. 4) Color change measured quantitatively.

HIV-RNA in CSF and plasma was quantified by different polymerase chain reaction (PCR) methods. In Paper I, the Roche Amplicor monitor assay (versions 1.0 and 1.5, Hoffman La-Roche, Basel, Switzerland) was used. In Papers III and IV, either the Roche Amplicor Monitor version 1.5, the Roche Taqman assay (version 1 or 2) or the Abbott RealTime HIV-1 assay (Abbott Laboratories, Abbot park, Illinois, USA) were used. The use of different methods was due to the retrospective nature of the study, where PCR methods with higher sensitivity evolved during the study period, but also due to regional differences in analytical methods between the clinical centers involved.

CD4+ T-lymphocyte count as well as WBCs and albumin in CSF and blood were analyzed with methods used routinely in local clinical laboratories.

3.2.3 Statistical analyses

Median and interquartile range (IQR) was used for general descriptive statistics in all papers. Values were $\log_{10}$-transformed when appropriate. Statistical analyses were performed using either Prism© version 5 (Graphpad Software Inc, La Jolla, CA, USA) or IBM SPSS Statistics©.

Paper I

Differences between groups were analyzed either by ANOVA with Tukey's post hoc test for multiple comparisons in the case of parametric variables, or with the Kruskal-Wallis test followed by Dunn's post hoc test when normality could not be assumed. Spearman's rank correlation coefficient and linear regression was used to assess dependency between biomarkers. Principal component analysis was used to determine the combined trends of all amyloid and tau biomarkers. Finally, receiver-operator characteristics were also performed to determine the sensitivity and specificity of respective biomarker.

Paper II

To test statistically significant differences between the groups, Kruskal-Wallis analysis followed by Dunn's post hoc test was used. In order to determine potential correlations between CSF WBCs and the biomarkers analyzed, Spearman's rank correlation coefficient was performed.

Paper III

In the cross-sectional part, variables were compared using the Mann-Whitney test. Non-linear Loess regression was used to assess the relationship between CSF NFL and CD4+ cell counts. Log10 CSF NFL and CD4+ cell counts were fitted using the function Log10 CSF NFL = b1 + b2/(CD4 + b3). Due to a partial overlap of individuals in the cART and NA groups, a linear mixed effects model was used to investigate the relationship between age and NFL. A multiple linear regression analysis with backward elimination was performed to analyze possible predictors of increased NFL. In the longitudinal part, Wilcoxon Matched-Pairs Signed Rank test compared NFL before and after treatment.

Paper IV

ANOVA with Tukey's multiple comparisons post hoc test was used to determine differences between groups. Pearson correlation and linear regression was used when analyzing dependency between tau proteins, HIV-RNA, and neopterin. To assess potential effects of p-tau and age, a general linear model was used.

4 RESULTS

4.1 Paper I

With an aging HIV-infected population, sensitive diagnostic methods for HAND as well as differential diagnostics regarding other types of dementia will be increasingly important. Measurement of CSF biomarkers is an appealing diagnostic alternative. In Paper I, we analyzed amyloid metabolites (sAPPα and -β, $A\beta_{1-42}$), t-tau, and p-tau.

4.1.1 Amyloid metabolites

For a presentation of amyloid metabolite distribution across groups, see Figure 7. sAPPα and -β were significantly lower and at comparable levels in the groups with ADC and OI compared to NA, AD, and controls ($p < 0.001$ for all). No difference in sAPP concentrations was seen between the subgroups of OI.

Levels of $A\beta_{1-42}$ showed great variability in the ADC group, but did not differ significantly from any other group including AD. OI as a group exhibited lower $A\beta_{1-42}$ as compared to the NA group ($p < 0.001$). When separating the subgroups of OIs, only cryptococcal meningitis differed significantly from the NA ($p < 0.05$).

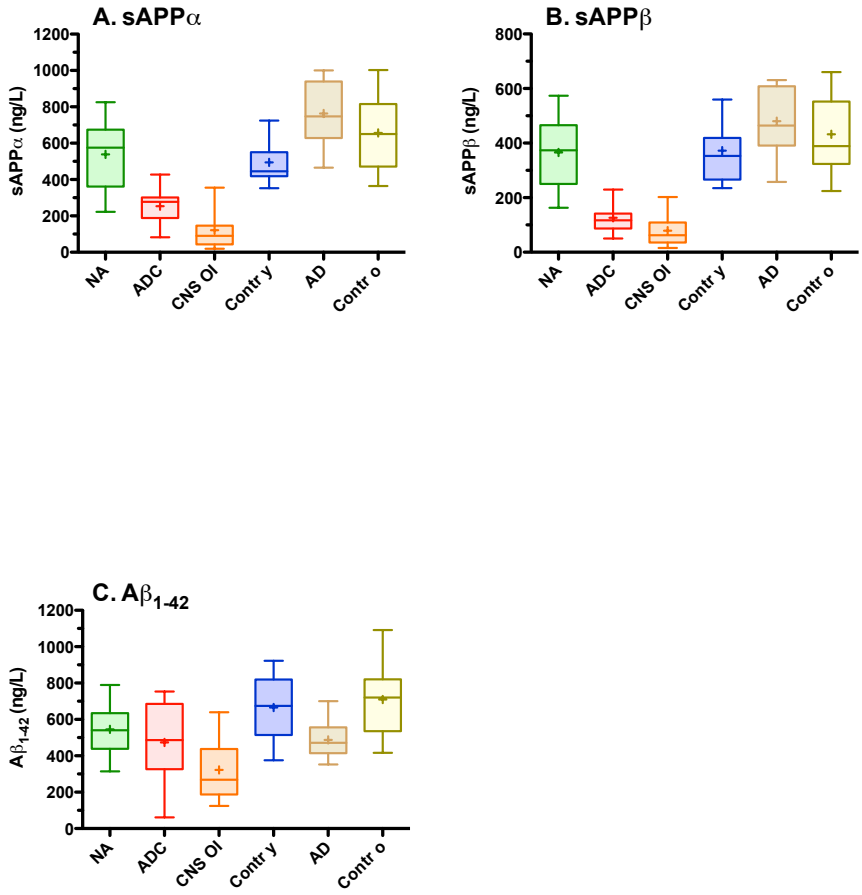

Figure 7. Paper I: Levels of sAPPα, sAPPβ, and Aβ1-42 across study groups.

4.1.2 Tau metabolites

Tau metabolite distribution across groups is outlined in Figure 8. Levels of t-tau, indicative of axonal damage, were increased in ADC as compared to NA ($p < 0.05$) and younger controls ($p < 0.001$). However, great variability of t-tau within the ADC group was noted. Obvious increases of t-tau between the OIs were not seen as compared to other HIV-positive groups and controls. P-tau did not differ within the HIV-infected groups, nor were the HIV-positive patients different from young controls. A physiological aging effect, as

reflected by p-tau, was noted, with significant increases in the older control group, compared to the younger controls ($p < 0.01$). As expected, AD stood out, with significant increases of both t-tau and p-tau in comparison to all other groups.

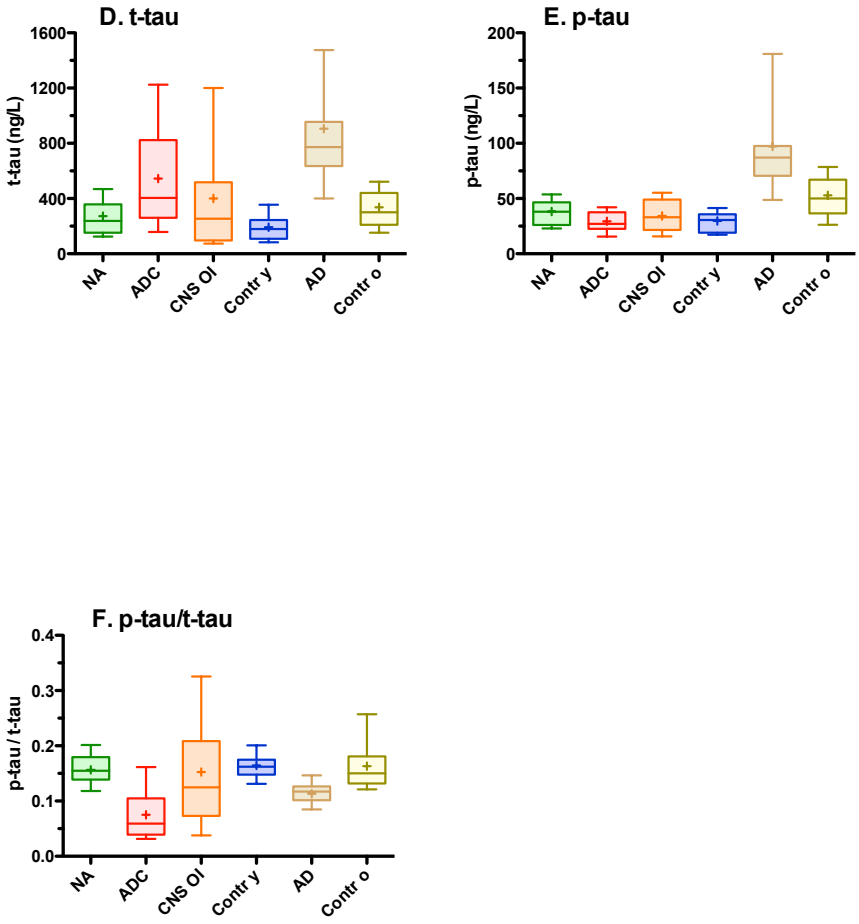

Figure 8. Paper I. Levels of t-tau, p-tau and the ratio of p-tau over t-tau across groups.

4.1.3 Principal component analysis

To determine the compound effect of all biomarkers across groups, principal component analysis was used. This revealed a visual separation, where ADC and OIs were mainly clustered in the upper left quadrant, while AD was located in the upper right quadrant. NA and controls were generally distributed in the lower left and right quadrants.

4.1.4 Receiver-operator characteristics

To determine the use of amyloid and tau as potential discriminative markers for ADC as compared to other HIV-infected subjects, receiver-operator characteristics (ROC) were performed in order to establish sensitivity, specificity and relevant cut-off values. ADC and OIs exhibited similar levels of biomarkers, but OIs can be readily excluded by other methods such as neuroradiology, clinical presentation and specific analyses of the relevant pathogen. With OIs excluded from the ROC analysis, both sAPPs proved to be sensitive and specific markers for discriminating ADC. Cut-off values at 333ng/L for sAPPα and 205 ng/L for sAPPβ were determined. Sensitivity and specificity for sAPPα at the levels mentioned were 90.5% and 85% respectively. For sAPPβ, sensitivity was 90.5% and specificity 90%. $A\beta_{1-42}$, t-tau and p-tau were neither sensitive nor specific enough to use as individual markers in discriminating ADC.

4.2 Paper II

In Paper I, the amyloid and tau metabolites had a similar distribution in both ADC/HAD, and OIs. To further determine if the perturbations were attributable to an HIV-specific effect, or were a general feature of neuroinflammation, we analyzed sAPPα and β, $A\beta_{1-42}$, t-tau, and p-tau in HIV-negative patients with CNS infections. Bacterial meningitis and HSV-1 encephalitis, both severe infections with a fulminant inflammatory response were assessed together with lyme neuroborreliosis, an infection that generally exhibits a smoldering, moderate inflammatory response. AD, HAD, and healthy controls were also included for comparison. For a summary of biomarker concentrations across groups, see Figure 9.

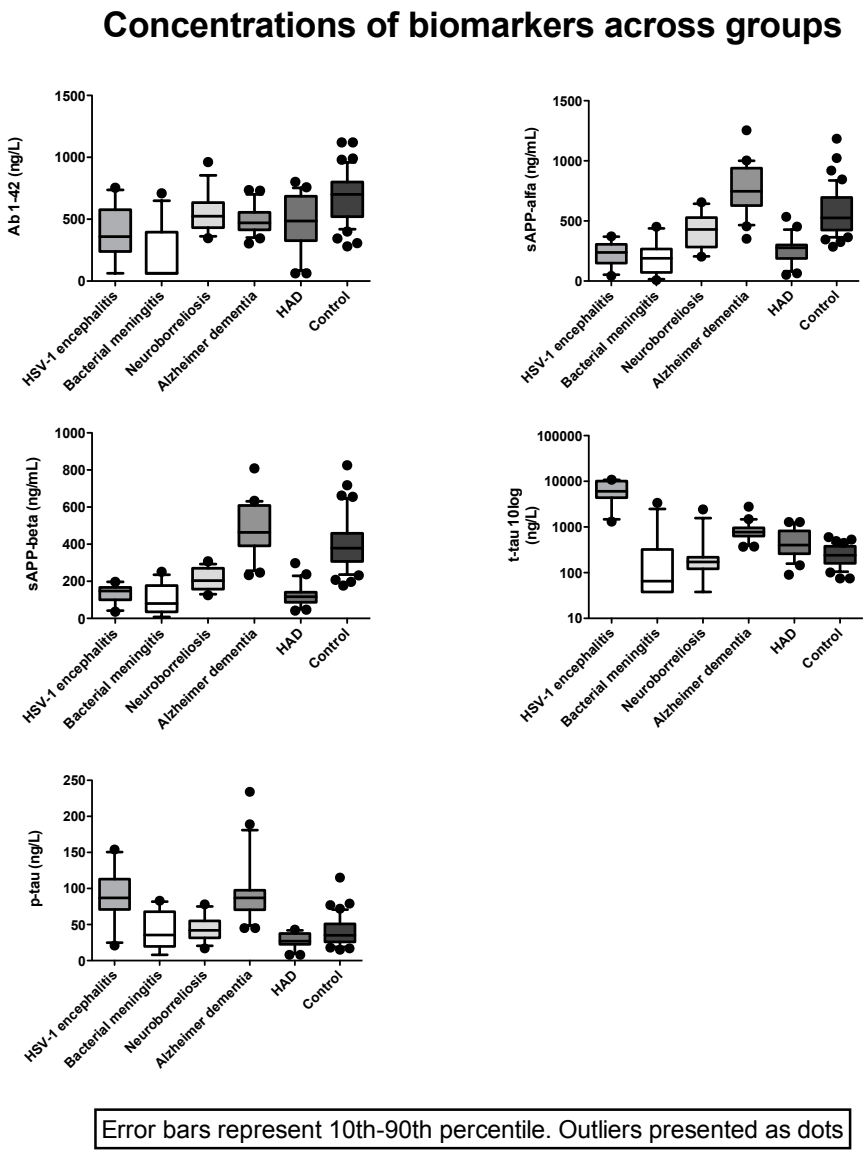

Figure 9. Paper II. Levels of amyloid and tau metabolites across study groups

4.2.1 Amyloid metabolites

The bacterial meningitis group exhibited the lowest concentrations of amyloid metabolites and showed clearly significant decreases in sAPPs and $A\beta_{1-42}$ as compared to healthy controls ($p < 0.001$ for all). Levels of amyloid metabolites in HSV-1 encephalitis were also decreased as compared to controls (for sAPPs $p < 0.001$, for $A\beta_{1-42}$ $p < 0.01$). Neuroborreliosis showed no obvious decrease in $A\beta_{1-42}$, although a slightly significant decrease of sAPPβ was seen as compared to controls. As expected, HAD decreased in sAPP concentration ($p < 0.001$), but a decrease in $A\beta_{1-42}$ was also seen in comparison to controls ($p < 0.05$). Not surprisingly, $A\beta_{1-42}$ was decreased in AD as compared to controls ($p < 0.01$) and sAPPs were slightly increased. No significant differences were seen between bacterial meningitis, HSV-1 encephalitis, neuroborreliosis, and HAD.

4.2.2 Tau metabolites

T-tau levels were highest in the HSV-1 encephalitis group, with significant increases compared to CNS infections, controls, and HAD ($p < 0.001$, $p < 0.05$ for HAD). No significant difference was seen between HSV-1 and AD when comparing levels of t-tau. T-tau concentrations in the AD group were increased compared to controls, as previously known as well as when comparing the AD group to bacterial meningitis and neuroborreliosis ($p < 0.001$ for all). P-tau concentrations in HSV-1 encephalitis were at similar levels as in AD and were significantly increased compared to bacterial meningitis ($p < 0.05$), HAD ($p < 0.001$) and controls ($p < 0.01$). HAD showed decreased concentration of p-tau compared to controls ($p < 0.001$), but not with regard to bacterial meningitis and neuroborreliosis.

4.3 Paper III

In Paper III, low-level changes in CSF NFL, a marker for axonal disruption, was measured with a sensitive method across groups of untreated, NA HIV-infected patients with different blood CD4+ T-cell counts, HAD patients, subjects on suppressive cART, and controls. Also, NFL was measured before and after cART initiation in a subset of patients. Since milder forms of HAND are common among both treated subjects and patients without obvious decline in everyday function, we hypothesized that NFL might

reflect a smoldering axonal injury in all HIV-infected patients, and possibly also signs of premature or accelerated brain aging.

4.3.1 NFL across groups

The HAD group exhibited clear increases of CSF NFL compared to all other groups, including NA patients with comparable levels of CD4+ T-cells ($p < 0.001$). In all, 93% of the HAD patients had NFL above the upper age-adjusted normal reference. Among the NA patients, the population with CD4+ levels below 50 cells /mL blood had higher NFL levels compared to all other CD4+ strata ($p < 0.001$), and 69% of CD4+ < 50 cells/mL exhibited elevated NFL compared to the reference. Increased NFL was relatively common in the groups with higher CD4+ T-cells as well, but to a lesser extent with increasing CD4+ T-cells.

4.3.2 Correlation of CSF NFL with CD4+ T-cell levels

Another way to describe the connection between levels of CSF NFL and CD4+ T-cells is by correlation and nonlinear regression. When looking at NA patients, a strong correlation of increased NFL with declining CD4+ T-cell counts were found ($p < 0.001$), but NFL and CD4+ T-cells did not correlate significantly in the group with suppressive cART. Using a Loess regression method showed a decline of NFL levels at a CD4+ T-cell count above approximately 250 cells/mL (Figure 10).

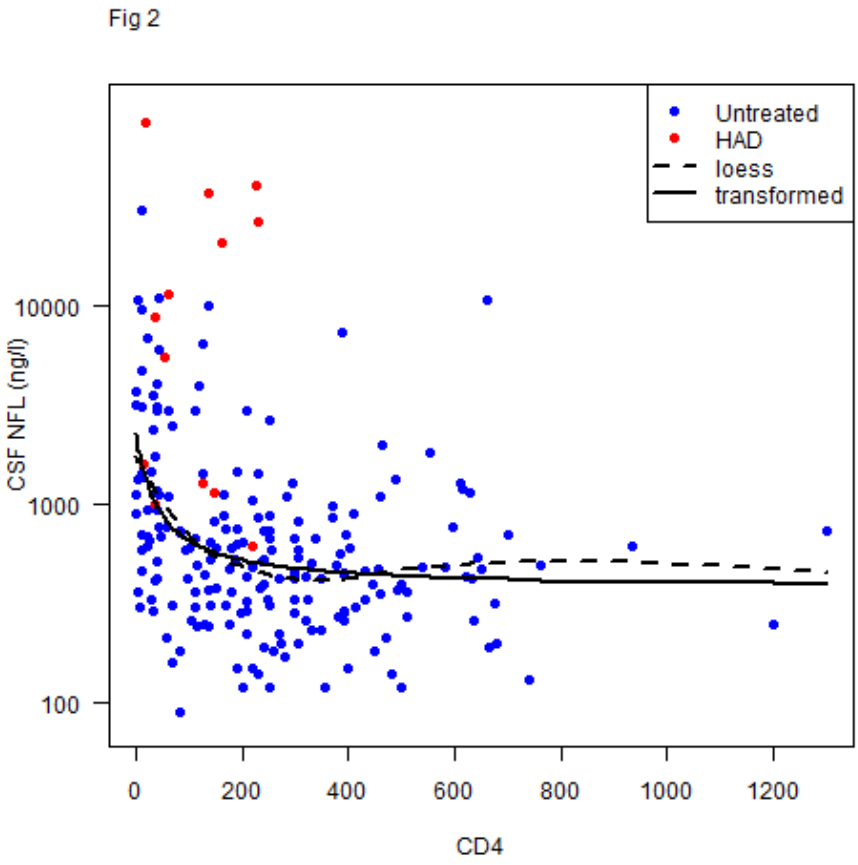

Figure 10. Paper III. Loess regression showing an inverse correlation with increased levels of CSF NFL when CD4 T cell levels drop below 250 cells/mL. NFL log10-transformed.

4.3.3 NFL as a marker for premature or accelerated aging

A mixed effects model was performed to analyze group differences of CSF NFL and age between NA patients, those on suppressive cART, and those who were HIV-negative. This analysis was done to determine whether NFL could be used to reflect premature or accelerated aging in the HIV-infected groups. In the NA group, NFL levels were equivalent to those of HIV-negative people being 18.5 years older. Notably, patients on suppressive

treatment exhibited increased CSF NFL that corresponded to an age level 3.9 years older as compared to controls (Figure 11).

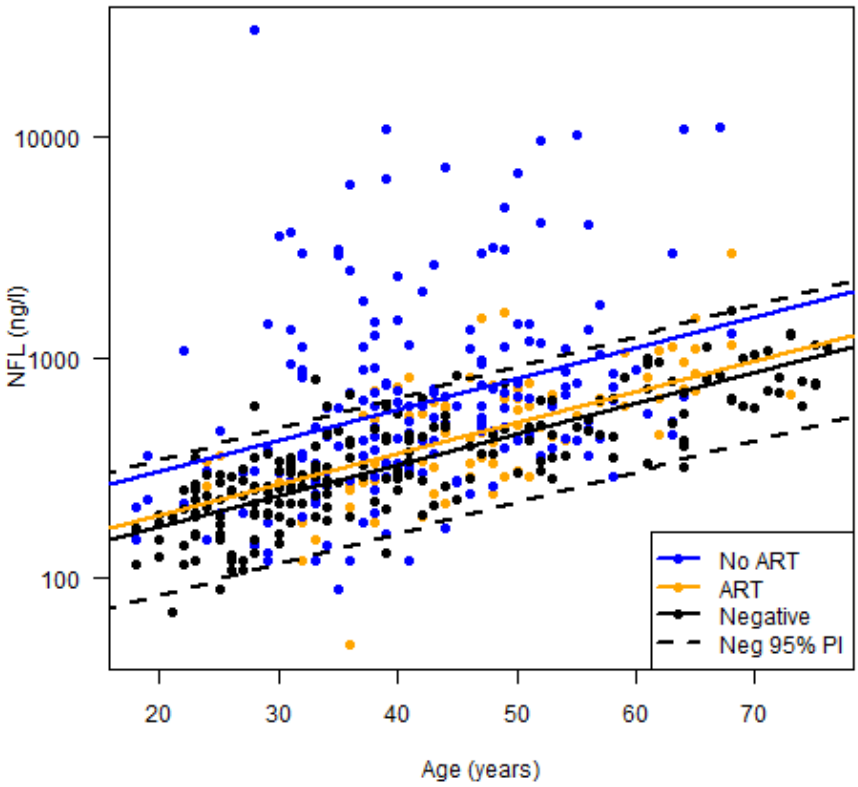

Figure 11. Paper III. Mixed linear effects model comparing levels of CSF NFL to age in HIV-infected neuroasymptomatic patients with cART, without treatment, and HIV-negative controls. NFL-levels in the untreated neuroasymptomatic group corresponded to HIV-negatives being 18.5 years older, whereas patients on cART had an NFL increase corresponding to HIV-negatives being 3.9 years older. NFL log10-transformed.

4.3.4 Predictors related to CSF NFL

A multiple non-linear regression was performed on HIV-patients with suppressive cART and untreated NAs. In the untreated group, age, CD4+ T-cell counts, CSF neopterin, CSF WBC count, and CSF/plasma albumin ratio were possible predictors of NFL in both univariate and multivariate analysis. In the treated group, age, plasma HIV-RNA before initiation of treatment, and CSF/plasma albumin ratio stood out as possible independent predictors.

4.3.5 Treatment effect on CSF NFL

In order to assess the effects of cART initiation on axonal disruption, CSF NFL was sampled before and after treatment initiation in 78 patients. In total, 63% of the patients decreased their CSF NFL concentrations after treatment initiation. In the subset with NFL elevated above the age-adjusted upper normal reference pre-treatment, 81% reduced their NFL levels after treatment initiation.

4.4 Paper IV

By contrast to CSF NFL, CSF p-tau generally does not increase in HIV-infected patients. As shown in Paper I, t-tau is increased in HAD patients There is however a physiological increase of both p- and t-tau with aging. In paper IV, we hypothesized that increases in CSF p-tau might occur at earlier ages in HIV-infected subjects, indicative of premature or accelerated brain aging.

4.4.1 Physiological aging as reflected by CSF p-tau

In our initial material, 342 HIV-negative controls were included. When using a Loess regression method, an obvious increase in p-tau was seen around the age of 50. To minimize the physiologic age effect on p-tau (since the control group was considerably older than the HIV-infected groups), all patients above the age of 50 were excluded (Figure 12).

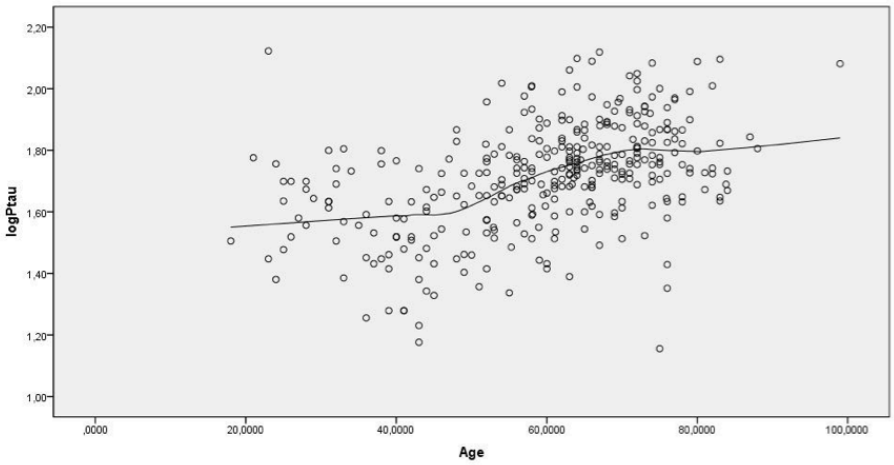

Figure 12. Paper IV. Loess regression evaluating p-tau (log10-transformed) to age in HIV-negative healthy controls. A physiological increase in p-tau was apparent, starting approximately at the age of 50 years.

4.4.2 Tau metabolites across groups

The HIV-infected group with suppressive cART exhibited significantly lower levels of CSF p-tau compared to the HIV-negative controls ($p < 0.05$), but not in relation to NA and HAD. No other differences were noted in p-tau across groups. CSF t-tau levels were increased in HAD versus all other groups ($p < 0.001$). The cART and NA groups had lower levels of t-tau in relation to the HIV-negative controls ($p < 0,01$) (Figure 13).

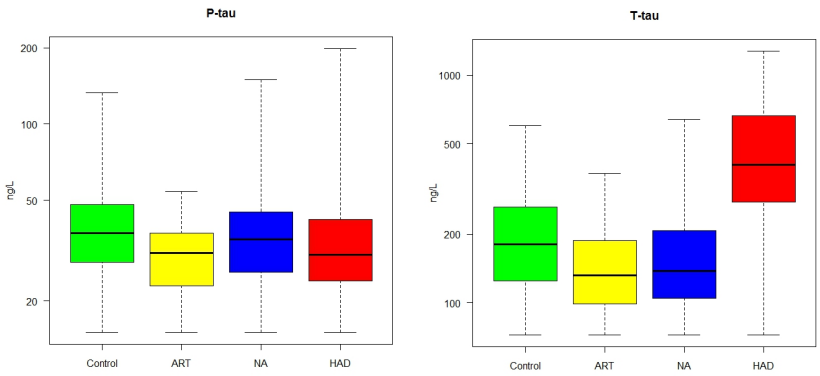

Figure 13. Paper IV. Levels of p-tau and t-tau across groups.

4.4.3 P-tau in relation to age

When using a general linear model to assess the dependency of p-tau to age, no differences were seen between groups.

4.4.4 Correlation of tau metabolites

P-tau correlated strongly with t-tau ($p < 0.0001$, r = 0.55), but not with any of the other biomarkers analyzed.

T-tau correlated with CSF neopterin ($p < 0.001$, r = 0.24) and CSF HIV RNA ($p < 0.05$, r = 0.16).

5 DISCUSSION

The neuropathogenesis of HAND is complex and only partly understood. In severe HAD, a typical pattern of mainly subcortical structural changes with progressive disease, such as atrophy and vacuolation of white matter, and infiltration of inflammatory cells (perivascular macrophages, multinucleated cells, and astrocytes) is seen [56]. In ANI and MND, structural changes are not commonly noted. With widespread use of cART, HAD is a rare presentation of HAND, but ANI and MND remain prevalent in the HIV-infected population. Several factors seem to influence development of HAND. Viral proteins (gp120 and Tat), cytokines, and chemokines produced by infected macrophages, TNFα, IL-6, and IL-8 for example, and other substances such as arachidonic acid have a direct toxic effect on neurons [91,92]. Furthermore, the BBB is compromised due to actions of viral proteins, chemokines, and cytokines, increasing the influx of monocytes into the CNS [93]. Logically, cART limits the effects mentioned above due to suppression of HIV, but it seems that cART does not suppress viral replication and the inflammatory processes in CNS completely.

Due to a long asymptomatic phase following primary HIV infection, a relatively large number of patients remain undetected. In Sweden, approximately 7-10% of those newly diagnosed presented with AIDS in 2014 [18]. Estimates from the UK suggest that one in five people with HIV are undiagnosed. Approximately half of all patients present late in the course of infection, either with clinical symptoms of AIDS, or a CD4+ T-cell count below 350 cells/mL [94]. It is reasonable that the numbers are even higher in low-income countries with an epidemic spread of HIV, limited access to medication and poor availability of testing. As previously discussed, HIV causes chronic neuroinflammation that is at least in part responsible for the progressive destruction of neurons and the development of cognitive and motor deficits. Consequently, we may be observing an ongoing neuronal degeneration in patients on suppressive cART, or the clinical symptoms may merely be reflecting residual brain damage from earlier insults during untreated HIV.

CSF NFL and neopterin

Although previous CNS damage from untreated HIV/AIDS or opportunistic infections probably plays a part in HAND, as indicated by the relation of

HAND development and CD4 nadir [42,95], our research indicates that the process of neurocognitive decline in HIV patients is also due to an ongoing immune activation and axonal degeneration exerted by HIV in the CNS. CSF NFL is increased in HAD, reflecting the presence of axonal disruption. Moreover, NFL is also increased in neuroasymptomatic HIV-infected patients without treatment and patients on suppressive treatment with cART, indicating subclinical inflammation and axonal degeneration. An increasing tendency for NFL concentration is noted with the severity of the disease, where HAD exhibits the highest levels, followed by neuroasymptomatics with CD4+ T-cells < 250 cells/ml, neuroasymptomatics with CD4+ T-cells > 250 cells/mL, and patients on cART. NFL decreases in most patients after cART initiation, probably due to decreased levels of CSF HIV-RNA and consequently less inflammation. Our results are in line with other studies of NFL in CNS HIV [68,69,96]. It should be emphasized that NFL is not a specific marker for HIV-related disease, but rather a signal for axonal damage in general, as seen in several other neurological conditions [97-100].

Neopterin, a marker for intrathecal macrophage and microglial activation, is increased in HAD compared to NA and cART groups ($p < 0.001$ for all). All NA strata, regardless of CD4 levels have significant increases in neopterin compared to patients on cART ($p < 0.001$ for CD4 levels below 200, $p < 0.01$ for CD4 levels above 200). Also the cART group exhibits signs of immune activation, as a majority showed increased levels compared to the upper normal reference of 5.8 nmol/l (median 6.9, IQR 4.9 to 8.8), concurrent with a previous study [101]. Neopterin correlates with NFL, further strengthening the argument of the immune response to HIV as a main driver.

With biomarker evidence of immune activation and axonal inflammation, the question remains whether it is possible to conclude that HIV in the CNS causes premature or accelerated aging of the brain. In HAD there is sound evidence that HIV-induced neuroinflammation is responsible for cognitive decline [40,56,72,102], but the use of biomarkers reflecting neuronal injury as a predictor for premature brain aging is still debateable in milder forms of HAND. Elevated CSF NFL in neuroasymptomatic HIV patients was shown to predict development of HAD in a case-controlled study [69], supporting the hypothesis that axonal disruption is an important factor in HAND development. In the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) study cohort comprising over 1500 patients with an HIV diagnosis, thorough NP testing is performed routinely. HAD is a rare

diagnosis, accounting for 2.4% of all patients after adjusting for confounders. However, milder presentations of HAND are common, with a prevalence of 32.7% for ANI and 11.7% for MND [60]. In a subset of 48 untreated HIV-infected patients from the CHARTER cohort, increased CSF NFL was found in a majority of HIV patients, but significant differences were not seen between ANI, MND, and neuroasymptomatics. HAD, although in a low population of three patients, showed significant increases in NFL [103].

In our material, we related CSF NFL to age and found that untreated neuroasymptomatics exhibited NFL levels similar to HIV-negative controls who were 18.5 years older. Cognitively intact patients on suppressive cART had levels equal to controls who were 3.9 years older. These findings, suggest increased axonal disruption in neuroasymptomatic patients. This could fuel a faster progression to cognitive decline and incomplete inhibition of neuroinflammation with cART. However, it is hard to correlate this finding with ANI and MND for two reasons: First, we did not perform routine NP testing. Therefore, a number of patients classified as neuroasymptomatic might in fact fulfill criteria for mild HAND if thoroughly tested. Second, the study design was cross-sectional and retrospective. To assess NFL as a predictor for premature/accelerated brain aging, patients need to be followed longitudinally with regular NP testing, CSF biomarker sampling, and possibly neuroradiological assays to detect structural damages.

Amyloid and tau metabolites

HIV-related perturbations of the amyloid metabolism are not fully understood, but several pieces of the puzzle have been investigated. Mouse-models and in-vitro studies have identified the HIV viral proteins gp120 and transactivator of transcription (tat) as important factors. Gp120 induces release of microglia-derived inflammatory cytokines (TNFα and interleukin 1β). These cytokines might stimulate APP production. Tat seems to be associated with an increase of intraneural deposition of APP and $A\beta_{1-42}$, but also has an effect on reduced CNS clearance of $A\beta_{1-42}$ [104-106].

Of the amyloid metabolites in our work, sAPPα and β were significantly decreased in HAD, HIV-associated opportunistic CNS infections, and HIV-negative patients with CNS infections. Decreased $A\beta_{1-42}$ was not a consequent finding in HAD in Paper I, although CNS OIs exhibited

decreased levels. However, those HAD patients and HIV-negative patients with acute bacterial meningitis and herpes simplex type 1 encephalitis in paper two showed significant decreases. The results contrasted the biomarker pattern with low $A\beta_{1-42}$ and normal sAPPs seen in AD, and suggest a different pathogenetic mechanism in HAD, HIV-related OIs, and acute CNS infections in HIV-negative patients, where the common denominators seem to be immune activation and neuroinflammation.

Although not fully understood, one explanation for the low levels of sAPPs in the HIV-infected patients cited above could be due to axonal deposition of APP, thereby reducing the available substrate for enzymatic degradation into sAPP [107]. Whether the same mechanism has been observed in HIV negatives with CNS infections has to the best of my knowledge not been studied, but a biomarker pattern of low sAPPs and $A\beta_{1-42}$ has been noted in multiple sclerosis [108]. Other speculative causes of decreased sAPPs could be inhibition of secretase activity, thereby limiting the degradation of APP into its soluble metabolites; perturbed transport of metabolites to the extracellular domain; or possibly because of an altered clearance from the CNS.

Whereas a decrease of $A\beta_{1-42}$ due to sequestration in amyloid plaques is a common finding in the brain of AD patients, previous studies of patients with HIV/AIDS show conflicting results [109,110]. In our material, we found both normal and decreased levels that varied greatly in two papers. Some studies aimed at CSF amyloid metabolites in HIV showed normal levels of $A\beta_{1-42}$ in HIV-infected patients [72,111], whereas other researchers found a decrease in patients with HAD [74,112]. Recently, in the first case report presenting a previously neuroasymptomatic HIV-infected patient who developed AD, the biomarker pattern showed an Alzheimer's disease-like profile, with decreased $A\beta_{1-42}$, normal sAPPs, and elevated tau proteins [77]. While it is hard to draw conclusions from one patient, it is a significant case, as combined HIV-infection and AD will most likely, be increasingly prevalent with an aging HIV-infected population on cART.

In our work, levels of CSF t-tau ranged from slightly decreased to increased in the HIV-infected groups. Although significantly higher in a majority of HAD patients as compared to neuroasymptomatic patients and HIV-negative controls, t-tau varied in HAD patients. Individuals with CNS OIs, mainly CMV encephalitis, had higher t-tau, but this was not a general finding in all patients. Neuroasymptomatic patients on and off cART exhibited normal to slightly decreased concentrations of t-tau. Conflicting results have previously been reported. Some papers indicate increased t-tau in HIV-infected patients, related to severity of brain atrophy as visualized by radiological means [111] or to HAD diagnosis [72,112], whereas others found normal to decreased levels in patients with varying degrees of HAND [74,113,114]. Increased t-tau primarily reflects a cortical axonal degeneration, and since HAD is mainly damaging subcortical structures, the axonal damages might not be clearly reflected by t-tau [56,73]. Although t-tau increases in a most patients with HAD in our material, it seems to be a less sensitive marker for HAND, including HAD, as compared to NFL. Among those CNS infections studied in HIV-negative patients, only HSV-1 encephalitis showed increased levels of t-tau. Probably, this was due to the widespread damages exerted by the virus on parenchymal brain structures, as opposed to the primarily meningeal inflammation exerted by bacterial meningitis and neuroborrelios.

Whereas t-tau is a signal for axonal disruption, p-tau reflects a pathological processing in which phosphorylation of tau induces a detachment from the microtubules, destabilizing the axon [115]. Although the mechanisms for pathological phosphorylation of tau are not completely understood, inflammation might be one factor [116]. Increased CSF levels and aggregates of p-tau in neurofibrillary tangles are common findings in AD, but this is not a general feature of HIV-infected patients with and without HAND. Furthermore, a physiological increase in both t-tau and p-tau is seen with aging. In our material, we found a clear increase of both tau metabolites in healthy controls over 50 years of age.

P-tau was not increased in any of the HIV-infected groups studied as compared to controls in our manuscripts. This is in agreement with a number of previous publications [72,74,111], although others have found increased levels of p-tau in HIV [112,117]. In fact, patients on cART showed slightly lower concentrations of p-tau compared to age-matched controls

in Paper IV, but there were no differences between the HIV-positive groups. When assessing p-tau as a marker for accelerated brain aging, we found no evidence for p-tau increases at earlier ages in HIV-infected patients, regardless of cognitive status or treatment. In conclusion, CSF p-tau is not, in itself, a reliable marker when examining HIV-related neuroinal damages, cognitive decline, and potential accelerated aging of the brain.

Unexpectedly, HSV-1 encephalitis exhibited increased p-tau at the same levels as AD. Although its significance is unclear, others have found an HSV-1 induction of tau phosphorylation in vitro [118]. T-tau increased to an even larger extent in HSV-1 encephalitis, and p-tau to t-tau ratio was not significantly different from HAD or AD. Presumably, the p-tau increase could also be a "spill-over" effect from largely increased t-tau.

Tau metabolites alone are difficult to use as diagnostic markers for HAND and for purposes of differential diagnostics. However, a combination of amyloid and tau metabolites distinguishes HAD from AD, indicating that a different processing takes place in HAD compared to AD (Table 5). OIs present a similar biomarker profile as HAD, but they can be readily diagnosed and excluded by specific laboratory analyses (bacterial cultures and PCR analyses for viruses) and by radiological methods such as MRI. Our results indicate that a combined assay of amyloid and tau metabolites can serve as a differential diagnostic alternative with regard to AD. When adding NFL and neopterin to the panel, the biomarkers may also be a useful diagnostic complement to NP testing of patients with of HIV-associated neurocognitive disorders.

	HAD	NA	cART	OI	AD
CSF NFL	+ + +	+ +	+	N/A	N/A
CSF t-tau	= / +	= / -	= / -	=/+	+ + +
CSF p-tau	=	=	= / -	=	+ + +
CSF sAPPa	- - -	=	= *	- - -	=
CSF sAPPb	- - -	=	= *	- - -	=
CSF Ab_{1-42}	= / -	=	=	= / -	- - -

*Table 5. Schematic summary of biomarker profiles across groups from all papers. HAD = HIV-associated dementia. NA = Neuroasymptomatic. cART = antiretroviral treated neuroasymptomatic. OI = Opportunistic infections. AD = Alzheimer's dementia. Increases denoted with +, decreases with -. Normal levels are denoted with =. N/A denotes "not available". * denotes data for comparison, adapted from Peterson, J et al, PLoS One,* ***9****(12): e116081.*

6 CONCLUSIONS

Combined measurements of amyloid ($A\beta_{1-42}$, sAPPα, and sAPPβ) and tau metabolites (t-tau and p-tau) in CSF distinguish HAD from AD and can be a useful approach for differential diagnostics between the two diseases. Although CNS OIs exhibit a similar biomarker pattern as compared to HAD, OIs can be readily excluded by using bacterial cultures, PCR detection, and radiological methods. The similarities in amyloid and tau metabolites in HAD and OIs suggest that the perturbations resemble each other and are possibly driven by neuroinflammation.

CNS infections in HIV-negative patients share an amyloid biomarker pattern of decreased sAPPs and decreased $A\beta_{1-42}$ with HAD and CNS OIs, suggesting an altered metabolism of amyloid precursor protein due to neuroinflammation. The perturbations were not specific for HIV. Acute, fulminant CNS infections exhibited a more pronounced change in amyloid metabolite levels, suggesting that severity of inflammation is of importance.

CSF NFL is a sensitive marker for assessing ongoing axonal damages in HIV-infected patients. Increased levels of NFL were seen in HAD, but also in neuroasymptomatic patients with or without cART, indicating that a subclinical axonal degeneration and immune activation, as measured by correlation to CSF neopterin, is a feature across the spectrum of HIV-infected patients. Albumin ratio correlates with NFL, suggesting compromised BBB integrity. Levels of NFL are decreased after cART initiation, but remain higher than controls. Furthermore, when comparing it with age, NFL is increased in neuroasymptomatic patients without treatment by an amount equivalent to HIV-negatives being 18.5 years older, which might indicate premature or accelerated aging of the brain in HIV-infection.

Increased CSF p-tau is not a general feature of HIV infection. Although a physiological increase of p-tau is noted in HIV-negative controls, we

found no evidence of increased levels of p-tau in younger individuals in the HIV-infected populations compared to controls.

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