Longterm effects in chronic HIV infection; clinical and laboratory studies

Lennert van den Dries

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Part I
Introduction

1

Chapter 1 General introduction and outline of this thesis

1

About the epidemic

History

The Human Immunodeficiency Virus (HIV) has infected more than 70 million people worldwide and has killed a staggering 35 million since the start of the epidemic [2]. In 1981, clinicians reported five homosexual men in 1981 presenting with pneumocystis jeroveci pneumonia, cytomegalovirus infection and mucosal candida [3]. Over the next years, a growing number of individuals presented with a syndrome of rare opportunistic infections, which was later named 'Acquired Immunodeficiency Syndrome' (AIDS). A fierce scientific race ended up in the discovery of a retrovirus being the causative agent of AIDS in 1983 [4,5]. The scientific community responded by searching for adequate treatment. Although the first active antiviral drugs were already available in the late 1980s, it took until 1996 before successful treatment was introduced. This treatment consisted of a combination of three antiretroviral drugs which suppress viral replication, abrogate immunological decline and prevent end-stage HIV disease, i.e. AIDS. To this date, combination antiretroviral therapy (cART) remains the cornerstone in HIV treatment. In the meantime, the epidemic continued with an estimated 36.7 million people around the world living with HIV at the end of 2015. Of these people, approximately 46% (17 million) were on antiretroviral treatment [6]. Compared to 2010, global cART coverage has risen tremendously, with an increase from 24% to 54% in eastern and southern Africa. In contrast, cART coverage in the the Middle East, Northern Africa and Eastern Europe has improved more modestly, with certain regions where still 7 out of 8 people living with HIV are not receiving treatment, despite being eligible [6,7].

Transmission

The virus incontrovertibly has its roots in non-human species and surveys of African apes identified the chimpanzee (Pan troglodytes troglodytis) as a prime suspect for the pandemic HIV-1 strain M. However, it still remains elusive how, when and where the HIV pandemic started. Chimpanzees from southern Cameroon infected with the Simian Immunodeficiency Virus (SIV) exhibit broadly cross-reactive antibodies against HIV, indicating close resemblance of both viruses in this region [8]. The oldest known proof of HIV infection dates back to 1959 and stems from a native inhabitant of Kinshasa (Congo). Genotypic analysis of this sample supports other large phylogeographic studies and places the spatial origin of the pandemic strain of HIV-1 to Kinshasa [9,10]. The most recent common ancestor is estimated to date back even further, around 1920. SIV likely crossed the species barrier at several occasions due to the consumption of chimpanzee meat (also referred to as bushmeat) by indigenous clans. In the first decades of the 20th century, the Sangha river (connecting Southern Cameroon with Kinshasa) provided an easy mode of

transportation for the export of rubber and ivory. Colonial cities and trading posts along this route grew larger and as a result of generalized social disruption in these area's, commercial sex work and venereal diseases flourished [11]. The combination of a high SIV prevalence in chimpanzees, bushmeat consumption intensity and accelerated urbanisation likely facilitated cross-species transmission of the virus in Kinshasa. In Guinnea-Bissau, another HIV strain (HIV-2) emerged and grew to epidemic proportion around 1955-1970. Its zoonotic origin is the sooty mangabey (Cercocebus atys atys). HIV-2 is less pathogenic and results in lower infectivity compared to HIV-1. Hence, the prevalence of HIV-2 is now mainly restricted to the region of Western Africa, its former colonial ruler (Portugal) and Portuguese migration destinations (e.g. Luxemburg) [12,13].

Recent advances

The devastating consequences of the HIV epidemic are unprecedented. The United Nations launched an ambitious campaign in 2014 to improve access to HIV testing and boost treatment coverage. Recent evidence suggests the need to treat people with HIV at a much earlier stage of infection; the test and treat strategy. Early treatment can prevent future non-AIDS related complications [14]. While historically the CD4 count would guide clinicians when to start therapy, current guidelines advise to initiate cART regardless of CD4 count. Early treatment is not only beneficial in terms of prognosis, it also prevents new infections by reducing a person's ability to transmit the disease, resulting in a societal benefit [15,16]. In analogy of these benefits that test and treat provides, the UN adopted a 'Fast Track strategy'. Its goal is to enable diagnosis in 90% of all people living with HIV; to start treatment in 90% of individuals with confirmed HIV diagnosis; and to induce viral suppression in 90% of this population by the year 2020. Early treatment has already resulted in a dramatic effect in the number of new HIV infections among children. As a result of treating HIV-infected mothers, an estimated 1.6 million new HIV infections have been averted since 1995. However, the total number of new infections per year on a global scale has not decreased since 2010 and remains around 2 million a year [17]. The inability to maintain linkage to care in low-income countries is likely a crucial factor in this observation. A promising breakthrough is the implementation of pre-exposure prophylaxis. Several major studies demonstrated a protective effect of antiretroviral chemoprophylaxis in high-risk seronegative individuals before potential exposure [18,19]. After studies indicating the cost-effectiveness of this approach [20], National legislative authorities around the world are currently implementing this new approach, or are on the brink of doing so.

About the virus

Structure

Viruses are carriers of genetic material and require a host organism to replicate. HIV taxonomically belongs to the family of retroviridae and is placed in the genus of lentiviridae. The highly structured genome of HIV consists of RNA and is organized in several open reading frames. Three regions (Gag, Pol and Env) encode large polyprotein precursors, which are

subsequently processed into mature proteins by viral or cellular proteases. These mature proteins are essential for virion structure (Matrix, Capsid and Nucleocapsid protein), enzymes (Protease, Reverse Transcriptase and Integrase) and surface proteins (gp120 and gp41). The remaining six regions (vif, vpr, vpu/vpx, tat, rev, nef) translate into regulatory and accessory proteins. Regulatory proteins are essential during replication, whereas the relevance of accessory proteins remains uncertain. Accessory proteins have been shown to enhance replication efficacy and play a role in host-virus interaction [21]. After a virion fuses with a mammalian host cell, RNA is converted to DNA by Reverse Transcriptase and is subsequently integrated into the host genome by Integrase. This unique quality of retroviruses is both a weakness and strength. Cells that contain integrated HIV DNA will harbor the virus until death, but the enzymes needed for this process can also be used as a target for specific classes of antiviral drugs. After integration, the fate of a replication competent virus is dictated by the activity of the cell. Transcriptionally active cells are more likely to express HIV. Both host and viral factors regulate transcription of HIV DNA. The process starts when host RNA polymerase II binds to the promoter region (Long Terminal Repeat), a region that flanks both sides of the integrated HIV genome. Transcription of the provirus (HIV DNA) initially starts out slowly but is amplified a hundredfold in the presence of Tat. At first, short completely spliced mRNA's are produced encoding mostly regulatory proteins, followed by larger incompletely spliced mRNA enabling the production of larger proteins and later the full-length unspliced mRNA is produced that acts as the virion genomic RNA. After assembly and budding, the mature virion consists of a combination of proteins and two copies of positive stranded RNA, protected from the outside environment by a cone-shaped cylindrical core and a lipid bilayer.

Biology

HIV is transmitted through unprotected sexual intercourse, mother to child transmission, intravenous drug use or blood transfusion. The major reservoir for HIV is CD4 T-cells, although cells of the myeloid lineage can also become infected. HIV enters the cell through the CD4-receptor and a chemokine co-receptor, CCR5 or CXCR4. The R5-tropic HIV-1 strains predominate during acute infection and infect both T-cells as well as myeloid cells [22]. X4-tropic variants are well able to infect T-cells, but are less efficient in myeloid cells [23]. During the acute phase of infection that typically lasts several weeks, a peak viremia of 106 to 107 RNA copies/mL is reached. CD4 T-cells are infected abundantly and a decrease in CD4 count is observed before equilibrium is obtained. A viral set point of around 30,000 copies/mL is obtained within the first 6-12 months. During this period of clinical latency CD4 T-cell levels slowly decrease.

Pathology

When CD4 T-cell levels drop below 200 cells/m3, the host immune system is unable to mount an effective response to otherwise harmless bacteria, fungi and viruses. Pathogens such as Cryptococcus, Cytomegalovirus and Pneumocystis Jiroveci (formerly known as Pneumocystis carinii) can induce symptomatic disease and this results in AIDS. Another phenomenon associated with AIDS is HIV-associated dementia. This devastating illness was widespread during the pre-

cART era with a prevalence rate of approximately 30-50% in patients with AIDS. HIV-associated dementia (or AIDS dementia complex) is characterized by massive viral invasion and inflammation of the CNS [24]. Although in vitro infection of neurons has been established in the past, HIV is generally not considered a neurotropic virus. Neurons lack the CD4 receptor and do not support further replication. In vivo studies irrefutably demonstrated that other cell types are in fact the reservoir for HIV in the CNS. Under homeostatic conditions, the CNS is an immune privileged site deprived of monocytes, T- and B-cells. Under abnormal conditions however, lymphocytes have been shown to pass the blood brain barrier [25]. Cells of the mononuclear lineage have also repeatedly been found at inflammatory sites and perivascular lesions and are likely to play a more vital role than lymphocytes. Studies performed in non-human primates have demonstrated perivascular macrophages as a source of HIV in the CNS [26]. Human post mortem studies have found HIV infected microglial cells in the CNS and compartmentalization of so-called macrophage tropic variants of HIV [27]. These observations support the notion of the CNS being a reservoir for HIV and the mononuclear lineage as a culprit for viral persistence. It remains elusive what the origin of these infected microglial cells are. However, these cells are associated with both nourishing and phagocytic characteristics. Viral invasion supposedly tips over the homeostatic balance that microglial cells maintain in the CNS. Irrespective of the mechanism, untreated HIV-dementia is a serious complication and is associated with a high mortality rate. Treatment with cART can prevent AIDS and is able to suppress the viral load in the body to below the threshold of detection. When treatment is interrupted, the viral load rebounds quickly and therefore cART should be administered life-long.

Beyond the virus

Non-AIDS related comorbidities

With the introduction of cART, HIV has been reverted to a chronic illness. However, in the last decade a multitude of studies demonstrated that illnesses not associated with AIDS are found more frequently in people living with HIV as compared to uninfected controls. These include (but are not limited to) myocardial infarction, metabolic abnormalities, anal carcinoma, coagulation abnormalities and cognitive abnormalities [28–32]. The exact pathophysiology of this phenomenon remains unknown but causes like medication, risk behavior profile, smoking and HIV-related factors have been proposed as causative factors. A specific interest is taken in the role of chronic immune activation, a condition characterized by elevated levels of inflammatory biomarkers [33]. Several hypotheses exist that attempt to explain chronic immune activation, such as microbial translocation [34], pyroptosis [35] and co-infections [36], but the mechanism is most likely heterogeneous and complex.

Neurocognitive disorders in HIV

Viral invasion of the central nervous system (CNS) is a well-known occurrence in HIV infection. The viral load in the cerebral spinal fluid (CSF) mirrors that in plasma, although it is generally 1

to 2 logarithmic scales lower in CSF. The CNS parenchyma of HIV-infected individuals exhibits the formation of multi-nucleated giant cells, and even though the viral load is undetectable, HIV can be detected in the central nervous system long after cART treatment has been initiated. For the treating physician, it remains the question whether these observations hold any clinical relevance. Mild cognitive changes are reported to be more prevalent in HIV compared to uninfected individuals [37] and can have a substantial impact on daily functioning. However, the methods for screening cognitive function applied in prior studies are being scrutinized in recent years [38]. The current gold standard to identify neurocognitive disorders in HIV is neuropsychological assessment. Neuroimaging studies have demonstrated increased white matter abnormalities in HIV-infected individuals. White matter abnormalities have been demonstrated in other neurologic disorders, but also have a strong association with increasing age and vascular co-morbidity. Although it seems tempting to link these abnormalities with cognitive disorders observed in HIV, to date no concrete evidence has been able to prove this statement.The presence of pro-inflammatory biomarkers in plasma [39] and CSF has been used in the past to identify those at risk for neurocognitive disorders, but so far these markers have not been shown to be useful in clinical settings. The main reason is that progression to more severe cognitive disorders in HIV is unpredictable. Therefore, clinicians are hesitant to change therapy based solely on the presence of neurocognitive abnormalities. However, when these abnormalities are present,European guidelines stress the need to monitor the viral load in the CSF to prevent viral failure. In the absence of (substantial) plasma viremia, a high viral load in the CSF could point towards compartmentalization. However, intermittent viremia has also been observed in around 10% of the HIV population without any major clinical complications [40]. In addition, considering the pro-thrombotic state during HIV and the increased prevalence of vascular complications, it remains a question whether micro-infarctions could cause HIV related neurocognitive disease.

Coagulation abnormalities in HIV

Infection and coagulation can be regarded as mutually influential. During severe septic shock and viral sepsis, consumptive coagulopathy occurs which can result in disseminated intravascular coagulation [41]. Vice versa, fibrinogen is an acute phase protein and has been linked to promote inflammatory processes [42]. Both coagulation and inflammation are strictly regulated biochemical processes with extensive enzymatic feedback loops. This homeostatic balance is compromised under pro-inflammatory conditions, as is the case during viral infection. Where some viruses can cause bleeding (e.g. dengue, Marburg and Ebola), other viruses are associated with pro-thrombotic complications, as is the case in HIV. Increased incidence of venous and arterial thrombosis in HIV has been reported in retrospective studies. Patients with HIV seem to be more prone to develop stroke and myocardial infarction, but identifying those at risk has proven to be a difficult task. Coagulation abnormalities in HIV infected patients are reflected by change in a wide variety of plasma pro-coagulant and anti-coagulant biomarkers. Most of these markers can be linked to a pro-coagulant status. Treatment with cART normalizes these markers to some extent, but not completely. In the normal process of aging, microvascular lesions can be seen in a large proportion of adults.

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Metabolic abnormalities

Compared to the relationship that inflammation has with coagulation, the effect of HIV on metabolic homeostasis is less well defined. The observation from large cohort studies that linked HIV to increased risk for myocardial infarction [28] have sparked researchers to investigate the vascular consequences of HIV. A large tri-continental study linked the occurrence of vascular events with the use of protease inhibitors [43] and abnormal lipid profiles in general in patients receiving cART [44]. Treatment seems to play a large role in the occurrence of metabolic abnormalities, but the influence of pro-inflammatory monocytes in plaque formation is currently under investigation [45].

Outline of this thesis

HIV and neurologic complications Without treatment, end-stage HIV infection is accompanied by a high prevalence of opportunistic infections. This prevalence has dropped dramatically after the introduction of cART. During effective treatment the burden of neurologic complications is also small, but not negligible. There is considerable debate about the prevalence, pathogenesis and clinical relevance of certain neurological complications such as stroke, neurocognitive disease and the residual risk of opportunistic infections. Chapter 2 focuses on the current body of knowledge about neurocognitive disorders in HIV. The literature is reviewed on the prevalence, pathogenesis and implications of cognitive disorders in HIV. In addition, clinically relevant topics such as stroke and pneumococcal meningitis in HIV infected individuals are discussed. Are people living with HIV prone to develop these co-morbidities? This question has been the subject of many studies in the past. To understand the interplay between HIV and the CNS, we first reviewed current literature; chapter 2 is directed towards finding an answer to this question and forms a stepping-stone towards the introduction of the TREVI study.

TREVI study

To study the impact of neurocognitive disorders in HIV, we designed a cross-sectional cohort study at the Erasmus MC. The goal was to assess the prevalence of cognitive disorders in a chronically infected and well-treated HIV population. An appropriate screening strategy as advised by the European AIDS Clinical Society was applied and assessed for sensitivity and specificity using the Neuropsychological Assessment as a gold standard. During 2012 to 2013, we set up the TREVI study, which was intended to assess neurocognitive disorders in the HIV population of the Erasmus MC. From a clinical perspective, the consequence of identifying cognitive deficits can be considerable. Patients diagnosed with cognitive disorders can be referred to a neurologist for further evaluation. Additional investigations include obtaining central spinal fluid to rule out compartmentalization of viral replication as well as imaging studies. Depending on the results and clinical course, it can even result in initiating/switching therapy. Therefore, it is crucial to determine the benefit and consequences of these screening strategies. In Chapter 3, we discuss

the results of screening for cognitive disorders in the TREVI study. Chapter 4 focuses on socio-economic characteristics of the patients involved in the TREVI study. It describes various factors involved in the quality of life and labor participation.

Experimental TREVI studies

Our understanding of HIV is based on the work of numerous researchers worldwide. Years of research have been invested in the development of therapeutic approaches and the identification of pathogenic mechanisms. These efforts have resulted in considerable progress, but many questions still remain unanswered. With the continuous introduction of revolutionary laboratory techniques, the scientific community is moving forward in its quest to understand HIV. In the TREVI study, we attempted to contribute to the knowledge about HIV. We stipulated relevant research questions about inflammatory, coagulation- and metabolic abnormalities, and set up basic experiments to test our hypothesis. We collected blood specimens during the TREVI study and isolated plasma and peripheral blood mononuclear cells (PBMC's). In Chapter 5 to 8 we describe the impact of chronic infection in people living with HIV on coagulation and metabolic abnormalities as well as chronic immune activation. Chapter 5 describes the concept of immune activation and the presence of this phenomenon in a well-treated HIV-infected population at different treatment intervals. This experiment was designed to assess the effect of long-term treatment on the expression of pro-inflammatory cytokines in PBMC's and plasma. In Chapter 6, we applied a novel tool to assess the risk for metabolic complications by measuring hair cortisol. Based on anthropomorphic and laboratory data, we identified patients with the metabolic syndrome and investigated whether this was correlated to cortisol levels. In contrast to uninfected individuals, people living with HIV at increased risk for the metabolic syndrome had low hair cortisol levels. This finding inspired us to initiate a follow up study. For this part, we tested the sensitivity of PBMCs of HIV infected individuals to corticosteroids. In Chapter 7 we zoom in on specific elements of the coagulation cascade. Von Willebrand Factor (vWF), an important protein in the induction of hemostasis, was measured in patients with and without a history of thrombosis. Using vWF as a predictive marker, we assessed whether symptomatic disease is linked to the level of this biomarker. For the last chapter of part II, we recruited pediatric HIV-infected individuals. Contracting HIV during childhood, when the immune system is still developing, has potentially detrimental effects. When left untreated, 50% of children develops AIDS and dies before the age of two [46]. Although treatment is initiated at an increasingly earlier age, morbidity and mortality remains disproportionately high in children, even in developed countries [47,48]. Although clinical studies are still in progress, this manuscript already contains the first experimental study in this specific population. In chapter 8 we evaluate the levels of circulating endothelial cells (CECs) in HIV-infected children. CECs represent the turnover rate and degradation of the endothelial lining of the vessel walls. Current literature already demonstrates an increase of CECs under pathological conditions, particularly cancer and sickle cell anemia. Using CECs as a correlate of vascular damage, we investigated the impact of chronic HIV infection on the endothelium.

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Chapter 2 The Central Nervous System and Chronic HIV Infection

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Abstract

The central nervous system (CNS) is important since it could act as a reservoir in chronic HIV infection, which has relevant implications in the perspective of eradication strategies. In addition, the CNS is a target organ for developing late complications due to chronic HIV infection. HIV enters the CNS at an early stage of infection but can still be detected even after a prolonged period of effective treatment with combination antiretroviral therapy (cART). Much remains unknown about if and how HIV infection affects CNS integrity and what the effect of cART is. Clinicians are confronted with studies that report an increase in HIV-related complications such as cognitive disorders, stroke and the risk for infections of the CNS. Current knowledge indicates a complex multifactorial pathogenesis. The literature is reviewed on current knowledge and insights from a clinical perspective.

2

Introduction

The central nervous system (CNS) is one of the "target organs" in chronic HIV infection and potentially acts as a reservoir. This is important in the context of ongoing and future eradication approaches and clinical trials. However, even during adequate treatment, a certain degree of inflammation remains detectable in the CNS. The association of chronic HIV and certain non-AIDS associated co-morbidities (e.g. cardiovascular disease) are apparent, but to what extent chronic HIV affects the CNS remains elusive. The possibilities for measuring effects in the CNS as a vital organ are limited and often indirect, which poses a challenge for clinical research. Regardless of the magnitude of the reservoir or inflammatory conditions, in the end the health care practitioner needs to evaluate the risk of specific CNS-related illnesses occurring in HIV-infected individuals. In this review we focus on clinical neurological manifestations in well-treated chronic HIV infection, what is known about the pathogenesis of HIV infection and the CNS, and possible implications for treatment. A specific interest is taken in the frequently reported but highly controversial subject of HIV-associated cognitive disorders, as well as stroke and infections of the CNS. Although the latter two subjects have been reported mainly in the context of immunosuppression, recent studies in chronically infected individuals demonstrate an ongoing burden of disease.

I. The CNS as a target organ for HIV

A. Infections of the CNS

From a historic perspective, HIV is closely related to opportunistic infections of the CNS. These include bacterial, parasitic, fungal and viral infections that occur primarily during immunosuppression, as is the case in AIDS. During end-stage HIV, the failure to maintain immunologic control against commensals like JC virus and Cytomegalovirus results in overt pathology. The lack of synergism between CD4 T-cells and other components of the immune system, like the cytotoxic response, results in poor effector function against pathogens like Toxoplasma Gondii and Cryptococci. Late diagnosis of HIV infected individuals is a crucial factor in the persistence of these opportunistic complications of the CNS in the post-cART era. Even in countries with advanced health care, today close to 60% of patients have a CD4 below 350 at the time of HIV diagnosis [49].

Remarkably, some causes of meningitis and encephalitis that are frequently encountered in immune competent patients are not commonly associated with AIDS. Herpes Simplex is often found in the cerebral spinal fluid (CSF) during encephalitis in immune competent individuals; however, this is not the case in AIDS patients, nor does literature support an increased prevalence in chronic HIV infection. However, invasive pneumococcal infections like bacterial meningitis do occur more often in HIV-infected individuals and these patients also have a higher chance on a worse outcome. Indeed, the susceptibility is most pronounced during immunosuppression, but individuals with normal CD4 counts under successful antiviral treatment seem to remain at

increased risk for bacterial meningitis [50]. The incidence of bacterial meningitis is 8.3 times higher in HIV infected individuals compared to uninfected individuals [51]. S. pneumoniae is the most common causative organism of bacterial meningitis in HIV-infected patients, just as in HIV-negative patients. Salmonella meningitis occurs more often in HIV-infected patients compared to HIV-negative patients [52].

There is not one unequivocal guideline for pneumococcal vaccination in HIV patients. Because of a higher susceptibility to pneumococcal infections, national guidelines in the United States advise pneumococcal vaccinations in HIV infected individuals [53]. Dutch national guidelines only recommend pneumococcal vaccination when there is concomitant intravenous drug abuse [54]. Because side effects of pneumococcal vaccinations are minimal, it seems justified to administer prophylactic vaccinations in this risk group [50], on the other hand, studies that evaluated efficacy of pneumococcal vaccinations in HIV infected individuals have conflicting results. In patients with CD4+ T-lymphocyte counts less than 500 cells/μL, a lower response to pneumococcal vaccination is to be expected [53].

B. Stroke and vascular changes

Only a limited amount of studies have been published comparing stroke incidence between well-treated HIV-infected individuals and uninfected controls. Cohort studies frequently include patients with cancer and opportunistic infections, both of which are known to independently increase the risk of stroke. As a result, the actual incidence of stroke is possibly overestimated. Overall, HIV is more commonly associated with an increase in ischemic strokes compared to hemorrhagic strokes. A large retrospective study from the US reported a higher incidence rate of ischemic stroke in HIV-positive versus HIV-negative individuals (5.27 vs 3.75 per 1000 person years of observation) [55]. After excluding cancer and co-infections, a large European cohort study still found an increased 10 year cumulative incidence in HIV infected individuals compared to matched uninfected controls (2.68 vs 2.07) [56]. In contrast, a large prospective multicenter cohort study showed no difference in stroke incidence when comparing HIV-positive men who have sex with men (MSM) to HIV-negative MSM [57]. Furthermore, the incidence of ischemic stroke and coexisting HIV-infection is increasing from 0.08% in 1997 to 0.18% in 2006, whereas the overall stroke hospitalization rate lessened by 7% [58].

Demographic characteristics and risk factors are known to influence stroke incidence. Traditional cardiovascular risk factors such as hypertension, old age and smoking remain significant predictors for stroke, also in HIV infected individuals. However, the increase in stroke risk in HIV infected individuals is most pronounced in a relatively young age group of <50 years [55]. The reported median age of stroke in HIV patients ranges from 42 to 48 years [58]. In lower-income countries the median age is even lower; a median age of 33 years is reported in a large retrospective study in 2000-2006 in South-Africa [59], but this likely reflects a high portion of

patients with immunodeficiency and opportunistic neurological infections. Besides traditional risk factors, several 'new' risk factors exist in the HIV infected population. Large retrospective studies on stroke in HIV-infected patients identified higher odds of stroke in non-Caucasians, and among individuals with liver and renal disease and cancer [60] compared to uninfected individuals with similar age and co-morbidity burden. In addition, a consistent association between stroke and HIV status persisted after adjusting for traditional ischemic stroke risk factors and co-morbidities, with the highest risk in patients with a baseline CD4 count below 200 cells/μL [60].

Stroke is the result of ischemia in the CNS due to thrombosis, hypoperfusion or hemorrhage. Vascular wall integrity is of paramount importance in the occurrence of stroke. Cerebral small vessel disease increases with age and vascular white matter changes can be detected in large population based studies [61]. In HIV, vascular changes to intracranial and extracranial vessels include the presence of cerebral aneurysms, atherosclerosis and inflammatory changes [62]. Stroke is often preceded by additional co-morbidities promoting plaque formation such as hypertension, diabetes and dyslipidemia. HIV infection exacerbates these factors, but to which extent is of current special interest. The prevalence of carotid plaques is higher within the HIV infected population and is related to the increased risk on cerebrovascular events [63].

Diagnosing stroke can be challenging because a broad differential diagnosis in HIV-positive patients exists, especially in the case of immunodeficiency. Mimics of stroke, such as seizures or opportunistic CNS-infections, should be excluded. Studies on the safety of thrombolysis in chronically infected individuals are lacking. Vasculopathy, aneurysms and other vessel wall-changes occur more frequently in HIV infected individuals [59,64] and could precede stroke. However, it seems probable that a large proportion of HIV-positive patients has a thrombo-embolic or atherosclerotic cause of stroke, similar to HIV-negative patients. In this case, thrombolysis is in accordance with current guidelines for treatment of ischemic stroke and can be advantageous. Aneurysmal changes in HIV infected patients are in most cases asymptomatic and only associated with micro-infarctions. However, if intracerebral hemorrhage is suspected, an aneurysmal bleeding has to be considered, similar to uninfected individuals [59].

The initiation of cART decreases the incidence of cardio- and cerebrovascular events. Statins have an additional beneficial effect in preventing cardiovascular events and stroke in HIV infected patients. This is also the case in the absence of dyslipidemia, but the number needed to treat is relatively high [65]. After ischemic stroke, prophylactic therapy should be initiated, although statins and cART can have interactions. Protease inhibitors inhibit the enzyme CYP3A4 and the combination with simvastatin and atorvastatin results in a higher risk for rabdomyolysis, myopathy and kidney failure. Preferred therapy is rosuvastatin, pravastatin or fluvastatin.

C. Cognitive disorders

In the pre-cART era, HIV-associated dementia occurred in the late stages of the disease in around 15% of patients, but after the introduction of cART in 1996 the incidence dramatically declined to around 2% [32]. Nevertheless, based on clinical observations there were indications that milder forms of HIV-associated cognitive impairment remained common, affecting the quality of life of patients with chronic HIV infection. A classification was introduced to categorize cognitive impairment based on the severity of the illness [66]. This classification, also referred to as the Frascati criteria, is predominantly used for research purposes. In this classification, HIV associated neurocognitive disorder (HAND) is used as an umbrella term covering HIV-associated dementia (HAD), minor neurocognitive disorder (MND) and asymptomatic neurocognitive impairment (ANI). The diagnosis of MND in the 2007 Frascati criteria requires a 1SD lower performance compared to normative data in two out of five domains, preferentially using the mean of two or more neuropsychological tests.
Studies in the post-cART era report different prevalence rates of HIV associated cognitive impairment, which probably reflect various interpretations of the Frascati algorithm, and different characteristics of the cohorts. Overall, when compared to uninfected control groups, the HIV infected group performs worse on neuropsychological testing [32]. Recent studies have expressed the concern that the Frascati criteria exaggerate the prevalence of impairment due to inadequate statistical interpretation and the use of inappropriate reference data [38,67]. Moreover, the prevalence of cognitive impairment greatly increases by considering asymptomatic impairment as abnormal, but in fact this entity holds unclear clinical relevance. This is illustrated by a large multicenter cohort study [32] which found no difference in prevalence of HIV associated cognitive impairment after 35 months of follow up. Over time, the study showed no unidirectional decline of symptomatology [68]. Remarkably, imaging studies have demonstrated that even despite the 'asymptomatic' title, in vivo microglial activation remains present [69].

A great deal of controversy exists about screening for HIV associated cognitive impairment. The 2015 guidelines of the European Aids Clinical Society (EACS) advise clinicians to screen for cognitive deficits, but so far, no tool has proven to be satisfactory. For instance, the (international) HIV dementia scale (iHDS) has a sensitivity and specificity of approximately 65%, which makes it at best a moderately useful screening tool [70].

A vital argument in the discussion about cognitive impairment is the implication for treatment. The clinical consequences of cognitive complaints involve additional diagnostic procedures as well as adjusting treatment. Additional procedures such as magnetic resonance imaging (MRI) of the brain or a lumbar puncture to determine viral escape in the CSF are suggested in the 2015 EACS guidelines. MRI studies of the brain are primarily performed to exclude other neurologic disorders. A frequent finding in HIV infected patients is atrophy of subcortical gray matter structures, as well as white matter abnormalities [71]. The clinical value of these findings so far is unclear. Examination of CSF viral load is aimed to rule out viral escape. Viral escape

defined as discordance between CSF and plasma viral load is considered a rare complication, and clinicians should consider optimizing therapy. Current guidelines call for active surveillance and modification of cART strategy when applicable, i.e. to switch to a potentially more CNS-active drug in case of cognitive impairment or viral escape. The role of the drug-specific CNS penetration effectiveness (CPE) remains a controversial topic. Switching to a regimen with a higher CPE score has been proven beneficial a number of studies, but only when discordance exists between viral load in the CSF and plasma [72]. Switching therapy based on minor cognitive abnormalities has not been proven beneficial [73].

II. CNS as reservoir

A. Viral persistence

HIV can be detected in the CSF as early as 8 days after suspected infection (Fiebig stage I). From this acute phase onward, the viral load in the CSF continues to mirror the plasma viral load, although generally 1 to 2 logs lower [74]. The CNS is considered one of the viral reservoirs in chronic HIV infection: a sanctuary where the virus can hide. The exact magnitude of the reservoir in the CNS during effective treatment is difficult to assess because brain parenchymal tissue cannot easily be obtained. However, available studies in humans show that replication competent virus can be detected in the CNS during the chronic phase [75]. In the CSF, transient viremia is reported in up to 10% of successfully treated and neurologically asymptomatic patients [40]. So far, little is known about the impact and clinical relevance of intermittent CSF viremia. However, the question rises as to what extent viral escape and intermittent CSF viremia overlap.

Much is unknown about the mechanism behind migration of HIV into the CNS, but increased transendothelial trafficking of HIV-infected monocytes in response to chemotactic signals has repeatedly been demonstrated. Therefore monocytes, rather than T-cells, form a major mode of transportation for HIV to the CNS and might support viral persistence [76]. These infected myeloid cells are found predominantly, but not exclusively, in perivascular regions [26]. Despite effective treatment with combination antiretroviral treatment (cART) and successful suppression of plasma viral replication, HIV can still be detected in the CNS during the chronic phase of infection. Microglial cells are a cellular reservoir for HIV as well, although the origin of these infected microglial cells remains under debate (i.e. monocyte derived versus residential). Astrocytes can harbor HIV, but for reasons unknown these cells do not support synthesis of new virus. Neurons are not infected in vivo because they lack appropriate receptors for HIV.

Viral suppression is seen rapidly upon treatment initiation and prevents AIDS related morbidity and mortality [14]. Early initiation of treatment might reduce the extent of reservoir formation, but more research is needed to answer this question. Another benefit of early initiation of treatment is transient HIV remission, as was demonstrated in the Visconti study and the case of the Mississippi baby [77,78] .

B. Chronic inflammation

The association of chronic HIV infection and the array of CNS-related pathologic and clinical symptomatology has proven to be complex. The causes are most likely multifactorial and a number of aspects potentially influence clinical outcome, such as lifestyle factors, the degree of immunosuppresion, past opportunistic infections and direct viral cytotoxicity. However, in the recent decade it has become evident that a chronic inflammatory state seems to add to the burden of disease in HIV. Patients exhibit chronic inflammation and diminished immune function which can be demonstrated by monocyte and T-cell activation, endothelial cell dysfunction, and increased coagulation [79,80]. In the CNS, a persistent pro-inflammatory phenotype and accumulation of microglial cells and (perivascular) macrophages has repeatedly been demonstrated [24]. Several mechanisms have been identified to drive this process, for example microbial translocation and pyroptosis [34,35]. Certain co-infections that are more frequent in HIV-infected individuals, can contribute to this process, e.g. hepatitis C and cytomegalovirus [36]. Finally, even though the benefits of cART far outweigh the possible risks, there are also potential side effects of cART. Data from in vitro and animal models have demonstrated mitochondrial damage and subsequent oxidative stress [81]. Although CSF biomarkers of neuronal damage and imaging studies in humans support the presence of CNS injury in HIV infected patients, a possible additive role of cART toxicity remains uncertain. Immune activation markers in plasma drop substantially within the first year of treatment, although complete normalization is generally not achieved [80]. Early treatment will prevent a low CD4 nadir, might potentially diminish chronic immune activation and could also potentially lower the prevalence of co-morbidities, also in the CNS.

Discussion

HIV infects the CNS at an early stage and can be detected even after years of successful treatment. Direct viral toxicity compromises the integrity of brain parenchyma, but additional HIV-related risk factors, the presence of a chronic illness and several other known and unknown factors contribute to this process. For physicians, the question whether chronic HIV infection will adversely affect the CNS is of paramount importance. A great deal of controversy exists whether clinical outcomes such as cognitive dysfunction, stroke and meningitis are directly or indirectly related to chronic HIV infection. Regardless of this controversy, the incidence of neurological diseases seems to be increased when compared to uninfected individuals. Medical care for these neurologic illnesses transcends medical disciplines; suggesting the need for a multidisciplinary approach. Therefore, specialists previously unacquainted with HIV will need to get familiar with this chronic disability, especially neurologists.

Conclusion and future perspectives

This review aims to summarize all relevant data and current insights on HIV and the CNS in relation to clinical decision-making. However, in an area where treatment options are rapidly improving, many questions remain unanswered and need further research. Although studies on opportunistic infections have taken a back seat with the introduction of cART, a number of CNS-related infections are still prevalent during chronic HIV infection, albeit at a much lower rate. With a favorable prognosis of the HIV population in the Western world, the impact of illnesses affected by increasing age will be accentuated, such as stroke and cognitive disorders. A number of studies already investigated the benefit of early treatment initiation on co-morbidities. For the clinician it will be important to know how this affects the incidence of cognitive complaints, stroke and invasive pneumococcal infections. For stroke, the impact of statins and antiplatelet medication as primary prevention in HIV infected individuals has only been investigated in a limited amount of studies. The long-term benefit of pneumococcal vaccination has been proven in certain (non-HIV) subgroups; similar trends have been observed in HIV patients, but definitive data from representative large cohort studies are lacking. Last but not least, the question of fundamental importance is whether the CNS is a target organ for HIV and supports reservoir formation. The number of study initiatives aimed to eradicate HIV is gradually increasing and the results can be carefully interpreted as promising. However, it will remain crucial in the development of 'cure research' to address this potential sanctuary for HIV.

2

Part II

Part II TREVI Study; clinical studies in an adult population with chronic HIV infection

3

Chapter 3 Neurocognitive impairment in a chronically well-suppressed population; the TREVI study

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Abstract

Recent studies have indicated that the prevalence of HIV associated neurocognitive disorder (HAND) as reported in the literature does not correspond with clinical observations. Current guidelines advise the use of screening tools to detect patients eligible for Neuropsychological Assessment (NPA). We assessed the value of screening for cognitive abnormalities in a well-treated HIV population and investigated the association with clinical correlates. Patients with chronic HIV (N=388) were screened for cognitive complaints using the 3 Simioni questions and the international HIV dementia scale (iHDS). NPA was performed in a subset of patients (N=69). The sensitivity and specificity of current screening strategies were calculated; CD4, CD4 nadir, viral load, cART duration and the presence of co-morbidities were evaluated for associations with NPA result. A total of 127 (33%) reported cognitive complaints. The sensitivity and specificity of the Simioni questions was 82% and 24%, respectively. Adding the iHDS resulted in a sensitivity of 50% and a specificity of 73%. A CD4 nadir count <50 cells/m3 was associated with an abnormal NPA (p=0.01). Co-morbidities were more prevalent in patients with an abnormal NPA, although not statistically significant (p=0.276). Age, current CD4, viral load and cART duration were not associated with abnormal NPA.

3

Introduction

HIV associated neurocognitive disorders (HAND) are frequently reported in HIV infected individuals. The multicenter CHARTER study is one of the largest studies of HAND to date and reported a prevalence rate of 52%, which fluctuated depending on co-morbidity burden [32]. The large US Multicenter AIDS Cohort study reported a lower prevalence rate of 25-33% over a 5 year period [82]. The difference is likely a result of different cohort characteristics, but this high reported prevalence raised the question whether this actually reflects clinical observations in daily practice. Neuropsychological Assessment (NPA) is considered the gold standard to detect cognitive abnormalities. HAND is diagnosed by a score of at least 1 standard deviation (SD) below the means of normative scores in at least two out of five tested cognitive domains and is divided in three different subtypes of neurocognitive impairment. In asymptomatic neurocognitive impairment (ANI), there is no interference with everyday functioning. Patients meet the criteria for minor neurocognitive impairment (MND) when interference is present. The third entity, HIV associated dementia, is a rare complication estimated to strike 2% of the HIV infected population and is defined as a score of at least 2 SD below the means of normative scores [66]. The introduction of ANI was intended to identify a subgroup of HIV-infected individuals who actually have neurocognitive impairment despite the absence of functional decline in daily activities. However, major cohort studies did not show a clear unidirectional decline [68,82] so the question remains what the impact of diagnosing ANI is on daily functioning and prognosis. Recent studies have advocated for a more stringent definition of HAND, incorporating more conservative statistical analysis, removing ANI as an entity within the HAND spectrum and introducing better normative data [38,67].

The pathogenesis of cognitive deficits in HIV has been subject to a myriad of studies. It would be useful to characterize those patients at high risk for developing neurocognitive disorders. A high incidence of cortical atrophy and white matter abnormalities have been demonstrated in imaging studies, but a causal relationship with cognitive complaints has not unequivocally been demonstrated [83]. Although the distinct contribution of HIV in cognitive deficits is difficult to assess, the virus can be detected during acute infection, but also after years of treatment with combination antiretroviral therapy (cART) [40]. Increased markers of inflammation and central nervous system (CNS) injury have been demonstrated in a significant proportion of HIV infected individuals, with monocyte-related markers being most prominently linked to cognitive outcome [24,84,85]. In addition, low CD4 counts as well as viral burden have been linked to cognitive disorders in the past [32,83].

Cognitive screening is part of standard patient care, although not common practice. A number of screening tools exist to assess cognitive functioning, such as the Mini Mental State Examination [86], the Montreal Cognitive Assessment, the HIV dementia scale [87] and the more widely used international HIV dementia scale [88]. All screening tools are aimed to identify cognitive abnormalities and assist the clinician in deciding to refer the HIV-infected

patient for further neurocognitive evaluation. A perfect screening tool should include all patients at risk with a 100% accuracy, has to be easy to perform and unambiguous in interpretation. This is unfortunately never the case in real life and the importance of each factor has to be weighed.

Co-morbidities in HIV infected individuals have gained a great deal of attention in the recent decade. Although cART initiation greatly reduces AIDS related diseases, chronically infected individuals seem to remain at increased risk for other, non-AIDS associated illnesses such as cardiovascular disease, metabolic abnormalities and certain malignancies [14,43]. The exact etiology of this phenomenon has not yet been elucidated and little is known about the relationship with cognitive deficits.

The present study was aimed to address the controversial impact that cognitive disorders have in HIV infected individuals. We investigated the prevalence of cognitive deficits in a chronically, well suppressed cross-sectional cohort of HIV patients. We hypothesized that the most widely used screening tool for cognitive disorders in HIV, the international HIV dementia scale (iHDS) is sensitive to detect cognitive deficits. We also investigated clinical correlates of disease severity as predictors for HAND. Considering the potential impact of non-neurological co-morbidities, we also determined the burden of non-neurological co-morbidities in HIV infected patients with and without cognitive deficits.

METHODS

The TREVI study was designed to investigate the prevalence of cognitive disorders in a well-treated HIV population. We recruited patients attending the outpatient clinic of the Erasmus Medical Center in Rotterdam (the Netherlands) in consecutive order for a period of 1 year between December 2012 and December 2013. All patients had to master the Dutch or English language to appropriately compare the NPA. Patients with major neurologic co-morbidities that could affect the outcome of NPA were excluded. Patients were asked to participate in neurocognitive screening and sign the informed consent. Patients were asked if they had any cognitive complaints, based on the 3 Simioni questions as advised by the EACS guidelines [89]. This included any problems encompassing concentration, attention and/or memory that patients perceived in their daily lives. A trained research assistant screened patients using the iHDS. A cut-off score of 10 is currently applied to determine whether patients are indicated for further investigation [88]. Consecutive patients with a score of 10 or less and patients with a score of 10,5 and higher and inquired for their willingness to participate in NPA were randomly selected. An experienced neuropsychologist supervised the tests used in the NPA.

The test battery was adapted to the CHARTER study as much as possible to increase international validity [32]. The duration of the NPA is approximately two hours, and it contains 13 validated and commonly used tests. In brief, the NPA included the following tests (in this

order): Mini Mental State Examination, 15-word task (an episodic memory task, which measures learning abilities and memory consolidation), Trail making task [90] (attention, executive functioning), Stroop colour-word task [91] (attention, executive functioning), Fluency (language and/or semantic memory), Wechsler Adult Intelligence Scale-III [92] (visual-motor coordination, motor and processing speed), Rey Complex Figure Test [93] (visuoconstructive abilities, visual memory), Boston Naming Test [94] (word retrieval and language), WAIS-III (working memory, attention and concentration), Wisconsin Card Sorting Test [95] (attention, executive functioning and cognitive flexibility), Grooved Pegboard [96] (constructional praxis), Similarities (WAIS-III; verbal comprehension and reasoning), Block Design (WAIS-III, visuoconstruction). The scores from all tests were converted to z-scores. All z-scores were weighed and converted to a global deficit score. A global deficit score above 0.5 was considered abnormal.

The sensitivity of the iHDS was assessed in a 2x2 table by the ability to accurately predict NPA outcome, as represented by a GDS above or below the cut-off. Medical history of all participants was assessed for treatment duration and co-morbidities. Laboratory history was assessed for immunologic and viral parameters. CD4 (nadir) counts were grouped and GDS of these groups were compared using a Mann Whitney test. The amount of co-morbidities was scored and grouped based on dichotomous GDS (impaired vs normal). Co-morbidities that were non-chronic (e.g. a thrombo-embolic event or myocardial infarction) were only included if it occurred less than 6 months prior to inclusion. The proportion of patients without co-morbidities was compared between GD categories using a Pearson chi-square test.

Results

Patient characteristics

Out of a population of 1648 patients, we recruited a total of 400 patients for this study. Four patients were excluded during the study because the iHDS was not performed and 8 patients withdrew from the study. Out of 388 patients, 127 (33%) reported to have problems in concentration, attention and/or memory (figure 1). The mean age was 48 years (± 11), the majority of the patients were male (89%), the median CD count was 600 (IQR=450 – 780) and 326 (84%) had a viral load below 200 copies per ml (table 1a). From this cohort, 74 participated in the NPA; 5 patients were excluded from the analysis due to incomplete data. A total of 69 patients were used for analysis. Characteristics of this group (table 1b) were comparable to the entire cohort, although patients were older (53 ± 11). Detailed characteristics comparing patients with and without complaints are listed in table 2.

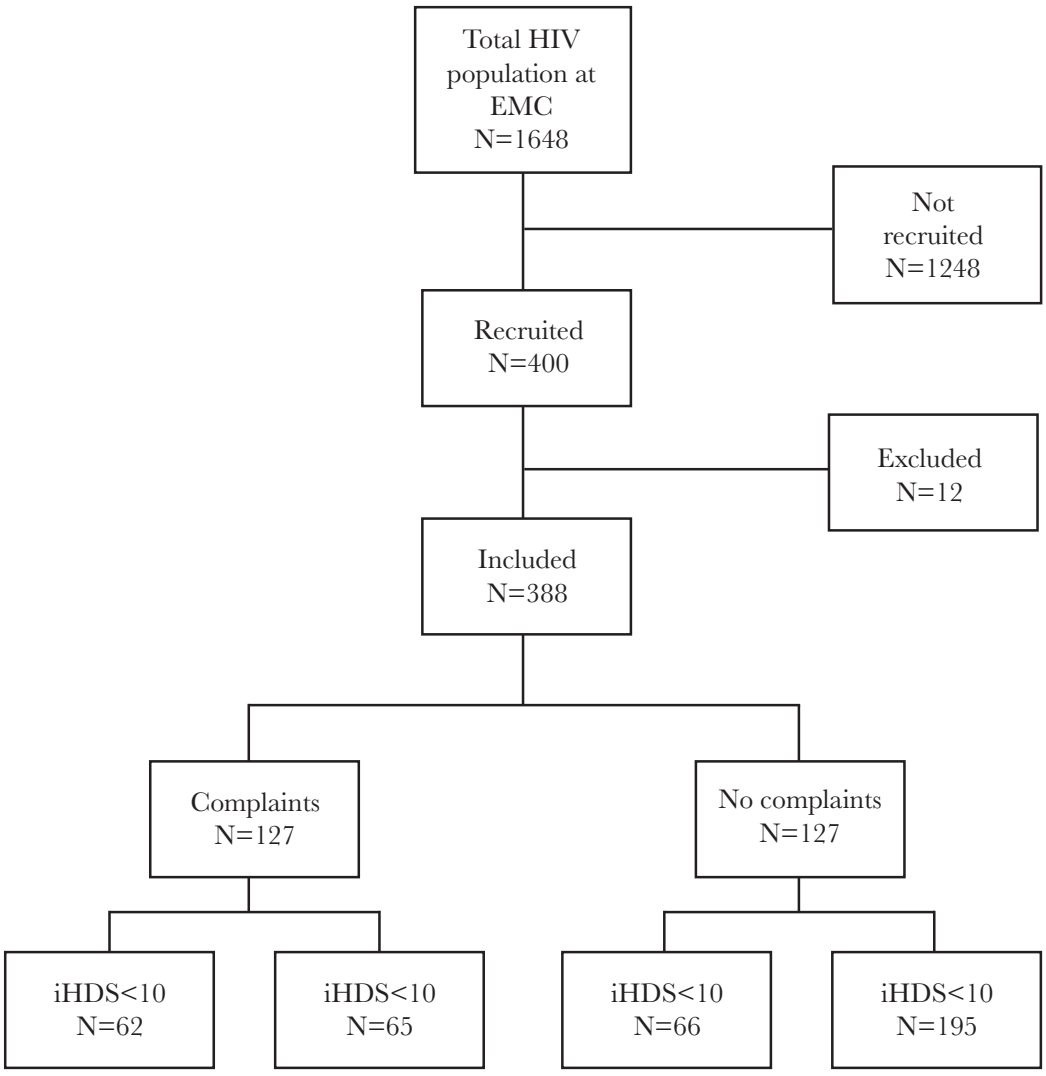

Figure 1. Flowchart of the TREVI study on neurocognitive impairment. Complaints were assessed by applying the 3 Simioni questions. iHDS: international HIV dementia scale.

Table 1a. Participants of the TREVI cohort (N=388)

Complaints	no. (%)	127 (33)
Age at inclusion	Mean (SD)	48 (11)
Male seks	no. (%)	344 (89)
Caucasian ethnicity	no. (%)	363 (94)
CD4	Median (IQR)	600 (450-780)
CD4 Nadir	Median (IQR)	240 (130-340)
Viral Load <200 co/ml	no. (%)	326 (84)

General characteristics of the TREVI cohort. All 388 patients underwent neurocognitive screening.

Table 1b. Participants in Neuropsychological Assessment (N=69)

Age	Mean (SD)	53 (11)
Males	no (%)	57 (82,6)
Caucasian	no (%)	63 (91,3)
iHDS < 10	no (%)	34 (49)
Complaints	no (%)	54 (78,3)
CD4 count	Median (IQR)	600 (430 - 740)
CD4 Nadir	Median (IQR)	220 (60 - 355)

General characteristics of TREVI cohort that underwent neuropsychological evaluation. Patients with and without complaints were included, both with normal and abnormal cognitive screening.

Table 2. Characteristics of NPA participants per complaint group.

Complaints		No (15)	Yes (54)	p-value
Age	Mean (SD)	60 (10)	51 (10)	0,005
Males	no (%)	12 (80)	45 (83,3)	0,763
Caucasian	no (%)	15 (100)	48 (88,9)	0,401
iHDS	Median (IQR)	10 (8,5 - 12)	10,8 (9 - 12)	0,544
<10	no (%)	9 (60)	25 (46,3)	0,348
CD4 count	Median (IQR)	560 (370 - 710)	640 (450 - 743)	0,272
CD4 Nadir	Median (IQR)	180 (50 - 230)	245 (65 - 373)	0,39
Viral load < 200 co/ml	Median (IQR)	14 (93,3)	49 (90,7)	0,753

General characteristics based on complaints expressed by patients. Complaints were assessed by asking the 3 Simioni questions.

Predictive value of screening

Of the 69 patients that underwent NPA, 54 patients reported cognitive problems in daily functioning. When applying the Simioni questions, 23 out of these 54 patients had an abnormal NPA. However, 5 out of 15 patients without complaints also had an abnormal NPA. This results in a reasonable sensitivity (82%), but a low specificity (24%). Adding the iHDS greatly improved the specificity (73%) but at the expense of sensitivity (50%). The iHDS on its own correctly predicted an abnormal NPA result in 19 out of 34 patients, but 9 out of 35 patients with a normal iHDS still had an abnormal NPA (table 3). The sensitivity of the iHDS alone was 68% and specificity 63%.

Clinical correlates

Patients with an abnormal NPA had a significantly lower iHDS score (p=0.009). Age, current CD4, viral load and cART duration were not associated with abnormal NPA outcome (table 4). The nadir CD4 counts of patients were grouped based on clinically relevant cut-of values (50, 200, 350 and 500). Patients with a CD4 nadir count <50 cells/m3 had a statistically lower GDS compared to patients with a CD4 nadir >50 cells/m3 (0.55 vs 0.18; p=0.01). No significant difference in GDS was detected in the groups with CD4 nadir count below 200 (p=0.334;), 350 (p=0.802) or 500 (p=0.780). In the group with abnormal NPA, a larger proportion had one or more co-morbidities (figure 2), although not statistically significant. In comparison, 70% of patients in the normal NPA group had no co-morbidities, versus 61 percent in the abnormal NPA group (p=0.276).

Table 3. Contingency table of complaints/iHDS vs NPA.

		GDS > 0,5	GDS < 0,5	Total
		Impairment	No impairment	
No Compaints	iHDS < 10 (Impairment)	5	4	9
	iHDS > 10 (No impairment)	0	6	6
Complaints	iHDS < 10 (Impairment)	14	11	25
	iHDS > 10 (No impairment)	9	20	29
	Total	28	41	69

A contingency table assessing complaints and the results of the iHDS compared to the gold standard, neuropsychological evaluation. A global deficit score (GDS) was calculated to determine which patients scored below average.

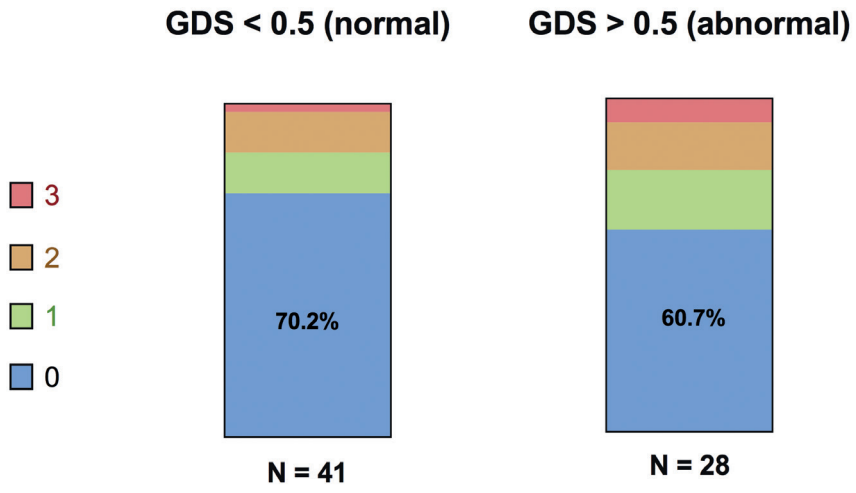

Figure 2. Distribution in number of co-morbidities in patients with normal and abnormal NPA scores. 70% in the group with normal NPA results had no co-morbidities vs 61% in the group with abnormal NPA results.

Table 4. Characterisics of NPA group per outcome.

		NPA		
		Normal (N=41)	Abnormal (N=28)	p-value
GDS	Median (IQR)	0,14 (0,05 - 0,25)	0,7 (0,59 - 0,82)	< 0,0001
Age	Mean (SD)	51 (11,5)	55,8 (9,5)	0,07
iHDS	Median (IQR)	11 (9,8 - 12)	9,8 (8,6 - 11)	0,009
Years on cART	Median (IQR)	2,7 (0,6 - 7,9)	4,7 (1,5 - 11,8)	0,122
Years since diagnosis	Median (IQR)	4,2 (1,7 - 13,9)	6,1 (1,9 - 14)	0,449
CD4 count	Median (IQR)	650 (485 - 815)	530 (403 - 720)	0,071
CD4 Nadir	Median (IQR)	250 (100 - 355)	165 (35 - 393)	0,165
Viral load	Median (IQR)	19 (19 - 28)	19 (19 - 19)	0,616

Discussion

This study was designed to evaluate the use of the international HIV dementia scale in a clinical setting and clinical factors associated with neurocognitive disorders. Current European guidelines stress the need to regularly screen for cognitive deficits in HIV infected individuals [89]. The 3 Simioni questions should guide clinicians in determining whether the patient should be referred for extensive neuropsychological evaluation. Considering that NPA is a laborious exercise it would be beneficial for both patient and clinician to have a screening test with a high specificity. Possible consequences of reduced cognitive function include lower quality of life and reduced drug adherence [97,98]. Therefore, penalty for missing cognitive dysfunction is considerable, and a sensitive test would be beneficial as well. We therefore investigated the use of the international HIV dementia scale, one of the most widely used HIV-specific screening tools. A third of our study population noted to have difficulties in concentration, attention or memory.

However, of the patients with complaints that underwent full neuropsychological investigation, 57% would prove to be unimpaired according to NPA results. Adding the iHDS filters out false positives, but also results in a disappointing low sensitivity (50%). We concluded that current clinical practice to detect neurocognitive disorders in HIV-infected individuals is unsatisfactory. Considering the impact that neurocognitive deficits can have on an individual, more sensitive and specific screening tools are needed.

In our study, a low CD4 nadir seems to be associated with poorer neurocognitive functioning.

In the CHARTER study, one of the largest cohort studies on HAND to date, CD4 nadir was also associated with poorer neurocognitive functioning [32]. This is comparable with results from other large cohort studies investigating HAND [99]. In addition, the CHARTER cohort demonstrated that a low CD4 nadir was also associated with a higher level of with white matter damage and variability in subcortical volumes [83]. This might be explained by irreversible damage caused by factors such as opportunistic infections, chronic immune activation, or a history of HIV encephalitis [24]. We found no differences when applying higher (clinically relevant) cut-off scores, indicating that this effect occurs below a certain threshold. Preventing severe immunosuppression could protect HIV-infected individuals from cognitive deficits. Other mechanisms of neurological damage could include vascular damage and other existing co-morbidities. We found a higher degree of co-morbidities in the group with cognitive deficits, albeit non-significant. It is also possible that direct viral cytotoxicity induces parenchymal damage. In vivo studies already demonstrated high viral loads in the cerebral spinal compartment during the chronic phase of infection [40]. There have even been studies linking viral burden in the CNS to ante-mortem cognitive functioning [100]. Although the plasma viral load was undetectable in the majority of patients in our study, we can't rule out the effect of cytotoxicity. We could not find any other clinical parameters that could help guide clinicians in determining which patients to screen.

The strength of this study is a clinical set-up in a relatively uncontrolled environment, reflecting a realistic outpatient setting. Clinicians are often confronted with vague symptoms in chronically well-treated HIV infected individuals. We implemented the application of current guidelines in a standardized way and identified the pitfalls in the evaluation of cognitive deficits. However, our study does have some limitations. First of all, it would have been optimal to perform NPA in all the included patients. However, we choose to perform NPA only in a subpopulation due to the extensiveness of this examination. We only included Dutch and English-speaking individuals to minimize the effect that language can have during NPA. Cultural aspects can influence the perception of cognitive complaints and might affect CD4 nadir because immigrants have shown to present relatively late in the course of HIV infection [49]. In addition, we only measured cross-sectional and have no information on whether individuals with cognitive complaints progress in their symptoms.

This study demonstrates the limitations of brief cognitive evaluation and rapid screening tests such as the international HIV dementia scale. Its intentional use is to identify those patients at risk for cognitive dysfunction. Based on our data, we would recommend caution when assessing a patient in a clinical setting. Ideally, all patients would have to be subjected to the gold standard of neurocognitive evaluation, i.e. NPA. However, the authors realize that this consequently causes a burden to both patient and neuropsychologist.

Conclusion

In a chronically well-treated HIV population, the amount of patients expressing cognitive complaints when asked is exceptionally high (33%). We used the iHDS, an internationally valid screening tool, to screen for cognitive deficits. We found a sensitivity of 68% for the iHDS, which decreased to 50% when applying this as an addition to the Simioni questions. The sole presence of complaints as indication of cognitive deficits resulted in an unacceptably low specificity (24%). The presence of a CD4 nadir below 50 cells/m3 was associated with a significantly lower GDS. We found no other clinical correlates associated with an abnormal NPA. We detected slightly more (non-confounding) co-morbidities in the group with an abnormal NPA but this was not significant. There is a need to improve neurocognitive screening and selection tools.

3

Chapter 4 Determinants of employment in people living with HIV in the Netherlands

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Abstract

Since HIV has become a manageable chronic disease, employment is of increasing importance for people living with HIV (PLWH). This study aimed to investigate the level of work participation among PLWH in the Netherlands, and the associated determinants of employment. For this study the baseline measurements of a longitudinal cohort study with a 2-year follow-up, the TREVI project, were used. The TREVI project aims to study cognitive function disorders among PLWH in relation to their employment, productivity, and social functioning. From December 2012 until December 2013, data on cognitive functioning, measured by the HIV Dementia Scale, and medical data derived from patient records were collected. Employment status and possible determinants of employment were assessed by a digital survey. Chi square analysis and multivariate logistic regression analysis were conducted in order to investigate the level of employment and associated determinants of employment. This cross-sectional study revealed significant differences in the level of employment compared with Dutch reference data: i.e. in the age group 40-54 years PLWH had a significantly lower employment rate than the general Dutch population. Multivariate analysis showed that employment was negatively associated with a lower or higher age (reference: 40-54 years), a longer period since diagnosis, problems with physical functioning, and a higher score on the HADS Depression. Having paid work at diagnosis was positively associated with employment. PLWH, particularly in the age of

40-54, in the Netherlands have a significant lower level of employment compared to the general population.

Counseling should address reduced psychological and physical functioning in order to improve the position of PLWH on the labor market.

Introduction

The life expectancy of people living with HIV (PLWH) has increased substantially since the introduction of combination antiretroviral therapy. In high-income countries, such as the Netherlands, HIV can nowadays be seen as a chronic disease. With reduced mortality and longer survival, the quality of life of PLWH has become increasingly important [1]. Participating in society and having a job is an important component of the quality of life and has proven beneficial for PLWH because it helps to structure life, leads to social contacts, provides identity and status, and helps to set targets and obtain resources [2]. Moreover, employment status is reported to be strongly related to better physical/mental health and quality of life among PLWH [3].

Employment is negatively affected by chronic diseases [4, 5]. Previous research on employment showed that the level of unemployment among PLWH is substantially higher than in the overall labor force with comparable demographic characteristics. PLWH contemplate returning to work but are still unable to be gainfully employed due to perceived barriers such as loss of income disability benefits [6-9]. Therefore, the impact of HIV on work participation appears to be substantial. In addition, PWLH often experience stigma and discrimination at work [10-12], or when returning to work after a period of work disability.

Moreover, various disease-related factors may influence a patient's employability. First, neurocognitive functioning may play a significant role in occupational success and maintenance. For example, van Gorp et al. showed that an index of learning and memory stood out as a robust predictor of finding employment [13]. Secondly, another symptom of HIV affecting a patient's ability to work is fatigue, which has a prevalence of about 20-60% in this patient population. Finally, depression and other psychosocial issues among PLWH are known to influence their ability to work [1, 3, 10].

In 2012, a multidisciplinary guideline on 'HIV and Work' was developed in the Netherlands [11, 14]. In this context, background studies (including a literature review, a qualitative study on work experiences of PLWH, and an expert panel) acknowledged the topicality and relevance of the above-mentioned determinants, and also highlighted the lack of studies addressing vocational outcomes among PLWH.

Therefore, this study investigates the level of work participation and related determinants among PLWH in the Netherlands. Apart from aiming to confirm determinants already described, we also explore the relevance of additional determinants emerging from the development of related guidelines in the Netherlands.

Methods

Study design

For this study the baseline measurements of a longitudinal cohort study with a 2-year follow-up, the TREVI project, were used. The TREVI project aims to study cognitive function disorders among PLWH in relation to their employment, productivity, and social functioning.

Study population

The population for the present study consists of PLWH attending the outpatient clinic of the Erasmus Medical Center (EMC; Rotterdam, the Netherlands). Patients were eligible for enrolment if they spoke adequate Dutch. Patients were excluded if they had a current opportunistic central nervous system infection, had current schizophrenia, current severe affective disorder believed to account for the subject's cognitive impairment, or a current neurological disorder such as epilepsy or multiple sclerosis.

Procedures

From December 2012 until December 2013, all eligible patients visiting the outpatient clinic of EMC were invited to participate in the TREVI project by their HIV physician or HIV nurse. If they were interested, patients underwent cognitive screening by a trained research assistant, using the International HIV Dementia Scale [15]. This is a standardized and internationally validated cognitive screening tool, which takes ± 2-3 min to complete. Patients with a score of 10 points (out of 12) or less, were considered for further evaluation of cognitive functioning. In addition, they received an informed consent document (with a random document number), information letter, and either an email with a web link to the questionnaire or a hardcopy questionnaire.

After the informed consent document was returned, we linked the document number to patient data, using a key that was in the possession of the principle investigator only. The questionnaire could be completed anonymously using a secured online survey system. Only the researchers had access to the survey responses. In case of non-response, a reminder was sent after 2 and 4 weeks.

The study was reviewed by the Medical Ethics Committee of the EMC and approved as not falling under the scope of the Medical Research Involving Human Subjects Act (WMO).

Outcome measures

Employment status was assessed by asking participants if they had a paid job at the time of completing the questionnaire.

Determinants of employment

Possible determinants for employment status were derived from a systematic literature review,

a qualitative study [11] and recommendations from the expert panel that was involved in developing the multidisciplinary guideline on 'HIV and Work' [14]. The following determinants were included in the questionnaire.

Background characteristics.

Gender, age, educational level, presence of children, sexual orientation, and marital status were assessed. Education level referred to the highest level of education completed and was divided into three categories: low (no, primary or lower secondary, and lower vocational education), middle (intermediate secondary and intermediate vocational education), and high (higher vocational education and university). Marital status was dichotomized as: married/cohabiting versus single (including divorced or widowed).

Medical status

Medical data (i.e., CD-4 count, CD-4 nadir, and viral load) were derived from patient records. Viral load measurements were divided into two groups: < 200 co/ml and ≥ 200 co/ml.

Work history

All respondents were asked about their work history over the past 12 months, e.g. if they had applied for another job. Furthermore, they were asked to indicate whether they had stopped or changed work since their HIV diagnosis.

Psychological functioning

Psychological functioning was measured with the anxiety and depression dimensions of the Hospital Anxiety and Depression Scale (HADS) [16]. The HADS contains 7 items relating to anxiety and 7 relating to depression. Items are answered on a 4-point Likert scale, resulting in a score of 0-21 on each construct (depression or anxiety). A score of ≥ 8 points indicates a psychiatric problem. At the intake of participants for the present study (at the outpatient clinic), scores on the International HIV Dementia Scale [15] were recorded.

General health and daily functioning

Health-related quality of life (HRQL) was measured with the MOS-HIV, a HIV-specific instrument consisting of 35 items addressing 10 dimensions of health (overall health, physical functioning, social and role functioning, cognitive functioning, pain, mental health, energy, distress, and quality of life) [17]. The subscales of the MOS-HIV were scored as summated rating scales on a 0-100 scale, with higher scores indicate better HRQL. From these 10 subscales, two summary scores were created: the Physical Health Summary score (PHS) and a Mental Health Summary score (MHS) [18].

Disclosure

The level of disclosure was measured by asking who had been informed about the HIV infection.

This question was asked for 13 different situations (e.g. sexual partner, close family, friends, neighbors, colleagues, in contacts with the healthcare sector, etc.). The answer options varied from (1) not disclosed at all to (6) full disclosure. The 13 different situations were reclassified into 4 groups: 1) sexual partner and close family, 2) social network, 3) work, and 4) contacts with financial and health sector. Also, a total disclosure score was calculated by summarizing the scores on all 13 different situations. The mean score for every group, and for the total sample was calculated, ranging from 1-6.

Stigma

Stigma was measured by asking about experienced stigma in the same situations as described for disclosure. The answer options were: never, rarely, sometimes, and often. An overall score for the experienced stigma in various situations was calculated by summarizing the scores on all these items, resulting in a mean score ranging from 1 (never) to 4 (often). Stigma was also measured by a selection of four items from the Berger Stigma Scale [19], of which a mean score was calculated ranging from 1 (completely disagree) to 5 (completely agree).

Lifestyle

Smoking, drinking, exercising and eating behavior were assessed by several questions, as in Rappange et al. [20]. Smoking was measured by asking 'Do you smoke?' (answer options: 'yes', 'no, not anymore', 'no, never smoked'). Drinking behavior was measured by asking about the number of alcoholic consumptions per week. Participants were categorized as 'no', 'moderate' or 'excessive drinker', based on Dutch guidelines [21]. Exercising was measured by the number of days per week with at least 30 min of exercise/day [22]. Eating behavior was measured by the number of days a week that balanced meals (a varied diet, rich in vegetables, fruit and whole-grain cereal products) were eaten [21].

Volunteer work and informal care

Respondents were asked to indicate whether they performed volunteer work or informal care at the time of the survey.

Statistical analyses

Statistical analyses were restricted to the participants who gave informed consent and had completed the full questionnaire. For all statistical analyses SPSS software (Version 22.0 for Windows) was used. Descriptive statistics were used to describe the characteristics of the study population. These descriptive data were analyzed for between-group differences with bivariate analyses (ANOVA) for numeric and continuous variables and Chi-square analyses for ordinal variables. A p-value < 0.05 was regarded as statistically significant.

Chi-square statistics were applied to compare the level of work participation among the

participants of this study with the general Dutch population [23]. These analyses were conducted for various subgroups (gender, age, educational level). A p-value <0.05 was regarded as statistically significant.

Logistic regression analyses were used to explore associations, expressed as odds ratios (OR) with corresponding 95% confidence intervals (CI), between the dependent variable employment status (having paid work for at least 1 h/week) and the possible determinants. Bivariate logistic regression was used to determine the single effects of all determinants of interest. Variables with a p-value < 0.1 were included in the multivariate analysis. Multicollinearity between constructs was measured by assessing the Variance Inflation Factor. A backward logistic regression technique was performed to determine the multivariate model with the best overall fit. In this analysis, independent variables with a p-value < 0.05 were retained in the final model. Variables were considered in blocks of related determinants (i.e., background characteristics, medical status, work history, cognitive functioning, general health, disclosure and stigma, lifestyle, volunteer work and informal care). The order of the blocks was determined based on the expected influence of the determinants, as observed in previous research. ORs, the 95% CI, and the change in the percentage of explained variance (incremental R square), are reported as results of the multivariate logistic regression analysis.

Results

Baseline characteristics

Of the estimated eligible patients (n=600) visiting the outpatient clinic of EMC [24], 400 were interested to participate in this study. Of these, 315 (79%) completed the survey. Informed consent was obtained from all individual participants included in the study. Table 1 presents the baseline characteristics of the study sample. The group consisted of 87% men/13% women, with a mean age of 48 years. Overall, the participants had a relatively high education level.

Table 1 Baseline characteristics of participants and differences between employed and unemployed participants at the time of enrolment in the cohort.

Variable	Total				Employed				Not Employed			
	N	%	Mean	SD	N	%	Mean	SD	N	%	Mean	SD
Total	315				206	65			109	35		
Gender												
Female	41	13			23	11			18	17		
Male	274	87			183	89			91	83		
Age*			48.1	10.6			45.7	8.9			52.6	12.1
Age group in years												
20-39*	63	20			46	22			17	17		
40-54*	168	53			126	61			42	42		
55-75*	84	27			34	17			50	50		
Marital status, % (n)												
Married or living together*	143	45			106	52			37	34		
Single, divorced or widowed*	172	55			100	48			72	65		
Has children												
No*	249	79			170	83			79	73		
Yes*	66	21			36	17			30	27		
Educational level												
Low*	73	23			38	18			35	32		
Middle*	106	34			72	35			34	31		
High*	36	43			96	47			40	37		
Sexual orientation												
Mainly attracted to men	268	85			179	87			89	82		
Both men and women	13	4			7	3			6	6		
Mainly attracted to women	33	11			19	9			14	13		
Medical status												
Months since diagnosis*			94.7	79.8			81.9	70.8			119.1	89.9
CD4 Nadir			260	173			268	171			245	178
CD-4			641	329			630	270			662	420

Variable	Total				Employed				Not Employed			
	N	%	Mean	SD	N	%	Mean	SD	N	%	Mean	SD
Viral load												
<200 co/ml	219	70			143	70			76	70		
≥200 co/ml	94	30			62	30			32	30		
Work history												
Work status at diagnosis*												
-No paid work	63	20			17	8			46	42		
-Paid work	251	80			188	92			63	58		
Change of work status since diagnosis* N=251,												
-No	140	44			113	60			27	44		
-Yes, I quit working	23	7			3	2			20	32		
-Yes, I reduced my hours	18	6			12	6			6	10		
-Yes, I changed my hours	4	1			4	2			0	0		
-Yes, I have another function	15	5			15	8			0	0		
Yes, I have another job	26	8			24	13			2	3		
-Yes, other	24	8			17	9			7	11		
Change of work status caused by HIV* N=112												
-No	64	20			57	75			7	19		
-Yes, because of physical problems caused by HIV	28	9			10	13			18	50		
-Yes, because of psychological problems caused by HIV	6	2			3	4			3	8		
-Yes, because of stigma/ discrimination at work caused by HIV	3	1			0	0			3	8		
-Yes, other	1	2			6	8			5	14		
General Health and daily functioning												
MOS-HIV: Physical Health Summary Score*			54.5	7.9			56.5	5.8			50.6	7.4

Variable	Total				Employed				Not Employed			
	N	%	Mean	SD	N	%	Mean	SD	N	%	Mean	SD
MOSHIV: Mental Health Summary Score*			51.5	8.8			53.8	7.6			47.1	9.4
Psychological factors												
HADS: Anxiety												
No*	238	76			175	85			63	58		
Possible*	30	9			18	9			12	11		
Probably*	47	15			13	6			34	31		
HADS Anxiety			5.0	4.0			4.0	3.3			6.9	4.6
HADS: Depression												
No*	255	81			184	89			71	65		
Possible*	28	9			13	6			15	15		
Probably*	32	10			9	4			23	21		
HADS Depression*			3.9	4.2			2.7	3.4			6.0	4.6
International HIV Dementia Scale*			10.8	1.3			11.0	1.2			10.4	1.5
Disclosure												
Total*			2.8	1.2			2.6	1.1			3.1	1.3
Sexual partner and close family			4.4	1.6			4.3	1.6				
Social Network*			2.3	1.3			2.1	1.1			2.7	1.5
Work			1.9	1.6			1.8	1.6			1.9	1.6
Financial and health sector			3.8	1.8			3.6	1.8			4.0	1.7
Stigma												
Experienced general*			1.4	0.6			1.3	0.5			1.6	.7
Experienced items Berger Scale*			2.2	1.1			1.9	1.0			2.6	1.2

Variable	Total				Employed				Not Employed			
	N	%	Mean	SD	N	%	Mean	SD	N	%	Mean	SD
Life style												
Smoking, % (n)												
-No	213	32			150	73			63	58		
-Yes	102	68			56	27			46	42		
Drinking												
-No*	82	26			49	24			33	31		
-Moderate*	207	66			145	71			62	58		
-Excessive	21	7			9	4			12	11		
Eating healthy			6.6	1.4			6.7	1.6			6.7	1.6
Exercise			5.2	2.3			5.1	2.3			5.4	2.3
Social security												
Receiving a benefit N=294												
-No*	217	69			193	95			24	26		
-Yes*	77	25			9	5			68	74		
Searching for another job n=296												
Searching for another job in the past 12 months												
No	222	71			158	78			64	70		
Yes	73	23			45	22			28	30		
Informal care												
Informal care family or roommate*												
No	300	95			200	98			100	92		
Yes	14	5			5	2			9	8		
Informal care other people*												
No	300	95			200	98			100	92		
Yes	14	5			5	2			9	8		
Volunteering*												
No	266	85			189	92			77	71		
Yes	48	15			16	8			32	29		

*p<0.05, n=number of participants, SD=standard deviation, Employed was defined as working ≥ 1 hour a week.

Employed versus unemployed participants

Almost two out of three participants (65.3%) had a paid job at the time of the survey. Compared with participants without employment, the 207 employed participants had a higher educational level (47% versus 37%). At the time of the diagnosis, 80% of the participants had a paid job. The work situation of 15% of the participants had changed because of HIV (e.g. reducing hours, changing hours, and another function). Overall, the scores on medical data were comparable between both groups, although those without employment had been diagnosed with HIV for a longer period of time (119 months versus 82 months).

Level of employment compared to the general Dutch population

The employment level for the total group of men and women was not significantly lower than that of the general Dutch population. Figure 1 shows the level of employment for the three age groups in the TREVI cohort compared to the general Dutch population. The level of employment for the group aged 40-54 years was significantly lower than that in the general Dutch population (75% versus 81%).

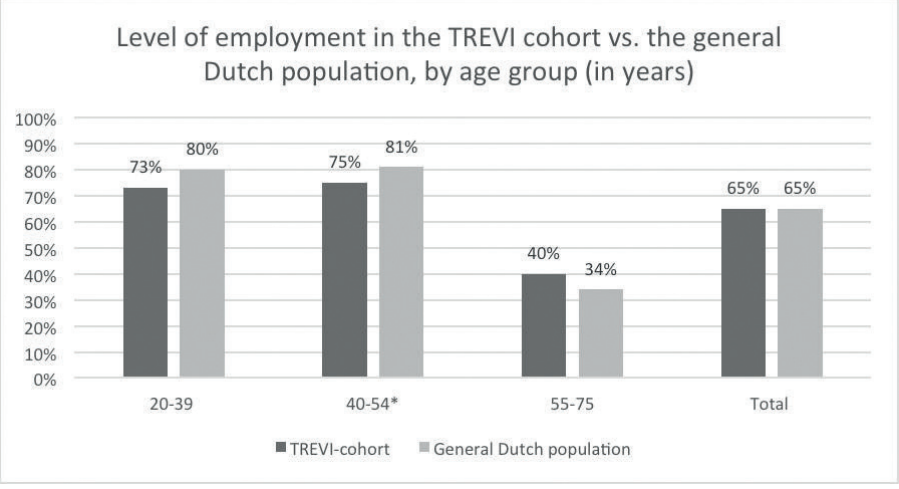

Figure 1 Level of employment in the different age groups (working at least 1 hour/week) $p<0.05$

Among men, the level of employment was significantly lower in the age groups 20-39 years (71% versus 82%) and 40-54 years (80% versus 87%) (Figure 2). No significant differences in employment were found between the TREVI cohort and the general Dutch population based on the level of education.

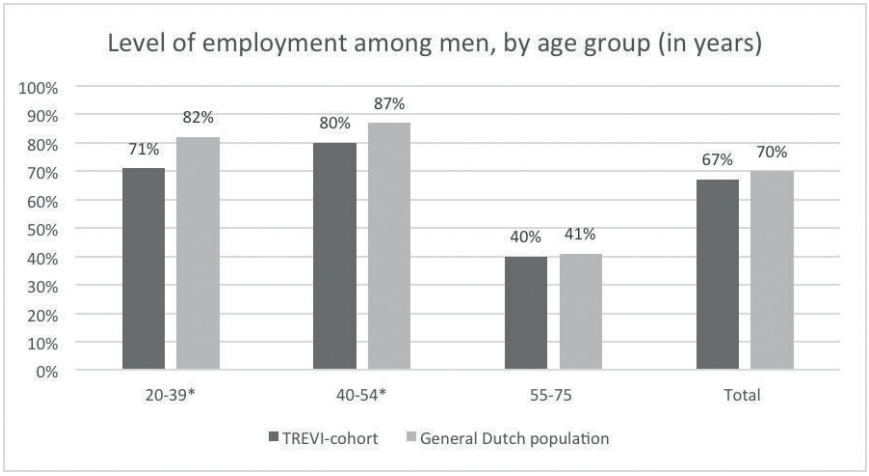

Figure 2 Level of employment among men in the different age groups (working at least 1 hour/ week) *p<0.05

The employed participants had a lower score on the HADS Anxiety scale, 6% was indicated as probably having an anxiety disorder (versus 31% of the unemployed participants). In addition, their score on the HADS Depression scale was significantly lower, 4% was indicated as probably having a depression (versus 21% of unemployed participants). Participants with employment reported a significantly lower level of experienced stigma on the items of the Berger Stigma Scale than unemployed participants (1.9 versus 2.6 on a scale from 1-5).

Characteristics related to employment status

Table 2 presents the results of the bivariate analysis.

Table 2 Bivariate logistic regression models for characteristics associated with employment (n=315)

Characteristics	OR	95% C.I.
Background		
Male (ref. female)	1.57	.81 - 3.06
Age	.94*	.91 - .96
Age in years (ref. 40-54 years)		
20-39	.90	.47 – 1.74
55+	.23*	.13 - .40
Marital status		
Married or living together (ref. Single, divorced or widowed)	2.06*	1.27 - 3.34
Has children (ref. no)	.56*	.32 - .97
Educational level (ref. low)		
Middle	1.95*	1.01 - 3.61
High	2.21*	1.23 - 3.98
Sexual orientation (ref. mainly men)		
Both men and women	.58	.19 - 1.78
Mainly women	.68	.32 - 1.41
Medical status		
Months since diagnosis	.99*	.99 - 1.00
CD-4	1.33	.380 - 1.51
CD4 Nadir	1.36	.21 - 8.72
Viral load (ref. <200 co/ml)	1,03	.62 - 1.71
Work history		
Working at diagnosis (ref. not working)	8.03*	4.32 - 15.09
General Health		
MOS-HIV: Physical Health Summary Score	1.10*	1.05 - 1.15
MOSHIV: Mental Health Summary Score	1.05*	1.01 - 1.09
Psychological factors		
HADS: Anxiety	.93	.85 - 1.02
HADS: Depression	.87*	.80 - .95

Characteristics	OR	95% C.I.
International HIV Dementia Scale	1.40*	1.15 - 1.70
Disclosure		
Total	.11*	.01 - 1.15
Work	1.77*	.97 - 3.22
Stigma		
Experienced general	.66	.38 - 1.14
Experienced items Berger Stigma Scale	.70*	.52 - .95
Life style		
Smoking (ref. non smoking)		
Yes	.35*	.20 - .63
Not anymore	.46*	.25 - .84
Drinking (ref. yes)		
No	.72	.43 - 1.22
Eating healthy	.96	.81 - 1.14
Exercise	.95	.86 - 1.06
Informal care		
Conducting informal care family or roommate (ref. no)	.49*	.24 - .98
Conducting informal care other people (ref. no)	.28*	.09 - .85
Volunteering		
Volunteering (ref. no)	**.20***	**.11 - .39**

OR=odds ratio, *p<0.1, CI=95% confidence interval

The following characteristics were significantly associated with employment: age, marital status, having children, educational level, months since diagnosis, work status at diagnosis, score on the MOS HIV Physical and Mental Health Summary, HADS Depression, International HIV Dementia Scale, total level of disclosure, disclosure at work, experienced stigma (using the Berger stigma scale), smoking, conducting informal care, and volunteering. For example, lower employment status was observed in PLWH who had a higher score on the HADS Depression scales (OR 0.87, 95% CI 0.80-0.95) and a lower score on the HIV Dementia Scale (OR 1.40, 95% CI 1.15-1.70).

Participants who had a paid job at the time of diagnosis (OR 8.03. 95% CI 4.32-15.09) were significantly more likely to have a paid job at the time of the survey.

Table 3 presents the results of the multivariate model of determinants of employment among PLWH.

	Step 1		Step 2		Step 3		Step 4		Step 5		Step 8	
	OR	**95% C.I.**	**OR**	**95% C.I.**	**OR**	**95% C.I.**	**OR**	**95% C.I.**	**OR**	**95% C.I.**	**OR**	**95% C.I.**
Block 1 Background characteristics												
Age (ref. 40-54)												
20-39	.68	(.42-1.75)	.67	(.32-1.41)	.59	(.27-1.33)	.50	(.22-.14)	.38*	(.15-.96)	.37*	(.14-.93)
55+	.28*	(.14-.53)	.29*	(.15-.56)	.30*	(.15-.62)	.35*	(.17-.74)	.21*	(.09-.50)	.22*	(.09-.51)
Marital Status (ref. Single, divorced or widowed)												
Married or living together	2.28*	(1.29-4.04)	2.26*	(1.22-4.20)	2.26*	(1.22-4.20)	1.99*	(1.10-3.76)	1.58	(.79-3.18)	1.62	(.81-3.27)
Months since diagnosis	.	.	.99*	(.99-.99)	.99*	(.99-1.00)	.99*	(.99-1.00)	.99*	(.99-1.00)	.99*	(.99-1.00)
Block 3 Work history												
Working at diagnosis (ref. not working)	.	.	.	.	8.58*	(3.92-18.78)	9.04*	(4.04-20.24)	8.07*	(3.39-19.23)	7.54*	(3.13-18.20)
Block 4 Cognitive functioning												
International HIV Dementia Scale	.	.	.	.	.	.	1.43*	(1.13-1.82)	1.25	(.96-1.62)	1.25	(.96-1.63)
Block 5 General Health (physical and psycho-logical functioning)												
MOS-HIV: Physical Health Summary Score	.	.	.	.	.	.	.	.	1.09*	(1.03-1.15)	1.08*	(1.02-1.05)
HADS: Depression	.	.	.	.	.	.	.	.	.89*	(.81-.96)	.87*	(.80-.95)
Block 7 Informal care and volunteering												
Volunteering (ref. no)	.	.	.	.	.	.	.	.	.	.	.45	(.17- 1.13)
Total R square	.12	.	.15	.	.29	.	.33	.	.46	.	.47	.
Incremental R square	.	.	0.03	.	.14	.	.04	.	.13	.	.01	.

*p<0.05
Additional information about the steps:
Step 1: Background characteristics: educational level and having children not included
Step 2: -
Step 3: -
Step 4: -
Step 5: General Health: MOS HIV MHS not included
Step 6: Did not add to the R square. In this step: Disclosure work, disclosure total, Experienced Stigma Berger Scale not included
Step 7: Did not add to the R square. In this step: Lifestyle: smoking not included
Step 8: Informal care and volunteering not included

All variables included in the model together explained 47% of the variance. Work status at diagnosis had the greatest influence on the R-square. In the final model, the variables age, months since diagnosis, score on the MOS HIV Physical Health Summary, work status at diagnosis, and the score on the HADS Depression were significant characteristics related to employment.

Discussion

This study contributes to a small but growing body of literature on PLWH and employment. The results show that PLWH in the Netherlands clearly have problems regarding their work participation and work situation.

In this study, at first glance the overall level of employment among PLWH in the Netherlands seems similar to the general Dutch population. However, on closer analysis, among an important part of the labor force (the age group 40-54 years), the level of employment of the TREVI cohort was significantly lower compared to the general Dutch population (relative reduction of 6%). Especially men in this age group (but also in the age group 20-39 years) had a significantly lower level of work participation (in the age group 20-39 years a relative reduction of 11%, and in the age group 40-54 years of 7%). Due to a lack of power it was not possible to provide more insight into the employment of women.

In contrast to earlier reports on PLWH [6, 8, 25], the overall level of employment in the Dutch sample was relatively high (65% in this study versus 40-55% in other studies); however, in those latter studies no distinction was made between subgroups. Compared to a previous Dutch study [26] the employment level in the TREVI cohort was low, especially in the youngest group (20-40 years; 73% versus 86%) and in the oldest group (55-75 years; 40% versus 48%). This may be related to the composition of the sample (e.g. distribution in education level) or to the status of the labor market/ economic situation, which may have been less favorable at the time of the survey.

Subsequently, we examined factors that could explain differences in employment among PLWH. It was found that age, months since diagnosis, work status at diagnosis, the MOS HIV Physical Health Summary score, and the HADS Depression score were factors significantly related to employment.

In the present study, being younger or older than the reference group was negatively related to employment. Previous studies also described that older people have less chance of getting a paid job [6-9, 27-33], which does not differ from the general population. The lower employment rate in our younger age group might be explained by the fact that younger PLWH do not yet have a stable working position and may still experience many changes in their personal life. There are

indications that fluctuations in the disease and uncertainty about the prognosis are negatively associated with employment [6, 9, 34]. Therefore, being HIV positive makes it harder to find and keep a paid job and start a career.
In our multivariate analysis a lower MOS HIV Physical Health Summary score and a higher HADS Depression score were significantly associated with unemployment. The role of the MOS HIV Physical Health Summary score confirms results from other studies, that physical limitations in PLWH are associated with decreased employment [2, 6, 8-10, 27, 29, 33, 35, 36]. With regard to depression the evidence remains contradictory; for example, Rabkin et al., Ezzy et al. and the present study support a negative association, whereas Lem et al. do not [8, 37, 33].

The relation between work history and actual level of employment among PLWH is not extensively studied. Only one qualitative study concluded that a gap in the resume of PLWH was a barrier for return to work [30]. In our study, having a paid job at the time of diagnosis was the strongest factor related to employment at the time of the survey. However, this result should be interpreted with caution because there could be other reasons why participants had no job at the time of diagnosis and of the survey. Nevertheless, because being employed at the time of diagnosis is an advantage for staying employed, it is important to make every effort to maintain work.

The results of our multivariate analysis do not confirm the role of some determinants described in other studies, such as impaired cognitive functioning, stigma and disclosure. Impaired cognitive functioning has been associated with problems at work and as a barrier for return to work [2, 9, 33, 38]. In our study a higher score on the International HIV Dementia Scale was not significantly related to unemployment. This may be explained by a limited variation in cognitive impairment in our HIV population. In future studies, comparison with the general population is also needed. In order to further investigate the relation between cognitive functioning and employment among PLWH, we will investigate the association between the results of neuropsychological evaluation and employment.
Stigma and disclosure are reported to be work-related issues affecting employment among PLWH [2, 6, 10, 29, 39-42]. However, in our study neither stigma nor disclosure remained in our multivariate model; additional studies are required for further insight into these determinants.

The present study has some limitations. First, since our data were cross-sectional, the observed relations cannot be interpreted as causal. However, many of our results are in line with other studies, indicating that these associations should be further explored in longitudinal research.

Second, some selection bias may have occurred as our sample included only Dutch-speaking patients from one outpatient clinic. For example, non-Dutch speaking migrants were not included in the study and this subgroup of the population may experience additional or different problems related to employment, such as stigma because of their ethnicity. On the

other hand, this selection can also be regarded as a strength of our study because it avoids other influencing factors, such as language difficulties. The present study had insufficient power to draw conclusions regarding female PLWH, who might have different experiences related to the keeping or finding a paid job. Therefore, because our results cannot be generalized to all PLWH in the Netherlands, additional studies are needed on HIV and work among other ethnic groups and women with HIV. Furthermore, because the majority of our participants were homosexual, they may have to deal with a double stigma related to their HIV status and their homosexuality.

In many countries loss of social services is an issue among PLWH [2, 10, 30, 35, 41]. In the Netherlands this is not necessary because in case of unemployment PLWH can apply for an unemployment benefit. However, in future research in other countries this possible determinant of employment, depending on the local insurance system, might be taken to account.

Furthermore, data on non-participation and non-response were not registered. Also, as employment was the main topic of this study, it is possible that mainly people with work (or interested in work) responded to the survey. If we have missed those individuals who function less well in the work situation, this may imply that the problem of employment among PLWH has been underestimated.

Finally, most data in this study were self-reported and (even though participation was anonymous), the responses may be subject to social desirability and/or recall bias.

In conclusion, by providing insight into the employment level and several factors associated with employment, this study highlights possible issues to be addressed in the vocational guidance of PLWH, and emphasizes the importance of surveillance of the work status and work-related problems of PLWH. Such information is important to provide proper counseling and may also be used for policy development, in work organizations, and even at the national level. Finally, the study underlines the need for longitudinal research to clarify the causality in the relationship between determinants and employment.

4

4

Part III
Trevi study; laboratory studies on the pathogenesis of chronic HIV infection

5

Chapter 5 Immune activation in prolonged cART-suppressed HIV+ patients is comparable to that of healthy controls

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Abstract

Sustained immune activation during chronic HIV infection is considered to augment co-morbidity and mortality. Effective combination antiretroviral therapy (cART) has shown to dampen immune activation especially during the first year cART, but the effects of long-term cART in patients without major comorbidities remains under-investigated. We performed a comprehensive analysis including cellular, intracellular and plasma biomarkers to study the effect of cART on immune parameters in 50 HIV patients. All patients were without major co-morbidities and grouped based on cART duration (0, 1, 3, 5, and 10 years). We included 10 matched healthy controls for comparison. The frequency of activated HLA-DR+CD38+ CD4 and CD8 T cells declined to levels observed in healthy controls after 1 to 3 years of cART initiation. The phenotype and ex vivo cytokine production of monocytes and NK cells were generally similar in HIV patients and healthy controls and remained unaffected by the duration of cART. Plasma IP-10 and TNF-RII were increased in untreated HIV patients, which normalized to levels comparable to healthy control levels in patients on cART. Plasma sIL-2R, D-dimer and CRP was not significantly different between groups. After the first year of cART, no additional effect on the level of inflammatory markers is observed in HIV infected patients without major co-morbidities. Residual immune activation status in well-treated HIV-infection is similar to levels observed in healthy controls.

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Introduction

With the introduction of combination anti-retroviral therapy (cART), HIV-related mortality and morbidity has decreased dramatically. As a result, studies investigating AIDS-related complications are substituted with studies focusing on long-term health consequences of HIV infection in patients with cART induced viral suppression [33,101]. These studies draw the picture that successful therapy does not completely reverse/normalize the inflammatory status induced by HIV infection since sustained activation of the immune system is observed which in turn leads to non-AIDS-related morbidity such as cardiovascular disease and non-AIDS related cancers.

Studies comparing immune parameters in HIV patients and healthy individuals have shown enhanced levels of the plasma cytokines IL-6 [102–105] and sCD14 [80,104,106,107], increased inflammatory profiles of monocytes [108–110] and enhanced frequencies of activated CD8+ T cells as determined by CD38 and HLA-DR expression [111–113] which is most pronounced during viremia and in case of poor immunologic recovery. However, not all studies were able to reproduce these findings and conflicting data or only subtle effects of HIV infection on monocytes and plasma cytokines were observed [80,107,114–117].

Several theories have been suggested to cause the sustained immune activation in HIV infection, which include microbial translocation, continued viral replication and pyroptosis [35,118]. cART-induced reduction of viral load is likely to diminish the degree of immune activation. Indeed, it has been shown that early after cART is initiated, markers of immune activation drop dramatically [80,119]. However, the long-term effects of cART treatment have been less well studied. One study showed that markers of immune activation fluctuate substantially over the course of chronic treatment [120], while another study demonstrated that T cell activation markers can reach levels comparable to uninfected controls as early as 6 months after treatment initiation [121].

The effect of long-term cART on immune activation after initial viral suppression remains under-investigated. The question whether immune activation persists in well-suppressed patients with good immunological recovery is of paramount importance, especially considering the suspected risks on increased mortality and morbidity. In the current study, we investigated the effect of long-term cART treatment on a comprehensive panel of biomarkers of immune activation in HIV patients. We investigated the temporal effect of cART in cross-sectional matched groups of well-treated HIV infected individuals. We found that after the first year of therapy-induced HIV suppression no additional effect on inflammation markers is observed in HIV-infected patients without any major co-morbidities. Importantly, the residual immune activation status in this population with well-treated HIV-infection is comparable to levels observed in healthy individuals.

Methods

Subjects Fifty individuals infected with HIV were recruited between 2012 and 2013 from the outpatient clinic of the Erasmus MC and participated in the cross-sectional TREVI cohort, a Dutch study focusing on neurocognitive disorders in patients living with HIV [30]. A selection of 10 individuals per group was performed based on the duration of cART treatment. The groups consisted of patients not receiving treatment, patients during the early phase of treatment (1 year on cART), patients during an intermediate phase of treatment (3 and 5 years on cART) and patients on long-term treatment (10 years of cART). A group of 10 uninfected otherwise healthy individuals was used as control (HC). Groups were matched on age, sex and (with the exception of the control group) smoking. Patient history was assessed and individuals with major co-morbidities, such as active hepatitis B or C and malignancies, were excluded. The presence of age related non-communicable diseases were reduced to a minimum in al groups. All participants provided written informed consent and the study was approved by the ethical committee of the Erasmus MC, the Netherlands.

Assessment of the frequency of leukocyte subpopulations Blood was collected in heparin BD Vacutainer® CPTTM tubes, and PBMC were isolated within 24 hours and cryopreserved in RPMI-1640 medium and DMSO (20%) for later use. Plasma was collected in 3.2% Citrated Vacutainer® tubes, spun twice and cryopreserved for later use. Viable PBMC were stained with the following antibodies: CD3-FITC/PE-Cy-7, CD45RO-PE, HLA-DR-PerCP-Cy5.5, CD8-APC-H7/FITC, CD4-PE-Cy7/APC-H7, CD38-eFluor450, CD14-PE/eFluor450, CD56-APC, CD19-APC-eFluor780, and CD16-eFluor450 (Biolegend/eBioscience/BD Biosciences). Flowcytometry was performed using the MACSQuant® (Miltenyi Biotec) and analyzed using Flowjo software (Treestar). The frequency of lymphocytes and monocytes was determined on the basis of their forward/sideward scatter (FSC-SSC) profile.

Intracellular cytokine staining Cytokine production by monocytes was determined as described before [122]. Briefly, PBMC were seeded at 0.25x106/250µl with serum free X-vivo media (Lonza) alone, or in combination with 2 ng/ml LPS Minnesota (TLR4 agonist, Sigma), or 1 µg/ml R848 (TLR7/8 agonist, Enzo Lifesciences). Cells were incubated for 2 hours and treated with 10 µg/ml brefeldin A (Sigma) to block protein secretion. After 7 hours of incubation, cells were fixed with 2% formaldehyde and stained for CD14. Cells were then permeabilized with 0.5% saponin and then stained with antibodies against MIP-1β-PE, TNF-PE-Cy7; MCP-1-APC; Tissue Factor (CD142)-PE, IL-6-FITC, IL-8-FITC. For the intracellular staining of NK and T cells, 0.5 x106 PBMC were incubated in a 96 wells plate for 1 hour in 200 µl RPMI-1640 medium with 5% FCS and subsequently fixed with 2% formaldehyde. Cells were then washed once with PBS, permeabilized with 0.5% saponin and stained with antibodies against TRAIL-FITC, granzyme B-PE, perforin-PerCp-Cy5.5, CD56-APC and CD3-eFluor450. 0.5 x106 PBMC were incubated in a 24 wells plate for 21 hours in 250 µl RPMI-1640 medium with 5% FCS in the presence or absence of IL-12 (0.25 ng/ml, Miltenyi) and IL-18 (50 ng/ml, R&D

systems). Cells were fixed, stained for CD3 and CD56, permeabilized and stained with antibodies against IFNγ-FITC [123]. Flowcytometry and analysis was performed as described earlier. All antibodies were purchased from Biolegend, eBioscience or BD Biosciences.

Assessment of plasma cytokines using multiplex immunoassays The ProcartaPlex human multiplex assay was used to detect cytokines in plasma (Affymetrix eBioscience). Targets included sIL-2R, interferon-gamma-inducible protein (IP)10, sTNFRII, IL-10, D-dimer, and CRP. The assays were analysed on the Ap54 microsphere based multiplex LUMINEX 100 FIDIS v3 using ProcartaPlex Analyst 1.0 Software.

Statistical analysis Statistical analysis was performed using SPSS software (IBM, v21.0). Cell populations of each sample were measured as percentage within the parent population and compared between subgroups. Continuous variables were assessed for normality and significance between groups was detected using a one-way ANOVA. A post-hoc correction for multiple comparisons was applied using Tukey HSD test when equal variances were assumed. For analysis of data with clear heterogeneity of variances, a Games-Howel test was applied.

Results

Table 1 displays the characteristics of the study population. All HIV patients and HC were male, and almost exclusively Caucasian. The mean age of patients combined was 45 years and did not significantly differ between the HIV groups and HC ($p=0.848$). The CD4 nadir count and viral load were highest at baseline (both $p<0.0001$). The CD4 counts were comparable across HIV groups ($p=0.978$). To minimize the effect of smoking as a potential confounder, we choose to balance this risk factor across the HIV groups. All patients were free from malignancies and active hepatitis co-infection. Only three patients had been previously diagnosed with comorbidities with a possible effect on inflammation. Two patients had well-controlled diabetes and one patient had minor myocardial ischemia 5 years before sampling for which he received adequate treatment. One diabetes patient was on cART for 3 years; the other diabetes patient and the myocardial ischemia patient were on cART for 10 years. None of the patients demonstrated significantly stronger inflammatory responses compared to other subjects.

Table 1. Basic characteristics of HIV infected cases and uninfected controls. Each group consisted of 10 individuals. N.a.= not applicable. HC= Healthy Controls. 0, 1, 3, 5, 10 = HIV groups with corresponding cART treatment duration (in years).

		Groups					
		HC	0	1	3	5	10
Years on cART	M (±SD)	0 (0)	0 (0)	1,1 (0,5)	3 (0,3)	5,2 (0,6)	10,8 (2,3)
Age	M (±SD)	44 (4)	45 (4)	42 (8)	44 (2)	45 (8)	45 (5)
Males	(%)	100	100	100	100	100	100
Caucasian	(%)	90	100	100	100	100	100
CD4 Nadir	M (±SD)	n.a	511 (209)	296 (151)	257 (131)	200 (77)	200 (99)
CD4	M (±SD)	n.a	629 (222)	601 (271)	630 (172)	624 (276)	667 (160)
VL below 200 co/ml	(%)	n.a	0	80	100	100	100
Years since diagnosis	M (±SD)	0 (0)	4,4 (2,8)	2,3 (1,2)	4,5 (1,8)	6,9 (2,4)	12,3 (2,4)
Smoking (%)	(%)	0	40	50	40	50	50

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Proportions of lymphocyte subsets not affected by prolonged HIV suppression by cART

Phenotypic characterization was performed to assess the impact of prolonged HIV suppression by cART on the percentage of blood lymphocyte populations. Figure 1 shows the proportion of CD4+ and CD8+ T cells, CD19+ B cells, NK cells, and NKT cells within the lymphocyte population. All HIV positive patients showed significantly lower CD4+ and higher CD8+ T cell percentages in comparison to healthy control subjects and these percentages did not normalize in patients with prolonged cART. Our patient population consists of chronically HIV-infected patients in which treatment was initiated when CD4 counts dropped below 350x10e6 CD4+ T cells/ml following previous guidelines [124]. The proportions of CD19+ B cells, NK cells, and NKT cells did not differ between untreated patients and those treated with cART for 1, 3, 5 or 10 years, and were comparable to those observed for healthy individuals.

Activation of CD4+ and CD8+ T cells reduced during cART induced HIV suppression

Next, we investigated the impact of duration of cART on the activation status of lymphocyte subpopulations. While no differences in CD45RO expression was observed in CD4+ and CD8+ T cells before and during cART (data not shown), the fraction of CD4+ and CD8+ T cells being HLA-DR+CD38+ were reduced in patients on cART and seemed to normalize to the low levels observed in healthy controls after 3 to 5 years (figure 1). Also in patients treated with cART for 1 year only, this effect was observed, albeit not significant (p=0.069).

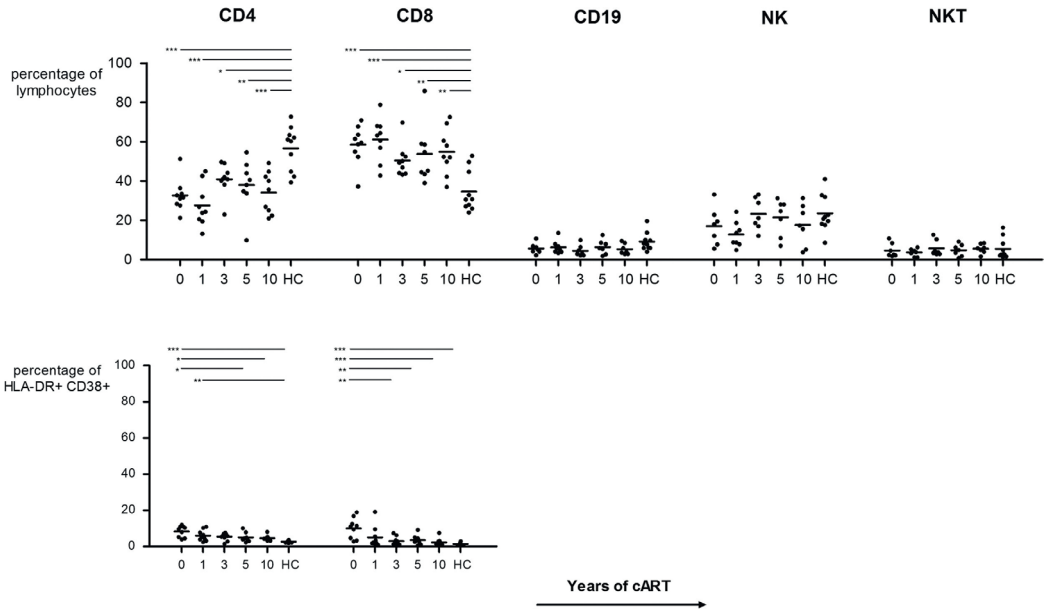

Fig. 1. Distribution of CD4, CD8, CD19, NK and NKT cells within lymphocyte populations. HC had more CD4 cells and less CD8 cells compared to all HIV+ groups. Below: the proportion of HLA DR positivity of corresponding CD4 and CD8 population. A significantly higher percentage of HLA DR+ CD38+ cells in the CD4 population of untreated HIV+ patients was found compared to patients more than 5 years on treatment and HC. The percentage of HLA+ DR+ cells in CD8 population was higher in untreated HIV+ patients compared to patients longer than 3 years on treatment.

0, 1, 3, 5, 10 = HIV infected patients in ascending order of antiretroviral treatment duration (0, 1, 3, 5 or 10 years of therapy). HC = Healthy Controls.

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

Monocytes seem unaffected by HIV infection and subsequent cART initiation

Circulating monocytes can be subdivided on the basis of expression of CD14 and CD16 into inflammatory (CD14++CD16+), patrolling (CD14+CD16+) and traditional (CD14+CD16-) monocytes. The proportions of inflammatory, patrolling and traditional monocytes seem unchanged by HIV infection, and cART initiation has no significant impact on their relative distribution (figure 2a). Also, the proportions of monocytes producing the pro-inflammatory cytokines TNF, IL-6, MIP-1β and MCP-1 (figure 2B) as well as the anti-inflammatory cytokine IL-10 (data not shown) were similar between healthy subjects and HIV patients, irrespective of treatment duration. Only when all HIV patients were pooled, significantly higher proportions of monocytes producing MCP-1 (p=0.003), IL-8 (p=0.008; data not shown) and Tissue Factor (p=0.011; data not shown) were observed as compared to healthy control subjects. However, it is important to note that the levels of induction of pro-inflammatory cytokines by monocytes of HIV-infected patients are variable.

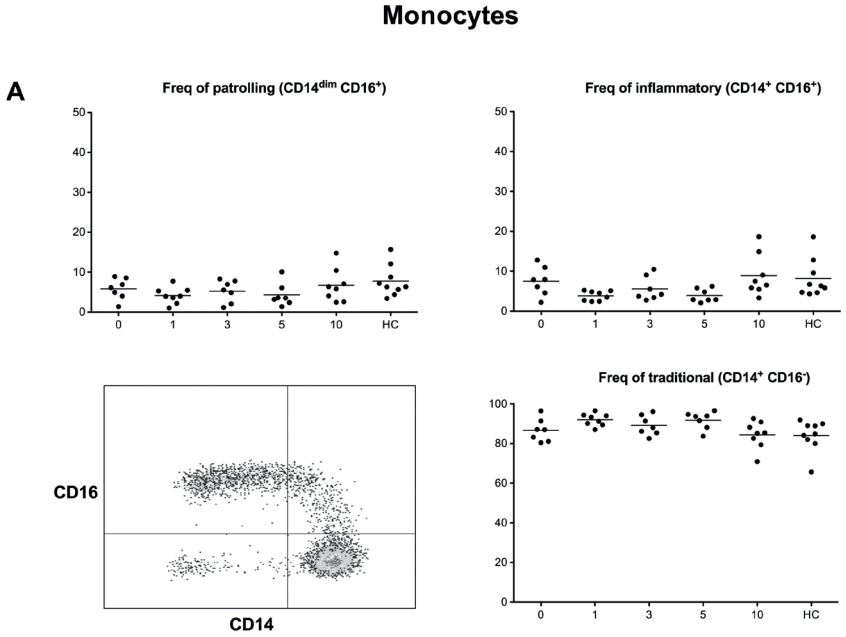

Fig 2a. Proportion of traditional (CD14+CD16-), inflammatory (CD14+CD16+) and patrolling (CD14dimCD16+) monocytes in otherwise healthy uninfected controls (HC) and patients with HIV infection in ascending order of cART treatment duration (0, 1, 3, 5 or 10 years of therapy). The variation of CD16 expression within groups was considerable but no statistically significant differences were observed between groups.

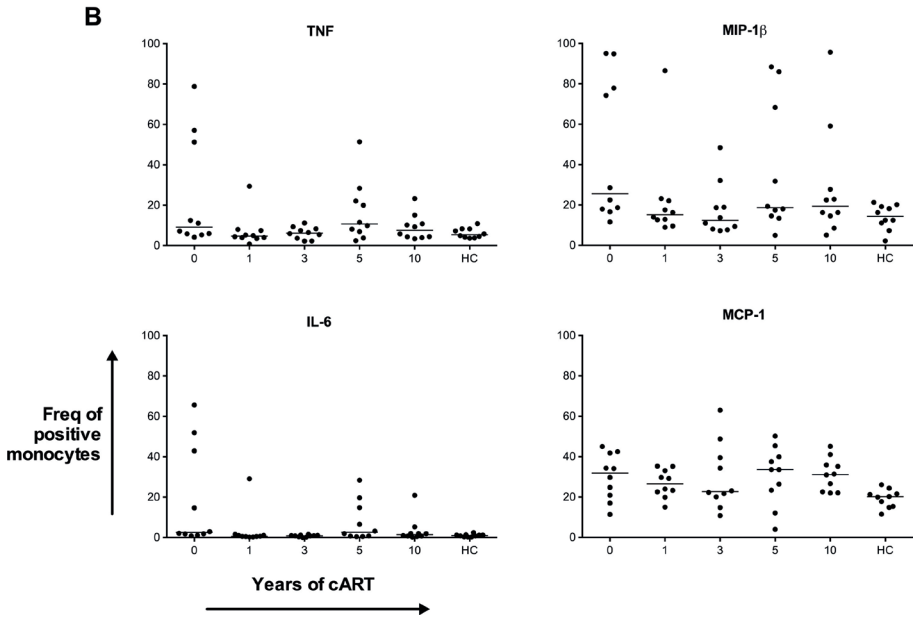

Fig 2b. Expression of inflammatory markers in monocytes of otherwise healthy uninfected controls (HC) and patients with HIV infection in ascending order of cART treatment duration (0, 1, 3, 5 or 10 years of therapy). No significant difference was found across groups in baseline intracellular levels of TNF, IL-6, MIP1β and MCP1.

NK cells seem unaffected by HIV infection and subsequent cART initiation

Under pro-inflammatory conditions, NK cells are able to mount a potent antiviral response, primarily mediated by IL-12 and IL-18. This results in the production of IFNγ, a cytokine crucial in controlling viral infections [125]. NK cells are able to induce killing of virus-infected host cells by self-recognition and subsequent production of enzymes, like perforin, granzyme B and membrane expression of TRAIL. Figure 3 illustrates the frequency of NK cells expressing TRAIL, granzyme B and perforin, and demonstrates no significant differences between expression of these markers in NK cells of patients compared to HC, albeit that the expression of granzyme B appeared higher in patients than controls. Also, no clear association with treatment duration was observed. The range of perforin expressing cells was high, probably reflecting a large intra-individual spread of this marker. To evaluate the function of NK cells, IFNγ production was induced with IL-12 and IL-18. The frequencies of IFNγ-producing NK cells were comparable in all HIV groups, and did not statistically differ from HC.

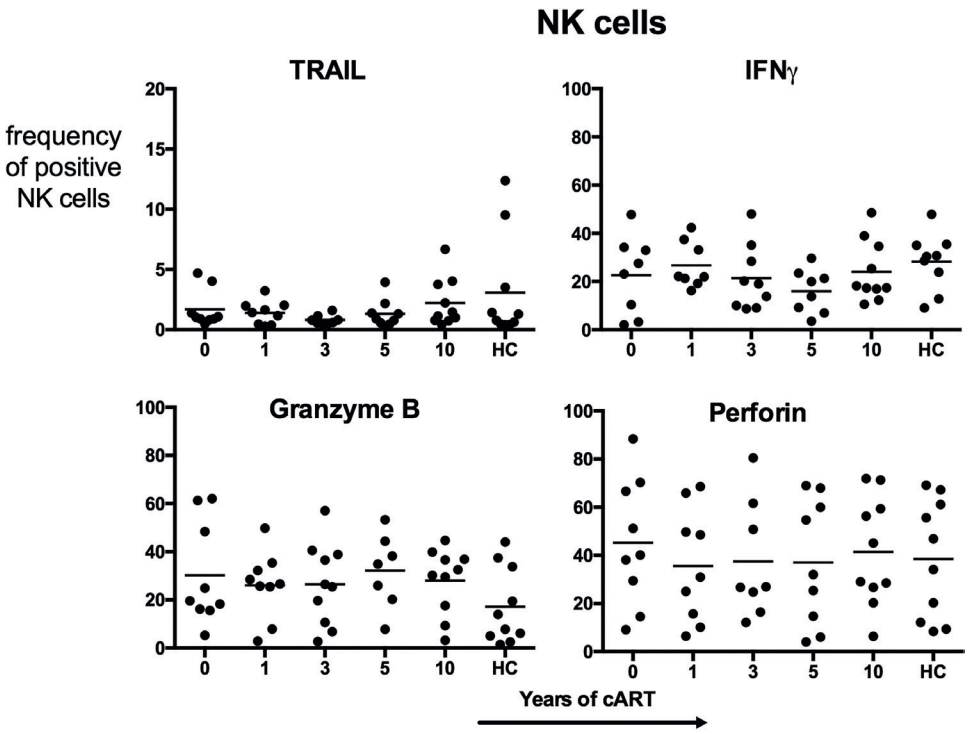

Fig 3. Intracellular markers associated with immune activation in NK cells. Intracellular expression of TRAIL, Granzyme, IFNγ and Perforin in NK cells. For the IFNγ assay, prior stimulation with IL-12 and IL-18 was performed. No uniderectional trend of immune activation level was observed with treatment duration.

TRAIL: TNF-related apoptosis-inducing ligand. IFN: interferon
* $p<0.05$
** $p<0.01$
*** $p<0.001$

Except for IP-10, the levels of inflammatory markers in plasma were not significantly elevated during untreated or cART suppressed HIV-infection.
Finally, plasma levels of various pro-inflammatory biomarkers were determined. As shown in Figure 4, plasma IP-10 levels were higher in untreated HIV patients compared to early, intermediate and long-term cART treated patients. sTNF-RII was higher in untreated HIV patients compared to HC. The plasma levels of sIL-2R and CRP showed a trend towards higher levels in the untreated group as compared to HC, but the levels did not significantly decline during the course of cART. Plasma D-dimer levels were not increased and displayed no change during the course of cART.

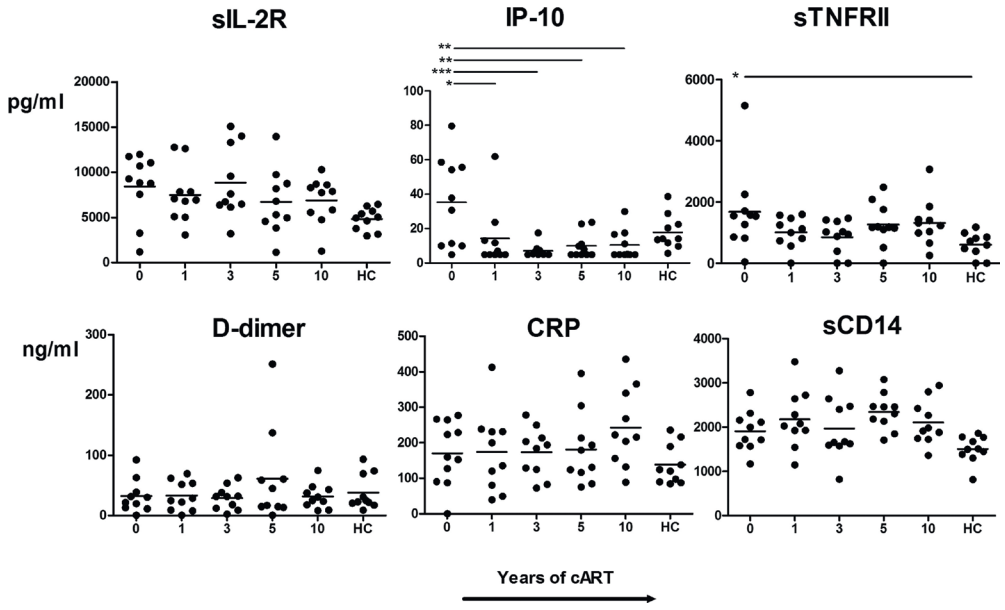

Fig 4. Plasma biomarkers measured in plasma of HIV infected patients in ascending order of treatment duration and healthy controls. The untreated group had significantly higher levels compared to patients on 1, 3, 5 and 10 years on treatment (IP10) and healthy controls (TNF-RII). Although sIL2R was generally higher in the HIV infected groups, no significance was achieved after multiple comparisons. D-dimer and CRP showed no clear association with treatment duration or HIV status. Results are in pg/ml.

sIL2R= soluble IL2 receptor. IP10=Interferon gamma-induced protein 10. TNF-RII= TNF receptor II. CRP= C-reactive protein.
* $p<0.05$
** $p<0.01$
*** $p<0.001$

Discussion

A number of studies have demonstrated that effective cART treatment of chronic HIV patients results in reduced activation of components of the patient's immune system. Most studies evaluated the immune effects during the first year after start of therapy. We now show the immune effects of HIV suppression up to 10 years after start of cART in a well-defined cross-sectional study cohort. Importantly, the selected patients are without any major co-morbidities, including hepatitis and malignancies, and reduced the number of individuals with age related diseases to an absolute minimum. Our findings show that after the first year of therapy-induced HIV suppression no additional effect on inflammation markers is observed in HIV-infected patients without major co-morbidities. Our observation of no or mild immune activation in suppressed HIV-infection questions the impact of ongoing inflammation on co-morbidity and mortality observed in HIV-infected patients without any previous major co-morbidities.

In line with previous studies, we observed that untreated HIV infection is associated with an increased expression of the activation markers HLA-DR and CD38 on CD4+ and CD8+ T cells, indicating occurrence of the persistent immune activation in untreated HIV-infected individuals [33,36,126]. Importantly, the T cell activation is not permanent, since the frequencies of HLA-DR+CD38+ T cells drop during the early stage (1 year) after treatment initiation and plateau during the intermediate (3 and 5 years) and long-term (10 years) phase of treatment. We did not detect a significant difference in this marker when comparing intermediate and long-term treatment to healthy controls. This is in line with a number of studies, although conflicting data exists. Some studies investigating the level of activated T cells in well treated HIV infected individuals report levels comparable to uninfected controls [111,112,121], others find a modest but significant increase [113,127]. These contradictory findings point towards additional confounding factors that influence the activation status of T cells in the absence of viremia. Behavioral and socio-demographic risk factors might play a role, as well as the timing of treatment initiation [128].

Different from the effect of chronic HIV infection on T cells, we were unable to find modulation of monocyte and NK cell frequencies, phenotype or function as a consequence of the ongoing infection, and as a result the additional effect of cART could not be assessed.
Little information is available in literature on the effect of long-term cART on NK cells, but a number of studies in monocytes have been performed. Our results are in line with studies these studies and show that monocytes do not exhibit an inflammatory profile [116,129–131]. In studies where increased CD16+ monocyte subsets are identified, these observations are often in the context of viremia [108,109,131].
We observed a large variation on the production of intracellular cytokines in monocytes between individuals. These outliers could represent patients that are at increased risk for illnesses associated with an activated myeloid phenotype. The presence of patrolling and inflammatory monocytes is strongly associated with cardiovascular disease in the setting of HIV infection

[45]. However, our patients show similar percentages of patrolling and inflammatory monocytes and only a minority of HIV-infected patients shows elevated production by monocytes of pro-inflammatory cytokines. Considering the absence of inflammatory profile in other subjects, we expect this phenomenon not to be HIV-related. It is tempting to speculate that HIV infection does not increase the risk of cardiovascular disease in patients in the absence of prior risk factors. Longitudinal studies are needed to formally address this issue.

In line with the effects of cART on T cell activation, we also observed that serum IP-10 and serum sTNFRII levels significantly declined during the first year after start of treatment, and normalized during continued treatment as compared to the levels observed in healthy individuals. Our results corroborate with a previous study where no additional effect of treatment was observed after one year of treatment [80]. However, others showed that the same plasma biomarkers have shown to correlate with morbidity and mortality [132–134]. In this context, IL-6 has been studied most extensively for its predictive value [132,135,136]. However, Borges et al. elegantly demonstrated using the data from these 3 major clinical trials representing close to 10,000 patients that plasma IL-6 can be influenced by a variety of other, non-HIV related factors like age, smoking and co-morbidities [105]. The aim of our study was to investigate the specific effect of cART on the level of immune activation. We therefor matched all patients on age and smoking status and excluded individuals with potentially confounding co-morbidities.

In conclusion, our findings suggest that in a HIV patient population devoid of clinically apparent co-morbidities, the modulation of immune parameters as a consequence of persistent HIV infection is relatively weak, with the majority of monocyte and NK cell markers as well as the serum biomarkers tested, resemble those in healthy individuals. cART reduced normalization of the T cell activation status and serum IP-10 and sTNFRII was observed early following treatment initiation, and no additional immune effects were observed upon continued treatment for up to 10 years. The data therefore questions the concept of chronic inflammation in HIV as the driving mechanism in the development of long-term co-morbidities in HIV as suggested by others [113,116,137], and leave room for alternative explanations.

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Chapter 6 The relation between long-term cortisol levels, glucocorticoid sensitivity and the metabolic syndrome in HIV-infected patients

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Abstract

Patients infected with the human immunodeficiency virus (HIV), have an increased risk of metabolic complications such as dyslipidemia, insulin resistance and hypertension; symptoms that are also associated with an excess of the hormone cortisol. We studied the relationship between long-term cortisol levels and metabolic syndrome (MetS) in HIV-infected patients. Cross-sectional study, performed in the outpatient clinic of infectious diseases of the Erasmus MC, University Medical Center Rotterdam, The Netherlands. Fasting blood samples and anthropometric data were collected in 126 HIV-infected patients. An ELISA-based technique was used to determine long-term cortisol levels in scalp hair. Cortisol levels were compared to 191 healthy controls. A higher risk of MetS was observed in HIV patients with a low hair cortisol (odds ratio lower vs. upper tertile 4.23, p = .04). Hair cortisol levels were not significantly

different between HIV patients and healthy controls (16.4 pg/mg vs. 13.5 pg/mg; p = .14). The risk of MetS was significantly higher in HIV infected patients in the lowest hair cortisol group compared with patients in the highest hair cortisol group. This finding contrasts with results from studies in uninfected individuals where a high cortisol level in hair is associated with metabolic syndrome. The results of this study suggest that these metabolic complications might be related to relative cortisol hypersensitivity in HIV patients. We therefor developed a novel simple and fast method to assess glucocorticoid sensitivity using only a few milliliters of blood. This assay confirmed our hypothesis.

Introduction

Since the introduction of combination antiretroviral therapy (cART) in 1996, the survival of patients suffering from HIV has increased dramatically. However, a higher incidence of metabolic complications has been observed in patients infected with the human immunodeficiency virus (HIV) as compared to the uninfected population (1-3). These complications include dyslipidemia, abnormal fat distribution (e.g. abdominal fat accumulation), hypertension, insulin resistance and type 2 diabetes mellitus (4,5).

Subsequently, metabolic derangements can result in the metabolic syndrome (MetS) and the HIV lipodystrophy syndrome (4,6). Although the exact mechanism of MetS in HIV has not been identified, a large tri-continental study demonstrated the additive effect of cART in developing cardiovascular complications. In this study, a 26 percent relative increased risk of myocardial infarction was observed per year of cART exposure (7). Many of the metabolic side effects that can occur in HIV patients are similar to symptoms caused by excessive glucocorticoid exposition, as seen in Cushing's Syndrome (CS). Patient with CS have excessive cortisol levels and frequently exhibit signs of dyslipidemia, insulin resistance, diabetes mellitus, hypertension, osteoporosis and abnormal fat distribution (e.g. buffalo hump and abdominal fat accumulation) (8,9). Because of the similarities between side effects of cART and CS, several studies have been performed to examine whether cortisol levels are elevated in HIV-infected patients. The results are conflicting; some studies find elevated levels of cortisol (10,11) whereas others contradict these findings (12,13).

Earlier studies used short-term cortisol measurements in serum, saliva or urine. These reflect cortisol levels of that specific moment in time varying from minutes up to a maximum of 24 hours. Cortisol is released in a circadian rhythm, with high cortisol levels in the morning and low levels in the evening (14). Furthermore, there is a daily variation in cortisol production due to stress, illness and physical activity (14). In comparison to serum, saliva or urine tests, cortisol measurement in scalp hair provides a better estimation of long-term cortisol levels. With this relatively novel method it is possible to measure long-term cortisol levels during one or several months, as the growth rate of scalp hair is one centimeter per month (15,16). This study is the first study that uses cortisol in scalp hair for a cortisol assessment in HIV patients.

The aim of this cross-sectional study was to investigate the relation between the hair cortisol levels and the presence and severity of the metabolic complications in HIV-infected patients. In addition, we aimed to investigate whether hair cortisol levels in HIV-infected patients differ from those in a healthy control group.

Methods

We included 126 HIV-infected patients who participated in the longitudinal TREVI cohort

study at the Erasmus University Medical Center in Rotterdam, The Netherlands. A control group consisting of 191 healthy subjects was used for comparison of the hair cortisol levels (16). Participants that used systemic corticosteroids or topical corticosteroids on the scalp were excluded from this study. All participants included in this study signed informed consent. Approval was obtained from the local ethical committee.

Body height, weight, waist circumference and blood pressure were obtained from the patients at the time of inclusion. Hair samples of approximately 150 hairs were collected from all participants, cut off as close as possible to the scalp at the posterior vertex. Fasting blood samples from 91 of the 126 HIV patients were obtained in a period of two weeks prior to four weeks after the hair sample collection. General risk factors such as alcohol and nicotine use were assessed with a questionnaire. Patient files were accessed to review medical history and medication use.

Fasting blood glucose, insulin, total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol and triglycerides were measured as well as CD4+ cell count and HIV RNA load. The degree of insulin resistance was estimated with the use of the homeostasis model assessment of insulin resistance (HOMA-IR) (17).

The diagnosis MetS was applied following the NCEP ATP III criteria (18). A diagnosis of MetS was established when three or more of the five following conditions were met: waist circumference >102 cm in men and >88 cm in women, blood pressure ≥135 (systolic) or ≥85 (diastolic) mmHg, triglycerides >1.70 mmol/l, HDL-cholesterol <1.03 mmol/l in men and <1.29 mmol/l in women and plasma glucose levels ≥6.1 mmol/l. In addition, blood pressure and blood glucose were also considered elevated in patients who used antihypertensive medication and/or glucose lowering medication, respectively. In patients who were on lipid-lowering therapy, triglycerides levels were considered too high (>1.70 mmol/l) and HDL-cholesterol levels were considered too low (<1.03 mmol/l in men and <1.29 mmol/l in women).

Analyses of glucose and lipids were performed according to standard laboratory procedures. Fasting serum insulin levels were determined by chemiluminescent immunoassays (Immulite® 2000 Automated Immunoassay Analyzer), HIV RNA concentrations were determined with CAP-CTM HIV-1 v2 (Roche diagnostics, Almere, NL).

Cortisol measurement was performed using the proximal three centimeters of the hair strands, corresponding with long-term cortisol levels during the preceding three months. The hairs were processed as described previously (16). Cortisol levels were measured with a commercially available ELISA kit for salivary cortisol (DRG Instruments GmbH, Marburg, Germany). A correction factor was used on all hair samples to correct for background signal caused by the hair matrix (19).

Mean hair cortisol levels between the patient group and the control group were compared using ANCOVA adjusting for gender and age. Correlations between hair cortisol and anthropometric and cardiometabolic parameters in the HIV patients were analyzed using bivariate and partial Pearson correlation analysis, controlling for gender and age. Continuous variables were logarithmically transformed where appropriate. Spearman correlation analysis was used to investigate the role between HIV RNA concentrations and hair cortisol levels. To assess the risk of MetS in HIV patients, hair cortisol levels were divided in tertiles (low, intermediate and high hair cortisol levels). Logistic regression was used to examine the relationship between hair cortisol and the risk of MetS. These analyses were adjusted for gender and age. All analyses were performed with the use of IBM SPSS for Windows, version 21 (SPSS Inc., Chicago, Illinois.) A p-value < 0.05 was considered as a statistically significant difference.

For the glucocorticoid (GC) sensitivity assay, we adapted a well known assay based on gene expression profiles in peripheral blood mononuclear cells (PBMC's). Because this strategy is time consuming and requires a high blood volume, we optimized an evealuated a novel simple and fast method to assess GC sensitivity requiring only a few milliliters of blood. A total of 31 patients with known hair cortisol levels were selected. Whole PBMCs were stimulated for 4 hours with 10-7, 10-8, 10-9, 10-10 or 10-11 M dexamethasone (DEX). GR function was assessed by intracellular staining of Glucocorticoid Induced Leucine Zipper (GILZ) and interleukin 2 (IL-2) followed by subsequent flowcytometric analysis. For IL-2 expression, cells were initially primed with 10ug/ml phytohaemagglutinin (PHA). Patients with MetS and low hair cortisol (group 1, N=7) were compared to all others (group 2, N=34).

Results

Patients' characteristics are shown in Table 1. Gender and age were significantly different in the patient group compared to the control group. Three patients with hair cortisol levels exceeding four times SD were excluded from this study. Although not reported at the first visit, one patient appeared in retrospect to have used topical corticosteroids on the scalp. The cause of increased hair cortisol levels in the other two patients remains unknown.

Of the 91 patients from whom fasting blood samples were obtained, 23 (25.3%) met three or more ATP III criteria and were thus diagnosed with the MetS. The most frequent MetS criterion in the HIV group was elevated systolic blood pressure (52.7%), followed by reduced HDL-cholesterol (43.9%) and hypertriglyceridemia (42.9%). The presence of enlarged waist circumference (14.3%) and elevated blood glucose levels (13.2%) were both less frequent (Table 2).

Table 1. HIV patients and control group characteristics

	HIV patients	Controls	p
n	123	191	
Male, n (%)	102 (82.9)	88 (46.1)	< .001
Age , mean (SD), years	47.3 (11.5)	36.4 (12.3)	< .001
BMI, mean (SD), kg/m2	23.8 (3.9)	24.4 (3.9)	.26
Hair cortisol, mean (SD), pg/mg	16.4 (16.8)	13.5 (13.5)	.14
MetS, n (%)	23 (25.2)		
Time since HIV diagnosis, mean (SD), years	7.4 (6.6)		
Receiving cART, n (%)	113 (91.9)		
Time since start cART, mean (SD), years	5.4 (5.7)		
CD4+ cell count x106/l, med (IQR)	550 (400 - 740)		
HIV RNA load < 200 co/ml, n (%)	101 (82.1)		
Ethnicity			
Caucasian, n (%)	115 (93.5)	170 (89.0)	
Other, n (%)	6 (4.9)	18 (9.4)	
Unknown, n (%)	2 (1.6)	3 (1.6)	
Antiretroviral therapy			
2 NRTI'sa + 1 NNRTIb, n (%)	86 (70)		
Receiving PIc, n (%)	18 (14.6)		
Other, n (%)	19 (15.4)		

med: median, IQR: interquartile range, cART: combined antiretroviral therapy, NRTI: nucleoside reverse transcriptase inhibitor, MetS: metabolic syndrome, NNRTI: non-nucleoside reverse transcriptase inhibitor, PI: protease inhibitor.

Table 2 Anthropometric and (fasting) metabolic parameters of the HIV patients (n=91).

Waist circumference males, cm	87.9 (±9.8)
Waist circumference females, cm	89 (±15.1)
Systolic blood pressure, mmHg	130 (94 – 187)
Diastolic blood pressure, mmHg	81 (±12)
Triglycerides, mmol/l	1.34 (.40 – 5.74)
HDL-cholesterol, mmol/l	1.22 (.53 – 2.24)
LDL-cholesterol, mmol/	3.34 (±1.00)
Total cholesterol, mmol/l	5.05 (2.3 – 8.1)
Glucose, mmol/l	5.20 (4.0 – 9.7)
Insulin, pmol/l	75 (7.0 – 385)
HOMA-IR	2.83 (.20 – 11.6)
MetS, n (%)	23 (25.2)

Normally distributed data are presented as mean (±SD), non-normally distributed data as median (range).
HDL: high-density lipoprotein, HOMA-IR: homeostatic model assessment insulin resistance, LDL: low-density lipoprotein, MetS: metabolic syndrome.

The mean hair cortisol levels in the patient group were not significantly different from the control group (p = .14), also after correction for gender and age (p = .20). However, there was a significant difference in the mean hair cortisol levels of HIV patients without MetS compared to the control group (17.3 pg/mg vs. 13.5 pg/mg, p = .03).

Hair cortisol levels were not correlated with age or gender in both the patient group and the control group. In the HIV group, there was a significant positive relation between hair cortisol levels and body mass index (BMI) (r = .180, p = .05). In the control group, no significant relation between hair cortisol and BMI was present (p = .18). There were no significant differences in gender, CD4+ cell count or HIV RNA load between the HIV-infected patients with MetS compared to the non-MetS patients. After correction for age there were no significant differences in time since first HIV diagnosis and duration of cART use between MetS and non-MetS patients (10.2 years vs. 6.4 years, p = .30 and 8.0 years vs. 4.6 years, p = .18 respectively). In both the HIV and control group no relation was found between hair cortisol levels, smoking status or alcohol use (p > .05).

We found no significant correlation between hair cortisol levels and LDL-cholesterol, total cholesterol, waist circumference, CD4+ cell count, insulin, glucose or HOMA-IR in the HIV patients. A positive correlation between hair cortisol and HDL-cholesterol was found in both the bivariate correlation ($r = .381$, $p < .001$) and in the adjusted correlation ($r = .341$, $p = .001$) (Table 3). In the HIV patients with a MetS diagnosis, the correlation between hair cortisol and HDL-cholesterol was stronger than in the total HIV group ($r = .751$ $p < .001$ in the non-adjusted model and $r = .769$, $p < .001$ in the model corrected for gender and age) (Figure 1).

Table 3. Bivariate and adjusted Pearson and Spearman correlations between hair cortisol levels and anthropometric and cardiometabolic parameters in HIV-infected patients.

	r	r (adjusted)b
BMI	.180*	.040
Waist circumference	.074	.029
Systolic bp	−.052	−.087
Diastolic bp	.067	.034
Glucose	.130	-.049
Insulin	.033	-.002
HOMA-IR	.032	0.00
Triglycerides	−.097	−.085
HDL-cholesterol	.381***	.341**
LDL-cholesterol	.007	−.107
Total cholesterol	.110	.054
CD4+ cell count	.103	.049
HIV RNAa	−.105	

a Spearman correlation analysis was used because of a non-normal distribution, b Adjusted for gender and age, bp: blood pressure, HOMA-IR: homeostatic model assessment insulin resistance, HDL: high-density lipoprotein, LDL: low-density lipoprotein.
* $p < .05$, ** $p < .01$, ***$p < .001$.

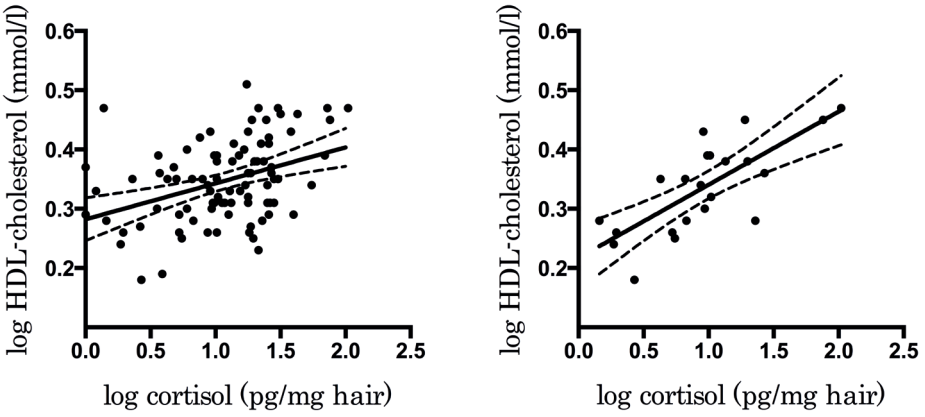

Figure 1. Correlation of hair cortisol with HDL-cholesterol in (a) the total HIV patients group (n=90, r = .381, p < .001) and (b) in the MetS diagnosed group (n = 22 r = .751, p < .001). The best-fit line and its 95% confidence interval (CI) are shown.

Hair cortisol levels were divided in tertiles (low-, intermediate- and high hair cortisol levels) and the odds ratio (OR) for MetS was calculated for each tertile. The highest hair cortisol tertile was considered the reference tertile. Cut off points were 8.33 and 19.35 pg/mg hair. In the unadjusted model, the OR for MetS was 2.9 (p= .09, 95% CI: 0.86 – 9.75) in the lowest hair cortisol tertile compared to the highest hair cortisol tertile and 1.5 (p= .56, 95% CI: 0.41 – 5.23) for the intermediate hair cortisol tertile compared to the high hair cortisol tertile. After correction for gender and age, the OR for the lowest hair cortisol group was 4.23 (p = .04, 95% CI: 1.09 – 16.47) and for the intermediate hair cortisol group was 1.65 (p = .48, 95% CI: .42 – 6.55) compared with the highest hair cortisol group (Figure 2).

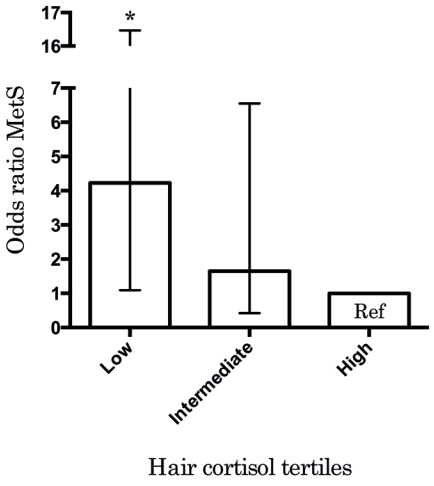

Figure 2. Odds ratios with 95% confidence interval for metabolic syndrome (MetS) diagnosis according to hair cortisol tertiles. Both in the unadjusted model, as well as after correction for gender and age (shown in figure) the risk of MetS was highest in HIV patients with the lowest long-term cortisol, as measured in scalp hair.
Ref: reference tertile, * $p < .05$.

For the expression of GILZ and IL-2, stimulation with high dose (10-7)M DEX resulted in a 14,6 fold increase in GILZ positive and a 1,3 fold mean decrease in IL-2 positive CD8 T-cells (data not shown). GILZ frequencies were significantly higher in HIV patients with both MetS and low hair cortisol (group 1) compared to others (group 2) after stimulation with 10-7, 10-8, 10-9 M DEX ($p<0,05$). There was no difference in the frequency of IL-2 producing cells between the two groups.

Discussion

This study has been the first to study long-term cortisol levels using hair analysis in relation to MetS in HIV-infected patients. Interestingly and in contrast to our hypothesis, we found that the odds for the MetS are 4.2 times higher in the patients group with the lower hair cortisol levels compared to the patients group with the higher hair cortisol levels. This result differs from the results from recent published studies in non-HIV infected subjects in which the risk of MetS was significantly higher in the highest hair cortisol quartile compared to the lowest hair cortisol quartile (20,21). Taken the literature into account, the results of this study could point toward a modulatory effect of cortisol in de development of metabolic complications in HIV-infected patients.

In addition, we found that hair cortisol levels in HIV-infected patients on average are not different from healthy persons. Interestingly, there is a positive correlation between hair cortisol and HDL-cholesterol that became even stronger when stratified for patients with MetS. These results are in contrast with previous observations in the (non HIV-infected) general population in which cortisol levels are negatively correlated with HDL-cholesterol (20,22).

The low hair cortisol in HIV patients with increased risk of MetS could hypothetically be explained by a hypersensitivity to cortisol. The effects of cortisol are mediated by the glucocorticoid receptor (GR), which is an important factor in determining cortisol sensitivity at a cellular level. Under physiological stress, including during HIV infection, GR function at tissue level is disturbed (23). Cortisol hypersensitivity is characterized by low systemic cortisol levels due to increased negative feedback action. Cortisol hypersensitivity could explain the signs of hypercortisolism in our cohort (23). Intracellular detection of GILZ and IL-2 as a correlate of GR sensitivity seems a promising tool and valuable alternative to the currently used gene expression assays. Using our novel flowcytometry based method; we demonstrated increased GR sensitivity in HIV infected individuals with MetS and low hair cortisol.

Several mechanisms have been described which could explain an increased cortisol exposure at the tissue level or cortisol hypersensitivity in HIV patients. The first possibility is that some HIV patients may have enhanced activity of the enzyme 11β- hydroxysteroid dehydrogenase type 1 (11β-HSD1). This enzyme converts the inactive metabolite cortisone in the active cortisol, resulting in increased cortisol activity at tissue level (24). Multiple pro-inflammatory cytokines such as TNF-alpha (TNF-α) and interleukine-1 beta (IL-1β) can increase the activity of 11β-HSD1 in vitro (25,26). A study concerning 30 HIV-infected participants showed that 11β-HSD1 mRNA concentrations in adipose tissue were three times as high in HIV-infected patients with lipodystrophy compared to HIV patients without lipodystrophy (12). Another possibility is the presence of the viral protein R (Vpr) in these patients. Vpr is a 96-amino acid virion associated accessory HIV protein that is required for replication of HIV in non-dividing cells (27). Vpr is a co-activator of the GR (28) and it is therefore plausible that it causes cortisol hypersensitivity.

We also looked if altered cortisol clearance can play a role in the results that are found in this study. Cortisol is partly metabolized in the liver by CYP3A4 enzymes. Many antiretroviral drugs can alter CYP3A4 activity. Protease inhibitors (PI's) can inhibit CYP3A4 enzymes, whereas almost all non-nucleoside reverse transcriptase inhibitors (NNRTI's) can induce CYP3A4 enzymes (31). In this study 18 patients were on a medication regime that consisted of a PI without a NNRTI (PI group) and 86 patients received one or more NNRTI's without receiving a PI (NNRTI group). We found no statistically significant difference in hair cortisol levels between the PI group and NNRTI group (18.0 pg/mg vs. 17.2 pg/mg, $p = .58$). Therefore, it seems unlikely that altered cortisol clearance mediated by cART influences the results found in this study.

The strength of our study includes a well monitored and representable chronically HIV infected cohort. All samples were collected after overnight fasting. In contrast to previous studies using single time point (saliva, serum) or short-term (24h urine) cortisol measurements, we used a relatively novel but well validated, method to determine long-term systemic cortisol levels in scalp hair (32). This method has been investigated within the context of a wide variety of clinical conditions such as (cyclic) Cushing's syndrome, cardiovascular disease and obesity (33-35).

Our study does have some limitations. The cross-sectional design does not allow us to assess whether a person with MetS has a propensity to remain in the lower tertile of cortisol over time. In addition, the observed associations do not determine whether the lower mean hair cortisol levels in HIV patients with MetS are cause or result of the metabolic complications in these patients. In the control group, no in depth phenotyping with respect to the metabolic syndrome was performed. The definition of MetS using the NCEP ATP III criteria is useful in clinical practice and is closely related to the Framingham risk score, a tool that has gained great interest in HIV care. However, some elements in these tools are based on snapshots of patient characteristics, such as systolic blood pressure, plasma cholesterol and glucose. Therefore, results should be interpreted in the context of patient care.

In conclusion, we found that relatively low long-term cortisol levels, measured in hair, are associated with an increased risk of MetS in HIV-infected patients. These results suggest that HIV patients with MetS and low hair cortisol levels may be cortisol hypersensitive. We performed further research by indirect ex vivo measurement of GR expression and bio-activity in HIV infected patients with MetS and low hair cortisol levels (36). We showed that MetS in HIV may be partially related to GC hypersensitivity, potentially leading to new treatment opportunities for these metabolic abnormalities. It would be interesting to elucidate the mechanism of cortisol metabolism and the exact role cortisol plays in the development of metabolic derangements in HIV-infected patients.

6

Chapter 7 von Willebrand Factor is elevated in HIV patients with a history of thrombosis

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Abstract

Arterial and venous thrombotic events are more prevalent in HIV infected individuals compared to the general population, even in the era of combination antiretroviral therapy. Although the mechanism is not fully understood, recent evidence suggests a role for chronic immune activation. We reviewed the Dutch National HIV registry database for HIV infected patients in Rotterdam with a history of arterial or venous thrombosis and calculated the incidence. We collected samples from patients with and without thrombosis and compared plasma levels of lipopolysaccharide (LPS), LPS binding protein (LBP), soluble CD14 (sCD14), and von Willebrand Factor antigen level (vWF). During a 10-year period, a total of 60 documented events in 14,026 person years of observation (PYO) occurred, resulting in an incidence rate of 2.50, 2.21 and 4.28 for arterial, venous and combined thrombotic events per 1000 PYO, respectively. The vWF was elevated in the majority of study subjects (mean 2,36 SD±0.88 IU/ml); we found a significant difference when comparing venous cases to controls (mean 2.68 SD±0.82 IU/ml vs 2.20 SD±0.77 IU/ml; p=0.024). This difference remained significant for recurrent events (mean 2.78 SD±0.75p=0,043).). sCD14 was positively correlated with LPS (r=0.255; p=0.003). The incidence of venous thrombosis was twofold higher in HIV infected patients compared to age-adjusted data from general population cohort studies. We couldn't find a clear association between immune activation markers to either arterial or venous thrombotic events. We observed a marked increase in vWF levels as well as a correlation of vWF to first and recurrent venous thrombo-embolic events.

These findings suggest that HIV infection is an independent risk factor for coagulation abnormalities and could contribute to the observed high incidence in venous thrombosis. This could be a reason to prolong anti-thrombotic treatment in HIV patients with a history of thrombosis.

Introduction

The delicate interaction between inflammation and coagulation has long been recognized and persists to play a pivotal role in numerous infections such as sepsis and viral hemorrhagic diseases. In chronic HIV infection, the hemostatic balance is tipped towards a more pro-coagulant status, resulting in thrombosis [138]. This is supported by both epidemiological and experimental data.

The annual risk on venous thrombosis in a representable European population below the age of 60 is slightly over 1 per 1000 person years of observation (PYO) [139] [140]. For arterial thrombosis, the annual risk is higher with incidence rates between 2-3 per 1000 PYO for myocardial infarction and around 0.65 per 1000 PYO for stroke [141] [142]. It is likely that HIV infection independently adds to this risk [143]. A nationwide study comprising of 4,333 HIV infected individuals reported an incidence of venous thrombosis in 3.2 per 1000 PYO [144]. In addition, events occur at a significantly younger age compared to the control population [145] [146]. Similar trends have been observed in myocardial infarction [147], stroke [148] and peripheral atherosclerosis [149]. A large tri-continental study determined an incidence of 5.7 per 1000 PYO for first cardio- or cerebrovascular event in a relatively young cohort [150] with cumulative exposure to cART as a major contributor [151] [43].

The exact pathophysiology of increased thrombotic activity in HIV remains unknown but recent publications advocate a role for chronic immune activation. In this hypothesis, HIV infection causes a loss of mucosal integrity in the gut together with CD4 T-cell depletion in local lymphoid tissue. This results in translocation of microbial products from the lumen to the circulation [34]. Bacterial endotoxins such as LPS are subsequently bound to pattern recognition receptors and trigger a potent inflammatory response in monocytes and macrophages [152] [153]. LPS, LBP and soluble CD14 levels have been found to correlate with a hypercoagulable state in chronic HIV, with or without combined cART [154] [134] [101] [155]. Although it is conceivable that immune activation accelerates clot formation, the exact mechanism remains to be elucidated.

In this study we addressed HIV as a common risk factor for both arterial and venous thrombosis and investigated chronic immune activation as the proposed driving mechanism. To test this hypothesis, we assessed the incidence of venous and arterial thrombotic events in a chronically infected HIV population. We compared coagulation (vWF), microbial translocation (LPS and LBP) and inflammatory parameters (sCD14) of patients with a past thrombotic event to patients without an event. We hypothesized that HIV infected individuals with a past thrombotic event have a higher exposure to microbial-driven immune activation.

Materials and Methods

Patients and study design

On February 25th 2013, we retrieved information from the Stichting HIV Monitoring (SHM) database, described elsewhere, which includes anonymized data obtained from treated and untreated HIV-infected patients, who have been followed in or after 1996 in our hospital [156]. Cases were defined as patients with a thrombotic event and a presumed or definite preceding HIV diagnosis. A preceding HIV diagnosis was presumed when the CD4 cell count was <200/mm3 within one year of the HIV diagnosis. Venous thrombo-embolic events included deep venous thrombosis (DVT), diagnosed by compression ultrasonography; or pulmonary embolism (PE), diagnosed by Computer Tomography (CT) pulmonary angiography. Arterial thrombo-embolic events included myocardial infarction (MI), diagnosed by electrocardiogram and cardiac biomarkers; cerebrovascular incident (CVA) or ischemic attack (TIA), diagnosed by neurological examination in combination with CT scan results; and claudication intermittens (CI) diagnosed by the ankle-brachial index. Cases were compared to randomly selected controls i.e. patients with HIV infection but no thrombo-embolic event in their history. This control population was comparable to the general HIV population in Rotterdam with respect to age and sex. All cases and controls were offered a questionnaire concerning classical risk factors. This research was approved by the local ehtics committee, patients had to sign an informed consent document to participate.

LPS, LBP, sCD14 and vWF measurements

We collected plasma from 65 cases and 65 control patients for analysis of LPS, LBP, sCD14 and vWF. The patient blood samples were collected in Ethylene diamine tetra acetic acid (EDTA). These were initially intended for viral load analysis and stored at -80° Celsius. The mean time from thrombotic event to blood sample collection was 6.2 years (SD±4.7). Samples were thawed only once to prevent protein degradation. LPS levels were measured using the Pyros Kinetix Flex® chromogenic endotoxin detection system (Associates of Cape Cod). Plasma samples were heat inactivated at 60°C and subsequently diluted with LPS free LAL H2O at concentrations varying from 1:20 to up to 1:400. Exact LPS quantities were derived from a standard curve of known control endotoxin concentrations. LBP was measured using a LBP ELISA kit® (Hycult biotech); soluble CD14 was measured using the Human sCD14 Quantikine ELISA Kit® (R&D systems); vWF antigen levels were measured with an in-house ELISA using DAKOPATTS antibodies and compared to the vWF antigen levels to commercially available pooled plasma from 20 or more otherwise healthy donors (CRYOcheck™).

Data analysis

For the calculation of incidence, we used available SHM data on all HIV patients in care at the Erasmus MC from February 25th 2003 to February 25th 2013. The incidence rate of an event was calculated as the number of documented cases occurring in a 10 year time period, divided by the total amount of unique person-years contributed to the cohort. Data was censored when

an event occurred, when the patient died or on the index date, whichever came first. Cases with both arterial and venous events were censored on whichever date came first, but were included in both calculations.

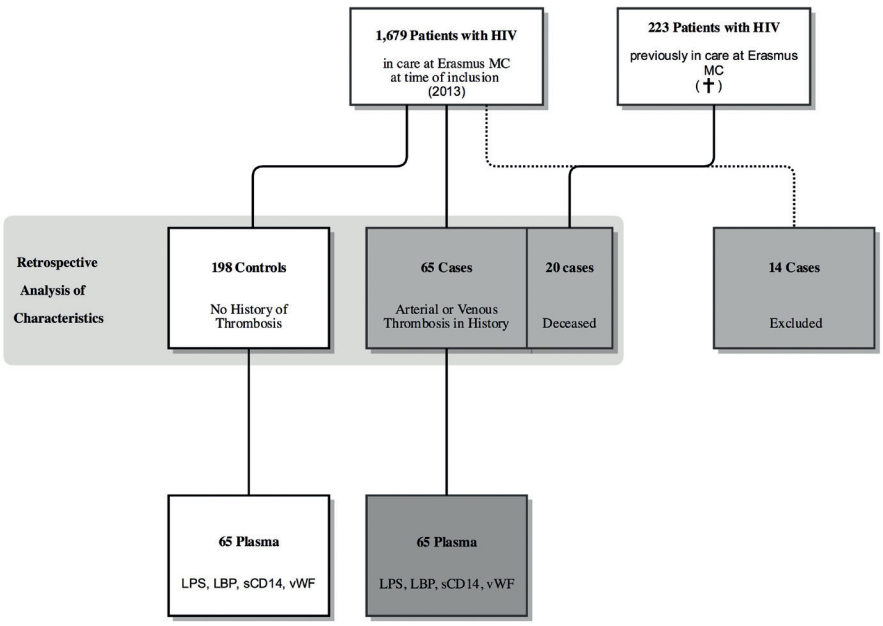
Normality of data was assessed by a Shapiro Wilk test and inspection of Q-Q Plots. Homogeneity of variance was assessed by Levene's Test for Equality of Variances. An independent T-test or Man-Whitney was performed for continuous variables when appropriate. vWF was normally distributed, and sCD14, LBP and LPS showed a non-parametric distribution. For categorical variables, a Chi-Square test was applied. Correlation analysis was performed using the Spearman's Rank-order test. A significance level of 0.05 was applied. All statistical analysis was performed using SPSS software, version 21 (IBM corp ©). Graphs were constructed using GraphPad Prism software, version 6.

Results

From February 25th 2003 to February 25th 2013, a total of 2,731 unique patients were registered in the Rotterdam SHM database, representing 14,026 person years. A total of 60 thrombotic events occurred during this 10-year period. The overall incidence of both arterial and venous thrombotic events combined was 4.28 per 1000 PYO. These rates were 2.50 and 2.21 for arterial events and venous thrombotic events, respectively. The incidence of patients with a recurrent event was 0.93 in the arterial group and 0.57 in the venous group per 1000 PYO when divided by the total amount of person years. When divided by the corresponding amount of person years in the specific event group only, the incidence increased to 95 in the arterial group and 73 in the venous group per 1000 PYO. In the group that had experienced a venous thrombosis, the incidence of patients that had a recurrence of a venous event was 30% after a period of 3 years.

At the time of inclusion on February 25th 2013, a total of 1679 HIV-infected patients were still in care out of which 79 were reported with a thrombotic event (see figure 1). Of these 79 patients, 10 patients were excluded based on a negative HIV test at the time of event and 4 patients refused participation, resulting in 65 available cases. In addition, we searched the database for patients that died between April 1st 2002 and November 22nd 20012 and had a history of thrombosis In total, 223 patients died during this period of which 20 had a documented thrombotic event in the past. Of these 85 patients, 37 were diagnosed with a PE of DVT; 41 with a MI, CVA or CI; and 7 had endured both a venous and arterial event.

Figure 1. Flow chart depicting the selection of patients for this study.



A random selection of the patients registered in the Rotterdam SHM database (as described earlier) was applied to select a control group of 198 HIV-infected patients. This group was comparable to the whole HIV population at the Erasmus Medical Center in respect to age and sex. When compared to the 198 controls at the time of observation, cases with arterial and venous events were significantly older (46.85 SD±11.10 vs 57.83 SD±11.01 years p<0.001 (arterial) vs 52.80 SD±11.21 p=0.007 (venous)), more likely to be of Caucasian descent (46.5% vs 70.7% p=0.005 (arterial) vs 67.6% p=0.018 (venous)) and were more likely to have history of hypertension (8.1% vs 39% p<0.001 (arterial) vs 21.6% p=0.013 (venous)) and malignancy (6.1% vs 19.5 % p=0.005 (arterial) vs 21.6% p=0.002 (venous)). Compared to controls, an arterial event was specifically associated with male sex (71.2% vs 90.2% p=0.011), a positive family history for cardiovascular events (17.7% vs 39.0% p=0.002) and diabetes mellitus (4.5% vs 22.0% p<0.001). There was no difference between cases and controls for traditional risk factors such as a high body mass index, smoking or immobilization. No difference was found for HIV related risk factors such as a low CD4 nadir count or protease inhibitor (PI) use (table 1).

Table 1. Patient Characteristics.

	Controls (N=198)		Cases (85)		P-value
Males (%)	141	(71.2)	70	(82.4)	*0.049*
Venous events only			28	(75.7)	*0.579*
Arterial events only			37	(90.2)	*0.011*
Both events only			5	(71.4)	*0.990*
Age (SD)	47	(11.1)	56	(11.0)	*<0.001*
Venous events only			53	(11.2)	*0.007*
Arterial events only			58	(11.0)	*<0.001*
Both events only			58	(7.7)	*0.008*
Caucasian (%)	92	(46.5)	61	(71.8)	*<0.001*
Venous events only			25	(67.6)	*0.018*
Arterial events only			29	(70.7)	*0.005*
Both events only			5	(71.4)	*0.005*
CD4 nadir (IQR)	180	(67.5-300)	170	(65-275)	*0.852*
Venous events only			190	(70-275)	*0.931*
Arterial events only			170	(60-315)	*0.874*
Both events only			140	(40-260)	*0.575*
HIV RNA undetectable at inclusion (%)	168	(84.8)	78	(91.8)	*0.114*
Venous events only			35	(94.6)	*0.113*
Arterial events only			36	(87.8)	*0.626*
Both events only			7	(100)	*0.265*
cART use	183	(92.4)	80	(94.1)	*0.610*
Venous events only			35	(94.6)	*0.640*
Arterial events only			38	(92.7)	*0.954*
Both events only			7	(100)	*0.449*
PI use	58	(29.3)	31	(36.5)	*0.233*
Venous events only			13	(35.1)	*0.477*
Arterial events only			16	(39.0)	*0.220*
Both events only			2	(28.6)	*0.967*

For patient characteristics of patients that underwent analysis of sCD14, LPS, LBP and vWF please see supplementary table 1. Patients with a thrombotic event in the past had a higher mean vWF antigen level when compared to controls (venous: 2.68 SD±0.82 IU/ml; arterial: 2.31 SD±1.10 IU/ml; both: 2.74 SD±0.90 IU/ml; controls: 2.20 SD±0.77 IU/ml). The difference in vWF antigen levels was significant in patients with a past venous event compared to controls (p=0.024, Independent samples T-test) (figure 2), which remained significant when comparing only recurrent events to controls (2.78 SD±0.75; p=0.043 Independent Samples T-test, figure 3). The significance of difference in vWF antigen level between cases combined versus controls varied upon the applied statistical test (p=0.047, Mann Whitney; p=0.071 Independent samples T-test). There was no clear connection between time since thrombotic event and the level of vWF antigen (see figure 4). There was no significant difference between cases and controls in sCD14 (median: 2.45 IQR±2.08 vs 2.31 IQR±1.36; p=0.341) and LBP (median: 20.19 IQR±17.56 vs 16.63 IQR±15.05 µ/ml; p=0.264) as well as LPS (median: 12.19 IQR±24.70 vs 10.76 IQR±23.58) although values tended to be higher in cases (figure 2).

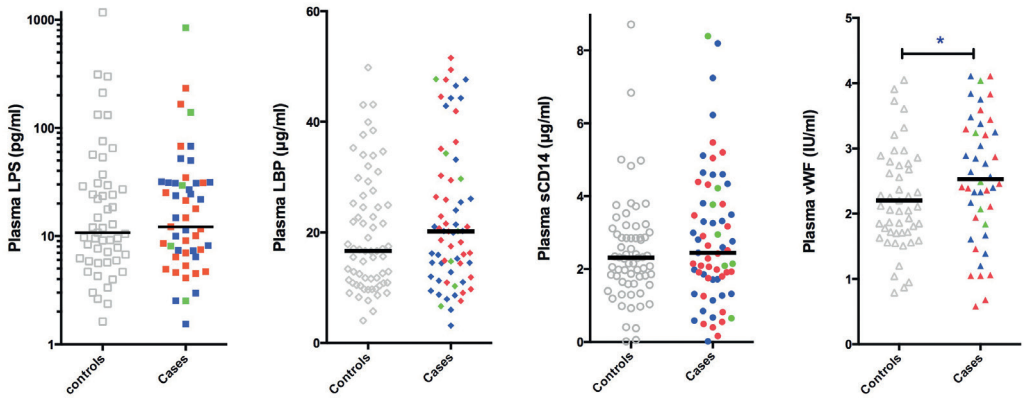


Figure 2. Plasma levels of LPS (N=101), LBP (N=130), sCD14 (N=130), and vWF antigen (N=93) in chronic HIV infected individuals with or without a history of arterial or venous thrombotic disease. Horizontal bar represents median for LPS, LBP and sCD14; and mean in vWF. Cases had a significantly higher vWF antigen level as compared to controls. Grey: controls

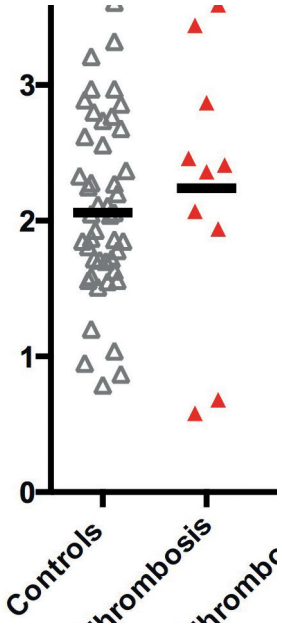


Figure 3. vWF antigen in individuals with HIV without thrombosis compared to HIV infected individuals with a recurrent venous or arterial trombotic event. Patients with a recurrent venous thrombotic event had a statistically significant higher vWF (p=0.043). vWF: von Willebrand Factor antigen level.

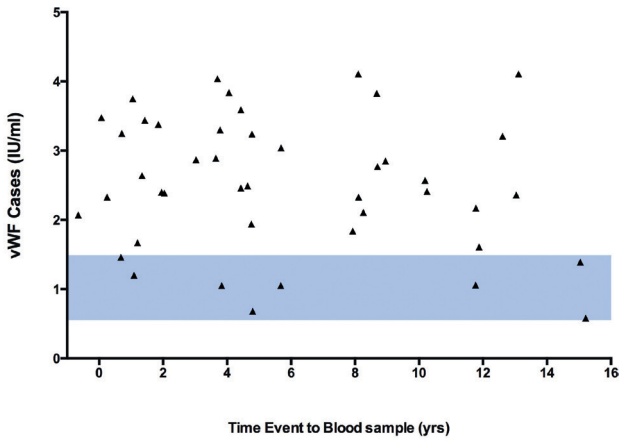


Figure 4. Time from event to blood sample collection plotted against vWF antigen level in HIV infected individuals with an arterial or venous event. The level of vWF antigen is considerably higher than the reference value. vWF= von Willebrand Factor antigen. Red: arterial events; blue: venous events; green: both arterial and venous events. Light blue area: reference value for vWF antigen level.

The only association detected was a positive correlation between levels of soluble CD14 and log 10 levels of LPS (p=0.003; r=0.255, fig 5A). There was also a weak trend toward positive correlation between plasma levels of vWF and sCD14 (p=0.078; r=0.184, fig 5D). The four other combinations of markers (vWF versus LBP; vWF versus log 10 LPS; sCD14 versus LBP; LBP versus log 10 LPS) were not correlated (all p values > 0.1).

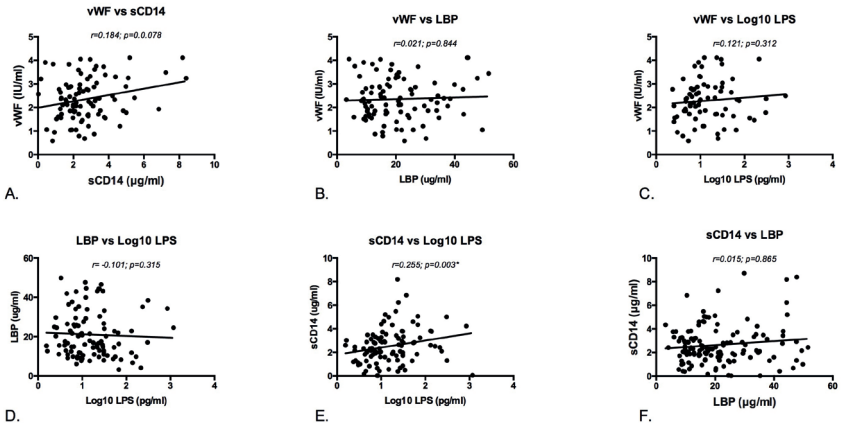


Figure 5. Correlation analysis of sCD14, LBP, LPS (log-transformed) and vWF. A Spearman's Rank-order test was used for analysis. Only sCD14 and LPS (log-transformed) had a statistically significant correlation (A). There was a weak trend toward positive correlation between vWF and sCD14 (D). LPS: Lipopolysaccharide; LBP: LPS binding protein; sCD14: soluble CD14; vWF: von Willebrand factor antigen level.

7

Discussion

Our results confirm previous observations of thrombosis being a prevalent co-morbidity in the HIV population, even in a cohort with prolonged c-ART treatment. Comparable to uninfected individuals, traditional risk factors such as diabetes, malignancy, hypertension and a positive family history continue to contribute to the development of an event, but additional factors play a role. This study was aimed to investigate the incidence of arterial and venous thrombosis in patients living with chronic HIV infection. In addition, we wanted to address what factors predispose HIV patients for the occurrence of a thrombotic event and by which mechanism.

To answer the first question, we performed a retrospective analysis. The incidence of arterial and thrombotic events combined was 4.28 per 1000 PYO. We found an incidence of 2.21 per 1000 PYO of venous thrombotic events, which is more than twofold higher compared to healthy controls from large cohort studies [140]. This result corroborates with literature where the risk on venous thrombotic disease is reported to be 2 to10-fold increased [157,158]. The variation in observed incidences between studies is probably related to the nature of these studies, mostly retrospective, the difference in diagnostic criteria and cohort characteristics such as immune status of patients. An increasing amount of evidence in the literature supports the notion that HIV status is associated with increased risk on myocardial infarction, stroke and venous thrombo-

embolism [159]. With the aging of HIV cohorts and related increased exposure to cART, it remains important to re-evaluate this incidence rate. The incidence rate for arterial events in our study was lower than previously found. Compared to the Data Collection on Adverse Events of Anti-HIV Drugs (DAD) Study Group, the incidence in our study is twofold lower [150]. The prevalence of risk factors such as smoking, diabetes and hypertension, were comparable in both studies. One difference is a high use of PI's in the DAD study cohort. PI's are known to cause metabolic abnormalities and could add in disease severity, although a direct causal relationship with arterial disease remains controversial [160].

In HIV infection, several well-defined coagulation abnormalities exist, such as Activated Protein C resistance, Protein S deficiency, increased D-dimer, Tissue Factor expression on monocytes and increased levels of vWF. In this study, we choose to examine vWF as a prognostic marker to investigate the predisposition to a thrombotic event. This protein is crucial in both primary and secondary hemostasis. It is produced almost exclusively in the endothelium and is stored as large multimers in vesicles called Weibel Palade bodies. The vWF antigen level in HIV patients was two-fold higher compared to the reference material (i.e. healthy donors), which implies an increased coagulation potential in all HIV infected patients. Several mechanisms could potentially result in a high level of vWF antigen. A delayed clearance of vWF, as is seen in conditions such as thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS), is unlikely the cause of high vWF antigen in HIV. The activity of ADAMTS13, the metalloproteinase responsible for clearing vWF, can be lower in HIV, but does not reach the levels needed for overt pathology (Jong et al, unpublished data) [161]. In addition, antibodies against ADAMTS13 are incidental in HIV related TTP [162]. It seems more logical to attribute the high vWF to increased transcriptional activity in endothelial cells due to chronic immune activation [163]. The production, storage and exocytosis of vWF are regulated through intrinsic and extrinsic factors such as, but not limited to nitric oxide, hypoxia, histamine, thrombin and other secretagogues. LPS, for instance, could be a potent stimulator of endothelial cells. Therefore, we explored the possibility of immune activation as a driving factor behind arterial and venous thrombosis. It is likely that monocytes rather than T-cells are responsible for the initial immunologic response to LPS translocation, considering these cells express CD14 and TLR4, the receptors for LPS. Although markers of coagulation and immune activation are partially restored upon initiation of cART, a complete normalization fails to appear [101] [79]. In the SMART study, a study aimed to investigate the benefit of a CD4 guided approach on treatment [164], markers of inflammation and coagulation such as IL-6, D-dimer and sCD14 were shown to be excellent predictors of atherosclerosis and mortality [165] [166] [134]. If immune activation is chronic and predisposes patients for morbidity, we expected to find higher baseline levels of these markers in patients with a thrombotic event in the past. Although levels of immune activation tended to be at the high end in all patients, we could not detect a significant difference between cases and controls. Correlation analysis revealed only a significant correlation between sCD14 and LPS (fig 5A). This could represent the increased inflammatory response of

monocytes on microbial products. It is reasonable to assume that immunologic and hemostatic factors can mutually influence each other. However, all the other associations we describe in this manuscript were non-significant.

Taken together, these data support the hypothesis of an increased activity in coagulation, although the exact mechanism remains to be elucidated. The presence of endothelial cell activation, increased fibrin formation and decreased anticoagulation, as observed in HIV infected patients, is compatible with a pro-thrombotic state [154] [167] and mirror markers of immune activation [168]. Additive pro-coagulant mechanisms such as increased tissue factor activity on monocytes as proposed by Funderburg et al [169] could be involved alongside endothelial cell activation. In the end, the model of chronic immune activation as proposed by Brenchley et al. [34], has given rise to new insights in the pro-thrombotic state in HIV. In the meanwhile, we should consider HIV as an independent risk factor for thrombosis. Possible strategies to further investigate immune activation as a causative mechanism could include the use of anti-inflammatory agents or early initiation of therapy. Further research on the specific interaction between endothelial cells and monocytes during HIV infection would be especially interesting. Caution has to be made in conferring primary prevention of thrombosis in HIV patients, since anti-coagulation has potentially serious side effects. It may be worth considering extending secondary prophylaxis in HIV patients with a history of venous thrombosis, since the recurrence rate is relatively high. Although we found a significant difference in vWF between venous recurrences and controls, the wide distribution of values prevents an accurate discrimination between these two, limiting vWF as a predictor for venous thrombosis.

The strength of our study is the detailed baseline demographic and clinical data recorded from a well-characterized population in the SHM cohort. Our study does have several limitations. The study is retrospective, relying largely on the quality of existing data. We are aware of the fact that our data reflect only documented thrombotic events, so the incidence calculated in our study probably underestimates the real number. The ideal study would be a prospective study with comparable person years of inclusion and blood samples before and after an event. We realize this is a laborious task considering practical issues. In addition, HIV-positive patients were mostly on cART, so the individual contribution of HIV infection and cART to immune activation biomarkers could not be distinguished. Our experiments could not establish a relationship between LPS, LBP, sCD14 and event status. A possible explanation could be the large interval between occurrence of the event and measurement of the parameters (mean = 6.2 years later). We would expect a higher level of immune activation markers around the time of the event. Although microbial translocation has gained much attention as a potential driver for immune activation, it should be stated other mechanisms have not been addressed in this study. We cannot exclude other potential mechanisms such as residual viral replication, CMV seropositivity and pyroptosis [35] as a driver for coagulation abnormalities.

In conclusion, our study confirms previous findings that HIV-infection results in a pro-thrombotic state, reflected by a high incidence in venous thrombotic events and a high percentage that experiences a recurrence. Although we did not see a clear association between markers of immune activation to event status, we did encounter a significant difference in vWF levels between patients with a past venous thrombo-embolism and those without. These data support the rationale to extend anticoagulant therapy once venous thrombosis has occurred. However, further investigation on e.g. fVIII and other pro-coagulants is needed. The wide distribution of vWF in our patient groups does not support the use of this marker as a clinical predictor for recurrent thrombosis in HIV patients.

	Controls (N=65)		Cases (N=65)	
Males (%)	54	(83)	54	(83)
Age (SD)	54	(11)	56	(11)
Caucasian (%)	32	(49)	47	(72)
CD4 nadir (IQR)	164	(40-250)	204	(70-305)
HIV RNA undetectable at inclusion (%)	57	(87,7)	60	(92)
cART use (%)	62	(95)	63	(97)
PI use (%)	25	(38)	18	(28)

Supplementary table 1. Patient characteristics of the 130 patients of whom samples were obtained. Cases and controls were age- and sex matched as a group.

7

Chapter 8 Circulating Endothelial Cells, as a marker for vascular damage, are increased in HIV infected children

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(Manuscript in preparation)

Abstract

Circulating endothelial cells (CECs) are mature endothelial cells discarded from the endothelium during normal homeostasis. An increased number of CECs is found in response to vascular damage, which has been demonstrated during chronic HIV infection in adults. Vascular damage might lead to vascular complications such as myocardial infarction and cerebrovascular events that are more prevalent in HIV infected adults. We hypothesized that CECs are also increased in HIV infected children. In addition, we investigated the potential correlation of CECs with metabolic parameters associated with increased risk on vascular damage. Peripheral blood was obtained from 40 HIV-infected children (age 5-20 years) at the ErasmusMC-Sophia children's hospital in Rotterdam, the Netherlands. Blood from 30 uninfected adults was used as control, as well as blood from 31 age-matched pediatric controls undergoing elective surgery. CECs were examined for associations with immunologic and metabolic parameters. The median CEC count per 4 mL was significantly higher in HIV infected children compared to uninfected adults (p=0,0001) but not compared to uninfected children (p=0.261). There was a significant but weak association between CECs and duration of antiretroviral therapy (r= -0,330; p=0,038). CECs are increased in treated HIV infected children compared to healthy adults, but not compared to children undergoing elective surgery in this study. We found a significant but weak inverse correlation with the duration of antiretroviral therapy.

8

Background

Vascular complications such as myocardial infarction and cerebrovascular events are more prevalent in the HIV population [28,144]. Although classical risk factors such as smoking, obesity and hypertension remain prominent predictors, other contributing factors seem to play a role in HIV. Increased tendencies of metabolic abnormalities like dyslipidemia have been demonstrated previously and a strong correlation has been established with the use of Protease Inhibitors [151]. In addition to antiretroviral therapy, chronic immune activation has been suggested as a potential driving mechanism in the occurrence of co-morbidities, but studies so far have not been able to prove this hypothesis [170]. Several small studies support the hypothesis that endothelial dysfunction associated with HIV viremia is an important contributor to the elevated cardiovascular disease risk in patients with HIV infection. [11]

The inner lining of the vasculature is composed of a continuous layer of endothelial cells. The function of the endothelium is complex and diverse. It acts as a barrier to separate the intravascular blood flow from the interstitium and regulates coagulation by the production von Willebrand factor. In addition, the endothelium promotes diapedesis of leucocytes during inflammation. Under normal conditions, vessel formation is established by sprouting of existing vessels (angiogenesis); through proliferation of resident or bone marrow derived EPCs (vasculogenesis); and splitting of vessels called intussusception. The exact embryonic origin of endothelial cells remains elusive, but studies have shown a close relationship to the hematopoietic lineage [171]. Several studies have investigated the correlation of endothelial subpopulations and cardiovascular outcome. Lopez et al [172] found a decreased number of Endothelial Progenitor Cells (EPCs) and an increased number of circulating endothelial cells (CECs)in HIV-infected adult patients. They suggest a model where damage of the endothelium in HIV infection is increased and repair diminished [173]. EPCs are regarded as endothelial cells with proliferative capacities but are difficult to distinguish from the hematopoietic lineage because both cell types share similar cellular markers. In contrast to Lopez et al, Costinuk et al [174] reported no difference in the number of EPCs between HIV infected and uninfected. At the other end of the spectrum, CECs are mature endothelial cells discarded from the endothelium during normal homeostasis. The degree of turnover of the vascular bed is relatively limited, represented by a low number of CECs. Therefor, enumeration of CECs relies upon sensitive techniques and accurate sample preparation. Flowcytometry has proven the best tool so far. Markers used to identify CECs include DAPI, CD105, CD146 and CD45, as well as morphological criteria such as size and whole intact cells [175].

An increased number of CECs is found in response to tumor related angiogenesis. Increased numbers of CECs have been used in studies to predict clinical outcome and treatment response. Low CEC numbers have been associated with a better clinical outcome in coloncancer [176–178] and prostate cancer [179]. In contrast, increased CECs have been associated with a clinical benefit in renal cancer and breast cancer [180–182]. CECs have also been measured in chronic HIV infection in adults. Three studies have been published so far. In addition to Lopez et al,

Falasca et al investigated the number of EPCs in two HIV patients before and after initiation of cART. They found a low level of EPCs in acute HIV and demonstrated a restoration after 6 months of therapy [183]. Guaraldi et al investigated the effect of switching from standard triple therapy to Ritonavir boosted Darunavir monotherapy (DRV/r) versus DRV/r and 2 nucleoside reverse transcriptase inhibitors (NRTIs). They randomized 30 patients and measured changes in EPCs and CECs and found no difference in levels after 24 an 48 weeks [184].

The clinical relevance of high CECs in HIV infection remains uncertain, but in light of the findings in adults, it is interesting to study the level of CECs in HIV infected children versus healthy controls since in children the confounding classical risk factors for vascular complications such as smoking, obesity and hypertension are present less frequently compared to adults.

Methods

We included 40 HIV infected children (age 5-20 years) that visited the outpatient department of the ErasmusMC-Sophia children's hospital in Rotterdam, the Netherlands between April 2014 and April 2015. The investigation was part of a larger study investigating neurocognitive function in children, and adults. Patients with an age younger than 4 years and older than 20 years were excluded, as well as patients exhibiting cognitive dysfunction. An adult control group of 30 healthy adults was recruited at the Erasmus MC, department of Internal Oncology. The pediatric control group consisted of 31 age-matched children at the Erasmus MC-Sophia Children's Hospital undergoing elective (relatively small) surgery. Patients were excluded in case of traumatic injury, apparent infections, fever, overt organ disease (chronic lung-, gastro-intestinal-, kidney- and liver disease), auto immune disease, immune deficiency, cancer as well as metabolic-, hematologic and endocrinological disorders and psychomotor impairment. Blood was sampled through venipuncture in the HIV- and adult group and through prior insertion of a venous catheter before surgery in the age-matched control group.

All patients and/or guardians as well as controls provided written informed consent. The local ethics committee approved the study. A 10ml EDTA collection tube was collected after at least one other collection tube was discarded. Patients had fasted for at least 4 hours before sample collection. For the control groups, we performed CEC enumeration only and fasting was not obligatory. Samples were stored at room temperature and processed within 8 hours after venipuncture. Blood peripheral mononuclear cells were isolated as described previously [185]. Briefly, the procedure included incubation with ammonium chloride to induce red cell lysis. After centrifuging, the supernatant was removed and the pellet was carefully re-suspended in PBS containing bovine serum albumin. Subsequently, cells were stained using a combination of monoclonal antibodies. Flowcytometric analysis was performed by selection on size, viability and expression of markers specific for CECs. We defined CECs as CD34pos, CD45neg, CD146pos and CD105pos.

For the HIV infected population, we reviewed medical history for cART duration, use of Protease Inhibitors (PI), CD4 count at the time of blood donation and metabolic laboratory markers (insulin, triglycerides, LDL, HDL). CECs were examined for associations with immunologic and metabolic parameters.

Statistical analysis was performed using GraphPad (GraphPad Software Inc. v6) and SPSS software (IBM, v21.0). Data was assessed for normality and a Shapiro-Wilk test when appropriate; a Mann Whitney test was applied to assess a significant difference between groups. For correlation analysis, a Spearman Rho test was applied.

Results

A total of 40 pediatric patients were included in the HIV group; 30 adult patients were included in the group of otherwise healthy adults and 31 pediatric patients were included in the group of children undergoing elective surgery. The median age in the HIV group was 10.1 years (IQR= 6.6 – 13.9); 19 out of 40 patients were male (47.5%); 36 patients (90%) had a viral load < 40 copies/ml at the moment of the blood sampling for this study, and 28 patients (70%) had a viral load <40 copies/ml at all 3 monthly outpatient department visits during the last year with a mean CD4 count of 1011 (±338) cells/m3 (table 1). A total of 28 children (70%) were on a PI containing regimen. In the pediatric patients undergoing elective surgery, the median age was 9.9 (IQR= 6.9 – 12.7) and 14 out of 31 were male (45%). The most common indications were internal and external ear surgery, (n=7) orchidopexia (n=4) and corrective skin surgery (n=3).

Table 1: General characteristics of the HIV infected children.

General characteristics			
Age	Median (IQR)	10.1	(6.6-13.9)
Males	No. (1%)	19	(47.5)
CEC	Median (IQR)	53	(21-135)
CD4	Mean (SD)	1011	(338)
>500	No. (%)	39	(977.5)
Viral Load <40co/ml	No. (%)	36	(90)
<40co/ml for 1 year	No. (%)	28	(70)
Years on cART	Median (IQR)	6.6	(4.8-11.4)
PI regimen	No. (%)	28	(70)

The median CEC count per 4 mL in the HIV infected group was 54 cells (IQR 21 - 135), which was significantly higher compared to adult controls (18 cells; IQR 8 – 40; p=0.0001, figure 1) but not compared to uninfected children undergoing elective surgery (80 cells; IQR 37-175). There was a significant difference between healthy adults and children undergoing elective surgery (p<0.0001). There was a significant but weak association between CECs and duration of antiretroviral therapy (r= -0.330; p=0.038). We found no correlation between number of CECs and the viral load at the moment of blood sampling for the study. We found no correlation of CECs with insulin, LDL, HDL, viral load, and CD4 T-cell count (table 2). Patients with a high CEC count (upper quartile) were more likely to have triglyceride levels above the appropriate age dependent cut-off compared to patients with a low CEC count (60% versus 18%; p=0.012). The use of protease inhibitors was less common in the group with a high CEC count (i.e. the upper quartile versus the rest; 50% versus 77%), although not statistically significant (p=0.111).

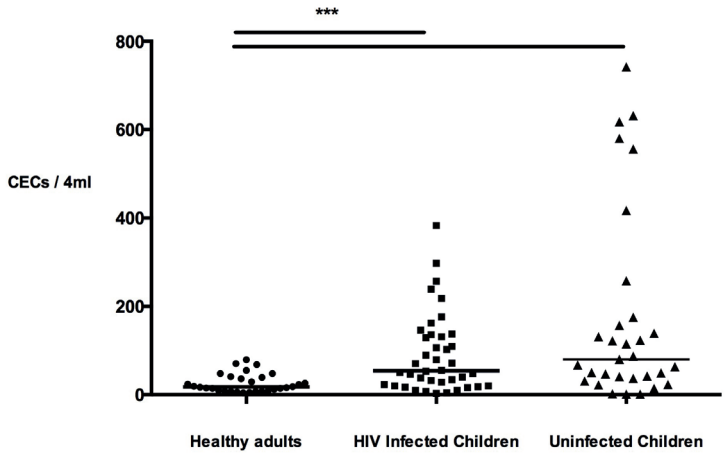

Figure 1. Enumeration of Circulating Endothelial Cells (CECs) in otherwise healthy uninfected adult individuals compared to HIV- infected children. ***p=0,0001

Table 2: Correlation of HIV associated factors and CEC levels.

Correlations to CECs	Spearman	Sig.
Age	-0.272	0.089
CD4	-0.011	0.947
Viral Load	0.028	0.866
Time on cART	-0.330	0.038
Triglycerides	0.146	0.382
Cholesterol	0.046	0.78
HDL	0.07	0.676
Chol/HDL	-3.03	0.081
LDL	-0.254	0.123
Insulin	0.049	0.768

Discussion

We hypothesized that CECs, a biomarker for vascular damage, associated with endothelial dysfunction in HIV infection, would be increased in HIV infected children as has been found in HIV-infected adults on a PI containing regimen. We found an increased number of CECs in HIV infected children when compared to healthy adults, but not compared to age matched children undergoing elective surgery. CECs are mature endothelial cells and represent increased vascular turnover or vascular damage, which could be associated with elevated cardiovascular disease risk, which has frequently been demonstrated in HIV. CECs have shown to be increased in HIV infected adults in other studies, but this phenomenon has not been studied in children, who relatively devoid of classical risk factors and potential underlying co-morbidities like cancer. We expected a lower enumeration of CECs in the group of children undergoing elective surgery. Although this population can't be considered as a healthy control, the co-morbidities for which these children underwent an operation were relatively insignificant and increased vascular turnover prior to surgery would be unexpected. The insertion of a venous catheter for blood donation could be a potential bias. It is conceivable that this causes disruption of the endothelial layer. Alternatively, another explanation for our observations could be that vascular turnover is actually increased during adolescence, perhaps representing normal vascular homeostasis during growth. A decreased number of CECs in context of these physiological processes would represent deterred vascular turnover.

In addition, we investigated the confounding effect of the use of Protease Inhibitors, agents associated with metabolic perturbations. Interestingly, we found an inverse correlation of cART duration and CEC enumeration, suggesting that the use of antiviral drugs is not likely the cause

of increased CECs. The specific effect of cART regimens could not be determined in this study due to insufficient power, but considering 70% of patients in this study were on PI's, it would be unlikely that PI's are responsible for the observed increase in CECs. Alternatively, this correlation might reflect the normalization of CEC numbers after acute HIV. This would support the hypothesis of Falasca et al where EPCs normalize after 6 months of antiretroviral therapy.

We found no correlation of CECs with HIV related- and metabolic markers (insulin, LDL, HDL, viral load, and CD4 T-cell count), although high CEC numbers were associated with high Triglyceride levels. Although this finding might suggest an association of CECs with metabolic abnormalities, more research is needed to confirm this observation and determine the underlying mechanism.

The strength of our study lies in a robust, high throughput assay to enumerate CECs and the application of this test in a population underrepresented in current literature, i.e. children and adolescents. Our study has a limitation however. The validity of our observation would be increased if we compared it to uninfected healthy age-matched controls in stead of children undergoing elective surgery.

In conclusion, CECs are increased in treated HIV infected children compared to healthy adults, but not compared to age-matched children undergoing elective surgery. We found a significant but weak inverse correlation with the duration of antiretroviral therapy, which might reflect the normalization of CECs after acute HIV infection. Patients with a high CEC count had an increased triglyceride level, which could at best be considered a surrogate end point for metabolic abnormalities.

8

Part IV
Summary and conclusions

9

Chapter 9
Summarizing discussion

New scientific discoveries are made on a daily basis and help increase our understanding of complex issues. These discoveries can change our point of view and are referred to in philosophy as breaking a paradigm. Paradigms are scientific concepts and are by definition related to the observer. For example, we know that microbes can cause disease, but this insight could not have evolved without the revolutionary discovery of the microscope. As mentioned in the introduction, HIV has probably existed much longer than we realized. The first patients died from AIDS long before we even knew the virus existed. Sufficient support from the (scientific) community is a prerequisite to break a paradigm. This in turn is facilitated by the availability of convincing evidence. Scientists need to challenge known concepts by delivering evidence to either support or break a certain paradigm. This thesis, like many others, was written to challenge our current view on certain scientific topic. It is divided in three parts: **part I** of this thesis (Chapter 1 and 2) provides a general introduction to HIV and the central nervous system (CNS). **Part II** (Chapter 3 and 4) focuses on clinical studies performed at the Erasmus Medical Center. **Part III** (Chapter 5 to 8) describes the basic laboratory studies performed with biological samples from HIV infected individuals.

Chapter 1 provides a general introduction to HIV pathophysiology, epidemiology and discusses the outline of this thesis. In **Chapter 2**, we discuss the proposed route of infection of HIV in the CNS and how it causes pathology, based on the results of a systematic review. A great deal of evidence points towards early infection of the CNS and subsequent persistence. It is uncertain whether increased longevity of infection causes complications in the long run. In addition, histopathological changes in the CNS also occur. Post mortem studies show that cells of the mononuclear lineage within the CNS can be infected with HIV and are fused together to form multinucleated giant cells. Also, HIV infected monocytes have been found in perivascular regions within the CNS. This might be interpreted as adverse, but the actual clinical relevance remains uncertain. Both patients and healthcare workers were confronted with HIV-related dementia during the pre-cART era. After implementation of effective treatment, overt neurologic complications became scarce. Although HIV can still be detected in the CNS after years of treatment, there seem to be few clinical consequences at the individual level. However, at the population level, an increased prevalence of stroke and pneumococcal meningitis is demonstrated compared to the general population. Considering the preventative measures that are available for these illnesses (i.e. vaccination and specific drugs), these subjects could prove interesting for future research initiatives. Neuropsychological disorders, albeit mild, have also been reported as being disproportionately more present in the HIV population. Although we have put this finding into a larger context by discussing potentially confounding factors, this

subject remains a topic that deserves more research. In **Chapter 3**, we focused on neurological complications by assessing the prevalence, related factors and impact of neurocognitive disorders in HIV-infected individuals. We evaluated the benefit of screening for neurocognitive disorders in 400 patients with HIV in a clinical setting. In addition, we investigated whether immunological and viral factors have an influence on neurocognitive outcome. Our study demonstrates the limitations of rapid screening in a clinical setting. One of our main findings was that a high number of patients expressed cognitive complaints (33%). Although roughly a third of our study population had difficulties with concentration, attention or memory, only 57% would prove to be unimpaired according to extensive neuropsychological testing. The international HIV dementia scale, an internationally well-validated screening instrument, proved ineffective in helping the clinician decide which patient to refer for neuropsychological testing. The most sensitive clinical correlate associated with poorer cognitive function was a low CD4 nadir, a marker that reflects a person's poorest (reported) immunological status. This is in line with several large cohort studies performed earlier in the United States. Therefore, preventing severe immunosuppression would seem knowledgeable. Although we found a higher degree of co-morbidities in the group with cognitive deficits, we cannot prove causality because of a lack of significance. This could be due to the relatively low number of patients (400) in our study. However, this finding might be interesting for future scientific endeavors. Cognitive disorders can play a vital role in the lives of HIV infected individuals. Social and work participation can be negatively influenced and in some cases, reduced compliance can be seen. In **Chapter 4** we investigated the vocational and socio-economic characteristics of our cohort. We specifically wanted to assess the effect of HIV on employment status. Although the employment status of HIV infected individuals generally corresponded well with Dutch socio-economic data, we found a remarkably lower employment rate among young seropositive males. A total of 15% of our cohort reported a need to change work hours or function after HIV diagnosis, from which we concluded that HIV definitively affects vocational status. These findings could assist counselors and policy developers for organizations where HIV-infected individuals work.

As illustrated in Chapter 3 and 4, the clinical outcome of patients is decided in large part through the efforts of patient and physician. Adequate treatment is available for our cohort and reduces the occurrence of complications, specifically neurologic disorders. However, the prevalence of non-AIDS related co-morbidities remains disproportionately high. Several theories exist as how this phenomenon is driven, ranging from personal attributable risk factors (e.g. smoking) to complex long-term direct- (e.g. pyropotosis) and indirect HIV-related effects (e.g. microbial translocation). One key similarity in all theories is the observation of a state of chronic immune activation. A persistent pro-inflammatory state seems to be present in HIV-infected individuals. In this final part of the thesis, we focus on the mechanisms behind certain physiological abnormalities. We collected biological samples from patients that participated in our clinical studies and were able to perform basic clinical laboratory studies. In **Chapter 5** we investigated the effect of long-term cART and the inflammatory phenotype. Most studies

evaluate immune activation during the first year of therapy. We measured immune activity in patients that were on cART for up to 10 years. Importantly, the selected patients are without any major co-morbidities. Biomarkers were measured in peripheral blood mononuclear cells (PBMC's) and plasma from patients participating in the TREVI cohort on 0, 1, 3, 5 and 10 years of treatment. Our observation of no or mild immune activation in suppressed HIV-infection questions the impact of ongoing inflammation. One of the major strengths in this study was the extensive panel of markers. We observed notable up-regulation in some pro-inflammatory markers (e.g. IP-10 and HLA-DR) but not all. Adequate selection of markers for scientific studies when investigating inflammation is therefore crucial. In **Chapter 6** we assessed the prevalence of metabolic syndrome and its relationship with long-term cortisol as measured in hair. Cortisol is popularly termed the 'stress' hormone and this reference holds true in many ways. High cortisol levels are associated with an increased risk of metabolic complications such as dyslipidemia, insulin resistance and hypertension, referred to as the metabolic syndrome. As chronic inflammation in HIV can be perceived as a state of chronic immunological stress, we hypothesized that cortisol would be increased in these patients as well. The mean levels of hair cortisol were similar in 91 HIV infected patients compared to a large group of controls. In contrast to our hypothesis, the relationship of cortisol and the metabolic syndrome (as defined by the international standard) was inverse. In non-HIV infected subjects, the risk of the metabolic syndrome is higher in patients with high cortisol. In our study, patients with low hair cortisol had a higher chance on the metabolic syndrome. This pattern corresponds best to the presence of glucocorticoid receptor (GR) hypersensitivity, which could be triggered by the (in)direct effects of HIV. We put this theory to the test by assessing the PBMC's of all the available patients on their capacity to respond to steroids in vitro. We used intracellular markers that are upregulated (GILZ) and downregulated (IL-2) upon dexamethasone stimulation. As hypothesized, we found higher levels of GILZ and lower levels of IL2 in patients with low hair cortisol upon exposure of dexamethasone, even at low concentrations. Although it seems tempting to conclude an increased GR sensitivity in all HIV patients, further validation is still necessary. Another interesting area that is linked to inflammation is coagulation. We adopted the hypothesis that depletion of CD4 T-cells in the gut-associated lymphoid tissue results in microbial translocation. Microbial products like lipopolysaccharide (LPS) are potent stimulators of the coagulation cascade. Considering the observation that venous and arterial thrombosis are more prevalent in HIV infected individuals, we investigated the role of von Willebrand Factor (an important coagulation factor), LPS and HIV in **Chapter 7**. We confirmed the increased prevalence of thrombosis in more than 14,000 HIV patient years by retrospective analysis. In addition, we aimed to provide a potential mechanism by evaluating the level of pro-coagulant markers and microbial translocation. Therefore we performed a case control study to investigate whether vWF or LPS would be increased in HIV patients with a thrombotic event, compared to HIV patients without an event. We measured vWF in plasma of HIV-infected individuals and found an elevation in patients with a history of thrombosis, especially in those with a recurrent event. Our experiments could not establish a relationship between LPS or LPS-related factors (LPS Binding Protein and

soluble CD14) with event status. vWF is produced in the endothelium, and increased production of this protein might represent activation of this cellular compartment. Taken together, this study supports the hypothesis of an increased activity in coagulation, although the exact mechanism remains to be elucidated. **Chapter 8** describes another aspect of vascular abnormalities in HIV, namely the role of circulating endothelial cells (CECs). The process of neovascularization and how to prevent this is currently under investigation in the oncologic research area because it offers potential targets for therapy. CECs can be considered the aged endothelial cell that is discarded in the circulation. Within the field of HIV, neovascularization as an entity holds little relevance, but measuring CECs in these patients might give insight why vascular complications are more prevalent in HIV. We analyzed the level of CECs in HIV-infected children because this population is relatively deprived of certain traditional risk factors (e.g. smoking). We found levels of CECs to be increased compared to healthy adults, but not compared to age-matched children undergoing elective surgery. This observation supports our previous statement that homeostasis in endothelial cells is disturbed, but to what extent remains uncertain. More studies are necessary to put our observation of increased CECs in patients with HIV into perspective, especially in relation to the development of adverse outcomes (e.g. cardiovascular incidents or stroke).

The initial focus of the research that is presented in this manuscript was to investigate the impact of neurocognitive complications in HIV infected individuals in the TREVI study. During the course of this research, several questions and opportunities to investigate indirect effects of HIV came along. Through the efforts of many researchers and clinicians, it was made possible to describe additional relevant topics that all hold relevance for chronic HIV infection. This thesis touches upon the presence and magnitude of coagulation abnormalities, metabolic perturbations and immune activation. We applied novel and innovative techniques and selected highly representative groups of patients.

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Chapter 10
Nederlandse samenvatting

Binnen de wetenschap worden continu nieuwe ontdekkingen gedaan. Een nieuwe doorbraak kan betekenen dat we op een andere manier het vraagstuk behandelen. Dit wordt vanuit de filosofie ook wel eens het doorbreken van een paradigma genoemd. Paradigma's zijn wetenschappelijke concepten die per definitie worden gevormd door de observator. Het concept dat bacteriën ziektes veroorzaken werd pas erkend na de revolutionaire ontdekking van de microscoop. Zoals reeds is aangegeven in de introductie bestond HIV al ruim voor de officiële ontdekking. Er stierven al mensen aan de gevolgen van AIDS voordat het virus werd geïsoleerd. Met de ontdekking van het Humaan Immunodeficiency Virus als veroorzaker van dit ziektebeeld werd een nieuw medisch en wetenschappelijk inzicht verworven. Eén van de voorwaarden om een paradigma te doorbreken is voldoende steun vanuit de wetenschap en maatschappij. Overtuigend bewijs is hiervoor onmisbaar. Een kritische blik is hiervoor onmisbaar en het is de taak van wetenschappers om bewijs te leveren om een paradigma te ondersteunen of te doorbreken. Het doel van dit manuscript is om wetenschappelijke concepten te toetsen en waar mogelijk een paradigma te ondersteunen of te doorbreken. Het boek is opgedeeld in 3 delen: **deel I** van dit manuscript (Hoofdstuk 1 en 2) is geschreven als algemene introductie en heeft tot doel de lezer te informeren over HIV en de gevolgen voor het centraal zenuwstelsel. **Deel II** (Hoofdstuk 3 en 4) richt zich op de klinische studies die verricht zijn in het Erasmus MC. **Deel III** (Hoofdstuk 5 tot en met 8) beschrijft de basale experimenten die verricht zijn met biologische materialen verkregen van HIV geïnfecteerde mensen.

Hoofdstuk 1 is bedoeld ter introductie en geeft de lezer inzicht over de pathofysiologie en epidemiologie inzake HIV en sluit af met een overzicht van dit manuscript. In **Hoofdstuk 2** bespreken we in een systematische review hoe HIV het zenuwstelsel infecteert en schade veroorzaakt. Uit recente onderzoeken blijkt dat HIV het zenuwstelsel in een vroege fase infecteert en in deze ruimte persisteert. Er bestaat nog onduidelijkheid hoe lang het virus aanwezig blijft onder adequate therapie en of er klinische nadelige effecten optreden. Histopathologische studies hebben aangetoond dat er op cellulair niveau veranderingen optreden na infectie. Uit post mortem studies in mensen en apen blijkt dat met name verandering optreedt in monocyten. Deze cellen fuseren dan tot zogenaamde multinucleaire reuscellen. Geïnfecteerde monocyten worden ook gevonden in bloedvaten die door het zenuwstelsel lopen. Hoewel dit als pathologisch beschouwd kan worden, is de werkelijke klinische relevantie nog onduidelijk. Zowel patiënt als clinicus werden voor de introductie van adequate therapie vaak geconfronteerd met het optreden van HIV-gerelateerde dementie. Dit fenomeen werd tot een zeldzaamheid gereduceerd toen patiënten met combinatie antiretrovirale therapie (cART) werden behandeld. Hoewel het virus nog jaren na adequate therapie kan worden gemeten,

lijken de klinische gevolgen per individu beperkt. Desalniettemin wordt op populatie niveau een verhoogde kans op een beroerte en meningitis worden aangetoond. Er zijn therapeutische mogelijkheden voor deze ziekten, zoals vaccinaties en medicatie, en zullen interessante onderwerpen vormen voor toekomstige onderzoeksinitiatieven. Daarnaast worden ook milde neuropsychologische stoornissen beschreven, maar deze lijken relatief mild vergeleken met de gevreesde HIV-dementie. Andere factoren dan HIV spelen ook een rol in het optreden van neurocognitieve stoornissen; dit is dan ook zeker een onderzoeksveld in ontwikkeling.
In **Hoofdstuk 3** bespreken we de TREVI studie. We onderzoeken het optreden van neurocognitieve stoornissen en de invloed van ziekte gerelateerde factoren in HIV geïnfecteerde mensen in het Erasmus MC. We constateren dat de huidige geadviseerde screening beperkingen oplevert. Een belangrijke bevinding is dat er relatief veel patiënten cognitieve klachten ervaren (33%). Uit deze groep blijkt echter maar 57% daadwerkelijk neurocognitief beperkt te zijn als een uitgebreid onderzoek wordt ingezet. Hoewel de internationale 'HIV dementia scale' een internationaal gevalideerd instrument is om cognitieve beperkingen op te sporen, blijkt deze niet effectief genoeg om de behandelaar te helpen in de keuze om patiënten te selecteren die gebaat zijn bij verder onderzoek. Overigens blijkt het CD4 nadir (een maat die aangeeft hoe diep iemand immuungecompromitteerd is geweest) geassocieerd met de aanwezigheid van een neurocognitieve stoornis. Dit komt overeen met eerdere grote studies uit de Verenigde Staten. Derhalve lijkt het voorkomen van immuundeficientie zinvol. Ook vonden we bij mensen met een cognitieve stoornis vaker een bijkomende ziekte (co-morbiditeit), maar er waren te weinig participanten binnen deze studie zodat statistische significantie uitbleef. Cognitieve stoornissen kunnen een grote beperking opleveren voor patiënten. Sociale contacten en arbeidsparticipatie kunnen hierdoor negatief beïnvloed worden. In **Hoofdstuk 4** onderzoeken we de arbeid gerelateerde en socio-economische karakteristieken van ons cohort. De invloed van de diagnose HIV op het hebben van een baan en het functioneren op de werkvloer staan hierin centraal. De arbeidsparticipatie binnen ons cohort is over het geheel genomen vergelijkbaar met de algehele Nederlandse populatie, maar jonge mannen lijken vergeleken met controle data vaker werkloos. Daarnaast valt op dat 15% van ons cohort aangeeft een aanpassing nodig te hebben in het aantal arbeidsuren of functie. Derhalve kan geconcludeerd worden dat HIV wel degelijk invloed heeft op de arbeidsparticipatie. Deze bevindingen kunnen als hulpmiddel dienen voor werkgevers zodat werknemers met HIV beter begeleid kunnen worden.

Zoals aangegeven in Hoofdstuk 3 en 4 is de behandeling van HIV een 'team effort'. Zowel de patiënt als de behandelaar spelen hierin een grote rol. Adequate behandeling van HIV heeft als effect dat er minder complicaties optreden, met name neurologische en neurocognitieve aandoeningen. Desalniettemin is de prevalentie van niet-AIDS gerelateerde ziekten (zoals hart- en vaatziekten, bepaalde vormen van kanker) hoger bij patiënten met HIV vergeleken met ongeïnfecteerde leeftijdsgenoten. Er zijn enkele theorieën over het ontstaan van dit fenomeen. Sommige factoren staan onder invloed van het gedrag (zoals roken), anderen lijken (in)direct onder invloed te staan van het virus (zoals pyroptosis en microbiële translocatie). Een veel

genoemde overkoepelende theorie is 'chronische immuun activatie', hetgeen inhoud dat er binnen het lichaam een constant pro-inflammatoir milieu bestaat. In het volgende deel van dit manuscript onderzoeken we mechanismen achter deze theorie. We gebruikten biologische materialen van patiënten uit ons cohort om basaal wetenschappelijke experimenten te verrichten. In **Hoofdstuk 5** bespreken we de effecten van langdurige therapie op het ontstaan van inflammatoire kenmerken. De meeste studies in de huidige literatuur kijken naar immuun activatie tijdens het eerste jaar van therapie. In onze studie includeerden we patiënten die op dat moment wel tot wel 10 jaar op antiretrovirale therapie stonden. De patiënten uit ons cohort waren relatief vrij van co-morbiditeiten. Biomarkers in het bloedplasma en op de oppervlakte van bepaalde bloedcellen (PBMC's) werden hiervoor gemeten bij patiënten die 0, 1, 3, 5 of 10 jaar op therapie zaten. De kracht van de studie zit in de diversiteit en uitgebreidheid van de onderzochte markers. Sommige markers bleken verhoogd, zoals IP-10 en HLA-DR, maar over het algemeen constateren we dat er relatief weinig tekenen van inflammatie bestaan bij chronische HIV infectie. Dit staat in contrast met eerder gepubliceerde studies. In **Hoofdstuk 6** bekijken we de prevalentie van het metabool syndroom bij patiënten met HIV en wat de relatie is met cortisol. Het metabool syndroom wordt gekenmerkt door dyslipidemie, insuline resistentie en hypertensie en is geassocieerd met een hoog cortisol. Cortisol wordt vaak het 'stress hormoon' genoemd en kan sinds kort worden gemeten in haren. Aangezien chronische immuun activatie geïnterpreteerd kan worden als een vorm van chronische stress, veronderstelden we dat cortisol verhoogd zou zijn bij patiënten met HIV. Het gemiddelde cortisol in 91 patiënten met HIV vergeleken met gezonde controles bleek echter gelijk. De correlatie van cortisol met het metabool syndroom bleek echter omgekeerd evenredig bij patiënten met HIV. Bij niet HIV-geïnfecteerde mensen is de kans op het metabool syndroom hoger indien het cortisol verhoogd is. In onze studie bleek dat HIV patienten met een laag cortisol een hogere kans hadden op het metabool syndroom. Deze observatie kan verklaard worden door de aanwezigheid van een overgevoelig glucocorticoid receptor (GR), mogelijk door het virus veroorzaakt. Om deze theorie te toetsen werd het effect van steroïden op PBMC's van dezelfde HIV geïnfecteerde patiënten gemeten. We gebruikten hiervoor markers die stijgen (GILZ) of dalen (IL-2) na blootstelling met dexamethason. We vonden een toegenomen stijging in GILZ expressie en daling in IL-2 expressie bij patiënten met een laag haar cortisol na blootstelling met dexamethason, zelfs bij lage concentraties. Hoewel deze bevinding onze theorie van GR hypersensitiviteit bevestigd, is nog meer validatie nodig om tot een definitieve conclusie te komen. Een ander interessant onderzoeksgebied is de relatie tussen inflammatie en stolling. Een bekende theorie binnen de HIV onderzoekswereld is het ontwikkelen van immuun activatie door microbiële translocatie. Als gevolg van HIV infectie kan het aantal CD4 T cellen in de darm drastisch verminderen. Dit resulteert in een verminderde barrière functie van de darm en leidt tot translocatie van bacteriële producten. Een voorbeeld van deze producten is lipopolysaccharide (LPS). LPS is een krachtige stimulus voor stollingsactivatie. Aangezien er een toegenomen kans is op het ontwikkelen van vasculaire complicaties bij HIV onderzochten we de relatie van LPS met von Willebrand factor, een bekend pro-coagulante stollingsfactor, in **Hoofdstuk 7**. We bevestigden de hogere

prevalentie van stollingscomplicaties in meer dan 14.000 patientjaren door retrospectieve analyse. Daarnaast onderzochten we de relatie tussen trombose en een toegenomen hoeveelheid vWF en LPS. Derhalve evalueerden we of hoogte van vWF door middel van een case-control studie. Het vWF bleek verhoogd in het plasma van HIV geïnfecteerde patiënten die in het verleden een veneuze of arteriële trombose hadden doorgemaakt, met name bij patiënten met een recidief. In onze experimenten konden we geen relatie aantonen tussen LPS of daaraan gerelateerde factoren (zoals LPS Binding Protein en soluble CD14) en het optreden van trombose. Het vWF wordt geproduceerd in het endotheel, een celtype dat de binnenkant van alle bloedvaten bedekt. Een toegenomen productie zou kunnen duiden op een activatie van dit celtype. Samengevat toont deze studie aan dat er een toegenomen stollingsactiviteit bestaat bij HIV, alhoewel het mechanisme nog onduidelijk blijft. In **Hoofdstuk 8** kijken we naar het optreden van vasculaire schade door HIV. We onderzochten de rol van circulerende endotheelcellen (CECs). Het lichaam is in staat nieuwe bloedvaten te formeren, hetgeen belangrijk is voor een toevoer van zuurstof en nutriënten. Binnen de oncologie wordt reeds uitgebreid onderzoek verricht naar mogelijkheden om neovascularisatie te remmen, aangezien dit ook tumorgroei verminderd. CECs kunnen beschouwd worden als verouderde endotheelcellen die afgegeven worden aan de bloedcirculatie. In de context van HIV is neovascularisatie niet een uitgesproken onderwerp, maar CECs zouden wellicht meer inzicht kunnen geven waarom vasculaire complicaties vaker optreden bij HIV. We onderzochten de hoogte van CECs in HIV geïnfecteerde kinderen, omdat deze populatie relatief vrij is van traditionele risicofactoren als roken. We constateerden dat de concentratie CECs toegenomen was in kinderen met HIV ten opzichte van ongeïnfecteerde volwassenen, maar niet ten opzichte van ongeïnfecteerde die een geplande operatie ondergingen. Deze observatie bevestigd het gegeven dat er een verstoring van de homeostase is van endotheelcellen bij HIV, maar in welke mate blijft onduidelijk. Meer studies zijn nodig om deze gegevens in context te plaatsen.

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Het onderzoek dat vooraf ging aan dit manuscript was initieel gericht op neurocognitieve complicaties bij patiënten met HIV binnen de TREVI studie. Gedurende het onderzoek kwamen verscheidene onderzoeksvragen en mogelijkheden naar de oppervlakte. Door de inzet van vele onderzoekers en clinici werd het mogelijk deze vraagstukken te behandelen. Mede hierdoor ontwikkelde zich een manuscript dat een variëteit aan relevante onderwerpen behandeld, zoals stolling, immuun activatie en metabole stoornissen. Al deze onderwerpen zijn reeds eerder onderzocht, maar niet in de vorm zoals het nu gepresenteerd wordt. We pasten nieuwe en innovatieve technieken toe op zeer representatieve groepen patiënten.

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Chapter 12 About the author

Lennert van den Dries was born in 1984 in the Netherlands and was raised partially abroad in Rwanda, central Africa. After finishing his pre-university education in The Hague, he started his medical training at the Vrije Universiteit in Amsterdam. After spending 4 years of medicine he went to Chicago to perform 6 months of research at the University of Illinois at Chicago (UIC). He studied chimerism in non-human primates after simultaneous solid organ transplantation and hematopoietic stem cell transplantation. He returned to the Netherlands to finish his internships and started in the field of Internal Medicine. After 16 months of residency, he started his PhD under the supervision of prof. E. van Gorp at the Erasmus Medical Center. During his time as a PhD candidate he completed the Research Master program 'Infection and Immunity'. He also worked at the outpatient Travel Clinic of the Erasmus Medical Center, which specializes in vaccinating immune compromised patients. He is currently working at the Elisabeth Medical Center in Tilburg.

PhD portfolio

Name:	Leendert Willem Johannes (Lennert) van den Dries
Erasmus MC Department:	Department of Viroscience
Research school:	Post-graduate molecular medicine
PhD period:	2012-2017
Promotor:	Prof. dr. E.C.M. van Gorp
Copromotor:	Prof. dr. A.M.C. van Rossum
Dr. K.S. Adriani	Uitlijnen: Co-promotor K. Adriani moet

Education

2012-2014:	'Infection and Immunity', biomedical research program; Erasmus Medical Center, school of Molecular Medicine.
2002-2009:	Medicine, 'Vrije Universiteit' Amsterdam, entry gained by competitive selection procedure.
1996-2002:	Pre-university education; 'Christelijk Gymnasium Sorghvliet', The Hague. Major: Physics and Health. Electives: economics, Latin.

In depth courses and education with certificates

2012/2016	Basic course on regulations and organisation for clinical investigators (BROK)
2013	Masterclass HIV, Virology education
2013	SPSS course
2012	Scientific English writing
2012	Survival analysis
2011	Treatment of patients with HIV/AIDS course
2011	Travel Medicine course accredited by the Dutch Center for Travel medicine

Teaching experience

2015 – 2016	Supplementary training for general practitioners ('Allemaal Beestjes'; translated: Bugs)
2012 – 2016	Frequent teacher on immunocompromised travelers at the NSPOH basic/ supplementary training for general practitioners/nurses.
2014 – 2016	Teacher at the Research Master 'Infection and Immunity', Erasmus MC, school of Molecular Medicine.

Oral presentations

2015	4th CCAS HIV/AIDS International Workshop, St. Kitts
2015	HIV in the context of occupational medicine (invited speaker), Amsterdam (AMC), the Netherlands.
2015	HIV and Immune Activation (invited speaker), Paramaribo, Suriname.
2015	34th meeting of the Dutch Society of HIV treating physicians (NVHB).

Poster presentations

2015	6th International Meeting on NeuroHIV, Matera, Italy.
2015	9th Dutch Conference on HIV Pathogenesis, Epidemiology, Prevention and Treatment (NCHIV).
2014	17th Annual meeting of the European Society for Clinical Virology, Prague, Czech republic.
2014	8th Dutch Conference on HIV Pathogenesis, Epidemiology, Prevention and Treatment (NCHIV).

Attended Conferences and symposia

2015	22nd Conference on Retroviruses and Opportunistic Infections, Seatle, MA, USA.
2014	HIV Drug Therapy Meeting, Glasgow, United Kingdom.
2013	North European Workschop on HIV infection in the CNS (HANSA), Berlin, Germany.

2013	59th Conference of the International Society on Thrombosis and Haemostasis, Amsterdam, the Netherlands.
2013	14th European AIDS Conference, Brussels, Belgium.
2013	European Congress of Virology, Lyon, France.
2013	5th International Meeting on NeuroHIV, Palermo, Italy

Extracurricular activities

2011 – 2016	Teacher at annual international education project 'Viruskenner'
2012 – 2013	SURE Conference committee ("Edges of Science: Visions of the Future in Biomedical Research").

Publications

Mahmud D, Nicolini B, **van den Dries LWJ**, Mahmud N, Arpinati M, Rondelli D. Human CD4(+)CD25(+) Cells in Combination with CD34(+) Cells and Thymoglobulin to Prevent Anti-hematopoietic Stem Cell T Cell Alloreactivity. Biol Blood Marrow Transplant. 2011 Jan;17(1):61-8.

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Chapter 13 Dankwoord

"If I have seen further it is by standing on the shoulders of giants." Een uitspraak die bekendheid heeft gekregen door Isaac Newton. Hij refereert hier naar de mogelijkheid om tot grotere hoogte gebracht te worden met behulp van de kennis van anderen die jou voorgingen. Ik heb tijdens mijn promotie inderdaad mijn horizon verbreed. Ik leerde een scala aan eigenschappen aan die ik mijn leven meeneem. In de eerste plaats wil ik mijn dankwoord richten aan de leescommissie: professor Boucher, professor Verbon, professor Meijers, dr. Nouwen, professor Robertson, professor Van Agtmael en professor Koopmans. Jullie hebben in de kostbare tijd die jullie bezitten de moeite genomen om mijn werk met kritisch oog te beoordelen, waarvoor mijn dank. Besides critically appraising my manuscript, some of you have travelled a great distance just to be at my defense, for which even more gratitude is in order. Dit werk was alleen nooit af geweest zonder de begeleiding van professor van Gorp. Beste Eric, jouw vertrouwen in mij is bemoedigend. Ik heb af en toe met de handen in het haar gezeten en telkens wist je op een immer kalme intonatie de hoofdzaken te benoemen. Je bent de manager van mijn promotie traject geweest. Jij hield het overzicht als ik (te) geconcentreerd was op een specifiek onderwerp. Je gaf me de ruimte om te proberen, om te falen en gelukkig ook succesvol te zijn. Daarvoor ben ik je erg dankbaar. Dit proefschrift zal een waardevol aandenken zijn van mijn promotietijd. Ook mijn co-promotor **Kirsten** wil ik hartelijk bedanken. Je bent grondig door mijn papers gegaan en hebt me geholpen om het overzicht te houden. Daarnaast ben je een heel toegankelijke en fijne collega. Dat je in alle drukte nog tijd wist vrij te maken om mij te begeleiden waardeer ik enorm. Beste Annemarie, **professor van Rossum** inmiddels, jou wil ik ook danken voor het vertrouwen dat je in me had. Rondom de afronding van mijn promotie neigde ik door alle drukte soms de binnenbocht te pakken, maar zoals je me leerde is het belangrijk om secuur te werk te blijven gaan. Als ik een opzet voor een artikel of poster naar je opstuurde kreeg ik die volledig rood terug. Met veel zorgvuldigheid bracht je belangrijke opmerkingen in mijn teksten aan. Dank voor je kritische blik; de lessen die ik van je heb geleerd zullen mijn carrière verrijken. Marco, jij bent tijdens en na mijn promotie een wereldvent gebleken. Je hebt een verfrissende en aanstekelijke invloed op mij gehad. 'Work hard, play hard' is jouw motto. De ritjes in jouw oude auto met Flora van Amsterdam naar Rotterdam waren legendarisch (en altijd met een homemade koffie erbij). Auto op de Kralingse zoom (gratis parkeren), metrootje naar de sportschool voor een Gladiator work out, proteïnshake erin en alsnog als eerste op het Erasmus MC. Je werkethos zou gepatenteerd moeten worden, de productie gaat dan gegarandeerd 200% omhoog. Samen legden we heel wat afstanden af; bijvoorbeeld naar buitenlandse congressen, tientallen kilometers rennen door de bagger tijdens een mudrace en natuurlijk de Marathon van Athene. Ik hoop jou en Odette (en natuurlijk Liz) nog vaker te zien in de toekomst. **Marco** en ik werkten in de 'kelder' van Viroscience op de 16e verdieping. Daar kwamen snel anderen op af. Aan het einde van de dag was er gelukkig vaak genoeg animo om te borrelen. Daar was dr. Dave vd Fever altijd wel bij. Samen met dr. Brooke kon je steevast rekenen op een diepgaande discussie over kosten-effectiviteit, de tour de France, integratiecursus of andere zaken waar ik eerlijk

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Als ik vertel over mijn tijd bij Viroscience kan ik natuurlijk niet de 'Exotics' vergeten. Aan het hoofd dr. **Byron Martina**, een sparringpartner als geen ander. Je weet de essentie van elke onderzoeksvraag onder de loep te nemen. Er bestaan geen aannames voor jou. De gesprekken in de late uurtjes zijn mij het beste bijgebleven… alsmede je flexibiliteit als de Salsa muziek aangaat. **Penelope**, your high heels will be remembered and if I ever encounter a silver haired bat I will think of you (and Rabies)! **Steffie**, dr. Lim, ik ben blij dat we elkaar blijven zien na ons vertrek bij het Erasmus MC. Het jaar 2017, waarin je je maatje hebt verloren, was een oneerlijke start voor jou. Ik hoop dat de zon weer gaat schijnen voor je. **Fasa**, what can I say about you. I'm not convinced you're completely fond of me, but I'm sure you don't dislike me. Maybe it's the involuntary Bro-Love we Dutch people like so much. I will give you the biggest hug when you get your PhD! Involuntary of course… **Petra**, je hebt een ijzersterke mening en altijd bereid me te helpen als ik een concentratie moest berekenen. Uiteraard kan ik **Jeroen** niet vergeten. Culinaire

hoogstandjes, gouden pipetduimpjes en een scala aan schuine moppen.

Buiten de Exotics en de 16e verdieping zijn er nog een hele hoop anderen die ik graag wil bedanken van Viroscience. Ik ga nu ongetwijfeld nog een hoop vergeten. Ik hoop dat die persoon zich dan realiseert dat er geen moment opzet in het spel is geweest. In de eerste plaats wil ik **Ab Osterhaus** bedanken dat hij mij überhaupt wilde aannemen. De sollicitatieprocedure bleek (zoals anderen vooraf al voorspelden) legendarisch; ik verliet je kantoor met hartkloppingen en een bezweet voorhoofd. Je kritische opmerkingen tijdens research meetings hield iedereen op de afdeling scherp. Dat je humor hebt liet je ook doorschemeren tijdens borrels en uitjes. Bij een Exotics uitje in Antwerpen ontving je ons zelfs in huis, met bizar lekkere oesters erbij. **Rob** (Gruters), ik kijk op naar de hoeveelheid kennis die je bezit en je brede interesse binnen de wetenschap. Je trots voor je muzikale familie steek je niet onder stoelen of banken. Je lijkt je prioriteiten goed op een rij te hebben. Ik wacht nog het moment af tot we jouw HIV vaccin in de kliniek gaan toepassen. Dank je voor je begeleiding bij mijn experimenten en ik hoop tot ziens. **Lineke**, super gezellig om jou ook nog te hebben meegemaakt. **Do**, I'm sure you'll make it in research! **Bri**, it has been a pleasure during our research master! **Sander**, wat doe je met je carrière; je zou de beste show-host ooit kunnen zijn en toch kies je voor Nature, Science en aanlokkelijke beurzen. **Rory**, **Caroline**, **Miranda**, **Arwen**, **Eefje**, **Oanh** en al die anderen zonder wie Sinterkerst of al die andere borrels niet geslaagd zouden zijn: bedankt!

Naast de afdeling Viroscience heb ik ook nog op andere afdelingen mogen meekijken. Op de 6e verdieping (Biochemistry) heb ik de fijne kneepjes van het pipetteren mogen leren van dr. **Tokameh Mahmoudi**. Thank you for you patience in teaching me how to handle experiments. Sometimes my monocytes would illuminate spontaneously when they shouldn't, at other times it remained dark under the microscope when I expected a discotheque. Your insight in HIV latency and how to reverse it is the truly impressive. **Elisa**, you have helped me deal with complex PCR protocols and arranging bicycle racing trips in Italy, grazie mille! **Michael**, ik heb je te weinig gezien door alle drukte rondom het einde van mijn promotie, maar je snoeiharde (en soms platte) Amsterdamse grappen zal ik voor altijd bij me dragen, I'm positive about that. **Tsung-Wai, Robert Jan, Mateusz** and **Khalid**, good luck with finding a cure for HIV! Van de research toren naar de klinische toren van het Erasmus. De groep van **Georgina** op 10e verdieping verzorgde een aanzienlijke bijdrage aan mijn onderzoek. Hartelijk dank voor jullie geduld als er wéér een patiënt op de niet afgesproken tijd langskwam en er wéér PBMC's geïsoleerd moesten worden. Jullie hebben aan de basis gestaan van mijn experimenten. De laatste personen van deze verdieping zijn eigenlijk per toeval op mijn pad gekomen (en gelukkig maar!). **André**, ons idee om PBMC's te gebruiken en er immuun activatie op te meten ontstond in Seattle bij een congres. Na een glas whiskey met **Mark** ging het idee rollen en na veel werk hebben we dit gave artikel gepubliceerd. **Anthonie**, je deed overkomen alsof de meest ingewikkelde FACS kleuringen kinderspel waren, bedankt voor de praktische lessen! **Casper**, je bent halverwege mijn promotie op onze afdeling gekomen. Je hebt keihard gewerkt en dat resulteerde in een promotie, huwelijk

en kort daarop een kind. Lekker bezig ouwe! **Simone**, je bent de stille motor achter de vaccinatiepoli! Op de afdeling kindergeneeskunde lopen 2 experts rond die ik niet ongenoemd wil laten. **Linda** en **Eline**, jullie besteden bewonderenswaardig veel aandacht aan jullie patiënten. Dat jullie breed georiënteerd zijn blijkt wel uit het feit dat jullie naast patiëntenzorg ook zonder problemen meedenken aan research initiatieven. Buiten het Erasmus om zijn er ook nog enkele 'buitenbeentjes' die hebben bijgedragen aan dit boekje. **Stephanie**, we zullen elkaar ongetwijfeld nog blijven spreken in de afronding van de TREVI-kinder studie. Laten we er een mooi artikel van maken! **Marlies**, het includeren van patiënten tijdens de TREVI studie was een hele klus. Met veel geduld hebben we er uiteindelijk 400 kunnen includeren. Een hele prestatie en wat mij betreft gaan we nog een keer naar St. Kitts om dat te vieren. Ik heb het als een toptijd ervaren! Ook bedankt **Harald** en **Pepijn**, ik zal jullie ongetwijfeld nog vaker ontmoeten. **Job** (van Exel), ook met jou was het fijn samenwerken! Ik wil ook extra aandacht besteden aan iemand die mij buiten mijn promotie heeft geïnspireerd. **Frank van Vliet** staat altijd (in werk en privé) voor iedereen klaar. Je kan je kwaad maken als iemand zich niet aan de afspraak houdt, of dat nou een student of een internationaal gerenommeerde professor is. Ook als ik dan moest rennen van de poli naar mijn les en ik kwam enkele minuten te laat dan wisten alle professoren van het Erasmus dat. Samen met Dr. **Nouwen** heb je een inspirerende master opgebouwd. Dank jullie beide voor de kans om hier aan mee te mogen doen.

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Wat mij betreft voeg ik nog twee mensen toe aan deze familie. Twee die ik al zo lang ken dat ik ze niet weg kan denken uit mijn natuurlijke habitat. In de eerste plaats **Cor Paul**. Corrie. CP. Knor. Je bent de oceaan overgevlogen om mijn promotie mee te maken. Toevallig ga je hierna snel trouwen, maar we weten allebei wat je échte motivatie was. Top dat je er bent. Ik ken je al sinds je nog maar 1 meter 90 was. Ook **Jan** hoort in dit rijtje thuis. Een vriend waar ik op kan bouwen. We zijn allemaal gegroeid, de één woont in Canada, de ander om de hoek. We kiezen allemaal een ander pad, maar als die samenkomt is het weer als vanouds.

Traditiegetrouw wil ik afsluiten met het dierbaarste wat er in mijn leven bestaat. Je bent mijn grootste fan en meest kritische publiek. Je nam de tijd om te luisteren naar mijn promotie perikelen, je las mijn artikelen en accepteerde de soms vreemde werktijden. Ik probeerde regelmatig muisstil naast je te kruipen na een geslaagde afdelingsborrel. Je ging mee naar Praag en trotseerde orkanen samen met Eric, Marlies en mij in St. Kitts. Als ik tijdens mijn promotie met een dilemma zat, probeerde je altijd de essentiële vraagstukken te vinden door me uit te dagen. Ik ben fan van onze gesprekken aan de keukentafel met een goed glas wijn. Inmiddels weten we elkaar met steeds minder woorden aan te voelen. Ik had het jou en mezelf gegund dat je ouders bij mijn promotie hadden kunnen zijn. Lieve **Tjitske**, je bent het mooiste hoofdstuk van mijn leven, ik ben gelukkig met je. Dank je wel.