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Trends in setpoint plasma HIV-1 concentration and CD4 cell count before and after introduction of effective antiretroviral treatment in the ATHENA national observational cohort

Luuk Gras

TRENDS IN SETPOINT PLASMA HIV-1 CONCENTRATION AND CD4 CELL COUNT BEFORE AND AFTER INTRODUCTION OF EFFECTIVE ANTIRETROVIRAL TREATMENT IN THE ATHENA NATIONAL OBSERVATIONAL COHORT

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Trends in setpoint plasma HIV-1 concentration and CD4 cell count before and after introduction of effective antiretroviral treatment in the ATHENA national observational cohort

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1

Introduction

Introduction

Human immunodeficiency virus (HIV) infection is one of the most devastating pandemics ever recorded in human history with 77.3 million infected people globally and 35.4 million people estimated to have died since the start of the pandemic from acquired immune deficiency syndrome (AIDS), the spectrum of opportunistic infections and diseases associated with HIV. In 2017 an estimated number of 36.9 million people were living with HIV, of whom 1.8 million were newly infected. Nine hundred and forty thousand people died in 2017 because of AIDS[1].

HIV can be found in semen, blood, cerebrospinal fluid, vaginal secretions, saliva, tears and breast milk of an infected individual[2-5]. Transmission can occur during unprotected sexual contact, blood-to-blood contact, during pregnancy or birth or through drinking breast milk. HIV infection is initially asymptomatic but causes depletion of the human immune system. If left untreated HIV infection eventually leads to death after a median of 7.9 to 12.5 years, depending on age[6]. Although the first antiretroviral drug was approved for treatment of HIV infection in 1987, it was not until 1996, when a combination of drugs with different mechanisms of action were combined into one regimen, that HIV disease changed from a lethal disease into a chronic condition. Provided HIV infection is timely diagnosed and combination antiretroviral therapy (cART) is started before the immune system is severely compromised and provided that treatment suppresses the virus in peripheral blood to below detectable levels, HIV infected individuals can have a near normal life expectancy[7]. However, with the treatment options currently available to the general HIV infected population HIV cannot be cured and treatment with antiretroviral drugs is lifelong.

In the studies included in this thesis we investigate changes in the concentration of HIV particles in peripheral blood, represented by the amount of HIV-RNA in plasma, and changes in the number of CD4 T and CD8 T lymphocytes in peripheral blood representing the level to which cellular immunity is affected. The concentration of HIV-RNA in plasma, also known as plasma viral load, and the CD4 cell count are two of the most important biomarkers in HIV disease progression. A higher plasma viral load is related to a higher rate of decrease in CD4 cell counts which in turn is related to a shorter time to AIDS. In chapter 2 we study trends over the course of the HIV epidemic in the Netherlands in the level of plasma viral load 9-24 months after HIV seroconversion and before therapy has started. We further study whether adaptation of HIV-1 to host genetic variations can account for these trends (chapter 3). In chapter 4 we study whether these trends are accompanied by a change in the rate at which CD4 cell counts decrease, and, as antiretroviral therapy should be started before the immune system is severely compromised, whether this influences the timing of antiretroviral therapy initiation (in terms of years after infection). In chapter 5 we study whether antigenic stimulation through

co-infection with hepatitis C virus (HCV) is associated with changes in plasma HIV load and CD4 cell counts in treated and untreated HIV infected individuals. Successful antiretroviral therapy reduces the plasma viral load and restores the CD4 cell count. We study the timing of the start of antiretroviral therapy and the ability of antiretroviral therapy to restore CD4 cell counts to levels seen in men-having-sex-with-men (MSM) without HIV infection (as reported in other studies) by modeling trends in CD4 cell count longitudinally after the start of antiretroviral therapy (chapter 6). We further study whether restoration of CD4 and CD8 cell counts to levels seen in HIV-negative individuals of the same age and gender is achieved when therapy is initiated at high CD4 cell counts (chapter 7).

HIV life cycle

Often only one single feature of the immune system, the number of CD4 T lymphocytes in peripheral blood (CD4 cell count), is used to predict residual time to morbidity and mortality. CD4 T lymphocytes are a type of white blood cells, named after the CD4 protein on the T-cell receptor of these cells. A naïve CD4 T lymphocyte needs to be activated to be capable of mediating immune protection. This involves contact with an infected cell that presents an antigen. Activated CD4 T lymphocytes will multiply and can release different types of cytokines, which act as messenger molecules between cells in different parts of the body. One of these messages is for CD8 T lymphocytes to become activated and clear the virus. In most human infections T cells either eliminate the virus or suppress it as a harmless persistent infection[8]. However, CD4 T lymphocytes are targeted and destroyed by HIV as part of its life cycle. HIV impairs the CD4 T cell response, as well as the response of uninfected CD8 T cells.

HIV infects T helper lymphocytes via binding to the CD4 receptor and a co-receptor (either CCR5 or CXCR4) on the cell surface (see Figure 1). Upon entry, viral RNA is converted into DNA by the HIV enzyme reverse transcriptase. Viral DNA is subsequently integrated into the DNA of the CD4 T lymphocyte by the HIV enzyme integrase. The infected cell can now be used to produce building blocks for new virions.

Over the course of the infection the number of CD4 cells decreases to very low levels and the immune system weakens resulting in an increased risk of developing AIDS.

Natural course of HIV infection

Within approximately three weeks after infection the virus concentration in plasma (the plasma viral load) reaches very high levels in the range of 10^6 to 10^7

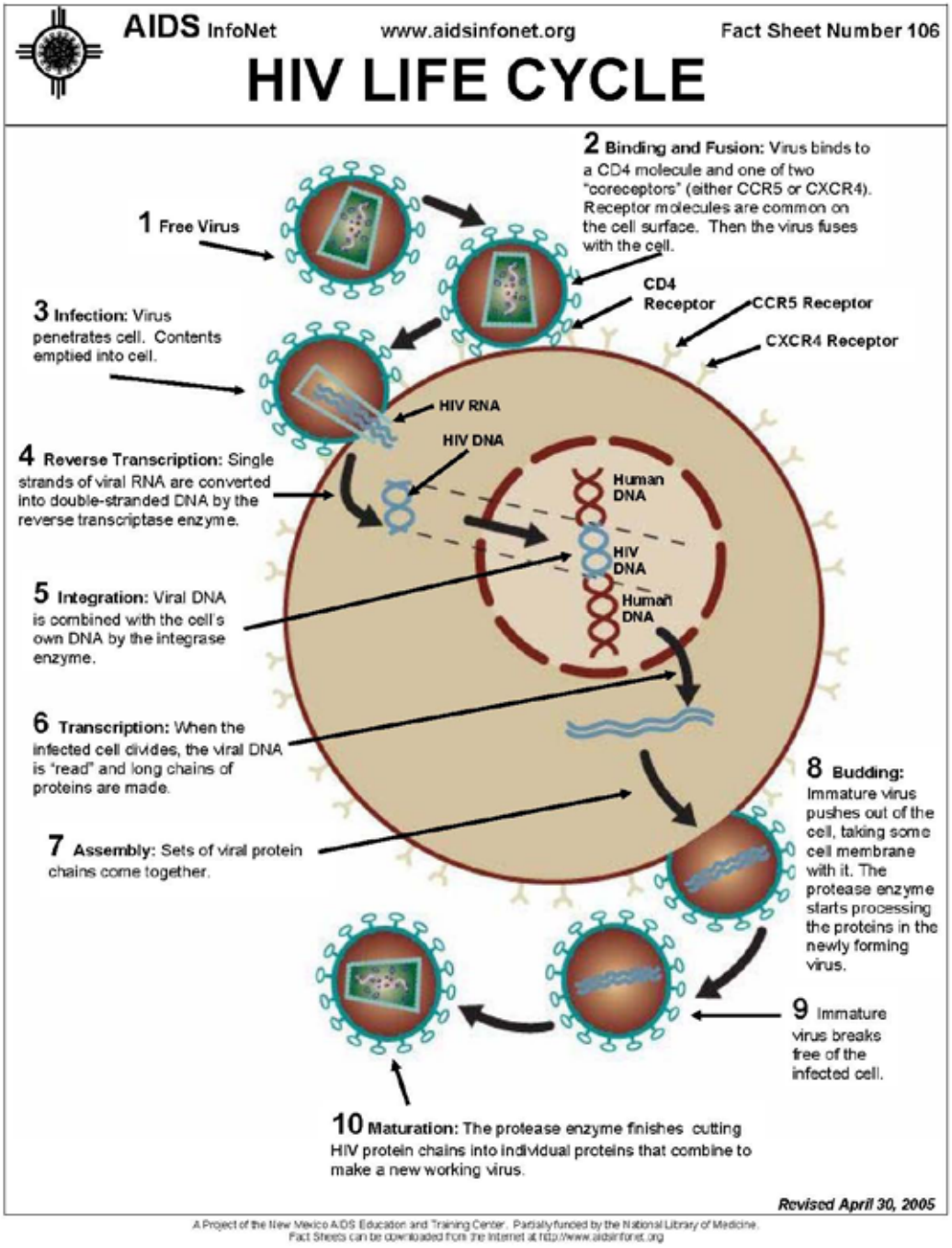

Figure 1. Life cycle of HIV. © 2016 International Association of Providers of AIDS Care.

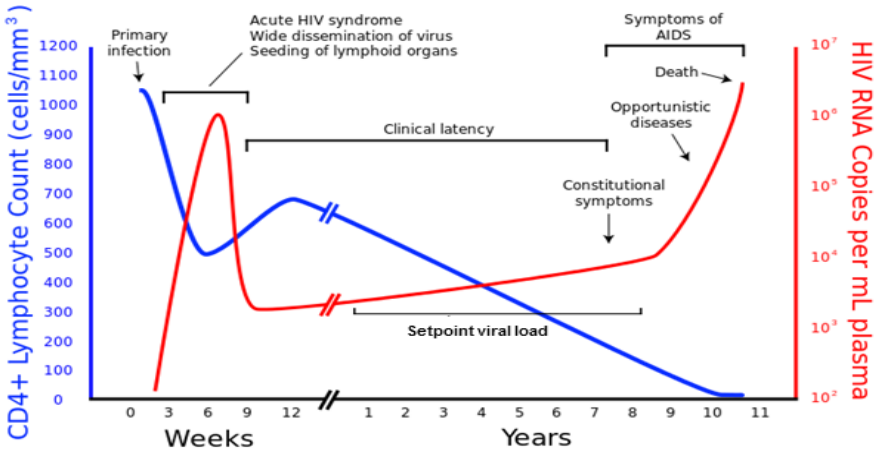

Figure 2. A generalized graph of the relationship between HIV copies (viral load) and CD4 counts over the average course of untreated HIV infection; any particular individual's disease course may vary considerably.

virus particles per milliliter[9-11] (see Figure 2). This is accompanied by a decline in CD4 cell counts, and, in most infected individuals, a flu-like illness and/or other symptoms such as fatigue, lymphadenopathy, headache, and rash[12].

A humoral immune response develops four to eight weeks after infection that can partly control HIV replication. Antibodies mainly target flee-floating HIV particles, although some may destroy HIV-infected cells. A cell-mediated immune response follows. Together, the humoral and cell mediated immune response partially control, but cannot stop HIV production. As a result, virus production and clearance reach a balance represented by a lower and more or less stable HIV-RNA concentration in plasma, the so-called setpoint viral load. Individuals with a lower setpoint viral load generally have a slower disease progression and a longer period of stable viral load[13]. The decline in CD4 cell count directly after HIV infection is followed by an increase, although only to levels lower than before infection[14,15]. During the years of the stable viral load, CD4 counts will gradually drop by an average of 50-90 cells/mm³ per year in asymptomatic individuals[16]. Once CD4 cell counts have dropped to less than 200 cells/mm³ the individual has an increased risk of AIDS. The median time from start of HIV infection to AIDS ranges between 7.7 to 11 years, depending on age[6].

Factors influencing progression

The rate of progression from HIV infection to AIDS varies widely between individuals and age is not the only factor affecting disease progression. In fast progressors AIDS occurs within 12 months from infection whilst other individuals can be infected for 20 years without progression to AIDS[17]. Disease progression is associated with the rate at which CD4 cell numbers decrease[18]. The rate of CD4 decrease is also highly variable and depends to a great extent on the level of the plasma viral load, which in itself depends on viral and host genetic factors.

Viral Factors

Viral "fitness" refers to the extent of adaptation by a virus for replication in a defined environment. The determinants of HIV-1 fitness are complex and include tissue tropism (the ability of a given pathogen to infect a specific type of tissue), immune system evasion, drug resistance, and viral intrinsic replication capacity (RC, usually measured in *in vitro* fitness assays). Virulence of HIV, defined as the ability to cause end-organ damage, differs between subtypes[19,20]. Certain HIV strains within subtypes have also been associated with slower disease progression[21]. After HIV transmission, individuals are infected with HIV strains using the CCR5-coreceptor. During the course of infection HIV increasingly uses other coreceptors to infect cells. The switch to the use of CXCR4-coreceptors is associated with an increase in viral load and a drop in CD4 cell count[22].

Changes in viral fitness can have huge implications for the HIV epidemic, including changes in HIV transmission rates[23], and changes in the rate of disease progression and CD4 cell count decline[24]. Plasma viral load setpoint, easier to measure than RC, is positively correlated with viral fitness and often used as a marker to study trends in viral fitness using cohort studies with participants for whom the date of infection can be reliably estimated. Because of its huge potential impact on the HIV epidemic, trends over time in viral fitness and its associated measures have been reported by cohorts from several countries. As conflicting results from other cohorts were published, we studied trends over the course of the HIV epidemic in the Netherlands in setpoint viral load, the accompanying CD4 cell count measured at viral setpoint and the CD4 cell count decline after HIV infection and before start of therapy in chapter 2 and 4 in this thesis.

Host factors

A number of host factors affecting HIV disease progression have been identified. Older age at HIV infection is associated with more rapid disease progression and shorter survival times[25,26].

A genetic variation in CCR5 coreceptor molecules (the proteins on the CD4 cell surface to which HIV binds before it enters the cell) influences both HIV susceptibility and disease progression. The CCR5-delta-32 mutation is relatively frequent among people from European ancestry (10-15% are heterozygous, and 1%

is homozygous). Homozygotes for the delta-32 allele appear to be resistant to HIV infection, although resistance is not complete[27] Infection rates in heterozygotes are near normal[28,29], but progression to AIDS is delayed[30].
The human leukocyte antigen (HLA) genes encode for proteins on the surface of cells that are responsible for regulation of the immune system in humans. Differences in HLA alleles have also been shown to influence HIV disease susceptibility[31] and disease progression[32-35]. In a genome-wide association study (GWAS) two loci were significantly associated with setpoint viral load[36]; one of them tagged by single-nucleotide polymorphism (SNP) rs9264942, located 35 kb upstream of HLA-C, the other SNP rs2395029 in HCP5. These were later confirmed in other cohorts[37-39]. In Chapter 3 of this thesis we investigate whether adaptation of HIV-1 to these host genetic variations may, at least partly, account for changes in setpoint viral load over calendar years in the Netherlands.

Co-infections

Co-infection with syphilis[40-42], malaria[43,44], herpes simplex virus[45], and other (opportunistic) infections[46] has been associated with an increase in HIV viral load and a decrease in CD4 cell count. Co-infection with hepatitis C virus (HCV) through blood-blood or sexual contact is frequent in HIV positive drug-users and homosexual men. Although initially asymptomatic, untreated HCV-infection can result in liver malignancy and liver cirrhosis. The impact of HCV co-infection on HIV disease progression has long been disputed, but results suggest an accelerated progression of HIV disease during HCV co-infection compared to HIV mono-infection[47]. In chapter 5 in this thesis we investigate the effect of acute HCV co-infection on CD4 cell count and plasma viral load in chronically HIV infected individuals.

Combination antiretroviral therapy and the risk of AIDS, HIV-related and non-related morbidity and mortality

The introduction of cART as part of standard HIV care in 1996 has changed HIV from a lethal infection into a chronic condition. cART suppresses viral replication, restores CD4 cell counts and as a result, slows down disease progression. In a European multicenter study in HIV-1 outpatient clinics the incidence of AIDS declined from 30.7 in 1994 to 2.5 per 100 person-years in 1998[48], whilst mortality also dramatically decreased[49]. As a result, the HIV infected population has aged and life expectancy in the general and HIV infected population is similar, provided cART is started before the CD4 cell counts have dropped to low levels[7,50]. Because of the aging of the HIV-infected population, the incidence of diseases associated with older age in the general population, such as cardiovascular disease, kidney and liver disease, malignancies, and neurocognitive decline, has increased. Studies have strongly suggested that the risk of these co-morbidities is higher in HIV-infected individuals compared to the risk in HIV-negative individuals of

the same age[51-55] and the risk of (some of) these diseases is higher when CD4 cell counts are lower. Furthermore, in virologically successfully treated HIV-infected individuals with high CD4 cell counts there continues to be a decreasing risk of mortality with higher CD4 cell counts (even for counts ≥500 cells/mm^3)[56]. Therefore, it is important to restore CD4 cell counts as quickly as possible to levels seen in HIV-uninfected individuals. But even though cART suppresses HIV, increases CD4 cell numbers and reduces immune activation, blood biomarkers reflecting chronic inflammation and immune activation remain higher compared to uninfected persons. cART turns (acute) HIV infection into a state of chronic inflammation and persistent immune activation. Low-level long-term immune activation and chronic inflammation are increasingly recognized as a common pathological basis contributing to an increased risk of a number of progressive and age-related diseases[57].

Although HIV-1 infection is thought to have been eradicated[58,59] in two HIV-infected individuals (one diagnosed with acute myeloid leukaemia and one with Hodgkin's lymphoma) who both received a stem cell transplantation using donors with a homozygous CCR5-delta-32 mutation, this cure is too risky for widespread application to the general HIV-infected population and cART cannot completely eradicate the virus from the infected individual. During cART some infected CD4$^+$ T cells return to a resting phase while harboring HIV and form a latent HIV reservoir that persists for the remaining life span of these cells[60]. These latently infected cells can be found in in brain, lymph nodes, blood, and gut-associated lymphoid tissue and evade detection and clearance by the immune system[61]. If at some point these cells are activated, HIV production will start again and without cART HIV rebound occurs[62,63]. Studies have therefore attempted to target the latent reservoir, however strategies such as developing drugs to reactivate the latent reservoir or gene therapy to inactivate HIV have not been successful so far[64-66].

Restoration of CD4 cell counts is strongly dependent on the CD4 cell count at which cART is initiated. Due to improvements in drug tolerability and safety, patients can remain on therapy for longer, which has contributed to a shift over time in initiating cART at an earlier stage of disease. Treatment guidelines by the US Department for Health and Human Services (DHHS) on when to start, which are generally followed in the Netherlands, have changed recommendations from starting cART when CD4 counts have dropped to below 200 cells/mm^3 to offering treatment to all HIV-infected patients in 2012[67]. This changed recommendation was based on expert opinion. Conclusive scientific evidence was provided by the START and TEMPRANO randomized clinical trials in 2015 which both showed a lower rate of serious AIDS and non-AIDS events in individuals who were offered immediate treatment compared to individuals in whom the start of treatment was deferred[68,69]. In chapters 6 and 7 in this thesis we investigate longitudinal CD4 cell count changes after the start of cART and study how baseline CD4 cell

count, age and other factors influence restoration. In chapter 7 we additionally study CD8 cell count and CD4:CD8 ratio changes after the start of cART.

Observational studies in this thesis

In all analyses presented in this thesis data from the AIDS Therapy Evaluation in the Netherlands (ATHENA) cohort was used. This cohort is maintained by the Stichting HIV Monitoring (SHM). The SHM was founded in 2001 as a result of the successful ATHENA project. The ATHENA project was set up following the introduction of combination therapy in 1996, which was prescribed to a large number of HIV infected individuals. At the time there was a fear that the combination therapy would fail because of drug resistance, as was the case previously with monotherapy. The ATHENA study was carried out from 1998 to 2001 among 3600 HIV-infected patients and showed that the combination therapy had dramatically positive effects: fewer people developed AIDS and fewer people died from AIDS[70]. This research was continued and extended to include all HIV-infected persons, leading to the foundation of the SHM. As of 2002 the SHM was officially charged by the Dutch Minister of Health, Welfare and Sport to monitor the HIV epidemic and the quality of HIV care in the Netherlands. HIV-infected individuals are followed in one of the 26 adult and 4 paediatric treatment centers in the Netherlands. Clinical and demographic data are collected from these individuals who have been in clinical care in or after 1996. At the end of 2017 19,582 HIV-infected individuals were under active follow-up[71]. The SHM participates in international collaborations that combine data from several cohorts to answer research questions that it cannot answer on its own. This includes collaborations which study the prognosis during cART of HIV-infected individuals not previously exposed to antiretroviral therapy, collaborations which study whether certain diseases are more common and possibly occur at a younger age in HIV-infected persons than in non-HIV-infected persons, collaborations which study risk factors of adverse events of antiretroviral therapy, and collaborations which study prognosis and outcomes in smaller subgroups of patients such as HIV-infected pregnant women and children, and children who are exposed to HIV *in utero*.

Additionally, in some analyses in this thesis, data from homosexual men included in the Amsterdam Cohort Studies on HIV infection and AIDS (ACS) were used. The ACS started in October 1984, two years after the first cases of AIDS had been diagnosed in the Netherlands. Enrolment started among homosexual men at high risk for HIV infection, with symptoms other than lymphadenopathy associated with HIV-1 or without symptoms, followed shortly by enrolment among drug users. Different enrolment waves followed, including HIV-negative and/or HIV-positive homosexual men. Because of regular follow-up visits, the timing of HIV infection could be reliably estimated in those that entered HIV

negative. All ACS behavioural data are collected on a six-monthly basis, and clinical data of HIV-1 positives are currently provided by the SHM. Data from the ACS made it possible to study the prevalence, incidence, and risk factors for HIV infection and AIDS, as well as the natural course of HIV-1 infection. Furthermore the ACS has carried out intervention studies and prevention programs[72]. The ACS and the ATHENA cohort together span almost the entire period of the HIV epidemic in the Netherlands.

In chapter 5 data from the ATHENA cohort was supplemented with data from HIV-positive individuals with acute HCV co-infection included in the MSM Observational Study of Acute Infection with Hepatitis C (MOSAIC) Study. In this prospective study in 6 Dutch HIV treatment centres individuals with HIV/HCV co-infection are matched to HIV-infected individuals without HCV infection. Goal of the study is to gain insight into risk factors for HCV (re-)infection, response to HCV therapy, and mortality and morbidity in HIV-HCV co-infected individuals.

Finally, we have used data obtained from the Antiretroviral Therapy Cohort Collaboration (ART-CC), an international collaboration of 21 observational cohort studies from Europe and North America which includes data from the ATHENA cohort. ART-CC was established in 2000 to examine the prognosis of HIV-1-positive, treatment-naive individuals initiating cART[73].

Randomized clinical trials (RCTs) vs. observational cohorts

All results presented in this thesis are based on data from observational cohort studies. In an observational cohort study a group of individuals is followed over time. An observational cohort study is well suited to provide descriptive information such as the incidence of disease or the natural history of disease. In an observational cohort study the researcher, after a sufficient length of follow-up, can study risk factors or interventions for disease by comparing characteristics of individuals who develop disease with those who do not develop disease. Risk estimates obtained from observational studies are associative, which means that two things are related, but need not be causally related.

In RCTs individuals are randomly assigned to an intervention or non-intervention group. The imbalance of other variables will be minimal between the two groups and therefore the estimation of the intervention effect is likely to be unbiased. In RCTs, estimates of the relation between intervention and outcome can be given a causal interpretation. When an association measure (obtained from an observational study) differs systematically from the corresponding causal effect of the intervention, we say that there is bias. Bias due to confounding in observational studies arises from the non-random manner of allocating the exposure. Therefore, the quality of the evidence provided by RCTs is generally

higher than the quality of evidence of observational studies. However, it is not always possible to perform an RCT; an RCT can be too time-consuming, too expensive or certain interventions can be considered unethical. Also, the effect of biological factors on disease outcome cannot be assessed by means of an RCT. In these cases observational cohort studies may provide a viable alternative.
A major challenge of observational cohort studies is to make comparisons that have a causal interpretation with minimal bias. Analyses using data from observational cohort studies can under some conditions lead to valid causal estimates. An observational cohort study in which disease incidence between risk factors or treatments is compared can be viewed as a conditionally randomized experiment (an experiment in which individuals are randomized to control and treatment groups within subgroups/strata as determined by patient characteristics and time, with possibly different randomization probabilities in the different subgroups/strata) if:

1. There are no unmeasured confounders; at each time point, the investigators have access to all variables which affect both treatment selection or the risk factor and disease outcome. With statistical methods the estimate can be adjusted to resolve the bias due to known confounders. After correction for the confounders, control and treated individuals or individuals at each risk factor level are exchangeable (conditional exchangeability); after confounder adjustment the individuals are comparable except for the treatment/risk factor. The assumption of no unmeasured confounding cannot be formally tested.
2. For every possible combination of confounder values, the probability of receiving each of the possible treatments or the probability of being in each of the possible risk factor categories is greater than zero (positivity) in the population of interest.
3. The consistency assumption requires that the exposure/risk factor or treatment is defined with enough specificity such that there are no two or more 'flavors' or versions of an exposure or treatment level that have a different effect on the outcome. For example, if a study has measured whether people have smoked in the past, whilst this may include e-cigarettes, cigarettes, and cigars and these have a different effect on the outcome, the composite 'smoking' has no meaning.

Under these three conditions, estimates obtained with observational cohort studies are indistinguishable from those obtained with randomized experiments[72] and a well conducted observational cohort study can provide effect estimates similar to RCT estimates[75,76].

Observational cohort studies can also have advantages over an RCT. Because of the controlled setting of a RCT its results might be too positive compared to when individuals are followed in a standard clinical setting. Furthermore, RCT participants often are a selection of individuals with characteristics which makes them

more likely to respond to the study drug (more healthy individuals); its results cannot be readily generalized to a target population with different characteristics. An observational cohort study with a less restricted inclusion of participants then has the advantage that its results are generalizable to a broader population. Observational cohort studies often have more missing data than randomized clinical trials (e.g. in baseline data). In four out of the six studies in this thesis results from a longitudinal analysis of data from observational cohorts are presented. Individuals participating in a longitudinal clinical trial are typically measured at the same set of pre-determined time points according to strict protocols whereas participants in an observational study are more likely to miss an appointment and may not be measured according to a fixed schedule but as they or their physician sees fit for them. Data in an observational cohort is also often more likely to be missing because of permanent withdrawal or dropout. It is important to consider the reason for data being missing (e.g. moving, death or being too ill) as not doing so may give biased results in a longitudinal analysis. Three types of missing data are generally differentiated[77].

1. Missing Completely At Random (MCAR). Dropout or missingness is independent of the observed and unobserved baseline and longitudinal measurements of interest.
2. Missing At Random (MAR). Dropout or missingness is related to the observed but independent of the unobserved baseline and longitudinal measurements of interest.
3. Missing Not At Random (MNAR). Dropout or missingness is related to unobserved baseline and/or longitudinal measurements (those longitudinal measurements that would have been observed if the individual had not dropped out).

If dropout or missing patterns are MCAR or MAR, standard longitudinal models can give unbiased estimates, provided that the longitudinal model is specified correctly with all relevant predictors, including transformations and interactions if necessary. However, when the dropout pattern is MNAR, standard longitudinal models give biased estimates and the dropout pattern needs to be modelled correctly together with the longitudinal measurements to obtain unbiased estimates of longitudinal patterns.

Outline of the thesis

Chapter 2 presents a study describing changes in setpoint viral load and CD4 cell count measured at viral setpoint in HIV infected individuals in the Netherlands between 1984 and 2007. Several cohort studies from different countries in Europe or North America have found an increase in setpoint viral load over time, suggesting a more virulent HIV epidemic, whereas other cohorts have not found such an increase. This was the first study to address these changes before as well as after the introduction of cART in 1996 in the Netherlands. **Chapter 3**

investigates the association of host genetic variations with setpoint viral load. The association between host genetic makers CCR5Δ32, HCP5 rs2395029 and -35HLA-Crs9264929 and setpoint viral load was compared between individuals with a seroconversion date between 1982 and 2002 and between 2003 and 2009. **Chapter 4** studies whether the changes in setpoint viral load over the course of the HIV epidemic described in chapter 2 coincide with trends in the rate of decrease in CD4 cell count before cART is started in individuals with evidence of a recent HIV infection. Modeling the natural course of HIV is hampered by cART as natural progression of disease in an individual patient stops by definition when cART is started. This can be viewed as a type of informative dropout. Chapter 4 also studies whether estimates depend on how this informative censoring is handled in the analysis. Part of the natural course of HIV after acute HCV co-infection is studied in **chapter 5.** The effect of acute HCV infection on shorter-term outcomes in individuals with chronic HIV infection (treated and not treated with cART) was investigated by modeling patterns in plasma viral load and CD4 cell count around HCV infection. In **chapter 6** we investigate CD4 cell count trajectories during long term virologically suppressive cART. In view of the shift in treatment guidelines towards an earlier initiation of cART, this chapter studies whether a timely start of cART (at higher CD4 cell counts) can restore CD4 cell counts to levels seen in HIV uninfected individuals (as reported in the literature) and which clinical and demographic factors influence CD4 cell count restoration. The association of risk factors and CD4 cell count restoration in individuals on virologically suppressive cART was further investigated in **chapter 7**. This chapter additionally investigates changes in CD8 cell count and the ratio of CD4 and CD8 cell count and compares these immunological changes to levels of CD4 and CD8 cell counts and their ratio obtained from Dutch and Danish HIV-negative individuals. **Chapter 8** discusses implications of the observed results.

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2

Viral Load Levels Measured at Setpoint Have Risen Over the Last Decade of the HIV Epidemic in the Netherlands

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Abstract

Background

HIV-1 RNA plasma concentration at viral setpoint is associated not only with disease outcome but also with the transmission dynamics of HIV-1. We investigated whether plasma HIV-1 RNA concentration and CD4 cell count at viral setpoint have changed over time in the HIV epidemic in the Netherlands.

Methodology/Principal Findings

We selected 906 therapy-naïve patients with at least one plasma HIV-1 RNA concentration measured 9 to 27 months after estimated seroconversion. Changes in HIV-1 RNA and CD4 cell count at viral setpoint over time were analysed using linear regression models. The ATHENA national observational cohort contributed all patients who seroconverted in or after 1996; the Amsterdam Cohort Studies (ACS) contributed seroconverters before 1996. The mean of the first HIV-1 RNA concentration measured 9–27 months after seroconversion was 4.30 $\log_{10}$ copies/ml (95% CI 4.17, 4.42) for seroconverters from 1984 through 1995 (n = 163); 4.27 (4.16, 4.37) for seroconverters 1996–2002 (n = 232), and 4.59 (4.52, 4.66) for seroconverters 2003–2007 (n = 511). Compared to patients seroconverting between 2003–2007, the adjusted mean HIV-1 RNA concentration at setpoint was 0.28 $\log_{10}$ copies/ml (95% CI 0.16, 0.40; $p<0.0001$) and 0.26 (0.11, 0.41; $p = 0.0006$) lower for those seroconverting between 1996–2002 and 1984–1995, respectively. Results were robust regardless of type of HIV-1 RNA assay, HIV-1 subtype, and interval between measurement and seroconversion. CD4 cell count at viral setpoint declined over calendar time at approximately 5 cells/mm^3/year.

Conclusion

The HIV-1 RNA plasma concentration at viral setpoint has increased over the last decade of the HIV epidemic in the Netherlands. This is accompanied by a decreasing CD4 cell count over the period 1984–2007 and may have implications for both the course of the HIV infection and the epidemic.

Introduction

During the asymptomatic phase of HIV-1 infection, virus production and clearance are believed to reach a balance reflecting a relatively stable level of HIV-1 RNA concentration in plasma. Whether this balance, or viral setpoint, is reached in all patients remains open to debate[1,2]. It is agreed, however, that with a higher HIV-1 RNA plasma level, progression to AIDS is more frequent[3], as is the rate of HIV-1 transmission[4]. A rising trend over time in plasma HIV-1 RNA concentration at setpoint might imply an increase in the efficiency of transmission[5,6]. Three observational studies found no evidence for such a change[7–9], whereas two studies did[10,11]. Contrasting results likewise come from studies of HIV-1 RNA replicative fitness at viral setpoint, thought to be positively correlated with HIV-1 RNA concentration in plasma[12,13]. One study suggested a lower replicative fitness in HIV-1 isolates obtained from patients infected in 2002–2003 compared to isolates from patients infected between 1986–1999[14], but samples were not matched for time since seroconversion. A similar study, using isolates obtained from participants of the Amsterdam Cohort Study and samples matched for time since seroconversion, found an increase in replicative fitness over time[15].
Here we present a study of changes in the mean HIV-1 RNA concentration and CD4 cell count at viral setpoint measured in patients who became seropositive between 1984 and 2007.

Results

Baseline characteristics of the included 906 patients are summarized in Table 1. CD4 cell counts were available for 811 (90%). Of the 906 total, 92% were male, 76% had homosexual contact recorded as the most likely transmission route, and 82% originated from W-Europe/N-America. Most patients from other regions of origin were from S-America/Caribbean. Only 2% were from sub-Sahara Africa.

Results of HIV-1 subtyping, using nucleotide sequences of the pol region obtained for HIV-1 drug-resistance testing, were available for 449 (50%) patients, and subtype B was found in 408 (91%). Infection with circulating recombinant form (CRF) 02_AG was found in 15 patients, CRF 01_AE in 8, subtype A in 5, subtype C in 5, subtype G in 4, subtype D in 2, subtype A1 in 1 and CRF 03_AB in 1 patient. Of 425 patients tested before antiretroviral therapy was started, 32 (7.5%) had at least one resistance mutation. In all 163 patients with seroconversion before 1996, the HIV-1 RNA concentration at setpoint was measured with assays using the NASBA technique. Overall, RT-PCR was most used (in 40% of the 906 total). HIV-1 RNA plasma concentrations measured at setpoint were below the lower quantitation limit of the assay used in 37 of 906 (4%) patients. In 19 patients (2%), HIV-1 RNA concentrations were above the upper quantitation limit of the assay used. The mean HIV-1 RNA concentration at setpoint in all 906 patients was

4.45 $\log_{10}$ copies/ml. It was 4.30, 4.27, and 4.59 $\log_{10}$ copies/ml in patients with an estimated seroconversion date between 1984–1995, 1996–2002, and 2003–2007, respectively. Table 2 shows the differences in mean HIV-1 RNA concentration according to estimated year of seroconversion, as obtained with unadjusted and adjusted regression models. Compared to patients with an estimated seroconversion date in or after 2003, the adjusted mean HIV-1 RNA concentration among patients seroconverting between 1996 and 2002 and before 1996 was lower by 0.29 $\log_{10}$ copies/ml (95% CI 0.16, 0.41; $p<0.0001$) and 0.27 (0.12, 0.42; $p = 0.0004$), respectively. Furthermore, the adjusted mean HIV-1 RNA concentration at setpoint was 0.32 (95% CI 0.12, 0.51) $\log_{10}$ copies/ml lower in women compared to men ($p = 0.002$). Patients infected with subtype B had on average a 0.40 (0.14, 0.67) $\log_{10}$ copies/ml higher HIV-1 RNA concentration ($p = 0.003$) than patients infected with non-B subtypes. The mean HIV-1 RNA concentration was 0.16 (0.00, 0.32) $\log_{10}$ copies/ml higher in patients from W-Europe/N-America compared to patients from elsewhere ($p = 0.04$). There were no significant differences in mean HIV-1 RNA concentration according to age at seroconversion ($p = 0.43$), HIV transmission group ($p = 0.95$), interval between seroconversion and viral setpoint ($p = 0.96$), or presence of a resistance mutation ($p = 0.92$).
To test whether the increase in viral setpoint could reflect changing use of various quantitative HIV-1 RNA assays over time, we added type of assay to the model. The differences in mean HIV-1 RNA concentration between different periods of seroconversion increased slightly (Table 2). Relative to seroconverters between 2003 and 2007, the mean HIV-1 RNA concentration was 0.31 $\log_{10}$ copies/ml (95% CI 0.18, 0.44; $p<0.0001$) lower for seroconverters between 1996 and 2002 and 0.40 (0.18, 0.63; $p = 0.0003$) lower for those seroconverting before 1996. The difference in HIV-1 RNA concentration measured with RT-PCR assays was on average -0.12 $\log_{10}$ copies/ml (95% CI -0.30, 0.07; $p = 0.21$) compared to NASBA assays and 0.04 (-0.09, 0.17; $p = 0.54$) compared to bDNA assays. The HIV-1 RNA concentration was on average 0.16 $\log_{10}$ copies/ml (-0.03, 0.35; $p = 0.10$) higher when measured with the NASBA technique compared to samples tested with assays using bDNA. HIV-1 RNA concentration measured using assays with a lower detection limit <400 copies/ml was 0.08 $\log_{10}$ copies (95% CI -0.24, 0.08; $p = 0.33$) lower than those measured using assays with a higher detection limit. However, the differences according to calendar year of seroconversion remained similar. Also, when analyses were stratified according to the type or sensitivity of the assay, results remained similar (results not shown).
Given scarce data on HCV or HBV co-infection in patients who seroconverted before 1996, we restricted analyses including such co-infections as a confounder to the 743 patients who seroconverted in or after 1996. Mean HIV-1 RNA concentration in patients with a HCV co-infection was 0.36 $\log_{10}$ copies/ml (95% CI 0.08, 0.64; $p = 0.01$) higher than in patients without a HCV co-infection. It was 0.09 $\log_{10}$ copies/ml (-0.16, 0.35; $p = 0.46$) higher in patients with a HBV co-infection compared to patients without. Differences in mean HIV-1 RNA concentration according to year of HIV-1 seroconversion remained similar, being

Table 1. Baseline characteristics.

	Estimated year of seroconversion			
	1984–1995	**1996–2002**	**2003–2007**	**Total**
Total	163	232	511	906
MSM from W-Europe/N-America, excluding non-B subtype	114 (71%)	143 (61%)	355 (66%)	612 (68%)
Gender				
Male	144 (88%)	206 (89%)	480 (94%)	830 (92%)
Transmission risk group				
MSM	119 (73%)	162 (70%)	410 (80%)	691 (76%)
Heterosexual	3 (2%)	49 (21%)	54 (11%)	106 (12%)
IDU	22 (13%)	7 (3%)	2 (0%)	31 (3%)
Other	17 (11%)	12 (5%)	18 (4%)	47 (5%)
Unknown	2 (1%)	2 (1%)	27 (5%)	31 (3%)
Region of origin				
W-Europe/N-America	134 (82%)	188 (81%)	420 (82%)	742 (82%)
Other	5 (3%)	40 (17%)	75 (15%)	120 (13%)
Unknown	24 (15%)	4 (2%)	16 (3%)	44 (5%)
Subtype				
B	59 (36%)	76 (33%)	273 (53%)	408 (45%)
Non-B	1 (1%)	8 (3%)	32 (7%)	41 (5%)
Sample not available	103 (63%)	148 (64%)	206 (40%)	457 (50%)
Resistance-associated mutation found				
At least one mutation	7 (4%)	5 (2%)	20 (4%)	32 (4%)
None	46 (28%)	78 (34%)	269 (53%)	393 (43%)
Sequence not available	110 (67%)	149 (64%)	222 (43%)	481 (53%)
Sensitivity of assay				
Standard	163 (100%)	104 (45%)	42 (8%)	309 (34%)
Sensitive	0	114 (49%)	443 (87%)	414 (46%)
Unknown	0	14 (6%)	26 (5%)	40 (4%)
Amplification technique of assay				
NASBA	163 (100%)	53 (23%)	44 (9%)	260 (29%)
bDNA	0	66 (28%)	175 (34%)	241 (27%)
RT-PCR	0	99 (43%)	266 (5%)	265 (29%)
Unknown	0	14 (6%)	26 (5%)	40 (4%)
HBV				
Negative	59 (36%)	201 (87%)	431 (84%)	691 (76%)
Positive	3 (2%)	14 (6%)	24 (5%)	41 (5%)
Unknown	101 (62%)	17 (7%)	66 (11%)	184 (20%)
HCV				
Negative	44 (27%)	188 (81%)	397 (78%)	629 (69%)
Positive	9 (6)	10 (4%)	20 (4%)	39 (4%)
Unknown	110 (67)	34 (15%)	94 (18%)	238 (26%)
Age at seroconversion in years median, (IQR)	34.4 (28.9–40.5)	33.8 (29.9–40.4)	36.4 (30.0–43.1)	35.2 (29.8–41.7)
Months between seroconversion and plasma HIV-1 RNA measurement, median (IQR)	11.6 (10.1–14.5)	10.9 (9.9–12.7)	10.9 (9.8–12.4)	10.9 (9.9–12.8)
Months between seroconversion and CD4 cell count measurement, median (IQR)	10.3 (10.0–11.1)	10.7 (9.8–12.2)	10.6 (9.7–11.9)	10.5 (9.8–11.8)

0.29 $\log_{10}$ copies/ml (0.17, 0.41; p<0.0001) higher in patients with seroconversion between 2003–2007 compared to 1996–2002. To avoid any bias arising from changes in the distribution of ethnicity, gender, and infection with non-B subtype over time, we focused on a homogeneous patient group of 612 MSM from W-Europe/N-America. Patients with a confirmed HIV-1 non-B infection were excluded. Figure 1 shows the HIV-1 RNA concentration at setpoint and at 12, 18, and 24 months after seroconversion and the estimated mean HIV-1 RNA concentration by calendar year of seroconversion. The dashed line in Figure 1a shows that the mean setpoint HIV-1 RNA concentration at the start of 1985 was 4.46 $\log_{10}$ copies/ml (95% CI 4.27, 4.65). It was 4.21 $\log_{10}$ copies/ml (4.09, 4.33) at its lowest value in 1995 and 4.88 $\log_{10}$ copies/ml (4.76, 5.01) in 2007. Estimates of differences in mean HIV-1 RNA concentration at setpoint according to the calendar year of seroconversion for this subgroup, shown in Table 2, are similar to those for the total of 906 patients. HIV-1 subtype was the only variable included in the adjusted model apart from estimated year of seroconversion. On further restricting the sample size to the 297 patients known to be infected with subtype B, we found similar differences in mean HIV-1 RNA concentration (results not shown). Finally, we looked separately at plasma HIV-1 RNA levels at 12, 18, and 24 months after seroconversion and found mean HIV-1 RNA concentrations of 4.50, 4.48, and 4.40 $\log_{10}$ copies/ml, respectively. Differences in mean HIV-1 RNA concentration at 12 and 18 months according to estimated year of seroconversion were similar to those obtained through models including the first HIV-1 RNA concentration between 9 and 27 months after seroconversion. In a final sensitivity analysis, including 751 patients with a maximum seroconversion interval of 6 months, the mean of the first HIV-1 RNA concentration taken after seroconversion was 0.48 $\log_{10}$ copies/ml (95% CI 0.26, 0.71; p<0.0001) lower for seroconverters before 1996 and 0.17 (0.00, 0.35; p = 0.05) lower between 1996–2002 compared to 2003–2007. The mean was 0.31 $\log_{10}$ copies/ml (0.06, 0.56; p = 0.02) lower for seroconverters before 1996 compared to 1996–2002.

In 811 patients with CD4 cell counts available between 9–27 months after seroconversion, the median count at viral setpoint was 520 cells/mm³ (IQR 390–680). Table 3 shows results of the linear regressions of CD4 cell count at viral setpoint. Mean CD4 cell count at viral setpoint declined throughout the period 1984–2007 by 0.025 cube root cells/mm³/year (95% CI 0.012, 0.038; p<0.0001), a decline of approximately 5 CD4 cells/mm³/year. Region of origin was the only other variable included in the adjusted model. Mean CD4 cell count at viral setpoint in patients from W-Europe/N-America with seroconversion between 2003–2007 was 507 cells/mm³ (485, 530), compared to 466 cells/mm³ (425, 509, difference p = 0.07) for patients from elsewhere. Table 3 shows results of regression analyses of CD4 cell count on a cube-root scale for our homogeneous patient group. The mean decrease over time of the first CD4 cell count 9–27 months after seroconversion and at 12, 18, and 24 months after seroconversion was 0.028 (95% CI 0.014, 0.041), 0.025 (0.011, 0.038), 0.027 (0.013, 0.041), and 0.021 (0.004, 0.038) cubic root cells/mm³/year, respectively. Figure 2 shows the estimates

Table 2. Mean (95% CI) differences in HIV-1 RNA concentration at viral setpoint (log_{10} copies/ml) according to time of seroconversion.

	Plasma HIV-1 RNA concentration	First 9–27 months	After seroconversion	at 12 months	at 18 months	at 24 months
	Patient group	All patients	Homogeneous patient group[a]	Homogeneous patient group[a]	Homogeneous patient group[a]	Homogeneous patient group[a]
	N	906	612[b]	552[c]	370[c]	315[c]
Unadjusted model	Year of seroconversion					
	2003–2007 (reference)	0.00	0.00	0.00	0.00	0.00
	1996–2002	-0.32 (-0.45, -0.20)	-0.44 (-0.59, -0.29)	-0.45 (-0.61, -0.29)	-0.35 (-0.54, -0.15)	-0.39 (-0.62, -0.16)
		p<0.0001	p<0.0001	p<0.0001	p = 0.0005	p = 0.0008
	1984–1995	-0.29 (-0.44, -0.15)	-0.37 (-0.54, -0.20)	-0.37 (-0.54, -0.20)	-0.38 (-0.61, -0.15)	-0.32 (-0.54, -0.10)
		p<0.0001	p<0.0001	p<0.0001	p = 0.001	p = 0.008
Adjusted model	Year of seroconversion					
	2003–2007 (reference)	0.00	0.00	0.00	0.00	0.00
	1996–2002	-0.29 (-0.41, -0.16)	-0.41(-0.67, -0.27)	-0.42 (-0.58, -0.26)	-0.33 (-0.53, -0.13)	-0.39 (-0.62, -0.16)
		p<0.0001	p<0.0001	p<0.0001	p = 0.001	p = 0.0008
	1984–1995	-0.27 (-0.42, -0.12)	-0.34 (-0.51, -0.17)	-0.34 (-0.51, -0.17)	-0.37 (-0.60, -0.13)	-0.32 (-0.54, -0.10)
		p = 0.0004	p<0.0001	p<0.0001	p = 0.002	p = 0.005
Adjusted model also including type of assay	Year of seroconversion					
	2003–2007 (reference)	0.00	0.00	0.00	0.00	0.00
	1996–2002	-0.31 (-0.44, -0.18)	-0.44(-0.60, -0.28)	-0.46 (-0.62, -0.29)	-0.34 (-0.54, -0.13)	-0.37 (-0.61, -0.13)
		p<0.0001	p<0.0001	p<0.0001	p = 0.001	p = 0.003
	1984–1995	-0.40 (-0.63, -0.18)	-0.55 (-0.82, -0.29)	-0.60 (-0.87, -0.32)	-0.40 (-0.76, -0.03)	-0.32 (-0.74, 0.11)
		p = 0.0003	p<0.0001	p<0.0001	p = 0.03	p = 0.15

a Homogeneous patient group: MSM from W-Europe/N-America. Patients with non-B subtype infection excluded.
b Adjusted for gender, region of origin, subtype, age at seroconversion, HIV transmission group, interval between seroconversion and viral setpoint, and presence of a resistance mutation.
c Adjusted for availability of subtype data.

back-transformed to the original scale. Mean CD4 count at 12 months was 592 (562, 653), 563 (523, 605) and 502 cells/mm³ (479, 527) for seroconverters between 1984–1995, 1996–2002, and 2003–2007, respectively. Estimated differences in CD4 count between seroconverters of 1996–2002 and 2003–2007 were greater in analyses of the homogeneous patients than in analyses of the total group.

Discussion

We found a rising trend over time in the HIV-1 RNA concentration at setpoint in patients infected in the last decade, with a complementary downward trend in CD4 cell count at viral setpoint.

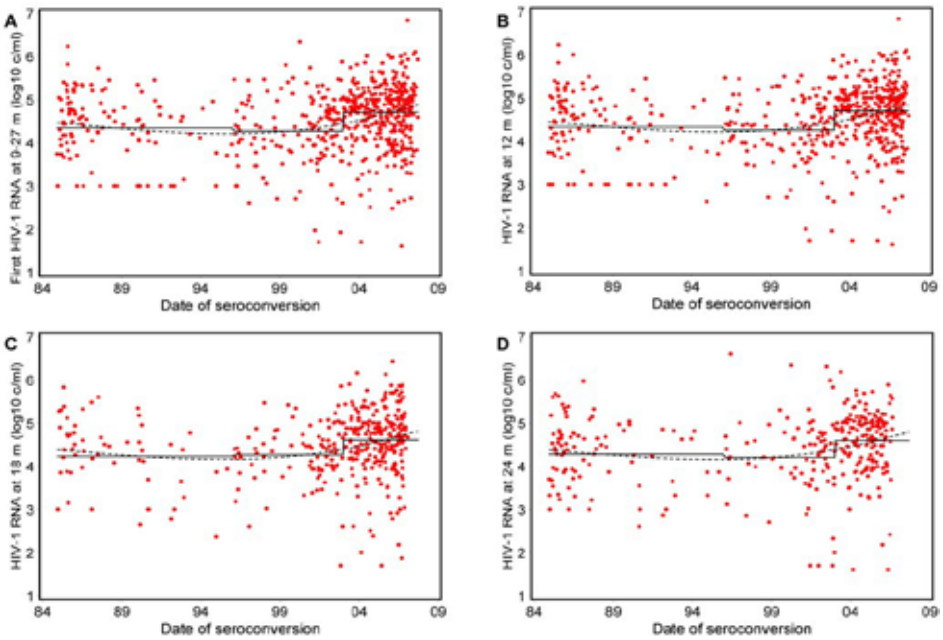

Figure 1. HIV-1 RNA concentration at viral setpoint and mean HIV-1 RNA concentration at each time period. In MSM patients from W- Europe or N-America with a proven or likely infection with subtype B: a) first HIV-1 RNA 9–27 months after seroconversion (n = 612), b) at 12 (n = 552), c) 18 (n = 370), and d) 24 months (n = 315). The solid black line shows the mean HIV-1 RNA concentration for patients with an estimated date of seroconversion from 1984 through 1995, 1996 through 2002, and 2003 through 2007 (as shown in Table 2). Dashed black lines are estimates obtained by continuous modelling of the estimated date of seroconversion using cubic splines.

Our results agree with those of the CASCADE study[10] and a recent study of the epidemic in Italy[11]. The CASCADE study found an increase in mean HIV-1 RNA concentration at viral setpoint of 0.035 $\log_{10}$ copies/ml/year over the period 1985–2002, although we found an increase only from 1996. Three other studies found no evidence for an increase[7–9]. Differences in patient selection, study period, and outcome definitions across these five studies might explain the discrepancies. In a study from the Swiss HIV Cohort Study (SHCS) and the Italian cohort study[8,11], all patients with a confirmed HIV-1 infection were selected. Other studies restricted patient selection to seroconverters with a maximum seroconversion interval of 6 months[9] or 12 months[7,10]. Herbeck et al.[7] looked at HIV-1 RNA concentration at setpoint in 384 homosexual patients with known seroconversion dates between 1985 and 2005, but most were infected before 1996. The study period in that study and the SHCS[8] might have been too short to find an increase in HIV-1 RNA concentration at setpoint over time. Outcome definitions of the five studies ranged from the first available measurement of HIV-1 RNA plasma concentration after seroconversion[9,10] to measurements at a later stage[7,8,11]. Because the exact moment of seroconversion is unknown, the former definition has the disadvantage of not knowing whether the measurement was taken during the peak HIV RNA concentration phase following infection, during the phase shortly before or after the peak, or during the setpoint phase. This type of measurement error most likely hampers the detection of significant changes over time. Admittedly, using measurements at a later stage can introduce bias, because patients who have started antiretroviral therapy early are censored from the analysis. Assuming that patients with a high HIV-1 RNA concentration and a low CD4 cell count will start therapy earlier than patients with a lower concentration and a higher cell count, results may be biased towards a lower HIV-1 RNA concentration and higher CD4 cell count at viral setpoint from 1996 onwards, especially in measurements taken 24 months after seroconversion.

This may explain our finding of a significant difference between patients who seroconverted before 1996 and those who did so between 1996 and 2002 with respect to the first HIV-1 RNA concentration after seroconversion and no significant difference when analysing the first HIV-1 RNA concentration taken 9–27 months after seroconversion. However, both analyses showed an increasing HIV-1 RNA concentration over time.

The decreasing trend in CD4 cell count at viral setpoint over time complements the increasing trend in HIV-1 RNA concentration. A similar decrease in CD4 cell count over time was likewise found in other studies[10,16,17]. Evidence of an increasing trend[18] or a stable level[7,19] in CD4 cell count might reflect a shorter study period.

Bias through systematic inclusion of correlated transmission networks is unlikely, as active enrolment of related partners was never in place during our entire study period. Other potential sources of bias which could have influenced our finding include the genetic heterogeneity of HIV-1, which can impede the

accuracy of quantitation, especially in early assays primarily designed to detect subtype B[20–22]. However, the effect persisted when we focused on a homogeneous group of MSM from W-Europe/N-America with a proven or highly likely subtype B infection. The higher mean HIV-1 RNA concentration in patients for whom we lacked subtype data points to some residual confounding because of infection with non-B subtypes. However, sensitivity analyses of patients known to be infected with subtype B found similar differences in mean HIV-1 RNA concentration at setpoint over time.

Disease progression has been shown to differ among patients with subtype A, C, D and G infection[23–25]. We found a higher mean HIV RNA concentration in patients with subtype B infection compared to non-B, as well as in patients from W-Europe/N-America compared to those with other origins. A recent study reported a higher setpoint virus load in patients with white ethnicity compared to black (mostly from sub-Sahara Africa), but no significant differences between infection with B and non-B subtypes[26]. Differences in the distribution of ethnicity and subtypes might explain this discrepancy.

Since only 30 of the 830 men we studied were infected through heterosexual contact, lack of power might have been a reason we found a non-significant difference in HIV RNA concentration at setpoint between MSM and heterosexually infected patients. The SHCS reported a significant difference between MSM and heterosexually infected patients[8]. Also in contrast to the SHCS, our study and others found a higher HIV RNA concentration at setpoint in female patients. The difference in HIV RNA concentration between men and women emerges only at lower CD4 cell counts[27], a factor that could explain these different results.

The HIV-1 RNA plasma concentration was measured with several assays. The distribution of the assays used has changed over the years, and we did not perform batch-wise re-testing of samples using only one assay. HIV-1 RNA concentrations measured within the dynamic range of the Versant HIV-1 RNA (bDNA) 3.0 assay are, on average, lower than those measured with the Cobas Amplicor assay (RT-PCR)[28–30]. The Amplicor HIV-1 Monitor assay (RT-PCR)[31] yields, on average, lower concentrations than the NASBA HIV-1 RNA QT assay, the only assay used in samples taken before 1996. This ranking was reflected in our analyses and might explain the more pronounced differences after adjusting for type of assay between seroconverters from 1984–1995 and from 2003–2007. In concordance with previous reports, the mean HIV-1 RNA concentration at setpoint was slightly higher when measured using assays with a lower detection limit of 1000 or 400 copies/ml compared to ≤50 copies/ml[32], but adjustment for assay sensitivity did not appreciably change our results. The changing distribution of assays is thus unlikely to explain the increase in mean HIV-1 RNA concentration at viral setpoint over time.

Techniques for measuring CD4 cell counts changed over time as well, leading to less test variability. Absolute CD4 cell counts were traditionally assessed using a dual-platform technique that has been gradually replaced by a single-platform technique introduced in the late 1990s. Others changes in flow

Table 3. Changes (95% CI) in CD4 cell count at viral setpoint (cells/mm³) using different models.

CD4 cell count	**First**	**9–27 months after**	**seroconversion**	**at 12 months**	**at 18 months**	**at 24 months**
Patient group	All patients	All patients	Homogeneous patient group[a]	Homogeneous patient group[a]	Homogeneous patient group[a]	Homogeneous patient group[a]
	Unadjusted	Adjusted[b]				
N	811	811	578	555	439	347
Median CD4 cell count, (cells/mm³)	520	520	530	530	490	480
Change in CD4 cell count at viral setpoint (cubic cells/mm³/year)	-0.025 (-0.038, -0.012)	-0.026 (-0.039, -0.013)	-0.028 (-0.041, -0.014)	-0.025 (-0.038, -0.011)	-0.027 (-0.041, -0.013)	-0.021 (-0.038, -0.004)
	p<0.0001	p = 0.0001	p<0.0001	p = 0.0004	p = 0.0002	p = 0.02
Difference in mean CD4 cell count (cube root cells/mm³)						
2003–2007 (reference)	0.00	0.00	0.00	0.00	0.00	0.00
1996–2002	0.18 (20.01, 0.37)	0.19 (20.01, 0.38)	0.31 (0.08, 0.54)	0.31 (0.07, 0.54)	0.50 (0.25, 0.76)	0.27 (20.05, 0.59)
	p = 0.07	p = 0.06	p = 0.008	p = 0.01	p<0.0001	p = 0.10
1984–1996	0.43 (0.20, 0.65)	0.45 (0.21, 0.68)	0.51 (0.26, 0.76)	0.45 (0.20, 0.70)	0.49 (0.24, 0.74)	0.35 (0.05–0.65)
	p = 0.0002	p = 0.0002	p<0.0001	p = 0.0004	p = 0.0001	p = 0.02

a Homogeneous patient group: MSM from W-Europe/N-America. Patients with non-B subtype infection excluded.
b Adjusted for gender, region of origin, subtype, age at seroconversion, HIV transmission group, interval between seroconversion and viral setpoint, and presence of a resistance mutation.

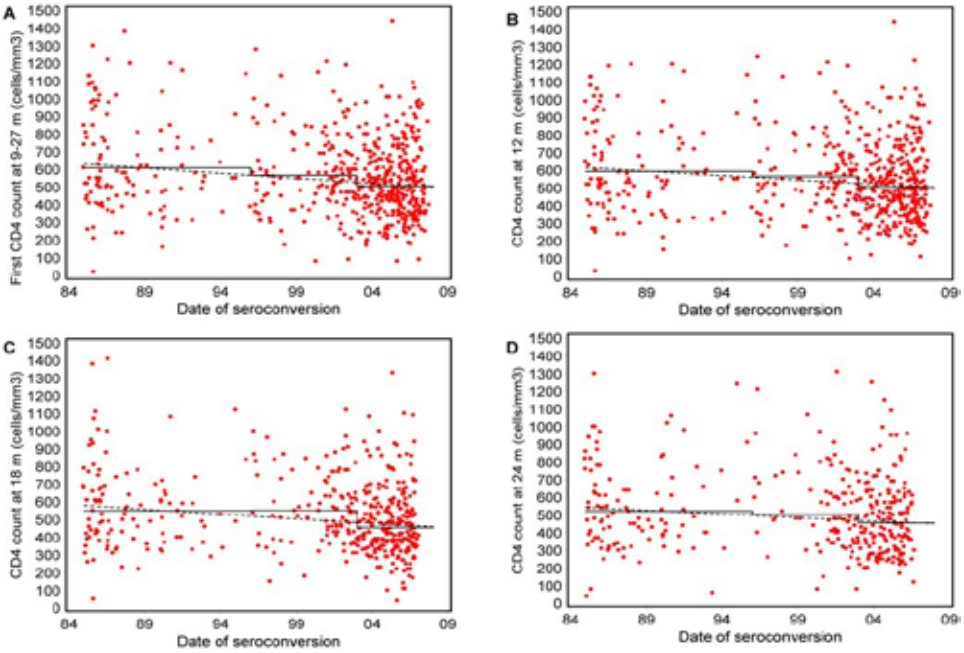

Figure 2. CD4 cell count at viral setpoint and mean CD4 cell count at each time period. In MSM from W-Europe/N-America with a proven or likely subtype B infection: a) first CD4 cell count between 9 and 27 months after seroconversion (n = 578), b) at 12 months (n = 555), c) 18 months (n = 439), and d) 24 months (n = 347). The solid black line shows the mean CD4 cell count for patients with an estimated date of seroconversion from 1984 through 1995, 1996 through 2002, and 2003 through 2007 (as shown in Table 2). The dashed black lines were obtained using linear models assuming a constant decrease between 1984 and 2007.

cytometry techniques over time include changes in the gating strategy and sample preparation. There is some evidence to suggest that CD4 cell counts turn out lower when measured using the single-platform technique. Also, CD45-SSC gating, more frequently used in later calendar years, may yield higher CD4 cell counts than CD45-CD14 gating, more frequently used in earlier calendar years[33]. For our study, these two potential biases may outweigh each other.

In samples obtained before 1996, quantitation followed storage at -80°C. No significant effect of freezing, storage, and thawing on HIV RNA recovery using the Amplicor HIV-1 Monitor and NASBA HIV-1 RNA QT assay has been reported[31,34,35]. Moreover, we observed an increase in mean HIV-1 RNA concentration at setpoint between periods 1996–2002 and 2003–2007.

The awareness level of physicians and patients to symptoms of acute HIV-1

infection has improved in recent years and could have influenced our findings. Setpoint HIV-1 RNA concentration has been shown to increase with the number of symptoms after recent HIV-1 infection[36,37]. In times when patients without symptoms were overlooked, the mean HIV-1 RNA concentration at setpoint was most likely overestimated. However, as data on symptoms were not collected, we could not investigate this further. The increasing number of patients included in our study over time positively correlated with increasing setpoint viral load levels. The contribution of resulting selection of higher viral load at setpoint in to facilitating the spread of HIV is a cause for alarm. However, other reasons for the higher number of study patients in more recent years include more sexual risk behaviour[38,39], more HIV-negative subjects who repeatedly test for sexually transmitted infections (STIs)[39–41], and a higher incidence of STIs[42]. Also, the start in 2003 of a randomized study of patients with primary HIV-1 infection has raised physician awareness. Therefore, the higher number of new infections in recent years included in our study cannot be ascribed solely to a higher setpoint viral load in those years. As for the other possibility, an ongoing phylogenetic study has found no indications that newly imported HIV-1 strains are responsible for the changes in setpoint HIV RNA levels.

Some evidence suggests that HIV-1 RNA concentration at viral setpoint is an adaptive trait and may change under the influence of selection[6]. Transmission of HIV-1 is thought to happen most efficiently very early, during primary infection, and late, during the symptomatic phase[4,43]. During the asymptomatic phase after primary infection, the probability of transmission may be smaller, but given the long duration of this phase, its contribution to the number of newly infected patients is probably substantial. Successful antiretroviral therapy, usually started some years after primary infection, suppresses HIV-1 RNA plasma concentration to levels at which the probability of transmission is considered minimal. Thus the period of infectiousness may largely be confined to the very early phase of infection, in which viruses that reproduce at a high level are selected. Such viruses could establish a high HIV-1 RNA concentration at setpoint in a new host[44]. For this hypothesis to hold, setpoint viral load between transmitter and recipient needs to be correlated. This correlation will be investigated in a future study, using data from transmission networks.

A significant increase in HIV-1 replication fitness over time has been described for the Amsterdam epidemic[15]. Follow-up molecular analysis should reveal which changes in the viral components are responsible for this increased replication capacity. An increase in HIV RNA concentration at setpoint may be also the result of adaptation of HIV to particular HLA molecules in the population. Kawashima et al. show that HLA molecules associated in 1983 with slow disease progression did not protect against disease progression in Japanese patients infected between 1997–2008[45].

In conclusion, we found an increase in the HIV-1 RNA plasma concentration measured at viral setpoint and a decrease in CD4 cell count in non-treated patients with a confirmed HIV-1 seroconversion during the last decade of the

HIV epidemic in the Netherlands. The higher HIV-1 RNA concentration could not be attributed to changes in subtype or assay used, but coincides with a higher proportion of treated HIV-1-infected patients. The implications of an increased HIV-1 RNA concentration at viral setpoint on disease progression and on transmission dynamics require further study.

Methods

Patient selection

The ATHENA observational cohort[46] includes anonomyzed data from all HIV-infected patients living in the Netherlands who receive care in one of the 24 HIV treatment centres. Initially, data were collected only from patients who had started combination antiretroviral therapy (cART). Data collection was extended in 2002 to include all HIV-infected patients, cART treated or untreated alike, who had been followed since 1996 in any of the centres. ATHENA patients are informed by their treating physician of an opt-out procedure. Ethical approval is not obtained, as data collection is part of HIV care. For our study, we also included data obtained from homosexual men (MSM) participating in the Amsterdam Cohort Studies (ACS)[47]. Informed written consent is obtained from all ACS participants, and the ACS has been approved by the Medical Ethical Committee of the Academic Medical Centre.

We selected patients with a maximum interval of one year between the last negative and first positive HIV-1 antibody test. The day of seroconversion was estimated as the midpoint between the last seronegative and the first seropositive test. Patients with negative or indeterminate Western Blot results in the presence of HIV-1 p24 anitigen or RNA were also selected, in which case the day of seroconversion was set one month prior to the date of the first positive antibody test. All patients in our study were at least 16 years old at the estimated day of seroconversion, had been sampled at least once for plasma HIV-1 RNA concentration between 9 and 27 months after seroconversion, and were antiretroviral therapy-naive. All patients who seroconverted before 1996 were participants of the ACS.

Measurements

First, we used the HIV-1 RNA concentration and CD4 cell count measured in peripheral blood sampled earliest in the 9–27 months after seroconversion as the measurements at viral setpoint. In addition, we selected results of the HIV-1 RNA and CD4 cell count measurements taken between 9 and 15 months (closest to 12), between 15 and 21 months (closest to 18), and between 21 and 27 months (closest to 24). Any measurements taken after the start of antiretroviral therapy were not used in any analysis. Assays were classified according to the amplification technique: nucleic acid sequence-based amplification (NASBA), reverse transcriptase-polymerase chain reaction (RT-PCR), and branched DNA signal amplification (bDNA). Assays using the NASBA technique were NASBA

HIV-1 RNA QT, NucliSens HIV-1 RNA QT, and NucliSens EasyQ (bioMe´rieux, Boxtel, The Netherlands). Assays using RT-PCR techniques were Amplicor HIV-1 Monitor, Cobas Amplicor, Cobas TaqMan HIV-1 (Roche Diagnostics, Pleasanton, CA, USA), LCx HIV RNA quantitative, and m2000rt HIV RNA (Abbott, Abbott Park, IL, USA). The only assay using the bDNA technique was Versant HIV-1 RNA version 3.0 (Siemens, Deerfield, IL, USA). Classification by amplification technique is very similar to classification by manufacturer. The RT-PCR assays made by Roche and Abbott were grouped together. Since the Abbott assay was used in only 59 patients who all seroconverted in 2006 and 2007 these assays could not be analysed separately. Finally, HIV-1 RNA assays were also classified as standard (having a lower detection limit of 1000 or 400 copies/ml) and sensitive (having a lower detection limit ≤50 copies/ml).

Statistical analysis

Parametric survival regression models with a normal error distribution were used to model changes in plasma HIV-1 RNA concentration at setpoint. When below the limit of detection, the value was regarded as interval-censored between 1 copy/ml and the lower detection limit. Values above the upper detection limit were right-censored at the upper detection limit. CD4 cell count at viral setpoint was modelled using linear regression models. CD4 cell counts were cube root-transformed to apply better to model assumptions. Estimated calendar year of seroconversion was modelled using 3 categories: <1996 (pre-cART), 1996–2002 and ≥2003. The post-cART cut-off was chosen so that each time period was sufficiently wide and included a sufficient number of patients. Estimated date of seroconversion was continuously modelled using restricted cubic splines with knots at the 5th, 50th and 95th percentiles. This allowed us to model date of seroconversion in a flexible manner and to avoid having to report results over year categories with few patients. Splines with 5 knots were also used but led to similar estimates. Potential confounders for analysis of HIV-1 RNA concentration at setpoint included gender, region of origin (W-Europe/N-America, other, and unknown), age at seroconversion, HIV-1 subtype (B, non-B, and unknown), transmission of drug-resistant virus (at least one mutation, none, and unknown)[48], interval between measurement and seroconversion, transmission risk group (men having sex with men [MSM], heterosexual, intravenous drug use [IDU], other, and unknown), co-infection with HBV of HCV (positive, negative, or unknown), sensitivity of assay (lower detection limit ≥400 and <400 copies/ml), and technique of the quantitative HIV-1 RNA assay used (NASBA, RT-PCR, and bDNA). Active HBV co-infection was defined as a positive HBsAg test. Chronic HCV co-infection was defined as a positive HCV RNA test or, if not available, a positive HCV antibody test. We used the first available test result up to 2 years after the estimated HIV-1 seroconversion date. Variables were retained in adjusted models if their overall p-value was <0.20.

To obtain results in a population as homogeneous as possible, we also ran models including only data from MSM from W-Europe/N-America. Patients with a proven

HIV-1 non-B infection were excluded. If the HIV-1 subtype was not determined, patients were included. In addition, we performed sensitivity analyses on patients with a confirmed subtype B infection. A final sensitivity analysis was performed on patients with a maximum seroconversion interval of 6 months, using the first HIV-1 RNA measurement after seroconversion as dependent variable. This analysis was performed in order to study the potential bias that results from censoring patients with an early start of antiretroviral therapy. Models were adjusted for time between the estimated day of seroconversion and the measurement using restricted cubic splines. Analyses were performed using SAS version 9.1 (SAS Institute, Cary, North Carolina, USA).

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Conceived and designed the experiments: LG FdW. Analyzed the data: LG. Wrote the paper: LG DB FdW. Commented on the paper: SJ MB AvS DB CF JML JP BB. Gave advice on data analysis: SJ MB AvS DB CF FdW.

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3

Rising HIV-1 viral load setpoint at a population level coincides with a fading impact of host genetic factors on HIV-1 control

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Abstract

Objective

Heterozygosity for a 32 base pair deletion in the CCR5 gene (CCR5wt/Δ32) and the minor alleles of a single-nucleotide polymorphism in the HCP5 gene (rs2395029) and in the HLA-C gene region (-35HLA-C; rs9264942) has been associated with a lower viral load setpoint. Recent studies have shown that over calendar time, viral load setpoint has significantly increased at a population level. Here we studied whether this increase coincides with a fading impact of above-mentioned host genetic markers on HIV-1 control.

Methods

We compared the association between viral load setpoint and HCP5 rs2395029, -35HLA-C rs9264942, and the CCR5wt/Δ32 genotype in HIV-1-infected individuals in the Netherlands who had seroconverted between 1982 and 2002 (pre-2003 seroconverters, n = 459) or between 2003 and 2009 (post-2003 seroconverters,n = 231).

Results

Viral load setpoint in post-2003 seroconverters was significantly higher than in pre-2003 seroconverters ($P = 4.5 \times 10^{-5}$). The minor alleles for HCP5 rs2395029, -35HLA-C rs9264942 and CCR5wt/Δ32 had a similar prevalence in both groups and were all individually associated with a significantly lower viral load setpoint in pre-2003 seroconverters. In post-2003 seroconverters, this association was no longer observed for HCP5 rs2395029 and CCR5wt/Δ32. The association between viral load setpoint and HCP5 rs2395029 had significantly changed over time, whereas the change in impact of the CCR5wt/Δ32 genotype over calendar time was not independent from the other markers under study.

Conclusion

The increased viral load setpoint at a population level coincides with a lost impact of certain host genetic factors on HIV-1 control.

Introduction

Disease progression after infection with HIV-1 is strongly associated with the amount of virus in blood[1]. In most infected individuals, virus production and clearance reach a balance approximately 18 – 24 months after seroconversion, reflecting a – temporary – relatively stable level of HIV-1 RNA in plasma, the so-called viral load setpoint. This viral load setpoint varies between individuals and is a strong and independent predictor of subsequent disease progression[2]. In several cohorts, certain host genetic factors, such as having a HLA-B57 or HLA-B27 type, have been associated with a lower viral load setpoint or a delayed clinical course of infection[3–5]. Heterozygosity for a 32 base pair deletion in CCR5, the gene that is coding for one of the HIV-1 coreceptors, is also associated with delayed disease progression and a lower viral load early in infection[6–8]. In the first genome-wide association study (GWAS) on HIV-1 control, the minor alleles of single-nucleotide polymorphism (SNP) rs9264942 35 kbp upstream of HLA-C and of SNP rs2395029 in HCP5, which is in high linkage disequilibrium with HLA-B57, were associated with a lower viral load setpoint[9], which was confirmed in other cohorts[10–12].

We and others have recently reported a rising trend over time of the viral load setpoint at a population level, which could signal an accelerating clinical course of infection[13–15]. The underlying mechanism for this increase is unclear but may reflect an adaptation of the virus to its environment. Indeed, due to its high replication rate, error-prone reverse transcription, and lack of proof reading, HIV-1 is known to rapidly accumulate mutations that can be positively selected, allowing rapid escape from host immune surveillance[5,16–21] and from antiretroviral drugs[22,23]. In addition to intrapatient escape from host defense mechanisms, adaptation of HIV-1 at the population level has been observed as well[24,25]. Continuous pressure by host genetic factors may result in viral adaptation at the population level and thus changed virus replication. Here we studied whether the increasing viral load setpoint over calendar time at a population level could be explained, at least partially, by a fading impact of host genetic factors on HIV-1 control.

Methods

Study populations

The SHM-HIV-1 Host Genetics study (SHM is the abbreviation of 'stichting HIV monitoring', which is the Dutch translation for the Netherlands HIV-monitoring foundation) included patients with an accurately estimated date of seroconversion (midpoint of a period of maximum 180 days between last HIV-negative and first HIV-positive), an age of more than 18 years at enrollment,

with viral load data available or anticipated to become available at around 18 – 24 months after seroconversion (viral load setpoint), and with information on treatment history available. Patients who initiated combination antiretroviral therapy (cART) before a viral load setpoint measurement was obtained were excluded from the study participation and sample collection. The patients were selected from existing cohort studies, which allowed us to create two groups of HIV-1-infected individuals who differed for calendar period of seroconversion (pre-2003 and post-2003 seroconverters). This date was chosen according to the study by Gras et al.[15] in which a significant and substantial difference of HIV-1 RNA concentration at setpoint was identified when patients who seroconverted between 2003 and 2007 were compared with individuals who seroconverted between 1996 and 2002 and before 1996.

Pre-2003 seroconverters

Individuals who seroconverted before 2003 (n = 459) were selected from the Amsterdam Cohort Studies (ACS; n = 335)[26] and from the AIDS Therapy Evaluation in the Netherlands (ATHENA) cohort (n = 124)[15]. The ACS enrolled participants between October 1984 and February 1988 and a total of 1013 asymptomatic men who were living in the Amsterdam area and who reported at least two homosexual contacts in the preceding 6 months were included in the prospective ACS on the prevalence and incidence of HIV-1 infection and risk factors for AIDS. At entry in the ACS, 239 men tested positive for HIV antibodies, five of whom refused to participate further. For these seroprevalent individuals, an imputed seroconversion date (on average 18 months before entry into the ACS) was used[27]. Of the 774 HIV-1 negative men, 131 subsequently seroconverted during active follow-up until 1996. Seroconversion date for these seroconverters was estimated as midpoint between last HIV-negative and first HIV-positive test result. None of the 365 seropositive men received treatment in their first 30 months after infection, thus excluding an effect of therapy on viral load setpoint. DNA for genotyping was available from a random sample of 335 (88%) cohort participants who subsequently were included in this study. Data on HLA type obtained by serology and/or PCR were available for all 335 individuals. All individuals with HLA-B*5701 (n = 17) carried the minor rs2395029 HCP5 allele, confirming the nearly complete linkage disequilibrium between these variants. Of these 17 individuals, eight fulfilled our definition of long-term nonprogressors (>10 years of therapy-naive asymptomatic follow-up with stable $CD4^+$ T-cell counts and >400 $CD4^+$ T cells/µl in the ninth and 10th year after seroconversion). This study population has been described previously[10].

The ATHENA observational cohort[15] includes anonymized data from 1998 onwards of all HIV-infected patients living in the Netherlands who visited one of the 25 HIV treatment centers. Twenty of the 25 participating hospitals were willing to ask their patients to participate in the SHM-HIV-1 Host Genetics study. From a total of 217 individuals with a known or accurately estimated date of seroconversion

based on last HIV-negative and first HIV-positive measurement, a total of 124 individuals fulfilled all entry criteria for the SHM-HIV-1 Host Genetics study and gave informed consent and blood for DNA isolation and genetic studies.

Post-2003 seroconverters
Individuals who seroconverted after 2003 were selected from the Primo-SHM cohort, a multicenter prospective cohort study in the Netherlands with 13 participating hospitals, which from 2003 onwards started to enroll individuals with laboratory evidence of primary HIV infection, defined as having a negative or indeterminate western blot in combination with detectable plasma HIV-1 RNA, or a period of less than 180 days between their last HIV-negative and first HIV-positive test result[28]. For this latter group, the midpoint between last HIV-negative and first HIV-positive test result was used as estimated date of seroconversion.

All 13 participating hospitals were willing to ask their patients to participate in the SHM HIV-1 Host Genetics study. For 24 individuals (10.3%) who were enrolled later in the epidemic, HLA typing was performed. One of these individuals carried an HLA*B5701 allele and also the minor HCP5 rs2395029 allele. For these post-2003 seroconverters, cART was available according to the Netherlands treatment initiation guidelines based on CD4 cell count and viral load (http://www.nvab.org/ richtlijnhiv), precluding categorization based on the clinical course of infection. None of these individuals fulfilled the definition of elite controller (more than 1 year follow-up with viral load less than five copies HIV-1 RNA/ml plasma).

In the end, 231 patients, from a total of 304 individuals, who seroconverted after 2003 and who fulfilled all entry criteria gave informed consent for genetic studies.

All studies are conducted in accordance with the ethical principles set out in the declaration of Helsinki and were approved by the institutional Medical Ethics Committee of the Academic Medical Center of the University of Amsterdam and the Boards of Directors and Medical Ethics Committees of participating hospitals in this multicenter study.

Genotyping
For this study, we analyzed SNPs rs2395029 (HCP5), rs9264942 (HLA-C), and the CCR5wt/Δ32 genotype. For the 335 pre-2003 seroconverters, enrolled in the ACS, genotype data for SNP rs2395029 (HCP5) were available from a GWAS that used the Illumina Infinium Human-Hap300 BeadChip (Illumina, San Diego, California, USA)[29] Manuscript was recently published. For the remaining 124 pre-2003 seroconverters and the 231 post-2003 seroconverters, SNP rs2395029 was analyzed by PCR amplification using Taq DNA polymerase (Invitrogen, Carlsbad, California, USA) and primer pair FW: 5'-CGAACTCCTCCTACCCTCATTGTG-3' and RV: 5'-CGTGGGTCCAGATACCAAGG-3'. Restriction digestion with XcmI (1 h 37°C;

New England Biolabs, Ipswich, Massachusetts, USA) results in a undigested PCR product of 327 bp or a 133 bp and a 194 bp product after restriction digestion, depending on nucleotide G or T, respectively, at SNP position. The rs2395029 HCP5 genotype as determined with the Illumina platform was confirmed for six samples [three homozygotes for the major allele (MAJ) and three heterozygotes] by the PCR as described above. SNP rs9264942 (-35HLA-C) was determined in all individuals using the predeveloped ABI TaqMan allelic discrimination-based technology with an ABI7900 Sequence Detection System (ABI, Foster City, California, USA), and the CCR5wt/Δ32 genotype was determined by PCR analysis of the CCR5 gene region as described previously[30].

Virological assays

Samples collected prior to 1997 were tested in the nucleic acid sequence-based amplification (NASBA) HIV-1 RNA QT (NASBA, bioMerieux, Boxtel, the Netherlands). Samples with a viral load below the quantification threshold of the NASBA assay (1000 copies/ml, n = 68) were re-tested in 2008 using the RealTime HIV-1 assay (Abbott Laboratories, Abbott Park, Illinois, USA), which has a threshold of 40 copies/ml. Details on the assays used after 1997 for viral load measurements in individuals from the ATHENA cohort and the primo-SHM study have been described previously[15]. Viral load setpoint was defined as the stable level of HIV RNA load around 18 – 24 months after seroconversion (using the mean of all measurements in that period) after establishing that all viral load measurements in the period of 15 – 27 months after seroconversion were within a maximum 0.5 $\log_{10}$ bandwidth. Viral load measurements for 180 of 870 initially selected study participants did not match one or more of the above criteria and no viral load setpoint was calculated. Individuals with viral load setpoint information who chose not to participate in this study did have a similar viral load setpoint when compared with individuals who were included in this study (data not shown). Furthermore, was the viral load setpoint in the post-2003 seroconverters who refused to participate in this study (n = 89) significantly higher when compared with pre-2003 seroconverters who refused to participate (n = 64) {4.44 [95% confidence interval (CI) 4.26 – 4.62] $\log_{10}$ copies/ml versus 4.10 (95% CI 3.98 – 4.20) $\log_{10}$ copies/ml, $P = 1.1 \times 10^{-3}$}.

Statistical analyses

Viral load data were analyzed after $\log_{10}$ transformation. A Student's t-test was used to test for association of the SNP rs2395029 and the CCR5wt/Δ32 genotype with viral load setpoint. To test for association of SNP rs9264942 with viral load setpoint, we used a one-way analysis of variation (ANOVA) test. We used unadjusted (including seroconversion period and each SNP separately) and adjusted linear regression models to test the association of the three SNPs and seroconversion period with viral load setpoint. Interaction terms between each SNP and each seroconversion period were included to test whether the effect of each SNP on viral load setpoint had changed over time. Analyses were performed

using SPSS 17.0 software (SPSS Inc., Chicago, Illinois, USA) and SAS version 9.1 (SAS Institute, Cary, North Carolina, USA). Power calculations for association analysis were performed using G*Power 3.1.2[31].

Results

Patients

Baseline characteristics of all study participants are summarized in Table 1. The pre-2003 seroconverters (seroconversion date between 1982 and 1 January 2003) were mainly men (95.6%), 95.6% of whom were MSM and 3.2% were heterosexual. Most of the women were infected via heterosexual contact (80%) and 10% of women were infected via injecting drug use. The pre-2003 seroconverters originated mainly from western Europe (96.3%) and 80.8% were known to be infected with a subtype B HIV-1 variant. In the group of post-2003 seroconverters (seroconversion between 1 January 2003 and 2009), 92.6% were men of whom 89.7 and 7.9% were MSM and heterosexual, respectively. All the women were infected via heterosexual contact. The infecting HIV-1 subtype was B in 50.6% of the individuals, another subtype in 3.0% of individuals, and not determined in 46.3% of the individuals; 86.1% were born in western Europe.

Genotype distribution for -35HLA-C rs9264942, HCP5 rs2395029, and CCR5wt/Δ32 in pre-2003 and post-2003 seroconverters

We determined the prevalence of the minor allele of SNP rs9264942 in the HLA-C gene region and rs2395029 in the HCP5 gene region and of the CCR5Δ32 allele in our study population. No significant differences were observed in genotype frequencies between the groups of pre-2003 and post-2003 seroconverters, or with the noninfected Hapmap population of European descent (Table 2). No deviations from Hardy – Weinberg equilibrium were observed (data not shown).

Impact of host genetic factors on viral load in pre-2003 and post-2003 seroconverters

We first analyzed whether the observed increase in viral load setpoint at a population level[13 –15] could be confirmed in our study population. The viral load setpoint, defined as the stable HIV RNA load around 18 – 24 months after seroconversion, was indeed higher in the post-2003 seroconverters as compared with the pre-2003 seroconverters [4.44 (95% CI 4.33 – 4.55) $\log_{10}$ copies/ml versus 4.17 (95% CI 4.10 – 4.25) $\log_{10}$ copies/ ml, $P = 4.5 \times 10^{-5}$].

Previous studies have demonstrated a strong association between a lower viral load early in infection and the minor alleles of rs9264942 (-35HLA-C) and rs2395029 (HCP5), or with heterozygosity for a 32 bp deletion in the CCR5 gene (CCR5wt/Δ32)[6,8,9,31]. The same associations were found for our pre-2003 seroconverters: setpoint viral load was highest in -35HLA-C wild-type homozygotes and

Table 1. Characteristics of the pre-2003 and post-2003 seroconverters.

Estimated year of seroconversion			
	1982 – 2002	**2003 – 2009**	**P**
Total	459	231	
Region of origin			5.5×10^{-6}
Western Europe	442 (96.3%)	199 (86.1%)	
Other	16 (3.5%)	31 (13.4%)	
Unknown	1 (0.2%)	1 (0.4%)	
Sex			7.3×10^{-2}
Male	439 (95.6%)	214 (92.6%)	
Female	20 (4.4%)	17 (7.4%)	
Transmission risk group			2.9×10^{-3}
MSM	420 (91.5%)	192 (83.1%)	
Heterosexual	30 (6.5%)	33 (14.3%)	
DU	3 (0.7%)	0 (0.0%)	
Other	1 (0.2%)	1 (0.4%)	
Unknown	5 (1.1%)	5 (2.2%)	
Subtype			2.1×10^{-15}
B	371 (80.8%)	117 (50.6%)	
Other	6 (1.3%)	7 (3.0%)	
Not determined	82 (17.9%)	107 (46.3%)	
Mean age at SC in years	34.9 (±7.5)	37.4 (±9.3)	9.84×10^{-1}

DU, drug users; SC, seroconversion.

Table 2. Prevalence (%) of genotypes in pre-2003 seroconverters, post-2003 seroconverters and healthy Hapmap population.

	rs9264942 -35HLA-C			**rs2395029 HCP5**			**CCR5wt/Δ32**		
	MAJ	**HZ**	**MIN**	**MAJ**	**HZ**	**MIN**	**wt/wt**	**wt/ Δ32**	**Δ32/ Δ32**
SC 1982 – 2002	47.7	42.3	10.0	94.1	5.9	0.0	80.6	19.2	0.2
SC 2003 – 2009	47.2	43.3	9.5	93.5	6.5	0.0	85.3	14.7	0.0
Hapmap	42.4	47.5	10.1	90.0	10.0	0.0	81.0	18.0	1.0

Prevalence of genotypes was not significantly different between pre-2003 and post-2003 seroconverters (Fisher's exact test). HZ, heterozygotes; MAJ, homozygotes for the major allele; MIN, homozygotes for the minor allele; SC, seroconversion.

significantly lower in individuals who were heterozygous or homozygous for the minor allele ($P = 7.7 \times 10^{-3}$; Table 3). Also, the viral load setpoint was significantly lower in pre-2003 seroconverters carrying the minor allele of SNP rs2395029 in HCP5 (n = 27) than in those who were homozygous for the major allele (n = 432, $P = 5.4 \times 10^{-4}$; Table 3). Finally, pre-2003 seroconverters with a CCR5Δ32 allele [either with a heterozygous (n = 88) or homozygous (n = 1) genotype] had a significantly lower viral load setpoint than those carrying two CCR5 wild-type alleles (n = 370, $P = 5.2 \times 10^{-3}$).

In post-2003 seroconverters, viral load setpoint in individuals homozygous for the minor allele of rs9264942 (-35HLA-C) was also significantly lower than the viral load setpoint in heterozygotes and homozygotes for the wild-type allele ($P = 9.9 \times 10^{-4}$). Surprisingly, however, the protective effect associated with the minor allele of rs2395029 in HCP5 or a CCR5wt/Δ32 genotype was absent in post-2003 seroconverters. The viral load setpoint in post-2003 seroconverters was not significantly different in carriers of the minor allele of rs2395029 in HCP5 (n = 15) when compared with carriers of two major alleles (n = 216, $P = 4.7 \times 10^{-1}$), and no difference in viral load setpoint was present between CCR5wt/Δ32 heterozygotes (n = 34) and individuals with a CCR5wt/ wt homozygous genotype (n = 197, $P = 6.3 \times 10^{-1}$).

Adjusted linear regression model for the analysis of fading impact of host genetic markers on viral load setpoint over calendar time

To test whether the associations of the three genotypes with viral load setpoint were independent of each other, adjusted linear regression analysis was performed. None of the associations between the three genetic markers and viral load setpoint changed significantly as compared with the unadjusted analyses (Table 4).

Additionally, we tested whether the observed changes in the association between each genetic marker and viral load setpoint between pre-2003 and post-2003 seroconverters were independent of the effect of the other two genetic markers by including interaction terms between seroconversion period and all of the three genotypes in the adjusted model. This model found no significant evidence that the association between rs9264942 -35HLA-C and viral load setpoint had significantly changed over time ($P = 1.7 \times 10^{-1}$; data not shown). However, the association between viral load setpoint and SNP rs2395029 in HCP5 had significantly changed, also after adjusting for a potential effect of the other two genetic markers under study (MAJ – heterozygotes in pre-2003 versus post-2003 seroconverters: 0.51 versus -0.06 $\log_{10}$ copies/ml; $P = 4.0 \times 10^{-2}$). Although the association between CCR5wt/Δ32 and viral load setpoint was only observed in pre-2003 seroconverters and absent in post-2003 seroconverters, there was no evidence that the effect had changed significantly over time ($P = 2.6 \times 10^{-1}$; data not shown).

Table 3. Association between host genetic markers and setpoint HIV-1 viral load in pre-2003 and post-2003 seroconverters.

	Viral load setpoint						
rs9264942 -35HLA-C		**MAJ**		**HZ**		**MIN**	
	n	**Mean (95% CI)**	**n**	**Mean (95% CI)**	**n**	**Mean (95% CI)**	**P**
SC 1982 – 2002	219	4.29 (4.18 – 4.40)	194	4.09 (3.97 – 4.20)	46	3.96 (3.75 – 4.17)	7.7×10^{-3}
SC 2003 – 2009	109	4.60 (4.46 – 4.74)	100	4.39 (4.22 – 4.56)	22	3.90 (3.48 – 4.31)	9.9×10^{-4}
rs2395029 HCP5		**MAJ**		**HZ**			
	n	**Mean (95% CI)**	**n**	**Mean (95% CI)**			**P**
SC 1982 – 2002	432	4.21 (4.13 – 4.28)	27	3.65 (3.29 – 4.00)			5.4×10^{-4}
SC 2003 – 2009	216	4.45 (4.34 – 4.56)	15	4.29 (3.82 – 4.76)			4.7×10^{-1}
CCR5 wt/Δ32	**wt/wt**	**wt/Δ32 + Δ32/Δ32**					
	n	**Mean (95% CI)**	**n**	**Mean (95% CI)**			**P**
SC 1982 – 2002	370	4.22 (4.14 – 4.31)	89	3.95 (3.79 – 4.12)			5.2×10^{-3}
SC 2003 – 2009	197	4.45 (4.33 – 4.57)	34	4.38 (4.15 – 4.60)			6.3×10^{-1}

P values from association testing using Student's t-test for rs2395029 and CCR5 wt/Δ32, or using one-way analysis of variation (ANOVA) for rs9264942. CI, confidence interval; HZ, heterozygotes; MAJ, homozygotes for the major allele; MIN, homozygotes for the minor allele; n, number; SC, seroconversion.

Table 4. Unadjusted and adjusted linear regression model for the analysis of association between host genetic markers and setpoint HIV-1 viral load in pre-2003 and post-2003 seroconverters.

	Unadjusted		Adjusted	
	Difference (95% CI)	**P**	**Difference (95% CI)**	**P**
rs9264942 -35HLA-C				
MAJ	0.45 (0.34 – 0.56)	1.0×10^{-4}	0.40 (0.29 – 0.51)	1.0×10^{-4}
HZ	0.25 (0.14 – 0.36)	2.0×10^{-2}	0.22 (0.11 – 0.33)	4.5×10^{-2}
MIN	0		0	
rs2395029 HCP5				
MAJ	0.42 (0.29 – 0.55)	1.0×10^{-3}	0.33 (0.20 – 0.46)	1.0×10^{-2}
HZ	0		0	
CCR5wt/Δ32				
wt/wt	0.21 (0.13 – 0.29)	1.0×10^{-2}	0.22 (0.14 – 0.30)	6.0×10^{-3}
wt/Δ32	0		0	

CI, confidence interval; HZ, heterozygotes; MAJ, homozygotes for the major allele; MIN, homozygotes for the minor allele; SC, seroconversion.

Discussion

At a population level, HIV-1 viral load setpoint seems to be increasing over calendar time[13–15], albeit not confirmed by others[33–35].

In our study in HIV-1-infected individuals in the Netherlands, we observed a significantly higher viral load setpoint in individuals who became HIV-1 infected in the period 2003 – 2009 as compared with individuals who became infected in the period 1982 – 2002. The observed rise in viral load setpoint over calendar time in the Dutch population is in agreement with the results of the CASCADE study[13] and a recent study of the epidemic in Italy[14]. Three other studies found no evidence for the increase in viral load setpoint[33–35], and differences in patient selection, study period, and outcome definitions across these studies might explain the discrepancies.

Moreover, we observed that associations between certain host genetic factors and a lower viral load setpoint were restricted to pre-2003 seroconverters. In this group, the minor alleles of rs9264942 -35HLA-C and rs2395029 in HCP5 and a CCR5wt/Δ32 genotype were all associated with a lower viral load setpoint as compared with the group with the respective wild-type genotypes. In post-2003 seroconverters, the protective effect of the minor allele of rs9264942 -35HLA-C was preserved, but the protective effect of the minor allele of rs2395029 in HCP5 and a CCR5wt/Δ32 genotype was no longer present in this group. The effect of carrying a minor allele of SNP rs2395029 in HCP5 had significantly changed over time, independent from the other two markers under study, whereas the change in impact of the CCR5wt/Δ32 genotype on HIV-1 control over calendar time was not significant.

Assuming an effect size for HCP5 rs2395029 of 0.67 (like in the pre-2003 seroconverters), a minor allele frequency (MAF) of 0.1, and type 1 error rate of 0.05, we had 83% power to detect a significant association in the 231 post-2003 seroconverters. However, assuming an effect size for CCR5wt/Δ32 of 0.7, a MAF of 0.2, and type 1 error of 0.05, we had 73% power to detect a significant association in this group. Thus, the post-2003 seroconverter group size may have been too small to observe this effect, which could also explain why we did not observe a significant change in the association of the CCR5wt/Δ32 genotype on viral load setpoint over calendar time.

The rise in viral load was originally observed by comparing pre-2003 and post-2003 seroconverters[15]. We also explored changes in viral load setpoint by comparing the same groups of seroconverters now separated by another calendar date of seroconversion. No significant differences in viral load setpoint were observed for individuals who seroconverted before or after 1996 [4.22 $\log_{10}$ copies/ml (95% CI 4.14 – 4.31) versus 4.30 $\log_{10}$ copies/ml (95% CI 4.20 – 4.30),

$P = 2.1 \times 10^{-1}$]. Dividing the groups based on seroconversion before or after years later than 2003 was not feasible because of sample size limitations in the late group.

Individuals who started cART after 1 January 1996 before a viral load setpoint measurement was obtained were excluded from further analysis, which occurred more often in the group of post-2003 seroconverters. Although this might have introduced a potential bias in our study, the fact that these individuals had to initiate cART implies that their viral load was most likely high. In other words, the mean viral load in the group of post-2003 seroconverters may have been even higher had these individuals been included with a pretherapy viral load measurement. Moreover, the distribution of genotypes under study was not different between pre-2003 and post-2003 seroconverters or between our seroconverter cohorts and the Hapmap population, also arguing against a bias in our patient selections.

HIV-1 subtype, region of origin, and mode of transmission were less homogeneous in the post-2003 seroconverters. These factors may have influenced disease progression[36], SNP prevalence, and/or viral load setpoint[34]. However, when the analysis was limited to the post-2003 subgroup of MSM from western European descent infected with a documented subtype B HIV-1, the protective effect of the minor allele of HCP5 rs2395029 and CCR5wt/D32 heterozygosity on viral load setpoint remained absent. Moreover, SNP genotype distributions were not significantly different between the pre-2003 and post-2003 seroconverters (Table 2).

Several different assays were used to determine HIV-1 RNA plasma load and batch-wise retesting was not performed. Samples with a viral load below the quantification threshold of the NASBA RNA QT assay (1000 copies/ml, n = 68) were re-tested in 2008 using the Real-Time Abbott HIV-1 assay. It has been reported that the mean HIV-1 RNA concentration at setpoint was slightly higher when measured with older assays that have a lower limit of detection of 1000 or 400 copies/ml as compared with the more sensitive assays with a lower limit of detection of 50 copies/ml[37]. This would imply that the difference in viral load setpoint between pre-2003 and post-2003 seroconverters would have been even more pronounced had all samples been retested with the more sensitive Abbott Real-Time assay. Furthermore, adjustment for assay sensitivity did not appreciably change viral load setpoint results in the study by Gras et al.[15].

The minor allele variant of SNP rs2395029 in HCP5 is in strong linkage disequilibrium with HLA-B57, which has been associated with long-term asymptomatic survival[3–5,38] and data are accumulating that HLA-B57 is in fact the causal genotype associated with HIV-1 control tagged by HCP5 rs2395029[32,39]. Interestingly, adaptation of HIV-1 to HLA has been described[24] and in contemporary

seroconverters in the Netherlands, we have evidence for a selective loss of epitopes that are presented by more protective HLA-types such as HLA-B57 and HLA-B27 as compared with epitopes presented by HLA alleles that have not been associated with relative protection from disease progression[25]. These studies indicate that population-level adaptation of HIV-1 to host defense mechanisms has indeed occurred, probably explaining the fading impact of rs2395029 in HCP5 on HIV-1 viral load setpoint.

Since 48% of the post-2003 seroconverters presented themselves with an acute infection, there might be a bias in this group for symptomatic infections that have been associated with a higher viral load[40]. Unfortunately, no data are available on symptoms during acute infection for post-2003 seroconverters. Although differences in the severity of acute infection between pre-2003 and post-2003 seroconverters cannot be excluded, similar minor allele prevalence was found for all three host genetic factors under study in both groups, indicating that the post-2003 seroconverters are unlikely to be an a priori genetically more susceptible group.

The absence of an association between a CCR5wt/Δ32 genotype and a lower viral load setpoint in post-2003 seroconverters may point to adaptation of HIV-1 to its host. We and others have indeed reported the intrapatient evolution of CCR5 using (R5) HIV-1 variants toward improved usage of coreceptor CCR5, which is reflected by a decreasing level of resistance of HIV-1 to inhibition by RANTES in the course of the infection[41–43]. It is tempting to speculate that the HIV-1 variants with increased ability to use CCR5 are more successfully transmitted and that this explains why the lower CCR5 expression levels in individuals with a CCR5wt/Δ32 genotype are no longer rate-limiting. Interestingly, a more efficient use of CCR5 may also explain why the replication rate of HIV-1 obtained early after transmission has increased over calendar time in the Dutch epidemic, thus possibly contributing to the observed increase in viral load setpoint[44]. Alternatively, an unusually high prevalence of CXCR4-using variants in the post-2003 seroconverters may have accounted for the absence of the protective effect of the CCR5wt/Δ32 genotype. Although we do not have data on coreceptor use for all post-2003 seroconverters, a low prevalence of CXCR4-using viruses (4 – 6%) was observed in three recent cohorts of seroconverters including a subgroup of the post-2003 seroconverters (n = 46) during the period 2003 – 2008[45–47]. As this low prevalence of CXCR4-using viruses in recent seroconverters is similar to that reported for historical seroconverters, it is unlikely that increased CXCR4 use explains the absence of the protective effect.

The association between -35HLA-C rs9264942 and viral load setpoint has remained the same over calendar time. The -35HLA-C minor allele is associated with high HLA-C cell surface expression, which may account for an overall better antigen presentation to cytotoxic T cells or improved recognition by natural killer

cells[48]. The preserved protective effect of this genotype suggests that to date HIV-1 cannot escape from the mechanism associated with it. It is tempting to speculate that escape would come at a too large fitness cost to the virus[49–51].

Our study focused on an HIV-1 subtype B-infected population in the Netherlands, which may limit the implications of our findings. However, the adaptation of HIV-1 to HLA, which was demonstrated in multiple cohorts[24], seems to imply that the host adaptation of HIV-1 is a more general phenomenon. Furthermore, we recently reported that early HIV-1 variants from people who seroconverted in the beginning of the epidemic were more resistant to neutralizing antibodies than early HIV-1 variants from individuals who became infected more recently[52], suggesting adaptation of HIV-1 at a population level also to the humoral immune response.

Importantly, our findings also imply that associations from GWAS on HIV-1 control that are not replicated in cohorts that differ in the age of their HIV-1 epidemic are not necessarily false positives in the discovery cohort, but may be due to adaptations of the pathogen to its host over calendar time.

The consequences of a higher viral load setpoint for the epidemic may be serious, as it facilitates transmission[53,54]. The reduction of HIV-1 transmission, which may be achieved by reducing viral load by cART, may be counteracted by a higher viral load prior to the initiation of therapy due to the fading impact of host genetic factors that originally controlled the virus. From this point of view, even earlier initiation of cART may be warranted, as has been suggested recently[55,56].

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Conflicts of interest

There are no conflicts of interest.

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4

Has the Rate of CD4 Cell Count Decline before Initiation of Antiretroviral Therapy Changed over the Course of the Dutch HIV Epidemic among MSM?

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Abstract

Introduction
Studies suggest that the HIV-1 epidemic in the Netherlands may have become more virulent, leading to faster disease progression if untreated. Analysis of CD4 cell count decline before antiretroviral therapy (ART) initiation, a surrogate marker for disease progression, may be hampered by informative censoring as ART initiation is more likely with a steeper CD4 cell count decline.

Methods
Development of CD4 cell count from 9 to 48 months after seroconversion was analyzed using a mixed-effects model and 2 models that jointly modelled CD4 cell counts and time to censoring event (start ART, <100 CD4 cells/mm^3, or AIDS) among therapy-naïve MSM HIV-1 seroconverters in the Netherlands. These models make different assumptions about the censoring process.

Results
All 3 models estimated lower median CD4 cell counts 9 months after seroconversion in later calendar years (623, 582, and 541 cells/mm^3 for 1984–1995 [n = 111], 1996–2002 [n = 139], and 2003–2007 seroconverters [n = 356], respectively, shared-parameter model). Only the 2 joint-models found a trend for a steeper decline of CD4 cell counts with seroconversion in later calendar years (overall p-values 0.002 and 0.06 for the pattern-mixture and the shared-parameter model, respectively). In the shared-parameter model the median decline from 9 to 48 months was 276 cellsmm^3 for 1984–1995 seroconverters and 308 cells/mm^3 for 2003–2007 seroconverters (difference in slope, p = 0.045).

Conclusion
Mixed-effects models underestimate the CD4 cell decline prior to starting ART. Joint-models suggest that CD4 cell count declines more rapidly in patients infected between 2003 and 2007 compared to patients infected before 1996.

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Introduction

The higher the HIV-1 plasma level, the more likely progression to AIDS is[1] and the higher the chance of HIV-1 transmission[2]. Previously, we found an increasing trend over time in plasma HIV-1 RNA concentration at setpoint, i.e., 9 to 27 months after HIV-1 seroconversion and an accompanying decreasing trend of CD4 cell count measured at viral setpoint[3]. In the Amsterdam Cohort Studies (ACS), a higher replicative fitness of HIV-1 isolates obtained from participants with seroconversion in more recent years was found[4]. Some cohorts have reported a similar increase in HIV-1 RNA concentration and decrease in CD4 cell count at viral setpoint over time[5–7], whereas others found no evidence for such changes[8,9]. The effect of higher viral load and lower CD4 T-cell count at setpoint on progression to AIDS or death is difficult to study in the combination antiretroviral therapy (cART) era as these endpoints are hardly observed in effectively treated patients. A surrogate marker of disease progression is the rate of CD4 cell decline in patients not on therapy. Since individuals with low CD4 cell counts are more likely to start antiretroviral therapy (ART), the presence of informative censoring needs to be considered. Mixed-effects models, the standard method of analysis of longitudinal data, can give biased estimates when censoring (here: the start of ART) is not at random and depends on unobserved CD4 cell counts[10]. Joint-modeling of longitudinal CD4 cell counts together with the censoring process can give unbiased estimates, under certain assumptions. Therefore, we investigated trends in CD4 cell count decline between 9 months and 4 years after HIV seroconversion, using regression models that make different assumptions about the censoring pattern.

Methods

Patient Selection

Data were obtained from men having sex with men (MSM) participating in the ACS or the AIDS therapy evaluation in the Netherlands (ATHENA) cohort. ACS recruitment started in 1984, mainly among MSM living in or around Amsterdam. Inclusion criteria varied over time[11]. The ATHENA cohort has been described elsewhere[12] and includes anonymized data obtained from treated and untreated HIV-infected patients, who have been followed in or after 1996 in any of the 25 HIV treatment centers and also includes ACS participants in care in or after 1996. From both cohorts, MSM from Western-Europe and North-America were selected with a maximum interval between the last negative and first positive HIV-1 antibody test of 1 year. The day of seroconversion was estimated as midpoint between both tests. Also included were MSM with serological evidence of acute infection in which case the day of seroconversion was set 1 month prior to the date of the first positive test. All MSM with seroconversion before 1996 were from the ACS. At seroconversion, all patients were 16 years or older. Patients had at least one CD4 cell count between 9 and 27 months after seroconversion while

being ART-naïve. Subtype infections other than subtype B were excluded. MSM without subtype determination were included, since in our cohort the prevalence of subtype B infection among MSM from Western-Europe or North-America is 95% and therefore highly likely[12].

Ethics Statement

Informed written consent is obtained from all ACS participants, and the study has been approved by the Medical Ethical Committee of the Academic Medical Centre. Ethical approval in the ATHENA cohort is not obtained as data are collected from patients as part of HIV care. Patients can opt-out after being informed by their treating physician of the purpose of collection of clinical data.

Outcome

CD4 cell counts in peripheral blood measured between 9 months and 4 years after seroconversion were used to model CD4 cell count decline. The 4-year threshold ensured a similar follow-up period for subjects seroconverting in early and in later years. Only CD4 cell counts obtained from samples taken prior to any of the following 3 dates were included for analysis: date of first starting ART, first date CD4 cell count had dropped below 100 cells/mm^3 (other studies[5,8] have censored CD4 cell counts after this date because of the possibility of an accelerated decline), and the date 1 year prior to AIDS diagnosis (CD4 cell count decline may accelerate around this date[13,14]).

Statistical Analysis

Trends in CD4 cell decline over time were analyzed using 3 models with different underlying assumptions. All models assumed a linear decline and included a random slope and intercept for each patient. Age at seroconversion (linearly modelled and centered at 35 years of age) and timing of seroconversion (either in 3 categories 1984–1995, 1996–2002, 2003–2007, or as a continuous variable using natural splines with knots at January 1993, 2000 and 2005) were included as fixed covariates. cART was introduced in the Netherlands as standard of care in 1996 and this motivated our first group 1984–1995 (pre-cART era). The second cut-off was chosen a-priori so that each time period was sufficiently wide and included a sufficient number of patients. We used 3 different statistical models: 1) Mixed-effects models. These models can provide unbiased estimates if censoring only depends on the observed CD4 cell counts, given covariates included in the model (the censoring process is missing at random (MAR)). If instead censoring depends on unobserved CD4 counts, such as the underlying subject-specific CD4 cell count trajectories, estimates will be biased. Joint-modeling of longitudinal CD4 cell counts and the censoring process can give unbiased estimates under certain assumptions[15]. We used two joint-modeling approaches: 2) pattern-mixture models; and 3) shared-parameter models[16].

Pattern mixture model. Pattern-mixture models quantify the longitudinal outcome conditionally on the timing of the censoring event. The censoring event

was either ART initiation, CD4 cell count dropping below 100 cells/mm³ or an AIDS diagnosis (in which case CD4 cell counts were censored from the date 1 year before the AIDS diagnosis). We used seven distinct patterns: censoring between 9–21, 21–27, 27–33, 33–39, 39–48 months, lost to follow-up (including suicide). A seventh pattern was no censoring event within 48 months. The basic probability structure of the model is:

$$p(CD4_i, cens_pattern_i \mid \theta) = p(CD4_i \mid cens_pattern_i, \theta)\, p(cens_pattern_i) \quad (1)$$

With i an arbitrary individual and θ a categorical variable representing the 3 seroconversion periods 1984–1995, 1996–2002 and 2003–2007. The joint distribution in (1) is obtained by multiplying pattern-specific probabilities of CD4 trajectories $p(CD4_i \mid cens_pattern_i, \theta)$, modelled via a random effects model, by weights according to the probability distribution, $p(cens_pattern_i)$. The parameter estimates of the CD4 trajectories, and their dependence on θ, are obtained via likelihood maximisation. The weights from the second term are obtained by dividing the number of patients in each pattern and in each period by the total number of patients in each period[17].

Shared parameter model. The basic probability structure of the model is:

$$p(CD4_i, T_i \mid \theta) = p(CD4_i \mid \theta)\, p(T_i \mid CD4_i) \quad (2)$$

with i and θ as above and T_i the time to the censoring event, $p(CD4_i \mid \theta)$ in (2) represents a longitudinal random effects model for the longitudinal CD4 cell counts and $p(T_i \mid CD4_i)$ a survival model for the time to censoring due to ART initiation, CD4 cell count dropping below 100 cells/mm³ or an AIDS diagnosis. Individuals that had complete follow-up were treated as censored cases. The survival model was regressed on the underlying CD4 cell count and age. We used a Weibull accelerated failure time model, but a Cox proportional hazards model gave similar results. In the shared-parameter model, the association between the censoring times and CD4 cell counts is modelled via a set of random effects. Conditional on the random effects, the longitudinal CD4 cell counts are independent of the censoring time, CD4 cell counts were cube root transformed to comply with normality assumptions. Both terms contribute to the likelihood, hence a joint likelihood was maximized.

Averaged mean estimates from the pattern-mixture model were estimated using PROC MIXED in SAS 9.2 (SAS Institute, Cary, NC). The obtained standard errors are slightly underestimated because the size of the patterns are assumed fixed. To correct for the uncertainty of pattern sizes, corrected standard errors can be obtained using the delta method[17,18]. Shared-parameter modeling was done using the R (R Development Core Team 2010) package JM[19]. SAS and R syntax is given in Supporting Information S1.

Results

In total, 606 patients were included in the study: 111 with seroconversion between 1984 and 1995, 139 between 1996 and 2002, and 356 between 2003 and 2007. Of all MSM, 9 (8%) out of 120 with seroconversion between 1984 and 1995, 122 (47%) out of 261 with seroconversion between 1996 and 2002, and 165 (32%) out of 521 with seroconversion between 2003 and 2007, were excluded because either the date of ART initiation, the date CD4 cell count had dropped below 100 cells/mm^3, or the date 1 year prior to diagnosis of AIDS was before 9 months after seroconversion. A last negative HIV test within 1 year of the first positive test was available for 524 patients. The remaining 82 patients had serological evidence of acute infection (3 seroconverters between 1984 and 1995, 15 between 1996 and 2002, and 64 between 2003 and 2007).

Baseline characteristics are shown in Table 1. Subtype was not determined in 330 patients (54%). The percentage of samples measured using a single-platform method gradually increased from 0% prior to 1997 to 94% in 2010. Timing of the first sample taken after 9 months after seroconversion was similar between the 3 seroconversion periods. Overall, the median first CD4 cell count at 9 months was 540 cells/mm^3 but it decreased with later periods of seroconversion. The proportion of patients with a censoring event increased over time. ART was started in 295 patients, in 30 patients AIDS was diagnosed, and in 14 patients CD4 cell count declined to below 100 cells/mm^3.

Intercepts (estimated CD4 cell count at 9 months after seroconversion) were similar between the 3 models (see Table 2). All models showed a significant association of a lower CD4 cell count at 9 months with seroconversion in later calendar years. Estimates of the decline in CD4 cell count obtained with the mixed-effects model did not differ significantly between periods of seroconversion (overall p-value = 0.56). However, in the pattern-mixture model there was a significant association of an increasing CD4 cell count decline with seroconversion in later years ($p = 0.002$) whilst in the shared parameter model there was a borderline trend ($p = 0.06$). In both joint-models, the decline for 2003–2007 seroconverters was significantly higher than for 1984–1995 seroconverters. In the shared-parameter model, the difference was 0.12 $\sqrt[3]{\text{cells}}$/mm^3/year (95% CI 0.00, 0.24). Estimates obtained with the pattern-mixture model showed a similar trend, although the rate of decline was steeper across all seroconversion periods compared to the shared-parameter model. In both joint models the decline did not differ significantly between seroconversion periods 1996–2002 and 2003–2007. Figure 1a–c shows the estimated decline in CD4 cell count graphically, on the original scale. When modelled using splines (Figure 2), the association between year of seroconversion and slope was borderline significant in the shared-parameter model ($p = 0.06$) but not in the mixed-effects model ($p = 0.32$). As expected, the estimates from the mixed-effects and the shared-parameter model differed least during the period when ART was not available. Shared-parameter model estimates suggest that the CD4 decline

became smaller over time before 1993 and became larger thereafter. However, 95% confidence intervals were wide.

There was no significant association between age and the mean CD4 cell count 9 months after seroconversion in any of the 3 models. Nor did the mixed-effects and pattern-mixture models find an association between age and the slope of decline in CD4 cell count after 9 months (-0.03 $\sqrt[3]{\text{cells}}$/mm^3/year, 95% CI -0.08, 0.02 and, 0.01 $\sqrt[3]{\text{cells}}$/mm^3/year, 95% CI -0.03, 0.05 per 10-year increase in age, respectively). The shared-parameter model estimated a stronger association between decline in CD4 cell count and older age; -0.06 $\sqrt[3]{\text{cells}}$/mm^3/year per 10-year increase in age, 95% CI -0.12, -0.01.
For a typical seroconverter, i.e., an individual 36 years of age at seroconversion, with a median CD4 cell count at 9 months, the median decline between 9 and 48 months (see Table 3) in the pattern-mixture model changed from 286 CD4 cells/mm^3 for 1984–1995 seroconverters (on average 88 cells/mm /year) to 363 cells/mm^3 for 2003–2007 seroconverters (112 cells/mm^3/year). In the shared-parameter model the estimated median decline was smaller (83 cells/mm^3/year for 1984–1995, and 90 cells/mm^3/year for 2003–2007 seroconverters) whereas the mixed-effects model decline estimates were substantially smaller (between 75 and 77 cells/mm^3/year across the 3 periods) compared to the pattern-mixture model estimates. Table 3 also shows that the pattern-mixture model estimated the median time from seroconversion to the 350 CD4 cells/mm^3 treshold was 2.2 years for 2003–2007 seroconverters compared to 3.8 years for 1984–1995 seroconverters. This compares to 2.7 years and more than 4.0 years for the shared-parameter model.

Discussion

Using joint-models, we found a trend of a steeper CD4 cell count decline before the initiation of antiretroviral therapy in HIV infected MSM since the early nineties. This suggests, together with the earlier reported higher viral fitness and the plasma viral load and lower CD4 cell count at viral setpoint, an increasing trend in HIV virulence in MSM followed in HIV treatment centers in the Netherlands over time[3,4]. Likewise, a recent meta-analysis concluded that virulence may have increased over the course of the HIV-1 pandemic[20]. The trend of a steeper CD4 cell count decline over time was only apparent when timing of censoring (because of ART initiation or disease progression) was jointly modelled with longitudinal CD4 cell counts and not when mixed-effects models were used. Mixed-effects models can provide unbiased estimates when the probability of the censoring event only depends on the observed CD4 cell counts (the MAR assumption), given covariates included in the model. Provided that the model for the censoring event is correctly specified, joint-models can provide unbiased estimates both when data are MAR, and when censoring is informative (when

Table 1. Characteristics of 606 included MSM living in the Netherlands with seroconversion between 1984 and 2007.

	Year of seroconversion			
	1984-1995	**1996-2002**	**2003-2007**	**Total**
	N=111	**N=139**	**N=356**	**N=606**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**Median (IQR)**
Age at seroconversion (years)	35.2 (29.7-42.1)	34.6 (30.2-41.1)	37.9 (31.6-43.8)	36.6 (30.6-43.1)
First CD4 cell count 9-27 months after seroconversion (cells/mm³)	580 (450-850)	550 (450-720)	510 (390-660)	540 (410-710)
Months between seroconversion and first CD4 cell count	10.3 (9.9-10.7)	10.7 (9.7-12.3)	10.5 (9.6-11.9)	10.4 (9.7-11.7)
Number of included CD4 cell count measurements	13 (10-14)	6 (4-8)	7 (4-9)	7 (4-10)
Number of included CD4 cell count measurements	13 (10-14)	6 (4-8)	7 (4-9)	7 (4-10)
	N (%)	**N (%)**	**N (%)**	**N (%)**
Timing of censoring				
9-21 months	6 (5%)	34 (24%)	76 (21%)	116 (19%)
21-27 months	4 (4%)	14 (10%)	44 (12%)	62 (10%)
27-33 months	12 (11%)	5 (4%)	29 (8%)	46 (8%)
33-39 months	4 (4%)	10 (7%)	33 (9%)	47 (8%)
39-48 months	10 (9%)	12 (9%)	46 (13%)	68 (11%)
No censoring within 48 months	67 (60%)	58 (42%)	116 (33%)	241 (40%)
Lost to follow-up 9-48 months	8 (7%)	6 (4%)	12 (3%)	26 (4%)
Reason of censoring within 48 months				
Start ART	14 (39%)	68 (91%)	213 (93%)	295 (49%)
AIDS diagnosis	15 (42%)	5 (7%)	10 (4%)	30 (5%)
<100 CD4 cells/ mm3	7 (19%)	2 (2%)	5 (2%)	14 (2%)

IQR: Interquartile range, MSM: men who have sex with men.

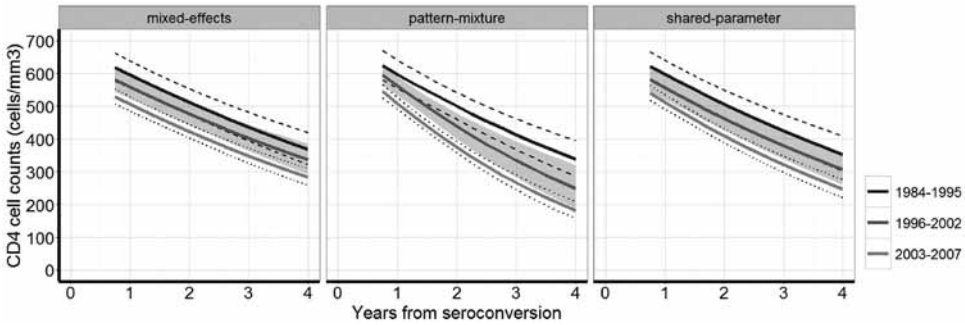

Figure 1. CD4 cell count for a typical patient (36 years of age), backtransformed to original scale by method of estimation. Dashed lines and shaded regions are 95% confidence intervals.

censoring depends on aspects of CD4 cell count that are not observed). The different results obtained from mixed-effects models and joint-models in this study, indicates that censoring CD4 cell counts due to ART initiation or disease progression is informative and estimates from standard mixed-effects models are probably incorrect. Estimation of parameters in joint models, compared to mixed-effect models, is more complex and joint models are currently not routinely used. Using the shared-parameter model, we estimated an annual CD4 cell count decline of around 90 cells/mm^3/year for 2003–2007 seroconverters. The pattern-mixture model estimated a steeper decline. The reported decrease among Italian patients included in the MASTER cohort[5] was less steep compared to our findings (approximately 55 cells/mm^3/year with a trend towards steeper decline in more recent years for MSM). The Swiss HIV Cohort Study (SHCS), using the same methodology as the MASTER cohort, found no significant evidence for a changing rate of decline between 1986 and 2002[8]. Analyses in both these studies were restricted to patients for whom at least 5 CD4 cell counts were available at least 200 days after the date of confirmed HIV infection. Furthermore, analyses were not restricted to patients for whom the date of seroconversion could reliably be estimated. Finally, estimates from the SHCS and MASTER cohort were obtained using mixed-effects models, without correction for informative dropout. The CASCADE collaboration[21] estimated the median time between seroconversion and reaching 350 CD4 cells/mm^3 in 30–35-year old MSM with seroconversion after 1996 to be 3.94 years, similar to our mixed-effect estimate of 3.8 years for 1996–2002 seroconverters. Our joint-model estimates of 3.1 and 3.0 years were substantially lower indicating the MAR assumption may not hold. The CASCADE collaboration did not find evidence for an increased rate of CD4 cell count decline in more recent calendar years. CD4 cell count decline was jointly modelled with survival time. However, no correction for informative censoring due to ART

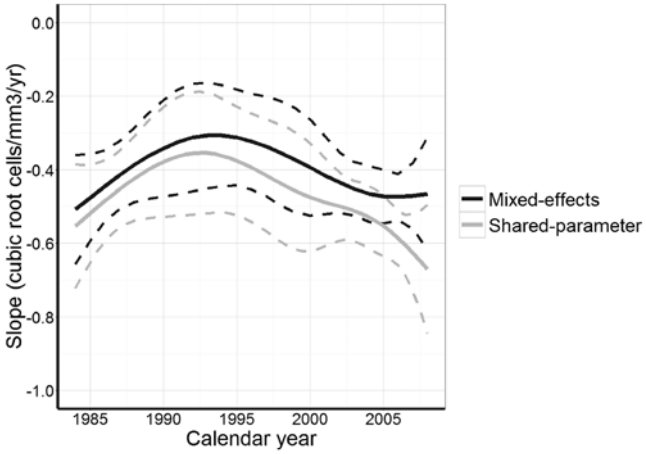

Figure 2. Mean slope (fat line) of CD4 cell count and 95% CI (dashed thin lines) in $\sqrt[3]{\text{cells}}/\text{mm}^3$/year according to calendar year of seroconversion estimated using a mixed-effects model and shared-parameter model.

initiation was made.

The reason for the less steep CD4 slope estimates and stronger effect of older age with CD4 cell count decline in the shared-parameter model compared to the pattern-mixture model is unclear, but an effect of age on either CD4 cell counts at 9 months on the slope, or both is to be expected, as shown by others[21,22].

Estimates obtained using shared-parameter models allowing the estimated underlying CD4 cell count to have a different association with censoring due to the start of ART and censoring due to disease progression (censoring because of <100 CD4 cells/mm^3, or 1 year prior to AIDS) were similar to those presented (results not shown). Furthermore, estimates from models censoring only CD4 cell counts because of ART initiation were also similar to our main results (results not shown).

Combined together, the ACS and ATHENA cohorts span almost the entire period of the Dutch HIV epidemic. A potential weakness is that ACS participants (all 1984–1995 seroconverters were ACS participants) might have different socioeconomic status, race, overall health status, or might otherwise be different to ATHENA participants. We attempted to minimize bias because of population changes by restricting inclusion to a very homogeneous population: MSM from Western-Europe or North-America and likely to be infected with subtype B. Most patients from both cohorts were seroconverters, and thus, had been tested for HIV before, suggesting a similar health seeking behaviour. Still, the possibility that our findings are the result of changes in patient characteristics over time cannot be excluded. The proportion of patients in our study identified through serological

Table 2: Estimates of mean CD4 cell count at 9 months after seroconversion and rate of decline in CD4 cell count between 9 and 48 months using 3 methods. In all models age at seroconversion (as a continuous variable, centered at 36 years of age) was included as an interaction term both with the CD4 cell count at 9 months and the slope.

	Mean CD4 cell count at 9 months after seroconversion, $\sqrt[3]{cells}/mm^3$ (95% CI)	Difference with 2003-2007 (95% CI)	p-value (overall)	Mean slope, $\sqrt[3]{cells}/mm^3/yr$ (95% CI)	Difference in slope with 2003-2007 (95% CI)	p-value (overall)
Mixed-effects model			(0.0005)			(0.56)
1984-1995	8.52 (8.32, 8.72)	0.43 (0.20, 0.66)	0.0002	-0.42 (-0.50, -0.33)	0.05 (-0.05, 0.16)	0.33
1996-2002	8.35 (8.16, 8.53)	0.25 (0.04, 0.47)	0.02	-0.43 (-0.52, -0.33)	0.04 (-0.07, 0.15)	0.47
2003-2007	8.09 (7.97, 8.21)	0.00		-0.47 (-0.53, -0.41)	0.00	
Pattern-mixture model			(0.002)			(0.002)
1984-1995	8.55 (8.36, 8.73)	0.37 (0.19, 0.55)	0.0008	-0.49 (-0.60, -0.37)	0.28 (0.17, 0.40)	0.0004
1996-2002	8.42 (8.23, 8.61)	0.24 (0.05, 0.43)	0.03	-0.65 (-0.85, -0.45)	0.12 (-0.08, 0.32)	0.30
2003-2007	8.17 (8.06, 8.29)	0.00		-0.77 (-0.87, -0.67)	0.00	
Shared-parameter model			(0.002)			(0.06)
1984-1995	8.54 (8.34, 8.73)	0.39 (0.16, 0.62)	0.0008	-0.45 (-0.55, -0.35)	0.12 (0.00, 0.24)	0.045
1996-2002	8.35 (8.17, 8.53)	0.20 (-0.01, 0.42)	0.06	-0.49 (-0.60, -0.38)	0.08 (-0.04, 0.20)	0.19
2003-2007	8.15 (8.03, 8.26)	0.00		-0.57 (-0.64, -0.50)	0.00	

CI: confidence interval.

Table 3. Estimated CD4 cell count decline (cells/mm^3) between 9 and 48 months and time between seroconversion and decline to 350 CD4 cells/mm^3 for a typical patient (aged 36 years) according to method of estimation and period of seroconversion.

		Method of estimation		
	Seroconversion period	**Mixed-effects**	**Pattern-mixture**	**Shared-parameter**
CD4 cell count decline between 9 and 48 months (cells/mm^3)	1984-1995	-249	-286	-269
	1996-2002	-244	-346	-274
	2003-2007	-246	-363	-293
Years between seroconversion and 350 CD4 cells/mm^3	1984-1995	≥4.0	3.8	≥4.0
	1996-2002	3.8	2.8	3.4
	2003-2007	3.0	2.2	2.7

evidence of recent HIV-1 seroconversion increased from 3% between 1984 and 1995 to 18% between 2003 and 2007. This could have biased our results if patients went to seek medical care because of severe acute seroconversion illness and would otherwise not have been diagnosed. The number of symptoms during primary infection is associated with viral load setpoint[23] and some specific signs with faster disease progression[24]. However, when these patients were excluded, similar results were obtained (results not shown).

Single-platform techniques to determine CD4 cell counts, gradually introduced in the late nineties, reportedly measure lower CD4 cell counts than dual-platform techniques[25]. We did not find a significant association between the standard CD4 cell count measurement technology at the time of the CD4 cell count measurement and the level of CD4 cell counts, and estimates in all 3 types of models remained similar (results not shown). However, this adjustment may have been incomplete as CD4 cell counts may have been measured with other techniques than the standard method. Factors influencing the number of measured CD4 cells which we could not control for include gating strategy, tobacco use and the time of day that the sample was taken[25–27].

Our study did not include patients who had started ART, or had a CD4 cell count below 100 cells/mm^3 within 9 months or AIDS within 21 months after seroconversion. As we had to exclude a high proportion of seroconverters in the cART era for this reason, this may have biased estimates for the 1996–2002 and 2003–2007 periods. Patients starting therapy soon after infection, outside the setting of a randomized clinical trial, are more likely to have low CD4 cell counts and/or a more pronounced decline. Hence our estimates of the slope in 1996–2002 and 2003–2007 may have been upwardly biased. However, one could also argue that 1996–2002 and 2003–2007 estimates may be downwardly biased

because patients with an early rapid CD4 cell count decline and starting ART before 9 months may have had a slower decline thereafter (because of regression to the mean effects) which is now not observed because of the early start of ART. Regression to the mean effects may have a dampening effect on bias resulting from exclusion of patients starting early treatment.
The probability of transmission of HIV is higher when the viral load is higher[28]. The duration of asymptomatic HIV infection increases with lower setpoint viral load[14]. Together these factors determine the expected number of new infections an infected person will cause during the course of infection[29]. Evidence suggests that the level of setpoint viral load may be largely determined by virus genotype[30] and that viral load between transmitter and recipient is correlated[31–35]. This may result in a selection of viral strains with higher setpoint viral load because the infectious period is shortened in the cART era (transmission during successful ART is absent or near zero[36]). The proportion of transmitters with high setpoint viral load among those transmitting HIV will increase and may lead, if viral load between transmitter and recipient is indeed correlated, to the observed increase in viral load and lower CD4 cell count at viral setpoint[3] and a steeper decline in CD4 cell count.
Another explanation of our finding may be changes in host genetic factors or adaption of HIV-1 to host genetic factors[37,38] or a rise in superinfection incidence, associated with a higher fitness[39]. Although dual infections can easily be missed, the incidence of 0% between 1984 and 1997[40] and 1 to 1.5% between 1996 and 2007 in Amsterdam patients[41,42] suggests that an increase over the years, caused by an increase in risk behavior[43], is unlikely to completely explain our findings.
In summary, to minimize bias it is important to jointly model timing of censoring because of ART initiation and/or disease progression in the analysis of longitudinal CD4 cell count trends. By doing so, we found a trend for a faster CD4 cell count decline in MSM with newly acquired HIV-1 infection between 2003 and 2007 compared to the pre-cART era. This could indicate an increase in HIV virulence over time. The higher rate of CD4 cell count decline, together with a lower CD4 cell count 9 months after seroconversion, results in a shorter time period between HIV infection and reaching the threshold of 350 CD4 cells/mm^3.

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Author Contributions

Conceived and designed the experiments: LG RBG FdW. Analyzed the data: LG RBG. Contributed reagents/materials/analysis tools: MB SJ. Wrote the paper: LG RBG. Revised the manuscript for important intellectual content: FdW BB JMP AvS DB CF.

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Supporting Information

Data from 3 patients, categorical variable 'DXXX' represents timing of censoring as shown in Table 1 (7 categories), continuous variable 'TDROPOUT' represents timing of dropout in years from 9 months after seroconversion ('TDROPOUT'=3.25 is 4 years after seroconversion) and 'DROPOUT' is a censoring indicator.

ID	YSERO	TT	CD4	CUBRTCD4	AGE10	DXXX	TDROPOUT	DROPOUT
1	1984-1995	0.08277	850	9.4727	0.48549	6	3.25	0
1	1984-1995	0.33209	650	8.6624	0.48549	6	3.25	0
1	1984-1995	0.57044	990	9.9666	0.48549	6	3.25	0
1	1984-1995	0.80332	680	8.7937	0.48549	6	3.25	0
1	1984-1995	1.06907	650	8.6624	0.48549	6	3.25	0
1	1984-1995	1.31839	760	9.1258	0.48549	6	3.25	0
1	1984-1995	1.49647	640	8.6177	0.48549	6	3.25	0
1	1984-1995	1.74853	540	8.1433	0.48549	6	3.25	0
1	1984-1995	2.01154	580	8.3396	0.48549	6	3.25	0
1	1984-1995	2.21976	500	7.9370	0.48549	6	3.25	0
1	1984-1995	2.47131	540	8.1433	0.48549	6	3.25	0
1	1984-1995	2.71995	490	7.8837	0.48549	6	3.25	0
1	1984-1995	2.97404	430	7.5478	0.48549	6	3.25	0
1	1984-1995	3.22541	360	7.1138	0.48549	6	3.25	0
2	1996-2002	0.03543	300	6.6943	-0.05644	3	1.78	1
2	1996-2002	0.37696	280	6.5421	-0.05644	3	1.78	1
2	1996-2002	0.79795	180	5.6462	-0.05644	3	1.78	1
2	1996-2002	1.04726	240	6.2145	-0.05644	3	1.78	1
2	1996-2002	1.29932	300	6.6943	-0.05644	3	1.78	1
2	1996-2002	1.56233	260	6.3825	-0.05644	3	1.78	1
2	1996-2002	1.73767	260	6.3825	-0.05644	3	1.78	1
3	2003-2007	0.05000	440	7.6059	0.05123	4	2.00	1

3	2003-2007	0.29932	460	7.7194	0.05123	4	2.00	1
3	2003-2007	0.54863	330	6.9104	0.05123	4	2.00	1
3	2003-2007	0.89304	410	7.4290	0.05123	4	2.00	1
3	2003-2007	1.16080	340	6.9795	0.05123	4	2.00	1
3	2003-2007	1.31381	350	7.0473	0.05123	4	2.00	1
3	2003-2007	1.44769	380	7.2432	0.05123	4	2.00	1
3	2003-2007	1.71849	350	7.0473	0.05123	4	2.00	1
3	2003-2007	1.94589	260	6.3825	0.05123	4	2.00	1

SAS syntax for the pattern-mixed model

This code gives averaged intercepts and slopes for each seroconversion period, as shown in Table 2. The estimated are weighted according to the proportional size of each pattern in each seroconversion period (the percentages in Table 1 divided by 100). The obtained standard errors are slightly underestimated because the size of the patterns are assumed fixed. To correct for the uncertainty of pattern sizes, corrected standard errors can be obtained using the delta method[17,18].

```
proc mixed data=data1 noclprint method=ml cl;

class id ysero dxxx;

model cubrtcd4=dxxx*ysero tt*dxxx*ysero age10 tt*age10 / noint so-
lution cl;

random intercept tt / subject=id type=un ;



estimate 'intercept 1984-1995' dxxx*ysero 0.054 0.036 0.108 0.036
0.090 0.604 0.072 0 0 0 0 0 0 0 0 0 0 0 0 0 0 / cl;

estimate 'intercept 1996-2002' dxxx*ysero 0 0 0 0 0 0 0 0.245
0.100 0.036 0.072 0.086 0.417 0.043 0 0 0 0 0 0 0 / cl;

estimate 'intercept 2003-2007' dxxx*ysero 0 0 0 0 0 0 0 0 0 0 0 0
0 0 0.213 0.124 0.081 0.093 0.129 0.326 0.034 / cl;

estimate 'slope 1984-1995' tt*dxxx*ysero 0.054 0.036 0.108 0.036
0.090 0.604 0.072 0 0 0 0 0 0 0 0 0 0 0 0 0 0 / cl;

estimate 'slope 1996-2002' tt*dxxx*ysero 0 0 0 0 0 0 0 0.245 0.100
0.036 0.072 0.086 0.417 0.043 0 0 0 0 0 0 0 / cl;

estimate 'slope 2003-2007' tt*dxxx*ysero 0 0 0 0 0 0 0 0 0 0 0 0 0
0 0.213 0.124 0.081 0.093 0.129 0.326 0.034 / cl;



estimate 'intercept 1984-1995 vs 2003-2007'

 dxxx*ysero 0.054 0.036 0.108 0.036 0.090 0.604 0.072 0 0 0 0 0 0
0 -0.213 -0.124 -0.081 -0.093 -0.129 -0.326 -0.034 / cl;

estimate 'intercept 1996-2002 2003-2007'

 dxxx*ysero 0 0 0 0 0 0 0 0.245 0.100 0.036 0.072 0.086 0.417
0.043 -0.213 -0.124 -0.081 -0.093 -0.129 -0.326 -0.034 / cl;
```

```
estimate 'slope 1984-1995 vs 2003-2007'

 tt*dxxx*ysero 0.054 0.036 0.108 0.036 0.090 0.604 0.072 0 0 0 0 0
0 0 -0.213 -0.124 -0.081 -0.093 -0.129 -0.326 -0.034 / cl;

estimate 'slope 1996-2002 vs 2003-2007'

 tt*dxxx*ysero 0 0 0 0 0 0 0 0.245 0.100 0.036 0.072 0.086 0.417
0.043 -0.213 -0.124 -0.081 -0.093 -0.129 -0.326 -0.034 / cl;

run;
```

R syntax for the shared parameter model

First a Cox proportional hazard model and a random-effects model on dataset 'data1' is fitted. The longitudinal model uses the data structure of multiple records per patient, as shown above. The Cox proportional hazards models uses one record per patient (dataset data1.id). The estimates obtained from the longitudinal and survival model are used as initial values for the shared-parameter model.

```
library("JM")

# CREATE DATASET WITH ONE RECORD FOR EACH PATIENT, TO BE USED FOR
THE SURVIVAL MODEL

data1.id <-data1[!duplicated(data1$ID),]

# ▸t the linear mixed effects and survival submodels separately;

▸tLME <- lme(CUBRTCD4 ~ TT * (YSERO + AGE10), random = ~ TT | ID,
data = data1)

▸tSURV <- coxph(Surv(TDROPOUT, DROPOUT ) ~ YSERO + AGE10, data =
data1.id, x = TRUE)

# Fit Joint Model

▸t.jm.weibull <- jointModel(▸tLME, ▸tSURV, timeVar = "TT", method
= "weibull-AFT-aGH", parameterization = "value", GHk=16)

summary(▸t.jm.weibull)
```

5

Changes in HIV RNA and CD4 cell count following acute HCV infection in chronically HIV-infected individuals

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Abstract

Objective
Little is known about the impact of acute HCV co-infection on HIV-1 disease progression. We investigated CD4 cell count and HIV RNA concentration changes after HCV infection in individuals chronically infected with HIV-1.

Methods
We selected individuals that had a last negative and first positive HCV RNA test less than 1 year apart. Bivariate linear mixed-effects regression was used to model trends in HIV RNA level and CD4 cell count from 2 years before the last negative HCV RNA test until the first of the following dates: start of anti-HCV medication, change in cART status and end of follow-up.

Results
At the estimated time of HCV co-infection, out of 89 individuals, 63 (71%) were cART-treated and 26 (29%) were not on cART. In persons on cART, median CD4 cell count declined from 587 to 508 cells/mm³ ($p<0.0001$) during the first 5 months after HCV infection and returned to 587 cells/mm³ after 2.2 years. Also, the probability of an HIV RNA >50 copies/ml peaked to 18.6% at HCV co-infection, with lower probabilities 6 months before (3.5%, $p=0.006$ compared to peak probability) and after (2.9%, $p=0.009$). In persons not on cART, no significant impact of HCV co-infection on trends in HIV RNA level or CD4 cell count were observed.

Conclusion
Acute HCV infection in cART-treated, chronically HIV-infected patients was associated with a temporary decrease in CD4 cell counts and increased risk of HIV viraemia >50 copies/ ml. This may increase the risk of further HIV transmission.

Introduction

Over the last two decades, the spread of hepatitis C virus (HCV) infection among HIV-1 infected individuals in the Netherlands has shifted from blood borne HCV infections among recipients of blood or blood products and injecting drug users (IDU) to sexually transmitted HCV infections among men who have sex with men (MSM)[1,2]. The effect of HCV on HIV disease progression has long been controversial, but results from recent studies suggest an increased risk of overall mortality in the cART era among HIV/HCV co-infected individuals compared to individuals with HIV mono-infection[3,4]. Furthermore, a meta-analysis found a poorer CD4 cell count response after 48 weeks on combination antiretroviral therapy (cART) in HIV/HCV co-infected individuals compared to HIV mono-infected individuals [5]. Since normalisation of CD4 cell counts while on virological suppressive cART may take several years[6], studying the impact of events influencing CD4 cell counts is important. The impact of acute HCV infection on HIV disease progression is still largely unknown. Only limited data are available, because in the traditional risk groups HCV infection by blood borne exposure occurs mostly before HIV infection. The objective of this study was to model trends in CD4 cell count and HIV RNA plasma concentration before and after acute HCV co-infection in individuals chronically infected with HIV.

Methods

Patient selection

We used data from individuals included in the AIDS Therapy Evaluation in the Netherlands (ATHENA) observational HIV cohort[7]. The ATHENA cohort includes anonymized data obtained from 20,781 treated and untreated HIV-infected patients (June 2012), who have been followed in or after 1996 in any of the 26 Dutch HIV treatment centers. Ethical approval in the ATHENA cohort is not obtained as data are collected from patients as part of HIV care. Patients can opt-out after being informed by their treating physician of the purpose of collection of clinical data. HCV RNA measurements were supplemented with those from 57 MSM from the HIV clinic of the Academic Medical Center in Amsterdam, with most individuals participating in the MSM Observational Study of Acute Infection with hepatitis C (MOSAIC study), a prospective study in which HIV- infected individuals with acute HCV infection are matched to two HIV-infected individuals without HCV[8]. All included individuals had a negative HCV-RNA plasma sample followed by a positive HCV RNA sample within 12 months. Individuals with a positive HCV antibody test result prior to the date of the first positive HCV RNA were excluded. The midpoint between the date of last negative HCV RNA result and first positive HCV RNA result was used as the date of HCV co-infection. The Siemens Versant HCV qualitative and Siemens Versant HCV RNA 3.0 assays were used to diagnose 47% and 31% of infections, respectively (Siemens Healthcare Diagnostics, the

Table 1. Demographic and Clinical Characteristics at the First Positive HCV RNA Test

	Without cART N (%)	On cART N (%)	Total N (%)
Total	26 (29)	63 (71)	89 (100)
Male gender	26 (100)	63 (100)	89 (100)
Year first HCV RNA positive			
1999–2005	6 (23)	8 (13)	14 (16)
2006–2012	20 (77)	55 (87)	75 (84)
HCV genotype			
Unknown	8 (31)	11 (17)	19 (21)
1	14 (54)	36 (57)	50 (56)
2	2 (8)	6 (10)	8 (9)
4	2 (8)	10 (16)	12 (13)
Transmission risk group			
MSM	23 (88)	60 (95)	83 (93)
Heterosexual	1 (4)	1 (2)	2 (2)
Injecting drug users	1 (4)		1 (1)
Unknown	1 (4)	2 (3)	3 (3)
Region of origin			
Netherlands	24 (92)	50 (79)	74 (83)
W-Europe/N-America	1 (4)	7 (11)	8 (9)
Other	1 (4)	6 (10)	7 (8)
Last HIV RNA at/before first positive HCV RNA test (log_{10} copies/mL)			
Missing		1 (2)	1 (1)
< 50		52 (83)	52 (58)
50–400		5 (8)	5 (6)
400–50,000	15 (58)	5 (8)	20 (22)
50,000–100,000	5 (19)		5 (6)
≥ 100,000	6 (23)		6 (7)

	Median (Q1–Q3)	Median (Q1–Q3)	Median (Q1–Q3)
HCV RNA conversion interval (months)	4.5 (3.4–6.5)	4.3 (3.0–6.2)	4.4 (3.2–6.4)
Age	41 (35–44)	44 (38–48)	43 (38–47)
CD4 cell count (cells/mm³)	470 (360–540)	550 (390–710)	510 (380–650)
Years on cART		6.5 (3.0–9.8)	
Known duration of HIV	1.9 (1.2–3.6)	7.3 (4.4–11.7)	5.9 (2.5–9.8)

Table 2. Number of Patients in Follow-up

	Years from Estimated HCV Co-infection					
	-2	**-1**	**0**	**1**	**2**	**3**
Untreated	4	18	26	7	6	3
On cART	30	51	63	18	14	11

Hague, the Netherlands). In the remaining 22% either unspecified or in-house assays were used.
To be sure that we only included individuals with a chronic HIV infection at the time of HCV co-infection, we excluded those with less than 6 months between HIV diagnosis and the estimated date of HCV co-infection. Two groups were considered: those who received cART at the first positive HCV RNA test and those who did not. Those not receiving cART at the first positive HCV RNA test could have received antiretroviral therapy in the past. Those receiving cART were required to be at least 6 months on cART at the first positive HCV RNA. Individuals with a change in cART status (untreated individuals starting cART or treated individuals interrupting cART) between the last negative and first positive HCV RNA test were excluded. Finally, we excluded one individual who was treated unsuccessfully with cART (high HIV viral load for multiple years) and was infected with a resistant HIV strain.

Outcome

Trends in CD4 cell counts and HIV RNA levels were modelled over time from two years before the last negative HCV-RNA test until the earliest of the following three events: start of anti-HCV medication (because of the effect of interferon on CD4 cell counts and HIV RNA), change in cART status after HCV infection (a therapy interruption of at most 1 month was allowed for CD4 cell count and 7 days for HIV RNA modelling) and end of follow-up. In addition, in cART-treated individuals, we excluded CD4 cell counts and HIV RNA measurements obtained within a period of 6 months after the start of cART, because changes in these markers are much larger during this initial phase of cART than after this period. In those individuals who were not receiving cART at HCV diagnosis but who previously had received cART, follow-up started from the moment cART was stopped.

Statistical analysis

Trends in HIV RNA and CD4 cell counts were modelled using bivariate linear mixed-effects models. The estimated HCV RNA conversion date was chosen as time origin. Average time trends were allowed to vary smoothly using natural cubic splines. To allow for flexible modelling of HIV RNA around HCV infection, 8 knots were chosen at time points -2.0, -1.0, -0.5, -0.25, 0, 0.25, 0.5, and 2.5 year.

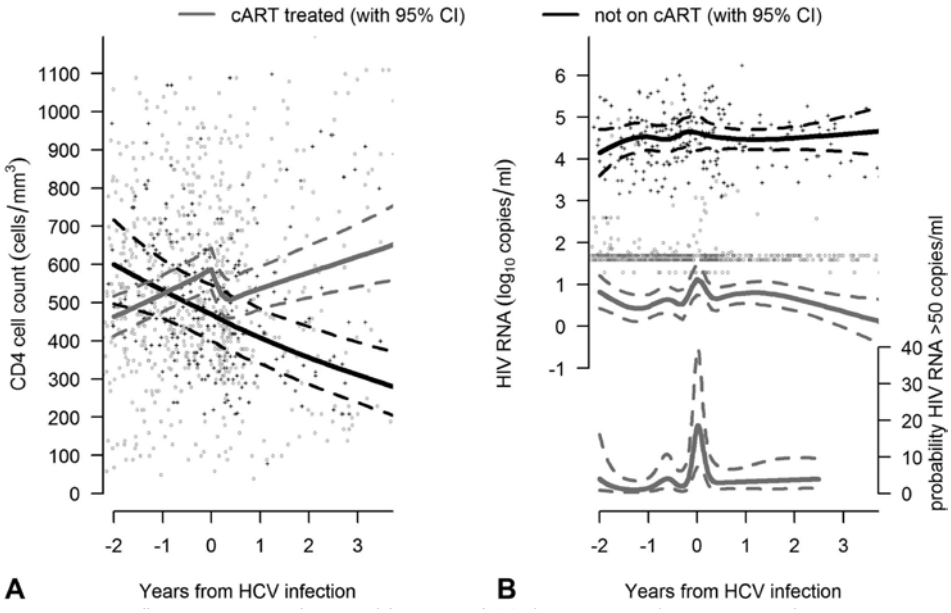

Figure 1: CD4 cell count trajectory and 95% confidence intervals (A) following acute HCV infection. HIV RNA plasma concentration, and probability of a concentration >50 HIV RNA copies per milliliter and 95% confidence intervals (B).

For individuals on cART we also modelled HIV RNA as a dichotomous outcome (≤ and >50 copies/ml) using a random effects logistic regression model and natural cubic splines as described above. The slope of cube root transformed CD4 cell counts were linearly modelled up to time 0 and thereafter with cubic splines with knots at 0.0, 0.25, 0.5, 1.0 and 2.5 year. For each individual, a random intercept and 2 random slopes (before and after HCV infection) were included. Results on the cube root scale were backtransformed to the original scale and apply to a 'typical' individual, i.e. an individual with zero for the random intercept and slopes. cART status was allowed to have an effect on the fixed intercept and slope parameters of CD4 cell counts and HIV RNA levels. The residual variation was assumed to be independent and to have constant variance within marker type (CD4 cell count and HIV RNA).
Data below the detection limit of HIV RNA assays were treated as censored responses. It may be confusing that in some analyses mean HIV RNA trajectories were at levels below the lower limit of detection of most assays currently available. The estimated mean depends to a large extent on the few observed values above the detection limit and the assumption that the HIV RNA is normally distributed. However, it is less biased than using the lower detection limit or the midpoint between 0 and the lower detection limit for censored observations[9], as is often done.

Taking the midpoint between the last negative and first positive HCV RNA ignores the uncertainty about the true date of HCV infection. Therefore, the estimated confidence intervals are likely to be too narrow. As a sensitivity analysis we created 5 datasets in which, for each individual, a random date of HCV infection was imputed between the last negative and first positive HCV RNA test (i.e., assuming a uniform distribution). These datasets were then analysed as described above and estimates were subsequently combined using methods for multiple imputation[10]. Standard mixed-effects estimates may be biased when starting anti-HCV medication depends on unobserved CD4 counts, such as the underlying subject-specific CD4 cell count trajectories. As a sensitivity analysis, longitudinal CD4 cell counts and time to censoring (due to start of anti-HCV medication or change in treatment status) were jointly modelled to check for the presence of bias due to informative censoring.
All modelling was done in R version 3.0.1 (R Development Core Team 2010) using the packages lmec[11] and lme4[12]. Joint models were fitted using the JM package[13].

Results

Of the 89 included individuals, 26 were without cART and 63 had been on cART for at least 6 months at the first positive HCV RNA test. Characteristics of the included individuals are shown in Table 1. All included individuals were men and 93% were in the MSM transmission risk group. Eighty-three percent of individuals were from the Netherlands and 9% came from either Western Europe or North America. For 84% of individuals, the first positive HCV RNA test was between 2006 and 2010. The median CD4 cell count at the time of the first positive HCV RNA test was 470 cells/mm^3 in untreated and 550 cells/mm^3 in cART-treated individuals. The median known duration of HIV infection at the time of the first positive HCV RNA test was 1.9 years in untreated individuals and shorter compared to the 7.3 years in cART-treated individuals. The median time between the date of the last negative and first positive HCV RNA test result was 4.5 months in untreated individuals and 4.3 months in cART treated individuals. Five individuals among the 26 who were untreated had had prior exposure to cART. In total 1023 CD4 cell counts and 909 HIV RNA measurements were included in the longitudinal analyses. Follow-up was censored within 6 months after HCV co-infection in 11 (42%) untreated and 28 (43%) treated individuals and within 1 year in 19 (73%) untreated and 45 (71%) treated individuals, primarily due to the start of anti-HCV medication. Table 2 shows the number of cART treated and untreated individuals in follow-up over time.

In cART-treated individuals, the median CD4 cell count increased from 463 cells/mm^3 (95% confidence interval [CI] 413-517) at two years prior to acute HCV infection to 587 cells/mm^3 (95% CI 534-644) at HCV infection. During the first 5 months after HCV infection, the median CD4 cell count of cART-treated

individuals declined significantly ($p<0.0001$) to 508 cells/mm^3 (95% CI 460-559). The median count increased thereafter and returned to above 587 cells/mm^3 after 2.2 years (Fig. 1A). In untreated individuals, median CD4 cell counts decreased from 600 cells/mm^3 (497-717) at two years prior to HCV co-infection to 290 cells/mm^3 (215-380) at 3.5 years after HCV co-infection. No significant change from a linear trend (on the cube root scale) around HCV co-infection was observed.

Between 6 months before HCV co-infection and 6 months after HCV co-infection, at least one HIV RNA measurement was available in 62 of the 63 cART-treated individuals. HIV RNA was >50 copies/ml at least once in 12 of these 62 individuals (19%). Highest observed viremia was 23,556 and 180,254 copies/ml in 2 individuals and 3,047 copies/ml or lower in the remaining 10.
Figure 1B shows that median HIV RNA in cART-treated individuals reached a peak of 13 copies/ml (95% CI 6-32) at the estimated time of HCV co-infection and was lower at 4 (95% CI 2-8) copies/ml 6 months before and 5 (3-9) copies/ml 6 months after HCV co-infection (test for no difference between 6 months before and at time of HCV co-infection $p=0.003$). Figure 1B also shows the estimated probability of observing a plasma viral load >50 copies/ml for a typical individual (i.e. all random effects zero). This probability was highest around the estimated date of HCV infection: 18.6%, 95% CI 7.1-39.6, while the probability of a plasma viral load >50 copies/ml 6 months before and after estimated infection was 3.5% (1.4-8.7) and 2.9% (1.2-7.1), respectively. The peak in the probability was significantly different from the probability 6 months before ($p=0.006$) and after ($p=0.008$) HCV co-infection.
The interval between the last negative and the first positive HCV RNA test result was shorter in cART treated individuals with observed HIV viremia (median 3.0 months) compared to treated individuals without observed HIV viremia (5.1 months). In untreated individuals the mean HIV RNA showed a gradual increase over time. Mean HIV RNA was only slightly higher around acute infection compared to 6 months before and after (test for no difference between 6 months before and moment of infection: $p=0.50$).

The sensitivity analysis in which the timing of HCV co-infection was estimated as a random time point between the last negative HCV RNA test and first positive HCV RNA test result yielded similar trends in mean CD4 cell count and 95% confidence intervals in cART-treated and untreated individuals to those obtained when the midpoint was used. The HIV RNA trend obtained with the 5 sets with randomly chosen date of infection was also similar to the midpoint results for untreated individuals. However, the peak in the HIV RNA trajectory for treated individuals was lower than the peak in the analysis using the midpoint (9 copies/ml (95% CI 4-19) versus 13 copies/ml (95% CI 6-32) copies/ml). Using the random time point as time of HCV co-infection, the mean HIV RNA 6 months before the peak was 4 copies/ml (test for no difference with the mean at the peak, $p=0.05$). Likewise, the highest probability of observing HIV RNA >50 copies/ml was also

lower (10.3% at 0.4 months, 95% CI 4.1-23.8, compared to 18.6%, 95% CI 7.1-39.6, when the midpoint was used). The difference between the peak probability and the probability of observing HIV RNA >50 copies/ml 6 months before the peak was not significant, p=0.08. Probabilities obtained using the two methods were similar at longer times before and after HCV infection (results not shown). In the second sensitivity analysis, joint model estimates were similar to estimates obtained with linear mixed models.

Discussion

The probability of HIV viremia >50 copies/ml in cART treated HIV-1 infected individuals was found to increase around the time of acute HCV co-infection. This was accompanied by a temporary decrease in CD4 cell count. In these individuals it took 2.2 years after acquiring HCV infection for the median CD4 cell count to return to levels similar to those seen before acute HCV infection. In individuals not on cART, however, no significant change in HIV RNA or in CD4 cell count trend around the time of HCV co-infection was observed. The small number of untreated persons may have contributed to not finding a significant increase in HIV RNA around the time of HCV co-infection. Moreover, it may be more difficult to observe an increase at high and more variable HIV RNA levels than at low HIV RNA levels.

Although the duration of increased HIV RNA viremia is likely to be short, these results may have important implications for the risk of onward transmission of HIV. Increased viral load in HIV-positive cART-naïve individuals has been associated with an increased risk of heterosexual transmission[14-17]. How these estimates can be extrapolated to risk estimates for homosexual transmission in cART treated MSM in the presence of an acute HCV infection is unknown. Given that the individuals in our study are likely to be involved in high-risk sexual behaviour[18], the risk of transmission of HIV to an HIV-uninfected partner shortly after HCV co-infection is likely to be increased, depending on the height of the viral load.

For cART-treated persons with a CD4 cell count which has not yet reached higher levels, a decline in CD4 cell count following HCV co-infection may have clinical consequences and could contribute to the observed increased risk of both all-cause mortality and HIV and/or AIDS-related death in the cART era in HIV/HCV co-infected individuals compared to HIV mono-infected individuals [4,19]. A meta-analysis and another recent study also found such an association for all-cause mortality, but not for AIDS defining events [3,20].

To our knowledge, no previous study has investigated the effect of acute HCV infection on HIV RNA and CD4 cell count in HIV-1 infected individuals. Studies

have reported increased HIV replication, decreased CD4 cell counts and increased CD4 cell activation after influenza vaccination, although other studies reported no such changes[21]. Changes have also been reported after pneumococcal vaccination[22], infection with syphilis[23-25], malaria[26,27], herpes simplex virus[28], and other opportunistic infections[29], and were seen in both cART-treated and untreated individuals. Generally, the increase in HIV RNA concentration after immunisation or infection (and in some cases following treatment of co-infection) was reversible, with a duration of increased HIV viremia of 8-9 weeks. This increase might be mediated through immune activation and subsequent amplification of HIV replication in the blood. Increased HIV viremia results in increased apoptosis of CD4 T cells, which may result in decline of CD4 counts[30]. Furthermore, the immune response to HCV may also have contributed to the decline in counts. Although after the initial rapid decline the CD4 cell count increases again in cART-treated individuals, it may be that chronic HCV infection contributes to an increased state of inflammation, as shown for HCV mono-infected individuals[31].
Thus, when CD4 cell counts decrease for no apparent reason or HIV RNA becomes detectable, among the possibility of intercurrent infection, co-infection with HCV should also be considered, especially in MSM. Early diagnosis of HCV is important in order to make a prompt start of anti-HCV medication possible and to minimize the risk of damage to the liver and onward transmission of HCV.

The estimated HIV RNA trends in treated individuals depended on the method used to estimate the time of HCV co-infection. The probability of observing HIV RNA >50 copies/ml and the peak in the HIV RNA trajectory were both lower in the sensitivity analysis in which the time of co-infection varied. Measurement error (in this case uncertainty around the true date of HCV co-infection) will generally result in bias towards zero (here: a lower peak). Were the exact timing of HCV infection known, the estimated probability of HIV RNA >50 copies/ml would have been higher. CD4 cell count trends were not sensitive to the method of determining the time of HCV infection, probably because these changes are less sudden and longer lasting.

Treatment with pegylated interferon and ribavirin has a depleting effect on the number of absolute CD4 cells[32] and was started within one year of HCV infection in 68% of individuals. The decline in CD4 cell count in treated patients in our study could not, however, be attributed to pegylated interferon therapy as follow-up was censored after the start of anti-HCV therapy. Furthermore, the results of the second sensitivity analysis, done to check for possible bias arising if starting anti-HCV medication were dependent on the underlying subject-specific CD4 cell count trajectories, shows that bias due to informative censoring is unlikely to play a major role.

HIV RNA assays associated with more frequent test results between 50-200

copies/ml in patients on cART such as the Roche Cobas TaqMan HIV-1 v1.5 and v2 assays[33] were not frequently used. HIV RNA in the first available sample at or after HCV infection was quantified with the m2000rt HIV RNA assay (Abbott, Abbott Park, IL, USA) in 30 samples (48%), with the Versant HIV-1 RNA version 3.0 (Siemens, Deerfield, IL, USA) in 22 samples (35%), with the Cobas Ultra Amplicor in 1 sample (2%), with the Cobas TaqMan HIV-1 v1.5 in 3 samples (5%) and v2.0 (Roche Diagnostics, Pleasanton, CA, USA) in 5 samples (8%), and for 2 samples (3%) the assay type was not available. There was no evidence that among the 8 samples measured with Cobas TaqMan HIV-1 v1.5 or v2 assays, HIV RNA concentration was more often >50 copies/ml than in the 52 samples measured with other assays (1/8 vs 11/52, chi-square test p=0.92). The type of HIV RNA assay is therefore unlikely to be the cause of the increased HIV viremia. We do not collect data on adherence. It may be that adherence has been sub-optimal for the individuals with high viral load. However, the 97% of treated individuals with viral load <50 copies/ml before and after estimated HCV infection, suggests that adherence at those times was high.

To limit the influence of changes in treatment status, we excluded individuals who started cART during the period of change from negative to positive HCV RNA, as well as those who interrupted cART for 1 month or more during the study period. This may have biased our results. For example, a HIV treatment naïve individual with a sudden CD4 cell count decline, prompting cART initiation after which acute HCV infection was diagnosed would be excluded from the present study. Therefore, it may be that our estimates for the trend in untreated individuals are upwardly biased, as individuals with sharp drops in CD4 cell count might have been excluded.

In conclusion, in chronic HIV infected individuals who receive cART, acute HCV infection was associated with a short-term increase in HIV viremia and a temporary decline in CD4 cell count, similar to changes seen after other infections or vaccinations. Dependent on the magnitude, these changes may have implications for further HIV transmission and clinical progression. No such changes were observed for individuals not on cART.

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6

CD4 Cell Counts of 800 Cells/mm³ or Greater After 7 Years of Highly Active Antiretroviral Therapy Are Feasible in Most Patients Starting With 350 Cells/mm³ or Greater

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Abstract

Objective
CD4 cell count changes in therapy-naive patients were investigated during 7 years of highly active antiretroviral therapy (HAART) in an observational cohort.

Methods
Three endpoints were studied: (1) time to ≥800 CD4 cells/mm³ in 5299 therapy-naive patients starting HAART, (2) CD4 cell count changes during 7 years of uninterrupted HAART in a subset of 544 patients, and (3) reaching a plateau in CD4 cell restoration after 5 years of HAART in 366 virologically suppressed patients.

Results
Among patients with <50, 50 to 200, 200 to 350, 350 to 500, and ≥500 CD4 cells/mm³ at baseline, respectively, 20%, 26%, 46%, 73%, and 87% reached ≥800 CD4 cells/mm³ within 7 years of starting HAART. Periods with HIV RNA levels >500 copies/mL and age ≥50 years were associated with lesser increases in CD4 cell counts between 6 months and 7 years. Having reached ≥800 CD4 cells/mm³ at 5 years, age ≥50 years, and ≥1 HIV RNA measurement >1000 copies/mL between 5 and 7 years were associated with a plateau in CD4 cell restoration.

Conclusions
Restoration to CD4 cell counts ≥800 cells/mm³ is feasible within 7 years of HAART in most HIV-infected patients starting with ≥350 cells/mm³ and achieving sufficient suppression of viral replication. Particularly in patients ≥50 years of age, it may be beneficial to start earlier than current guidelines recommend.

Introduction

The choice of when to begin highly active antiretroviral therapy (HAART) is based on a trade-off between the complications of long-term antiretroviral drug use[1–4] and the benefits of timely reversal of the deterioration of the immune system. Current guidelines[5–7] recommend HAART initiation before asymptomatic patients drop to 200 CD4 cells/mm³. Delaying the start of HAART until after this threshold (ie, in a relatively late stage of infection) is associated with faster disease progression and death as compared with starting when counts are still greater than 200 cells/mm³.[8–10] Further studies have concluded that the prognosis is improved when patients start HAART when CD4 counts are still greater than 350 cells/mm³.[11]
Residual HIV replication,[12,13] impaired thymic function,[14,15] advanced age,[16] enhanced T-cell activation,[17,18] apoptosis,[19,20] and, possibly, viral coinfection[21,22] have been associated with more limited immune restoration in patients on HAART. After an initial rapid increase, because of redistribution of cells trapped in the lymphoreticular system to the peripheral blood, CD4 cell counts may plateau after the first year of HAART.[23–28] These studies described follow-up of <3 years and included a mixture of pretreated and naive patients or a small number of patients. Studies with longer follow-up in naive patients likewise reported a plateau effect, however.[29,30] In contrast, no evidence of a plateau effect was found in patients who had suppressed plasma HIV-RNA levels to less than 1000 copies/mL.[31]
Here, we explore the capacity of patients on long-term HAART to improve CD4 cell counts. We assess how these improvements, 7 years after starting HAART, compare with CD4 cell levels in the non–HIV-infected population. In addition, we describe the determinants of reaching a plateau in CD4 cell restoration between 5 and 7 years of uninterrupted HAART.

Materials and methods

Study Population

Patients were selected from the AIDS Therapy Evaluation Project, Netherlands (ATHENA) national observational HIV cohort.[32] HAART was defined as a combination of 3 or more drugs from at least 2 drug classes or a combination of 3 or more nucleoside reverse transcriptase inhibitors, including abacavir or tenofovir. All patients were 16 years of age or older and had a recorded pre-HAART CD4 cell count. We performed 3 analyses.

Time to CD4 Cell Counts ≥800 Cells/mm³

In the first analysis, we used a longitudinally followed cohort of 5299 antiretroviral therapy–naive patients who initiated HAART between July 1, 1996 and December 31 2004 to analyze the probability of reaching CD4 cell counts 800 cells/mm³ in relation to pre-HAART values and other baseline characteristics.

Most studies[25,28,30] have used a threshold of 500 CD4 cells/mm³, the lower limit of the normal range in uninfected individuals. The mean observed CD4 counts in HIV-negative adults are 1050, 840, and 800 cells/mm³ for women, heterosexual men, and men who have sex with men (MSM), respectively.[33] Because our cohort is largely MSM, we chose ≥800 CD4 cells/mm³ as an endpoint. Potential predictors tested for association with time taken to reach the endpoint included pre-HAART CD4 count (<50, 50–200, 200–350, 350–500, and ≥500 cells/mm³), pre-HAART CD8 count (<600, 600–1300, and ≥1300 cells/mm³), pre-HAART HIV RNA plasma levels (<3, 3–4, and ≥4 $\log_{10}$ copies/mL), Centers for Disease Control and Prevention category C (CDC-C) events before starting HAART, age at starting HAART, gender, and region of birth (Western or Central Europe, North America, or Australia combined [WCE/NA/A] and sub-Saharan Africa, Caribbean, Latin America, Southeast Asia, and all other regions combined were tested for association with time taken to reach the endpoint). The CD4 cell count measured closest to starting HAART from 6 months before to 7 days after starting was selected as the pre-HAART CD4 cell count. Patients were allowed to change or interrupt regimens and were retained in the analysis regardless of the level of HIV RNA.

Long-Term CD4 Cell Response in Patients on Uninterrupted HAART for 7 Years

Second, we analyzed the immune system's maximum capacity to restore CD4 cell numbers in a longitudinally followed subcohort of 554 patients who started HAART between July 1, 1996, and June 30, 1998, and took HAART continuously for at least 7 years. These patients were also part of the first analysis; they were included regardless of whether they had HIV RNA measurements greater than detectable limits because we were interested in estimating the effect of periods of viremia on the rate of change in CD4 cell count.

We selected CD4 cell counts measured closest to weeks 24, 48, and 72 after initiating HAART (within a time frame of 3 months) and, subsequently, at 24-week intervals up to 360 weeks. Median increases in CD4 cell count at these time points were calculated and graphically summarized.

All CD4 cell counts measured between starting HAART and 7 years thereafter were longitudinally modelled. The same potential predictors as in the first analysis were tested for their association with slopes of CD4 cell counts over time. Given the smaller sample size for this analysis, the predictors were subdivided differently: region of origin (WCE/NA/A and all other regions combined), pre-HAART HIV RNA plasma concentration (<4.5 $\log_{10}$ copies/mL and ≥4.5 $\log_{10}$ copies/mL), and CD8 count (<1300 cells/mm³ and ≥1300 cells/mm³). To study the effect of viremia, we created a time- updated variable with values between 0% and 100% and denoting the percentage of time a patient's plasma HIV RNA concentration was ≥500 copies/mL after the initial 6 months of HAART. The value was 0% when a patient never had HIV RNA levels <500 copies/mL after the initial 6 months and 100% when a patient always had levels <500 copies/mL after the initial 6 months. We also tried to distinguish the effect of low-level viremia and high-

level viremia on the slopes of CD4 cell count using different cutoffs (500–1000, 1000–10,000, and >10,000 copies/mL). Given our inclusion criteria, however, the number of HIV RNA measurements greater than 500 copies/mL was limited, and we lacked statistical power to detect significant differences in slopes of CD4 cell count during periods of low-level and high-level viremia.

Decreases in CD4 Cell Count Between 5 and 7 Years in Virologically Suppressed Patients

The third analysis determined predictors for reaching a plateau in CD4 cell restoration between years 5 and 7. To counter random fluctuations in CD4 cell measurements, we used the individual slopes between 5 and 7 years derived from a longitudinal model similar to that used in the second analysis (ie, with 5 intercepts for the 5 pre-HAART CD4 cell strata and 4 slopes for the 4 time intervals but without any other covariates) to determine whether a patient had reached a plateau in CD4 cell restoration. This plateau can be interpreted as an on-average decreasing CD4 cell count between years 5 and 7. Included in this model was a subset of 366 patients on uninterrupted HAART who were among those included in the second analysis. Additional inclusion criterion for this subset was that all HIV RNA measurements between 6 months and 5 years after starting HAART were <500 copies/mL. Variables included in the analysis were the same as in the second analysis, with the exception of HIV viremia, which was now defined as at least 1 HIV RNA measurement ≥1000 copies/mL between 5 and 7 years after starting HAART (yes/no). This excludes HIV RNA measurements between 500 and 1000 copies/mL from the definition because these are unlikely to cause a plateau in CD4 cell restoration.

Statistical Analysis

The Cox proportional hazards model and Kaplan-Meier estimates were used for the first analysis of time to ≥800 CD4 cells/mm^3. Time was censored at the end of follow-up or time of death, whichever occurred first. The statistical model used for the longitudinal analyses was a mixed-effects model with a random intercept and 4 random slopes for each patient. A first-order autoregressive covariance structure was used to correlate intraindividual serial measurements. We divided the 7-year time period into 4 intervals: 0 to 6 months after starting HAART, 6 months to 3 years, 3 to 5 years, and 5 to 7 years; slopes were allowed to differ between them. The intervals were chosen by visual inspection of the graphs of median CD4 cell response. CD4 cell counts were square root transformed to comply with model assumptions. Slopes of CD4 cell count increase during each interval were estimated for the 5 pre- HAART CD4 cell count strata (<50, 50–200, 200–350, 350–500, and ≥500 cells/mm^3). The other variables were allowed to have 1 effect on the slopes between the start of HAART and 7 years thereafter (ie, the whole period) or to have an effect in each of the 4 previously stated time intervals. Model fit was determined by the Akaike Information Criterion statistic.[34] Logistic regression was used for the third analysis of decreasing CD4

cell counts between 5 and 7 years after first starting HAART. All calculations were performed using SAS 9.1.3 (SAS Institute, Cary, NC).

Results

Time to CD4 Cell Counts ≥800 Cells/mm³

The characteristics of the 5299 patients at the start of HAART are shown in Table 1. Most (76%) were male, 50% were MSM, and 63% originated from WCE/NA/A. The median HIV RNA concentration in plasma was 5.0 $\log_{10}$ copies/mL. A pre-HAART CD4 count of <200 cells/mm³ was found in 2703 patients (51%).

The time required to restore CD4 counts to ≥800 cells/mm³ was associated with a higher pre-HAART CD4 cell count. After 7 years of HAART, Kaplan-Meier estimates of the percentage of patients reaching ≥800 CD4 cells/mm³ were 20%, 26%, 46%, 73%, and 87% for those with a pre-HAART CD4 count of <50, 50 to 200, 200 to 350, 350 to 500, and ≥500 cells/mm³, respectively. Adjusted hazard ratios compared with those of patients with a pre-HAART CD4 count of 200 to 350 cells/mm³ were 0.26 and 0.46, respectively, for those with <50 and 50 to 200 cells/mm³ and were 2.84 and 6.79, respectively, for those with 350 to 500 and ≥500 cells/mm³ (Table 2). Female gender and higher pre-HAART HIV RNA levels were associated with a shorter time to CD4 cell counts ≥800 cells/mm³. Older age, Southeast Asian or sub-Saharan African origin, and HIV infection through intravenous drug use were associated with a longer time to this endpoint. There were no significant differences according to different pre-HAART CD8 cell count strata in adjusted models (P = 0.58).

Long-Term CD4 Cell Response in Patients on Uninterrupted HAART

Also in Table 1 are the demographic and clinical characteristics of the subset of 554 patients on uninterrupted HAART for 7 years. These patients started HAART between July 1, 1996, and June 30, 1998, and were among those included in the first analysis. Because the inclusion criteria for the second analysis implied starting HAART in earlier calendar years, there was a higher proportion of men, MSM, and patients originating from WCE/NA/A.

The median CD4 count increased from 221 (interquartile range [IQR]: 80–340) cells/mm³ at the start to 607 (IQR: 440–800) cells/mm³ after 7 years of HAART. The median CD4 counts at 7 years were 410, 548, 660, 780, and 870 cells/mm³ for those with pre-HAART CD4 counts of <50, 50 to 200, 200 to 350, 350 to 500, and ≥500 cells/mm³, respectively (Fig. 1A). Overall, increases were a median of 136 cells/mm³ during the first 24 weeks and leveled off over time to 40 cells/mm³ in weeks 96 through 144 and to 0 cells/mm³ in weeks 312 through 360. Median increases in CD4 cell counts after 7 years of HAART were between 367 and 410 cells/mm³ for the 4 pre-HAART CD4 count strata <500 cells/mm³, whereas increases were 287 cells/mm³ for patients in the ≥500 cells/mm³ stratum (see Fig. 1B; Wilcoxon test, P = 0.007).

Table 1. Characteristics at the start of HAART of 5299 antiretroviral therapy-naïve patients and of the 554 patients on uninterrupted HAART for seven years or more.

	Antiretroviral therapy-naïve patients starting HAART			Subset of patients on uninterrupted HAART for 7 years		
	N		**%**	**N**		**%**
Total	5299		100.0	554		100.0
Gender						
Male	4013		75.7	498		89.9
Region of origin						
WCE/NA/A*	3349		63.2	464		83.8
Sub-Saharan Africa	1090		20.6	36		6.5
Caribbean	182		3.4	12		2.2
Latin America	381		7.2	24		4.4
South-East Asia	171		3.2	9		1.6
Other	126		2.4	9		1.6
Transmission risk group						
Homosexual	2636		49.7	367		66.2
IDU	212		4.0	12		2.2
Heterosexual	1878		35.4	123		22.2
Other	573		10.8	52		9.4
Pre-HAART CD4 cell count (cells/mm³)						
<50	930		17.6	98		17.7
50-200	1773		33.4	155		28.0
200-350	1513		28.6	172		31.0
350-500	694		13.1	78		14.1
≥500	389		7.3	51		9.2
Pre-HAART clinical stage						
CDC-C	1494		28.2	147		26.5
	N	**Median**	**IQR****	**N**	**Median**	**IQR****
Age at starting HAART	5299	37	32-44	554	37.7	33.0-44.3
Pre-HAART HIV-RNA (log_{10} copies/ml)	4908	5.0	4.5-5.4	507	5.0	4.4-5.4
Pre-HAART CD8 cell count (cells/mm³)	4672	820	530-1220	488	903	560-1345
Pre-HAART CD4 cell count (cells/mm³)	5299	190	80-314	554	221	80-340
WCE/NA/A*	3349	200	80-330	464	240	80-340
Non-WCE/NA/A*	1950	175	70-290	90	180	80-290

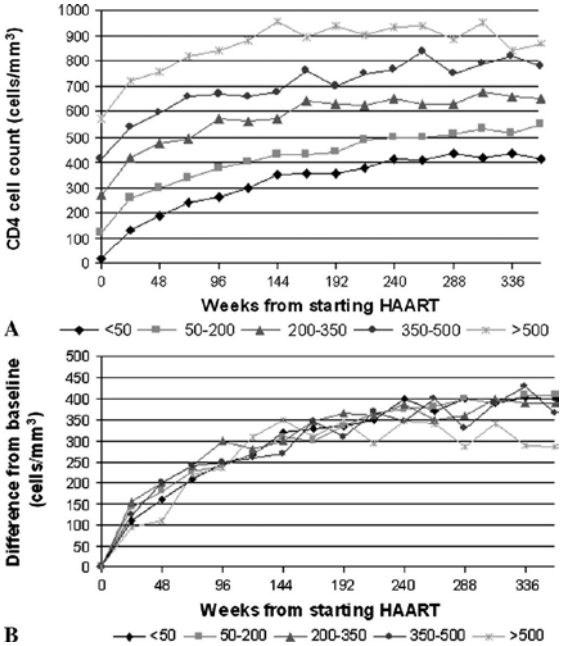
* WCE/NA/A: patients born in Western or Central Europe, North America or Australia. ** IQR: Interquartile range.

Table 2. Predictors of reaching ≥800 CD4 cells/mm³ after starting HAART.

	Univariate Hazard Ratio (95% CI)	P	Multivariate Hazard Ratio (95% CI)	P
Gender				
Male	1.00		1.00	
Female	1.10 (0.97-1.25)	0.14	1.26 (1.05-1.52)	0.01
Transmission risk group				
Homosexual	1.00		1.00	
Heterosexual	0.82 (0.72-0.92)	0.001	0.93 (0.78-1.10)	0.39
IDU	0.71 (0.52-0.96)	0.02	0.56 (0.42-0.77)	0.0003
Other	0.72 (0.59-0.88)	0.002	0.97 (0.79-1.21)	0.81
Region of origin				
WCE/NA/A*	1.00		1.00	
Sub-Saharan Africa	0.58 (0.49-0.69)	<0.0001	0.63 (0.51-0.77)	<0.0001
Caribbean	0.79 (0.57-1.08)	0.14	0.88 (0.63-1.21)	0.42
Latin America	0.85 (0.69-1.06)	0.16	0.91 (0.73-1.14)	0.41
South-East Asia	0.67 (0.47-0.94)	0.02	0.68 (0.48-0.96)	0.03
Other	0.92 (0.65-1.33)	0.68	0.94 (0.65-1.35)	0.73
Pre-HAART CD4 cell count (cells/mm³)				
<50	0.28 (0.22-0.36)	<0.0001	0.26 (0.20-0.34)	<0.0001
50 - 200	0.46 (0.39-0.55)	<0.0001	0.46 (0.38-0.54)	<0.0001
200 - 350	1.00		1.00	
350 - 500	2.76 (2.39-3.19)	<0.0001	2.84 (2.45-3.28)	<0.0001
>500	6.62 (5.67-7.73)	<0.0001	6.79 (5.79-7.95)	<0.0001
Pre- HAART clinical stage				
CDC-A, B	1.00		1.00	
CDC-C	0.69 (0.64-0.74)	<0.0001	1.07 (0.99-1.16)	0.09
Age at starting HAART				
Per 10 year increase	0.88 (0.84-0.94)	0.0001	0.92 (0.87-0.98)	0.01
Pre-HAART HIV-RNA (log_{10} copies/ml)				
<4.0	1.00		1.00	
4.0-5.0	1.12 (0.94-1.33)	0.23	1.51 (1.26-1.81)	<0.0001
≥5.0	0.91 (0.76-2.20)	0.21	1.81 (1.49-2.19)	<0.0001
Pre-HAART CD8 cell count (cells/mm³)				
<600	0.52 (0.44-0.60)	<0.0001		
600-1300	1.00			
≥1300	1.28 (1.12-1.46)	0.0002		

* WCE/NA/A: patients born in Western or Central Europe, North America or Australia.

Figure 1. Median CD4 cell count (A) and median difference between current CD4 and pre-HAART CD4 cell counts (B) according to pre-HAART CD4 cell strata of <50 (¤), 50 to 200 (n), 200 to 350 (:), 350 to 500 ( ), and ≥500 cells/mm (*) among a subset of 554 patients on uninterrupted HAART for 7 years.



Of 554 patients, 344 (62.1%) had HIV RNA plasma concentrations <500 copies/mL at all measurements taken between 6 months and 7 years after starting HAART. The remaining 210 patients had at least 1 HIV RNA result ≥500 copies/mL. In 80 of them, plasma concentrations were between 500 and 1000 copies/mL, which, in the majority (54 patients), occurred between 6 months and 3 years after the start of HAART. Periods of HIV viremia occurring after initial virologic success were found in 27.6% of the patients from WCE/NA/A and 42.2% of the patients (P = 0.006) with a non- WCE/NA/A origin.

The multivariate longitudinal analyses included 13,528 CD4 cell count measurements for the 554 patients. The median number of measurements per patient was 25 (minimum to maximum: 6–58). The model estimates can be interpreted as the slope or annual rate of change in CD4 cell count (on a square root scale). During the first 6 months, the slope of CD4 cell count was higher in patients with a pre-HAART HIV RNA measurement ≥4.5 $\log_{10}$ copies/mL than in those with <4.5 $\log_{10}$ copies/mL (P = 0.009). Furthermore, the slopes of CD4 cell count during the first 6 months were higher in women than in men (P = 0.003), in patients originating from regions other than WCE/NA/A (P = 0.04), and in patients with a pre- HAART CD8 count <1300 cells/mm³ as compared with ≥1300 cells/mm³ (P = 0.0007). The effect of body weight at the start of HAART on the slope of CD4 cell count was not significant in univariate or multivariate models (data not shown). Between 6 months and 7 years after starting HAART, the slopes did not differ significantly between men and women, between patients from various origins, or between

patients with various levels of pre-HAART CD8 cells or pre- HAART HIV RNA. The slopes of CD4 cell count between 6 months and 7 years were significantly higher in patients <50 years of age as compared with those ≥50 years of age at the start of HAART ($P < 0.0001$). There were no significant differences in CD4 cell count increases between 0 and 6 months according to age. Finally, during periods of viremia (HIV RNA >500 copies/mL after the initial 6 months of HAART), the slope of CD4 cell count was estimated to be lower than when HIV RNA levels were less than 500 copies/mL ($P < 0.0001$).
To facilitate interpretation, estimates from the longitudinal mixed-effect model were back-transformed from the square root scale to the usual absolute CD4 cell count scale. The estimated median CD4 count after 6 months of HAART was 448 (95% confidence interval [CI]: 419 to 478) cells/mm³ for a male reference patient of Western origin aged <50 years who started HAART with <1300 CD8 cells/mm³, 310 CD4 cells/mm³, and HIV RNA <4.5 $\log_{10}$ copies/mL. This is compared with 504 (459–551) CD4 cells/mm³ for a female patient, 483 (457–509) cells/mm³ for a patient with a pre-HAART HIV RNA level ≥4.5 $\log_{10}$ copies/mL, 403 (370–437) cells/mm³ for a patient with ≥1300 CD8 cells at the start of HAART, and 418 (381–456) cells/mm³ for a patient not born in WCE/NA/A. The estimated median CD4 count after 7 years of uninterrupted HAART for the reference patient was 704 (656–754) cells/mm³ compared with 585 (522–652) cells/mm³ for a patient aged ≥50 years at the start of HAART and 648 (598–701) cells/mm³ for a patient with viremia >500 copies/mL at all HIV RNA measurements between 5 and 7 years. The estimated median CD4 cell values and values at other time points are graphically depicted in Figure 2.

Decreases in CD4 Cell Count in Virologically Suppressed Patients

Finally, we selected 366 patients who took 7 years of uninterrupted HAART and in whom all measured HIV RNA levels between 6 months and 5 years after starting HAART were <500 copies/mL. Their distribution over the 5 pre-HAART CD4 cell strata and the other baseline variables was similar to that in the previous longitudinal analysis. The estimated median CD4 count at 5 years after the start of HAART for patients aged <50 years was 631 (IQR: 459–812) cells/mm³ and 489 (IQR: 412–725) cells/mm³ for those aged ≥50 years (Wilcoxon test, $P = 0.03$). In total, 150 patients had negative CD4 cell slopes between 5 and 7 years of uninterrupted HAART use. Variables independently associated with this outcome, as identified in a multivariate logistic regression analysis, are shown in Table 3 and were age ≥50 years at the start of HAART (odds ratio [OR] for a negative slope between 5 and 7 years = 3.01 [95% CI: 1.60 to 5.67] compared with age <50 years); at least 1 HIV RNA measurement ≥1000 copies/mL compared with all HIV RNA measurements <1000 copies/mL between 5 and 7 years after starting HAART (OR = 6.10 [95% CI: 2.12 to 17.51]); and, finally, a higher CD4 cell count at 5 years. Compared with patients with ≥800 cells/mm³ at 5 years, the OR for patients with <400 cells/mm³ was 2.23 (IQR: 1.10–4.51). The OR for patients with 400 to 600 CD4 cells/mm³ at 5 years was 1.07 (IQR: 0.54–2.11; $P = 0.85$), and the OR for patients with 600 to 800 cells/mm³ at 5 years was 1.08 (IQR: 0.54–2.15; $P = 0.82$);

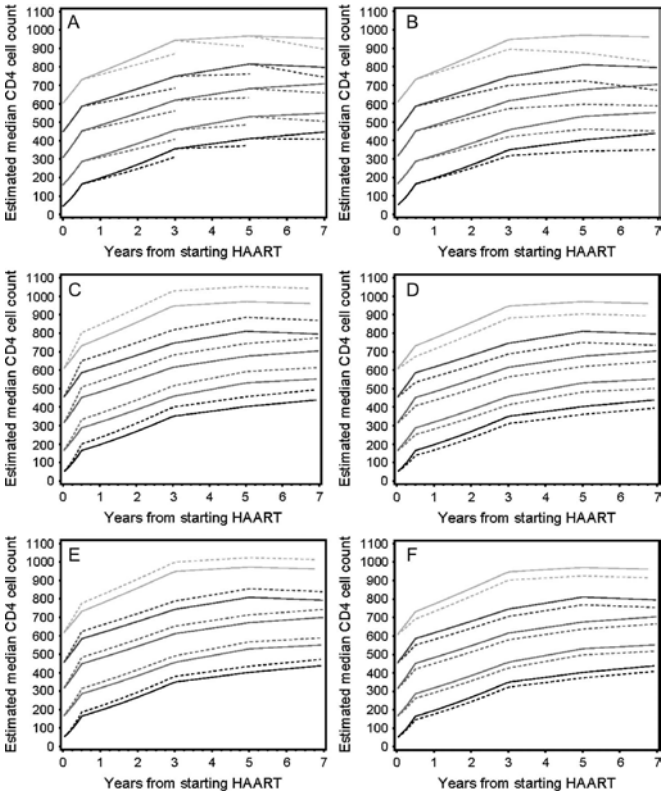


Figure 2. A–F, Estimated long-term median CD4 cell response according to the 5 pre-HAART CD4 cell count strata of ‹50, 50 to 200, 200 to 350, 350 to 500, and ≥Ê500 cells/mm³. The solid lines in each graph denote the median CD4 cell response for the reference patient: a male patient originating from WCE/NA/A who is ‹50 years of age at the start of HAART with a pre-HAART HIV RNA level ‹4.5 $\log_{10}$ copies/mL and a pre- HAART CD8 count ‹1300 cells/mm³ and in whom all HIV RNA measurements between 6 months and 7 years after starting HAART were ‹500 copies/mL. The dashed lines in A display the median CD4 cell response for patients with all HIV RNA measurements ≥500 copes/mL between 6 months and 3 years, between 3 and 5 years, and between 5 and 7 years after starting HAART. The dashed lines in the other parts of this figure display the median CD4 cell response for patients ≥50 years of age at the start of HAART (B), female patients (C), patients with a pre-HAART CD8 cell count ≥1300 cells/mm³ (D), patients with a pre-HAART HIV RNA level ≥4.5 $\log_{10}$ copies/mL (E), and patients not from WCE/NA/A origin (F).

neither was significantly different from those for patients with ≥400 cells/mm³ at 5 years. When age was included in the model as a continuous variable, the OR was 1.027 (95% CI: 1.003 to 1.052, P = 0.02) for each year by which the starting age was increased. In multivariate analysis, region of origin (P = 0.81), pre-HAART HIV RNA level (P = 0.55), or gender (P = 0.55) was not associated with negative CD4 cell slopes between 5 and 7 years, nor was the pre- HAART CD4 cell count (P = 0.66) after controlling for the CD4 cell count at 5 years.

Discussion

We performed 3 types of analyses. First, we evaluated determinants of CD4 cell count recovery up to 800 cells/mm³ in a cohort of HAART-naive patients. Second, we evaluated changes in CD4 cell count in patients on uninterrupted HAART for 7 years to determine the maximum capacity of the immune system to restore CD4 cell numbers. Finally, we determined predictors for decreases in CD4 cell count after 5 years of virologically successful uninterrupted HAART. The first 2 analyses showed that 7 years after starting HAART, patients starting with lower pre-HAART CD4 counts experienced less restoration of CD4 cell counts than patients starting with higher pre-HAART CD4 cell counts. The third analysis showed that the "plateau effect" found after long- term CD4 cell restoration is associated with achievement of CD4 levels in the normal range. Plateauing of CD4 cell counts at a less than normal range is associated with insufficient suppression of HIV replication and with older age at the start of HAART. The strength of this study is the long follow-up (7 years) in a large number of naive patients with a variety of pre-HAART CD4 cell counts. We did not look at differences in CD4 cell response between individual drugs or drug classes because that is beyond the scope of this report and is the topic of a future analysis.

The largest gains in the number of CD4 cells occurred in the first 6 months after starting HAART, presumably because of redistribution of CD4 cells from lymphoid tissue.[35] Thereafter, the rate of increase in CD4 cell counts gradually slowed. Between 5 and 7 years of uninterrupted HAART, CD4 cells still continued to increase in patients with a pre-HAART CD4 count less than 350 cells/mm³. Because of the slow rate of increase, however, restoration to CD4 cell levels ≥800 cells/mm³ is a lengthy process and may not be feasible for patients who start HAART with <200 CD4 cells/mm³.

The association between periods of HIV production despite HAART and reaching a CD4 cell plateau earlier and (thus) at a lower level confirms the importance of monitoring of HIV RNA and keeping plasma levels to less than 500 copies/mL. The single study[31] that did not find a plateau effect, in contrast to our study and others,[29,30] might reflect different levels of ongoing viremia or different age distributions among studies. The association of a lower CD4 cell count plateau with older age (≥50 years of age at the start of HAART) could reflect the lower normal CD4 cell range reported in older healthy individuals.[36–38] Larger CD4 cell gain in treated patients has previously been associated with younger age,[16,28] and less gain has been attributed to lower thymic function with older age.[14,15] Because patients with low CD4 cell counts remain at risk of developing new AIDS events after starting HAART, it may be appropriate to start antiretroviral therapy in older (ie, ≥50 years of age) patients earlier than in younger patients.

Factors associated with differences in CD4 cell response during the first 6 months of HAART include gender, region of origin, pre-HAART HIV RNA plasma levels, and the number of pre-HAART CD8 cells. These differences persisted over the

Table 3. Predictors of a plateauing CD4 cell count between 5 and 7 years after initiating HAART in 366 patients with HIV-RNA plasma concentrations <500 copies/ml between 6 months and 5 years of uninterrupted HAART

	No.	No. with plateau	Univariate OR (95% CI)	P	Multivariate OR (95% CI)	P
Gender						
Male	328	136	1.00			
Female	38	14	0.82 (0.41, 1.65)	0.58		
Transmission risk group						
Homosexual	238	100	1.00			
Heterosexual	84	33	0.89 (0.54, 1.48)	0.66		
IDU	7	3	1.04 (0.23, 4.73)	0.96		
Other	37	14	0.84 (0.41, 1.71)	0.63		
Region of origin						
WCE/NA/A*	311	127	1.00			
Other	55	23	0.93 (0.52, 1.68)	0.81		
Pre-HAART CD4 cells/mm³						
<50	70	29	1.18 (0.65, 2.17)	0.59		
50 – 200	88	33	1.00 (0.57, 1.78)	0.99		
200 - 350	115	43	1.00			
350 - 500	53	24	1.39 (0.72, 2.68)	0.33		
>500	40	21	1.85 (0.89, 3.83)	0.10		
CD4 cells/mm³ at 5 years						
<400	60	23	1.00		1.00	
400-600	108	40	0.95 (0.49, 1.81)	0.87	1.07 (0.54, 2.11)	0.85
600-800	105	38	0.91 (0.47, 1.76)	0.78	1.08 (0.54, 2.15)	0.82
≥800	93	49	1.72 (0.88, 3.35)	0.11	2.23 (1.10, 4.51)	0.025
Clinical stage at 5 years						
CDC-A, B	185	111	1.00			
CDC-C	181	39	0.89 (0.56, 1.43)	0.63		
Age at start of HAART						
<50 year	317	121	1.00		1.00	
≥50	49	29	2.35 (1.27, 4.34)	0.006	3.01 (1.60, 5.67)	0.0006
Viraemia between 5-7 years**						
None	346	135	1.00		1.00	
At least once	22	15	4.69 (1.66, 13.19)	0.003	6.10 (2.12, 17.51)	0.0008

* WCE/NA/A: patients born in Western or Cen\tral Europe, North America, Australia. ** at least 1 HIV-RNA measurement >1000 copies/ml.

study period. The long-term CD4 cell response is, however, largely determined by age and by the degree of HIV RNA suppression. The findings that patients from sub-Saharan Africa, and possibly from Southeast Asia, have a slower recovery to 800 CD4 cells/mm³ may indicate geographic variation in normal CD4 ranges but also differences in adherence. The latter may be confirmed by our finding of a higher proportion of patients experiencing periods of HIV viremia while on uninterrupted HAART. Normal CD4 cell counts in HIV-seronegative Dutch individuals are reported to be higher than in HIV-seronegative individuals from Tanzania, Ethiopia, Kenya, and China[39–44] but lower than in such individuals in Cameroon and Uganda.[45,46] Seropositive patients from Ethiopia experience a slower decline in CD4 cells than seen in Dutch patients, but time to AIDS is not significantly different between these Dutch and Ethiopian patients.[47] This suggests that immune restoration in patients originating from regions with low normal CD4 cell numbers might be slower than in patients with high normal CD4 cell numbers even if they are fully adherent.

The higher increase in CD4 cell count in women than in men follows most probably from the higher CD4 cell counts in uninfected women than in uninfected men[33,38] but might also reflect prescription of different antiretroviral drugs between men and women. As in other studies,[48–50] our patients with a lower pre-HAART CD8 cell count experienced higher rates of CD4 cell increase during the first 6 months. The reason for this relation is unclear, but it might be related to the level of CD4 and CD8 cell activation.[17] CD4 and CD8 cell activation was not measured in this cohort, however.

It is well established that patients with pre-HAART CD4 cell counts <200 cells/mm³ are much more likely to progress to AIDS or death.[10] Combined observational cohort data suggest that the long-term prognosis might be better for patients starting HAART when having ≥350 CD4 cells/mm³ as compared with 200 to 350 cells/mm³,[51] although the absolute risk difference is small. In our subset of patients who used HAART uninterrupted for 7 years, the restoration of CD4 cell counts was sufficient to minimize the risk for development of AIDS, even for those starting HAART with CD4 cell counts less than 200 cells/mm³. These patients were likely to be adherent, and those who died were excluded from the analysis. Therefore, the results of the longitudinal model and the analysis of the decrease in CD4 cell response after 5 years of HAART use cannot be generalized to all patients using HAART but do give an estimate of the immune system's maximum capacity for CD4 cell restoration during 7 years of therapy.

Conclusions

HAART restoration of CD4 cell counts in HIV-infected individuals to levels normally seen in uninfected individuals takes a long time and is not feasible within 7 years in most patients who initiate HAART with CD4 cell counts <350 cells/mm³. Patients ≥50 years of age when starting HAART and patients with

periods of viremia (HIV RNA level >500 copies/mL) experience smaller increases and are more likely to reach a CD4 cell plateau earlier and at a lower level. Given the better toxicity profiles of the currently used antiretroviral combinations, particularly in patients older than 50 years of age, it may be beneficial to start HAART earlier than current guidelines recommend.

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Appendix

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7

Determinants of Restoration of CD4 and CD8 Cell Counts and their Ratio in HIV-1-Positive Individuals with Sustained Virological Suppression on Antiretroviral Therapy

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Abstract

Background
An increasing number of HIV-positive individuals now start antiretroviral therapy (ART) with high CD4 cell counts. We investigated whether this makes restoration of CD4 and CD8 cell counts and the CD4:CD8 ratio during virologically suppressive ART to median levels seen in HIV uninfected individuals more likely and whether restoration depends on gender, age and other individual characteristics.

Methods
We determined median and quartile reference values for CD4 and CD8 cell count and their ratio using cross-sectional data from 2309 HIV-negative individuals. We used longitudinal measurements of 60,997 HIV-positive individuals from the Antiretroviral Therapy Cohort Collaboration in linear mixed-effects models.

Results
When baseline CD4 cell counts were higher, higher long-term CD4 cell counts and CD4:CD8 ratios were reached. Highest long-term CD4 cell counts were observed in middle-aged individuals. During the first two years median CD8 cell counts converged towards median reference values. However, changes were small thereafter and long-term CD8 cell count levels were higher than median reference values. Median 8-year CD8 cell counts were higher when ART was started with <250 CD4 cells/mm^3. Median CD4:CD8 trajectories did not reach median reference values, even when ART was started at 500 cells/mm^3.

Discussion
Starting ART with a CD4 cell count of ≥500 cells/mm^3 makes reaching median reference CD4 cell counts more likely. However, median CD4:CD8 ratio trajectories remained below the median levels of HIV-negative individuals, because of persisting high CD8 cell counts. To what extent these subnormal immunological responses have impact on specific clinical endpoints requires further investigation.

Introduction

Since 2012, US Guidelines have recommended offering antiretroviral therapy (ART) to all individuals diagnosed with HIV, regardless of their CD4 cell count.[1] As a result, an increasing number of HIV-1- positive individuals start ART at high CD4 cell counts. Furthermore, of those starting ART, a considerable proportion does so at a relatively old age. For example, in the Netherlands in 2015, 37% of those starting ART did so with a CD4 count of ≥500 cells/mm^3 and 23% of individuals newly diagnosed with HIV were 50 years or older.[2] Generally, the increase in CD4 cell count during virologically suppressive ART is less in older individuals.[3–9] This diminished recovery of CD4 cell count among older individuals has been attributed to lower thymic function.[10,11]
Lower CD4 counts with older age are also seen in healthy European HIV-negative populations, although the decrease seems to occur mainly at very advanced age.[12–16] CD4 cell counts have also been reported to differ according to smoking status,[17] gender,[13] the time of day of sampling,[18] season[19] and region of origin.[20,21]

Whilst CD4 cell count is considered the key prognostic factor for AIDS morbidity and mortality, some evidence suggests that the CD4:CD8 ratio also independently predicts time to death and to non-AIDS defining endpoints.[22–24] In the general population a CD4:CD8 ratio <1.0 is associated with mortality in very elderly people.[25] In HIV-positive individuals the ratio is decreased and low ratios are associated with pathological changes in the immune system such as immune activation, exhaustion, senescence and memory abnormalities.[26-28] The ratio increases rapidly during the first few years on ART and keeps increasing up to 15 years after starting ART, albeit slowly[29] and the ratio does not reach levels higher than 1.0 in two-thirds of individuals despite long-term viral suppression.[30,31]

We studied whether an early start, at high CD4 cell counts followed by long-term virologically suppressive ART, makes restoration to levels of CD4 and CD8 cell counts and the CD4:CD8 ratio seen in HIV-negative individuals more likely. We also investigated the effect of age and other factors on these immunological changes.

Methods

HIV-negative study participants

To obtain reference values we used 2309 cross-sectional CD4 and CD8 cell counts and CD4:CD8 ratios obtained from HIV-negative individuals recruited from the background population to the Danish HIV-cohort (either healthy staff or blood and stem-cell donors) and HIV-negative individuals from the Dutch AGE$_h$IV cohort (recruited either at the STI clinic of the Amsterdam Public Health Service or the existing Amsterdam Cohort Studies on HIV/AIDS). CD4 and CD8 cell counts

and CD4:CD8 ratios were used as dependent variable in 3 linear regression models including age and gender and their interaction as independent variables. We used the 25th, 50th and 75th prediction percentiles as the lower, median and upper reference values in graphs to put the immunological restoration during virologically suppressive ART in HIV-positive individuals into context. (See Text File SDC 1, Supplemental Digital Content, for further details on the selection and analysis of CD4 and CD8 cell counts and the CD4:CD8 ratio in HIV-negative individuals).

HIV-positive study participants

We used data from the Antiretroviral Therapy Cohort Collaboration (ART-CC; http://www.art-cohort-collaboration.org), an international collaboration of 21 cohort studies from Europe and North America that was established in 2000 to examine the prognosis of HIV-1-positive, treatment-naive individuals initiating ART, a combination of at least 3 antiretroviral drugs.[32] Participation of cohorts has been approved by their ethics committees or institutional review boards according to local regulations (see Text File SDC 2, Supplemental Digital Content, for a list of participating cohorts). We only included individuals who were 18 years of age or older and had a CD4 cell count and viral load measured at the start of ART. All included individuals had a decrease in HIV RNA viral load to below 400 copies per milliliter within 9 months from start of ART. In sensitivity analyses we changed the time limit to six months and cut-off to 50 copies per milliliter.

Outcome

We modelled longitudinal CD4 and CD8 cell counts and CD4:CD8 ratios after the start of ART. We excluded follow-up after an ART interruption longer than two weeks and after the first of two consecutive plasma viral load measurements ≥400 copies per milliliter. In sensitivity analyses we only included measurements until an ART interruption longer than one week or until the first plasma viral load measurement ≥400 copies per milliliter. Models including CD8 cell counts or CD4:CD8 ratios only included participants from the 14 cohorts which had collected data on these variables.

Statistical methods

Trends in CD4 and CD8 cell counts and their ratio were modelled via linear mixed- effects models (lme4 package[33] in R version 3.0.3[34]). CD4 cell counts were found to best comply with normality assumptions when square root transformed, CD8 cell counts when log transformed and the CD4:CD8 ratio when fifth root transformed. The trends over time since start of ART were modelled using restricted cubic splines with knots at 0, 0.1, 0.25, 0.5, 3 and 7.5 years. We used a random intercept and two random slopes (one slope between 0 and 6 months and one slope from 6 months onwards, with an unstructured covariance matrix) per individual as well as a random intercept for cohort.

Table 1. Demographical and clinical characteristics at the start of ART.

	N	%
Total	60,997	100.0
Gender		
Men	46,076	75.5
Women	14,921	24.5
Transmission risk group		
MSM*	24,591	40.3
Injecting drug use	4685	7.7
Heterosexual contact	23,335	38.3
Other/Unknown	8386	13.7
Age at start of ART (yr), median, IQR		39, 32-46
16-29	10,796	17.7
30-39	21,796	35.7
40-49	17,798	29.2
≥50	10,607	17.4
Region of birth		
Europe/North America	44,717	73.3
Caribbean / South America	3569	5.9
Sub-Saharan Africa	7729	12.7
Other/Unknown	4982	8.2
Year of start ART (median, IQR)		2007, 2004-2009
2001-2003	12,765	20.9
2004-2006	14,827	24.3
2007-2009	19,231	31.5
2010-2012	14,174	23.2
CD4 cell count at start of ART (cells/mm^3), median, IQR		246, 130-350
0-49	7301	12.0
50-99	4838	7.9
100-199	11,263	18.5
200-349	22,206	36.4
350-499	9575	15.7
≥500	5814	9.5
HIV RNA at start of ART (copies/ml), median, IQR		64,500, 14,300-182,800
<10,000	12,963	21.2
10,000-100,000	23,701	38.9
≥100,000	24,333	39.9
Smoking status at the start of ART, missing in 1683/16,650 (10.1%)**		
Non-smoker	8515	56.9
Current smoker	6452	43.1
Hepatitis C status at the start of ART, missing in 2093/21,176 (9.9%)**		
HCV-negative	17,072	89.5
HCV-positive	2011	10.5
CMV status at the start of ART, missing in 1922/8418 (22.8%)**		
CMV-negative	740	11.4
CMV-positive	6496	88.6

*MSM: Men who have sex with men, HCV: Hepatitis C virus, CMV: Cytomegalovirus
** Total number of participants in the subset of cohorts used in the analysis

All models included gender, region of birth (Europe/North America, Caribbean/South America, sub-Saharan Africa, and other regions), transmission risk group (men who have sex with men (MSM), injecting drug use (IDU), heterosexual, other and unknown), age, CD4 cell count and HIV RNA at the start of ART (measurement closest to the start of ART in the period 90 days before to 6 days after starting ART). CD4 cell count trends were also allowed to vary according to period of starting ART (2001-2003, 2004-2006, 2007-2009 and 2010-2012).
Because data on smoking status, CD8 cell count and hepatitis C virus (HCV) and cytomegalovirus (CMV) coinfection were not collected in all cohorts, we only used data from cohorts with at least 85% complete data on smoking status, CD8 cell count and HCV coinfection. For CMV coinfection we used data from the 5 cohorts with available data on CMV. Therefore, these variables were not evaluated together in one model but in separate models. For more detailed information on interaction terms and continuous covariables modeling, see Text File SDC 3, Supplemental Digital Content.

To help interpretation the fitted values were backtransformed to their original scale, where they can be considered as median values. They are graphically displayed for selected values of age and CD4 and CD8 cell count at the start of ART.

Results

HIV-negative population

Median CD4 and CD8 cell count in HIV negative participants decreased with older age while the median CD4:CD8 ratio was higher with older age (see Figures A-C SDC 4, Supplemental Digital Content). For a 37-year old male the median CD4 cell count was 830 cells/mm^3 and 1005 CD4 cells/mm^3 for a female. These

Table 2. Median (IQR) CD8 cell count and CD4:CD8 ratio at the start of ART for different CD4 categories.

CD4 cell count at the start of ART (cells/mm^3)	N	CD8 cell count, median, IQR (cells/mm^3)	CD4:CD8 ratio, median, IQR
0-49	3914	392, 233-610	0.05, 0.03-0.09
50-99	2704	600, 402-890	0.11, 0.08-0.17
100-199	6860	757, 525-1070	0.20, 0.14-0.28
200-349	13,654	900, 650-1250	0.27, 0.19-0.37
350-499	6206	995, 717-1394	0.37, 0.26-0.52
≥500	3967	1040, 753-1472	0.58, 0.41- 0.79
Any CD4 cell count	37,305	830, 560-1195	0.21, 0.11-0.34

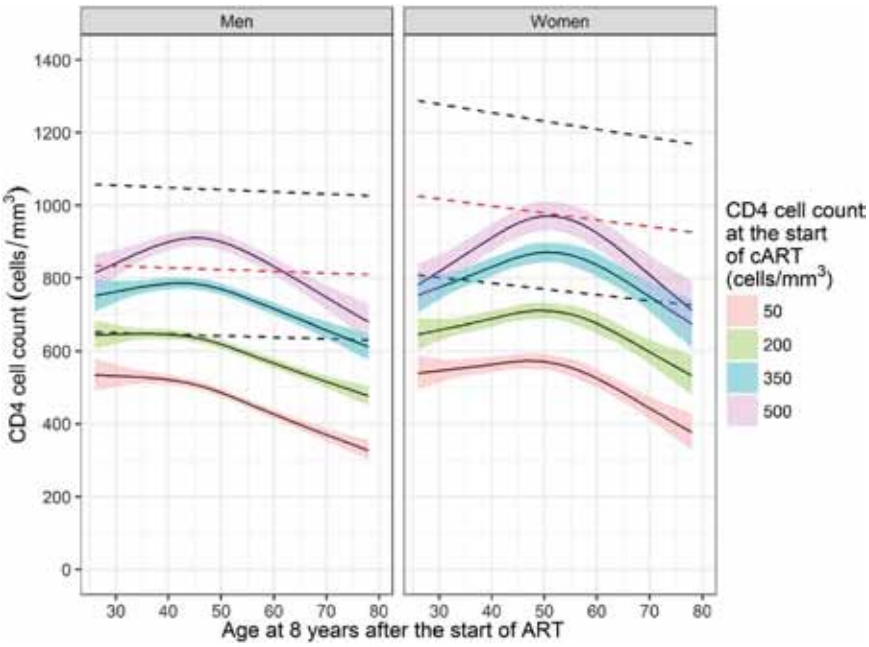

Figure 1. Median CD4 cell count at eight years of virologically suppressive ART (95% CI's in colour) in men and women by age at 8 years after the start of ART. Trends are shown for specific baseline CD4 cell counts of 100, 200, 350, and 500 cells/mm^3. Curves are for an average reference heterosexual individual born in Western Europe/North America starting ART between 2004 and 2006 with a plasma viral load of 4.81 $\log_{10}$ copies per milliliter and a random intercept and slopes equal to zero. Dashed lines show the lower, normal and upper reference CD4 cell counts by age as estimated in HIV-negative men and women.

values for the CD8 cell count and the CD4:CD8 ratio were 499 cells/mm^3 and 1.69 for males and for females 519 CD8 cells/mm^3 and 1.98, respectively. Modeling age using splines (see Figures SDC 5-7) gave a better data fit than modeling age linearly, but as the resulting trajectories were not consistently increasing or decreasing with higher age, we chose to model age linearly.

HIV-positive population

The majority of 60,997 included HIV-positive individuals were men (75%) and born in Europe/North America (73%), as shown in Table 1. Forty percent were in the MSM transmission risk group. Median age was 39 years (IQR 32-46). Median CD4 cell count was 246 cells/mm^3 (IQR 130-350). The median CD8 cell count in the subset of 37,305 individuals included in the analysis of CD8 cell count and

CD4:CD8 ratio was 830 cells/mm^3 and the median CD4:CD8 ratio was 0.21 (Table 2). Median CD8 cell count and CD4:CD8 ratio were both lower when ART was started at lower CD4 cell count.

CD4 cell count trajectories

We used 599,445 CD4 cell count measurements. The number of individuals with measurements after 2, 4, 6, and 8 years of virologically suppressive ART was 29,791 (49%), 16,679 (27%), 8836 (14%), and 4209 (7%), respectively. The median observed CD4 cell count at 8 years for those starting with a CD4 count of 0-49 (n = 728), 50-99 (n = 480), 100-199 (n = 1,025), 200-349 (n = 1359), 350-499 (n = 377), and ≥500 cells/mm^3 (n = 240) was 485, 507, 570, 667, 793, and 923 cells/mm^3, respectively.

Higher CD4 cell count at the start of ART was associated with higher median 8-year counts (Fig. 1). Only when ART was started with a CD4 count of 500 cells/mm^3 the median 8-year CD4 cell count in men reached the median reference value. CD4 cell count was nonlinearly associated with age. Middle-aged men showed higher median CD4 cell counts at 8 years compared to older and younger men when ART was started with a CD4 count of 350 or 500 cells/mm^3. Women showed a similar, but stronger pattern. Among those starting at a CD4 count of 500 cells/mm^3 45-year-old reference men (911 cells/mm^3, 95% CI 889-933) and 51-year old women (971 cells/mm^3, 95% CI 932-1011) reached highest median 8-year CD4 cell counts. Trajectories of women aged 20 years at the start of ART were initially higher than those of older women during the first 2 years of ART but flattened whilst trajectories of women aged 37, 54 or 70 years at baseline kept increasing (see Figure SDC 8, Supplemental Digital Content). Similar results were obtained when analyses were restricted to those who reached <50 HIV copies per milliliter within six months from starting ART. CD4 cell count trajectories were also similar according to start year of ART (results not shown).

CD4 cell counts at 8 years were lower with increasing baseline CD8 cell counts until 400 cells/mm^3, but the relation flattened off beyond 400 cells/mm^3 (see Figure SDC 9, Supplemental Digital Content in analysis additionally adjusted for baseline CD8 cell count in a subset of 37,305 individuals with CD8 cell counts available).

CD8 cell count trajectories

We used 374,985 CD8 cell count measurements from 37,305 individuals. The number of individuals with CD8 cell counts during virologically suppressive ART after 2, 4, 6, and 8 years was 18,316 (49%), 10,310 (28%), 5480 (15%), and 2525 (7%), respectively. The median CD8 cell count at 8 years was 765 cells/mm^3 (IQR 558-1040). For those starting with 0-49 (410 individuals remaining in follow-up), 50-99 (n = 281), 100-199 (n = 655), 200-349 (n = 794), 350-499 (n = 242), and ≥500 CD4 cells/mm^3 (n = 143) the median 8-year CD8 cell count was 800 (IQR 575-1100),

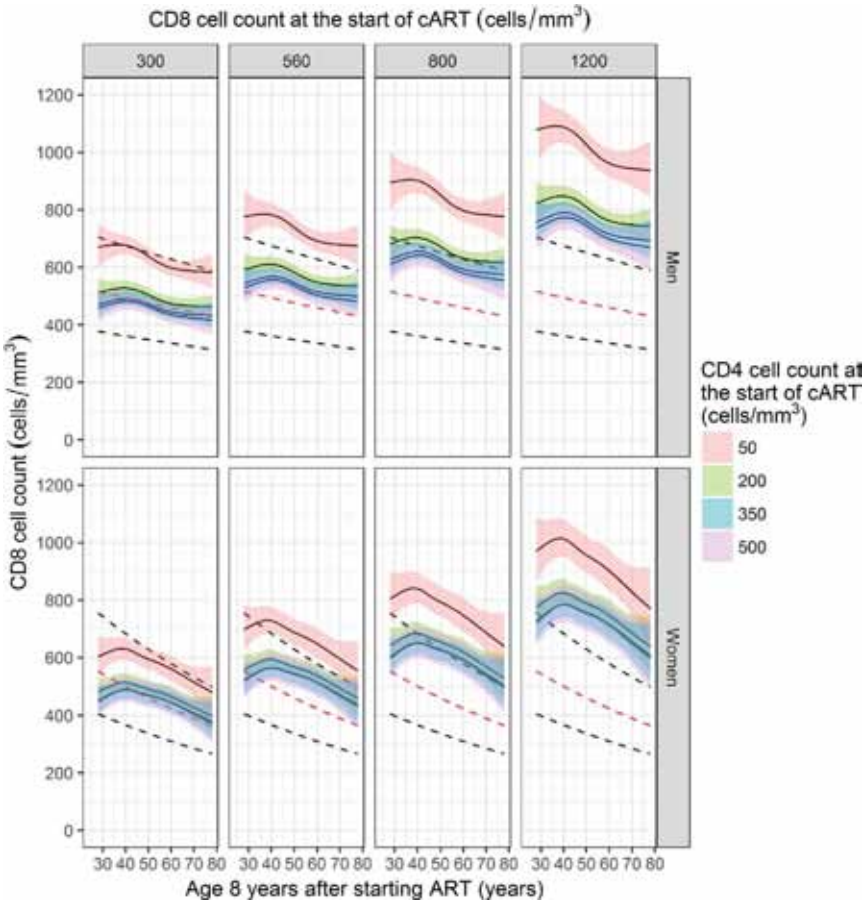

Figure 2. Median CD8 cell count after eight years of virologically suppressive ART by age, gender, and CD4 and CD8 cell count at the start of ART, for an average reference heterosexual individual born in Western-Europe or North-America and starting ART with 4.81 $\log_{10}$ copies per milliliter and random intercept and slopes equal to zero. Dashed lines show the upper, normal and lower reference CD8 cell count values.

770 (566-1075), 774 (570-1006), 740 (540-1030), 751 (558-1050), and 810 (557-1072) cells/mm^3, respectively.

Median CD8 cell counts at 8 years after the start of ART showed a similar downward trend with higher age as the trend observed in HIV-negatives (Fig. 2). This downward trend was not observed in men and women younger than 40 years, and median 8-year CD8 cell counts were similar across all ages below 40

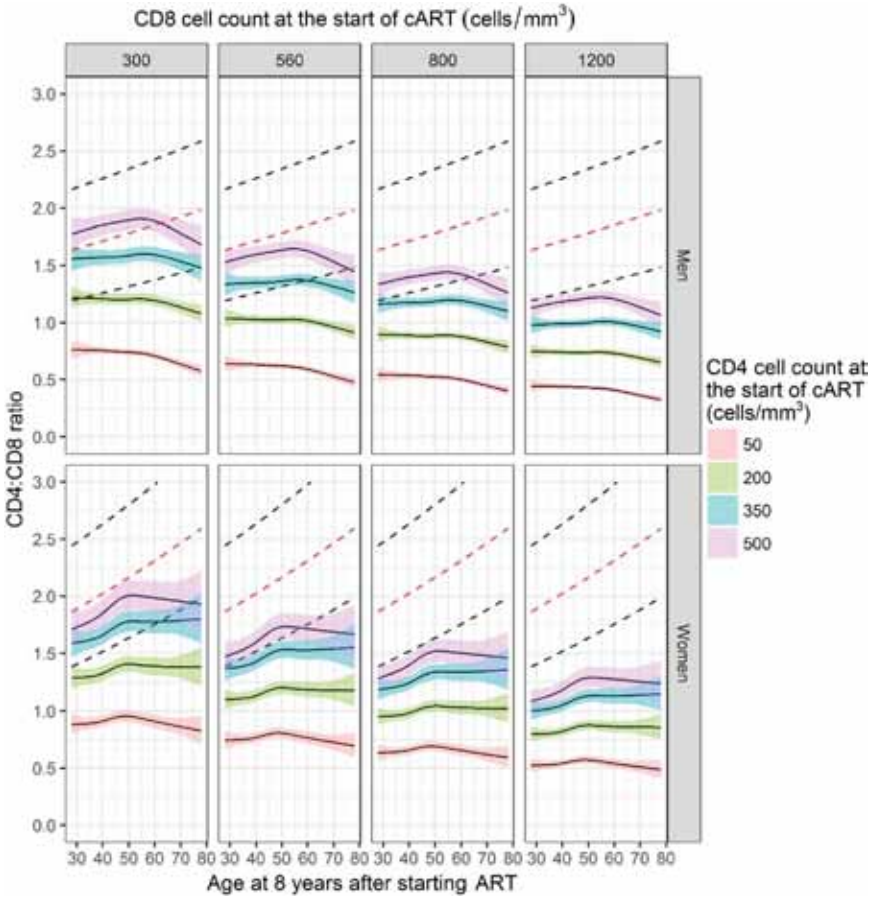

Figure 3. Median CD4:CD8 ratio at eight years after the start of ART (95% confidence intervals in color) according to age at eight years after the start of ART, gender, and CD4 and CD8 cell count at the start of ART. Median ratios shown are those for an average reference individual in the heterosexual transmission risk group, born in Europe/North America, 37 years of age at the start of ART, and a plasma viral load of 4.81 $\log_{10}$ copies per milliliter at the start of ART and random intercept and slopes equal to zero. Dashed lines show the upper, normal and lower reference CD4:CD8 ratio in HIV-negative men and women.

years of age. Higher CD8 cell counts at the start were associated with higher CD8 cell counts at 8 years. Median 8-year CD8 counts were similar to median reference values when ART was started with a CD8 cell count of 300 cells/mm^3 and a CD4 cell count of 200, 350 or 500 cells/mm^3. Similar median CD8 cell counts at 8 years were reached for those starting with a CD4 count of 200, 350 or 500 cells/mm^3.

However, median 8-year CD8 cell counts were higher when ART was started with a CD4 count of 50 cells/mm^3. The association between lower baseline CD4 cell counts and higher CD8 cell counts at 8 years starts from CD4 cell counts below approximately 250 cells/mm^3 (see Figure SDC 10, Supplemental Digital Content). Median CD8 cell count trajectories show a rapid decline in CD8 cell count during the first year when ART was started with a CD8 count of 800 or 1200 cells/mm^3 and a more gradual decline after 1 year. CD8 cell counts remained higher than the median reference range during the first 8 years (see Figure SDC 11, Supplemental Digital Content).

CD4:CD8 ratio trajectories

Median CD4:CD8 ratio at 8 years was 0.81 (IQR 0.57-1.11). The median 8-year ratio for those starting with 0-49, 50-99, 100-199, 200-349, 350-499, and ≥500 CD4 cell/mm^3 was 0.63 (IQR 0.45-0.85), 0.66 (0.47-0.87), 0.76 (0.54-1.01), 0.89 (0.64-1.19), 1.05 (0.75-1.38), and 1.09 (0.88-1.50) cells/mm^3 respectively.

Figure 3 shows that higher median CD4:CD8 ratios were reached when CD4 cell counts at the start of ART were higher and CD8 cell counts were lower (ie, the ratio at the start was higher). Among the combinations shown, median reference values were only reached in men younger than 60 years (at 8 years) who had started ART with the combination of a CD4 count of 500 cells/mm^3 and CD8 count of 300 cells/mm^3. Men and women with a CD4 count of 350 or 500 cells/mm^3 at the start reached median CD4:CD8 ratios ≥1.0 at 8 years, irrespective of the CD8 cell count at the start, except in men older than 60 years of age at 8 years.

Median CD4:CD8 ratio trajectories continued to increase during the first 8 years of ART (see Figure SDC 12, Supplemental Digital Content,). Ratio trajectories were higher in women compared to men.

Association of other variables with trajectories

The association between region of origin, transmission risk group, HIV RNA, smoking status and HCV and CMV coinfection at the start of ART and CD4 and CD8 cell count and ratio trajectories and levels reached at 8 years is discussed in text and shown in figures (see Figures SDC 13-15, Supplemental Digital Content, and Table SDC 16, Supplemental Digital Content). In short, CD4 and CD8 cell count after 8 years of virologically suppressive ART were higher in smokers compared to nonsmokers. Median trajectories of CD4 cell count and the CD4:CD8 ratio were lower in individuals infected by IDU compared to non-IDU. CMV-negative individuals showed a stronger decrease in CD8 cell count in the first year after starting ART compared with CMV-positive individuals and because CD4 cell count trajectories were fairly similar, CD4:CD8 ratio trajectories in CMV-positive individuals were lower compared with those in CMV-negative individuals.

Discussion

As ART is now both recommended and often started at high CD4 cell counts, we investigated whether median trajectories of CD4 and CD8 cell count and the CD4:CD8 ratio reach median reference values when ART is started with high (e.g. 500 cells/mm^3) and lower CD4 cell counts (such as 350, 200 and 50 cells/mm^3). During 8 years of virologically suppressive ART median reference CD4 cell counts (about 800 cells/mm^3) were reached only by men starting at high CD4 cell counts ≥500 cells/mm^3. Despite virologically suppressive ART CD8 cell count trajectories converged after two years to a stable level above the median reference value. Median reference CD8 cell counts were only reached when ART was started at low CD8 cell count (such as 300 cells/mm^3). As a result of persisting high CD8 cell counts median reference CD4:CD8 ratios were not reached, even when ART was started at high CD4 cell counts.

Measurements after interruption of ART of more than 2 weeks and after a confirmed HIV RNA >400 copies per milliliter were not included in the analysis. Our results are therefore not generalizable to all individuals who start ART but rather provide an estimate of the maximum capacity of the immune system to restore CD4 and CD8 cell counts and their ratio during long-term virologically suppressive ART. Our selection of individuals therefore will also include some immunological nonresponders, individual with nonperfect adherence, women during pregnancy and individuals with certain comorbidities or comedication which may impact CD4 and CD8 cell count trajectories. Furthermore, generalizability may be slightly impaired if those that interrupt ART or with virological failure have different CD4 or CD8 cell count trajectories before interruption or failure.
Median reference CD4 cell counts were only reached within 8 years in more than 50% of men when ART was started with high CD4 cell counts (such as 500 cells/mm^3). By definition, approximately 50% of individuals will have had a pre-HIV-infection CD4 cell count below the population median, and some even below 500 cells/mm^3. Hence, those who start ART with a CD4 count more than 500 cells/mm^3 are already a selected subgroup that probably had pre-HIV CD4 cell counts above the population median. Likewise, those who had lower-than-average CD4 cell counts before they acquired HIV are more likely to start ART with low CD4 cell counts. Ideally, one would like to compare CD4 cell counts during virologically suppressive ART with an individual's pre-HIV-infection CD4 cell count level but we did not have data on pre-HIV infection CD4 cell counts for the individuals in our study. The level of the first post-seroconversion CD4 cell count has been shown to be predictive for the CD4 cell count recovery after the start of ART.[35] However, also the timing of seroconversion, and post-seroconversion CD4 cell count were mostly unknown in our data.
The highest CD4 cell counts after 8 years of virologically suppressive ART were observed for women aged 51-52 years. Although in women we observed a peak CD4 cell count response during middle age at all levels of CD4 cell count at the

start, in men, a similar peak was only observed when ART was started at CD4 counts of 500 cells/mm^3 but not at lower CD4 cell counts. A similar nonlinear age-CD4 response pattern was found in a recent study.[36] Because we did not observe such a trend in the HIV-negative population, it is not clear whether an underlying biological process contributes to our findings. Adherence to ART has been shown to be less in younger individuals,[37] and differences in compliance may exist even when all HIV RNA measurements are below the limit of detection. Poorer adherence may explain the smaller increases in CD4 cell count after 2-8 years after the start of ART when ART was started at the age of 20 years compared with middle aged men and women. We aimed to select more adherent individuals in the sensitivity analysis with a stricter definition of virologically suppressive ART but results were similar to the main analysis. The peak in CD4 cell count response in women coincides with the mean age of natural menopause, but studies have found no evidence for a difference in CD4 cell count response after ART initiation between pre- and post-menopausal women with a virological response.[38,39]

A recent study found that CD8 cell count trajectories, adjusted for baseline CD4 cell count but not for baseline CD8 cell count, were very similar for those starting ART with a CD4 count of ≥200 cells/mm^3 but long-term CD8 cell count trajectories were higher with lower baseline CD4 count when ART was started with counts <200 cells/mm^3.[29] We show that reaching higher long-term CD8 cell counts is not only associated with lower baseline CD4 cell count but also with younger age and higher baseline CD8 cell count. CD8 cell counts at 8 years were lower with older age, similar, albeit at a higher value, compared to the trend in HIV-negative individuals. The association between higher baseline CD4 cell counts and lower CD8 cell count trajectories is counterbalanced by the association between higher baseline CD8 cell counts. Higher baseline CD8 cell counts usually accompany higher baseline CD4 cell counts, and are associated with higher CD8 cell count trajectories. Together, this results in long-term CD8 cell counts that are similar when ART is started with a CD4 count of 200 cells/mm^3 or higher. Lack of normalization of CD8 cell counts in HIV-positive individuals was also observed in a recent study by the Danish HIV Cohort.[40] Elevated CD8 cell count levels are suggestive of ongoing residual immune activation and residual HIV viremia, coinfections (such as CMV[41]), microbial translocation, loss of immunoregulatory responses and hypercoagulability are all thought to contribute.[42,43]

When ART was started at CD4 counts above 200 cells/mm^3 in men and 250 cells/mm^3 in women, median CD4 cell count trajectories reached ≥500 cells/mm^3 within 8 years of virologically suppressive ART. When ART was started at a CD4 count of 350 or 500 cells/mm^3, median ratios >1 were reached within 8 years. These cutoffs are frequently used to identify individuals at increased risk of morbidity and/or mortality. However, there continues to be an association of a lower risk of AIDS and death with a higher CD4 cell count, even above 500 cells/mm^3.[44,45] For the

CD4:CD8 ratio and CD8 cell count, these associations are less clear cut. Several studies have suggested that the CD4:CD8 ratio, independent of CD4 cell count, predicts time to death and non-AIDS-defining morbidity.[22–24,46] In addition, both low CD8 cell count in the first year after starting ART and increased CD8 cell counts >1500 cells/mm^3 at 10 years after the start of ART have been associated with mortality.[40] By contrast, a recent ART-CC study found only a small effect of CD8 cell counts and no significant effect of the CD4:CD8 ratio on all-cause mortality.[47] It may be that associations between either CD8 cell count or CD4:CD8 ratio and clinical outcome are only limited to specific causes of morbidity or death.

A limitation of our study was that analyses including data on CD8 cell counts, smoking and HCV and CMV coinfection was performed in different subsets of individuals which limits the interpretation of the results because we did not adjust for all covariates at the same time. Furthermore, there were few HIV-negatives older than 65 years. Therefore, comparisons to median HIV-negative values beyond 65 years is mostly based on extrapolation. Another limitation is that both HIV-negative cohorts were from a Western European population whereas CD4 cell counts are generally reported to be lower in sub-Saharan African populations.[48] In our study, CD4 and CD8 cell count and CD4:CD8 ratio trajectories in HIV-positive individuals from sub-Saharan Africa were all somewhat lower compared to those from Western Europe or North America. Whether these differences translate into differential risk for morbidity or mortality is difficult to investigate because of other socioeconomic and psychosocial differences.

Conclusion

Starting ART with a CD4 cell count of ≥500 cells/mm^3 makes reaching a CD4 cell count comparable to those seen in HIV-negative individuals more likely. However, even when ART is started with high CD4 cell count, median CD4:CD8 ratio trajectories remained below the reference levels of HIV-negative individuals, because of persisting high CD8 cell counts. To what extent these subnormal immunological responses have an impact on specific clinical endpoints requires further investigation.

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SDC 1

HIV-negative study participants

We estimated median reference ranges for CD4 and CD8 cell count, and the CD4:CD8 ratio using data obtained from two sources. One consisted of healthy individuals from the background population to the Danish HIV cohort recruited from healthy staff and blood and stem-cell donors. Secondly, we included HIV-negative participants from the AGE_hIV cohort study, recruited either at the STI clinic of the Amsterdam Public Health Service or the existing Amsterdam Cohort Studies on HIV/AIDS[26]. Each individual contributed one CD4 and CD8 cell count measurement.
Linear regression was used to obtain reference values for CD4 cell counts, CD8 cell counts and the CD4:CD8 ratio according to age and gender. Using the boxcox function in the R package MASS, residuals in the linear regression analysis of CD4 cell counts were found to best comply with normality assumptions when counts were 5th root transformed, whilst CD8 cell counts and the CD4:CD8 ratio were log transformed. The age variable was modelled linearly as well as via restricted cubic splines with varying (3-5) number of knots. Differences between models were assessed using the Akaike Information Criterion (AIC). We tested for differences between cohorts by including cohort as a covariate in the models. We used the estimated 25th, 50th and 75th prediction percentiles as lower, median and upper reference values in the analyses of immunological restoration after starting ART in HIV infected individuals.

Results

CD4 and CD8 cell count and CD4:CD8 ratio measurements were available for 2,309 HIV-negative individuals, 1,797 Danish blood and stem cell donors and healthy staff, and 512 participants in the AGE_hIV cohort study. Supporting information table S1 shows the distribution according to gender and age. Participants in the Danish blood donor study were mostly aged between 18 and 45 years whilst all of the HIV-negative participants in the AGE_hIV study were 45 years of age or older.
Median CD4 cell count declined with increasing age. The decline in CD4 cell count with increasing age was greater in women ($p=0.03$), than in men ($p=0.45$). Median CD8 cell count also declined with increasing age whilst the median CD4:CD8 ratio increased with increasing age. There was strong evidence for a decline in CD8 cell count and an increase in CD4:CD8 ratio with increasing age in both men ($p=0.0005$ and $p=0.0008$, respectively) and women (both $p<0.0001$). There was no evidence of a difference in CD4 or CD8 cell counts between the two cohorts. There was some evidence that the CD4:CD8 ratio in men from the Danish cohort was higher than that in men from the AGE_hIV cohort ($p=0.02$).

SDC 2

HIV-positive study participants

Cohorts included in this paper were AGEhiV the Netherlands, the Cohort of the Spanish HIV Research network (CoRIS), Spain; the French Hospital Database on HIV (FHDH); the Italian Cohort of Antiretroviral-naïve patients (ICONA); the Swiss HIV Cohort Study (SHCS); the AIDS Therapy Evaluation project, Netherlands (ATHENA); The Multicenter Study Group on EuroSIDA; the Aquitaine Cohort; the Royal Free Hospital Cohort; the South Alberta Clinic Cohort; The Danish HIV Cohort Study, Denmark; HAART Observational Medical Evaluation and Research (HOMER) Cohort, Canada; HIV Atlanta Veterans Affairs Cohort Study (HAVACS), US; Koln/Bonn Cohort, Germany; Osterreichische HIV-Kohortenstudie (OEHIVKOS), Austria; Proyecto para la Informatizacion del Seguimiento Clinico-epidemiologico de la Infeccion por HIV y SIDA (PISCIS), Spain; University of Alabama 1917 Clinic Cohort, USA; University of Washington HIV Cohort, US; VACH, Spain; Veterans Aging Cohort Study (VACS), US; and Vanderbilt-Meherry, US.

SDC 3

Statistical analysis

While ethnicity was known in 40% of individuals, region of birth was known in 84% of individuals and was therefore included in the analyses. In individuals with missing region of birth, information on ethnicity could be used to reclassify 353 individuals as having been born in Caribbean/South America, and 313 as having been born in sub-Saharan Africa. In addition, 4,584 individuals with white ethnicity were reclassified as having been born in Europe/North America and 988 with black ethnicity as having been born in sub-Saharan Africa (their CD4 cell count trajectories were similar to those born in sub-Saharan Africa). The remaining 3,445 individuals were classified into other/unknown region of birth.

Interaction and spline modeling

The association between continuous covariates and the trajectories of CD4 and CD8 cell counts and their ratio were modelled via splines, with knots approximately at the 2.5th, 27.5th, 50th, 72.5th and 97.5th percentiles (knots for CD4 cell count at the start were chosen at 10, 160, 260, 375 and 740 cells/mm^3, knots for CD8 cell count at the start at 190, 560, 830, 1200 and 1600 cells/mm^3 and knots for age at the start of ART at 22, 32, 39, 47 and 63 years). Log_{10} transformed plasma viral load was included linearly. Furthermore, we allowed the effect of region of birth on the time trend to differ by gender, and we allowed for three-way interactions between the time trend and i) age and CD4 cell count at the start of ART, ii) gender and age, and iii) gender and CD4 cell count at the start of ART. In these three-way interaction terms, time was modelled as a restricted cubic spline with knots at 0, 0.1, 0.25, 3, and 7.5 years, age at the start of ART as a restricted cubic spline with knots at 22, 39, and 63 years, and CD4 cell count at the start of ART as a restricted cubic spline with knots at 10, 260, and 740 cells/mm^3.

Figure SDC4A-C: Median (solid line), and 25th and 75th percentile (dashed lines) of A: CD4 and B: CD8 cell count and C: CD4:CD8 ratio in 1253 HIV-negative men (blue lines) and 1056 women (red lines). Individual observations are shown in blue dots for men and red dots for women.

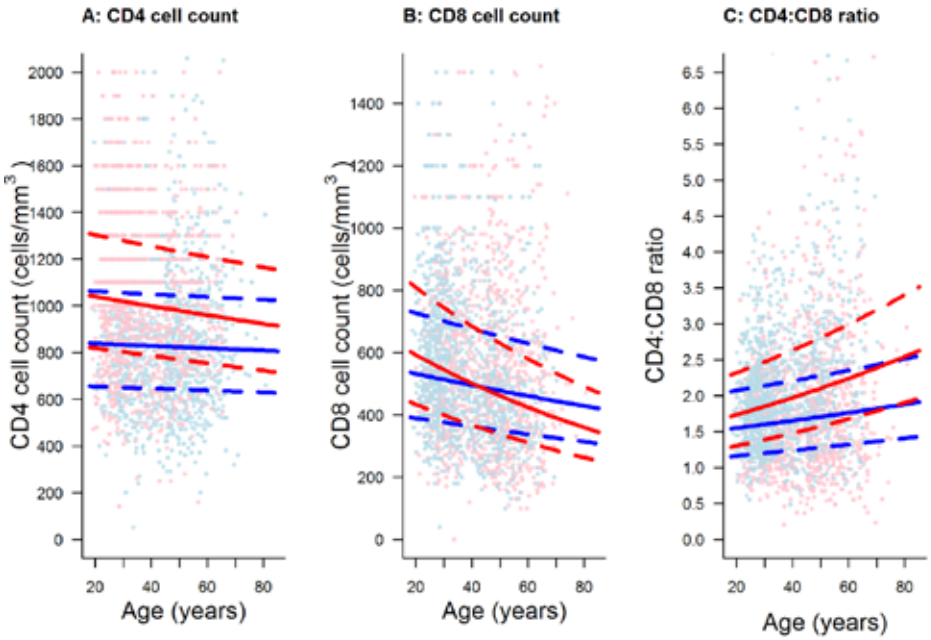
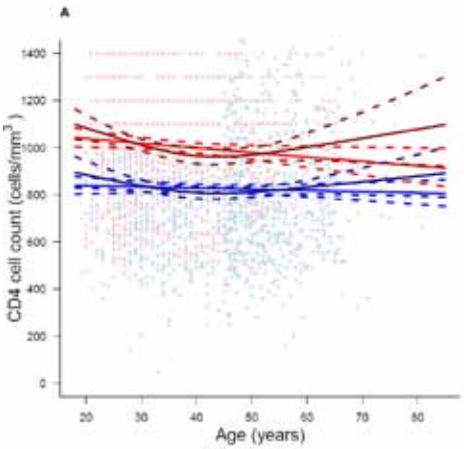
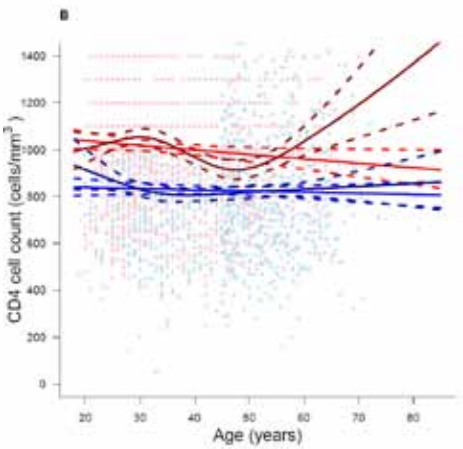


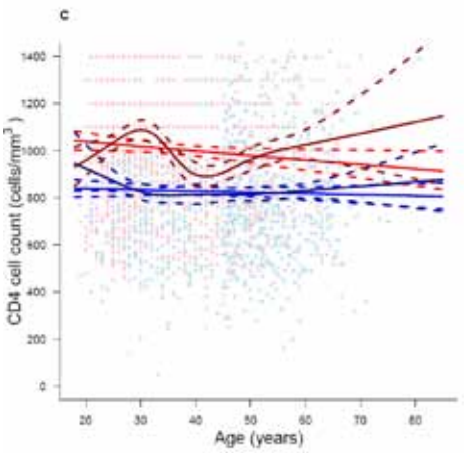
Figure SDC5A-C: Median CD4 cell counts (solid lines) and 95% confidence intervals (dashed lines) in HIV-negative men (dark blue) and women (dark red) obtained using linear regression models with age included as restricted cubic spline with 3 (A), 4 (B), and 5 (C) knots. In each figure we also show the estimates obtained using models that include age linearly on the transformed scale (blue lines for men and red lines for women). The model with 3 knots fitted the data significantly better than the linear (p=0.008), the model with 4 knots better than the 3-knot model (p=0.001) and the 5-knot model better than the-4 knot model (p=0.004).



3 knots (at 23, 39, and 62 years)

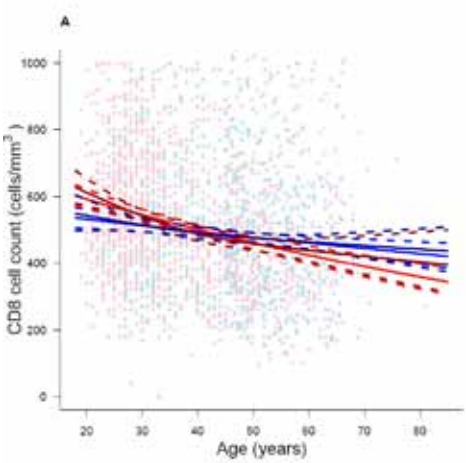


4 knots (at 23, 31,47 and 62 years).

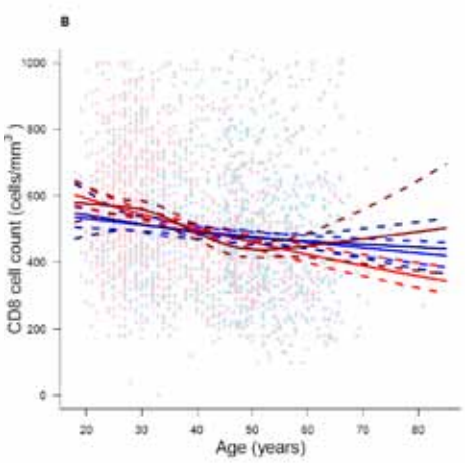


5 knots (at 23, 35, 50, 55, and 62 years)

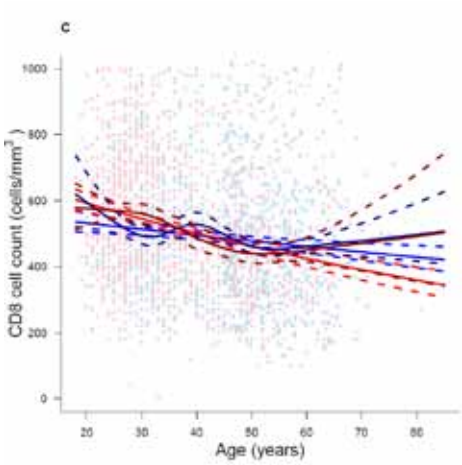
Figure SDC6A-C: Median CD8 cell counts (solid lines) and 95% confidence intervals (dashed lines) in HIV-negative men (dark blue) and women (dark red) obtained using linear regression models with age included as restricted cubic spline with 3 (A), 4 (B), and 5 (C) knots. In each figure estimates were obtained using models with age included as a linear predictor on the transformed scale are also shown (blue lines for men and red lines for women). The fit of the linear model was not significantly different from the 3 and 4 knot models and was slightly better for the 5 knot model compared to the linear model (p=0.03).



3 knots (at 23, 39, and 62 years)

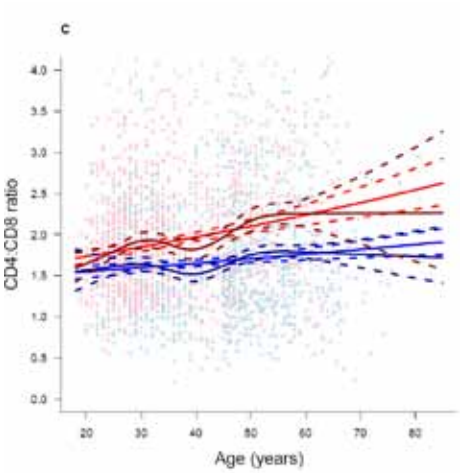


4 knots (at 23, 31, 47 and 62 years).

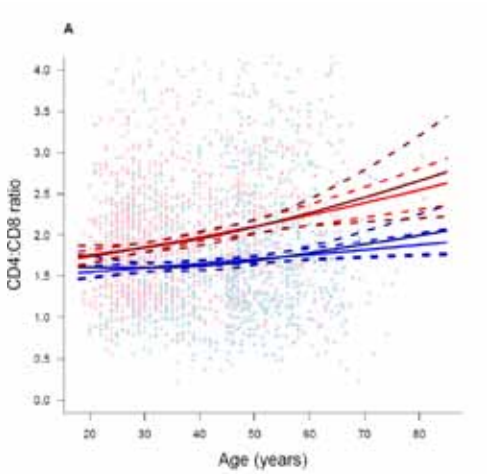


5 knots (at 23, 35, 50, 55, and 62 years)

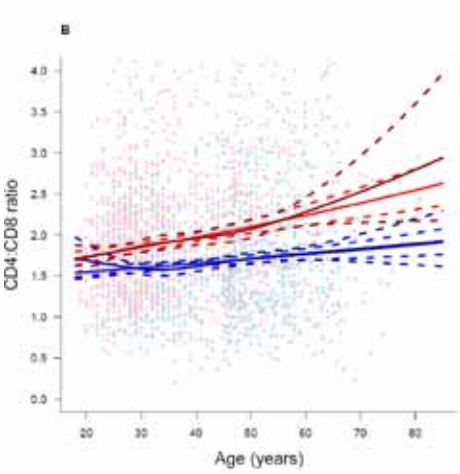
Figure SDC7A-C: Median CD4:CD8 ratio (solid lines) and 95% confidence intervals (dashed lines) in HIV-negative men (dark blue) and women (dark red) obtained using linear regression models with age included as a restricted cubic spline with 3 (A), 4 (B), and 5 (C) knots. In each figure estimates obtained using models including age as a linear predictor on the transformed scale are also shown (blue lines for men and red lines for women). The fit of the linear model was not significantly different from the 3 and 4 knot models and was slightly better for the 5 knot model compared to the linear model (p=0.02).



3 knots (at 23, 39, and 62)



4 knots (at 23, 31,47 and 62).



5 knots (at 23, 35, 50, 55, and 62)

Figure SDC8. Median CD4 count trajectories (95% confidence intervals in colour) during virologically suppressive ART according to age at the start of ART, gender, and baseline CD4 cell count at the start. Trajectories shown are those for an average individual (in the heterosexual transmission risk group, born in Western Europe/North America, starting ART between 2004 and 2006 with a baseline plasma viral load of 4.81 $\log_{10}$ copies/ml and with a random intercept and slopes equal to zero). Dashed lines show the estimated lower and median reference CD4 cell count.

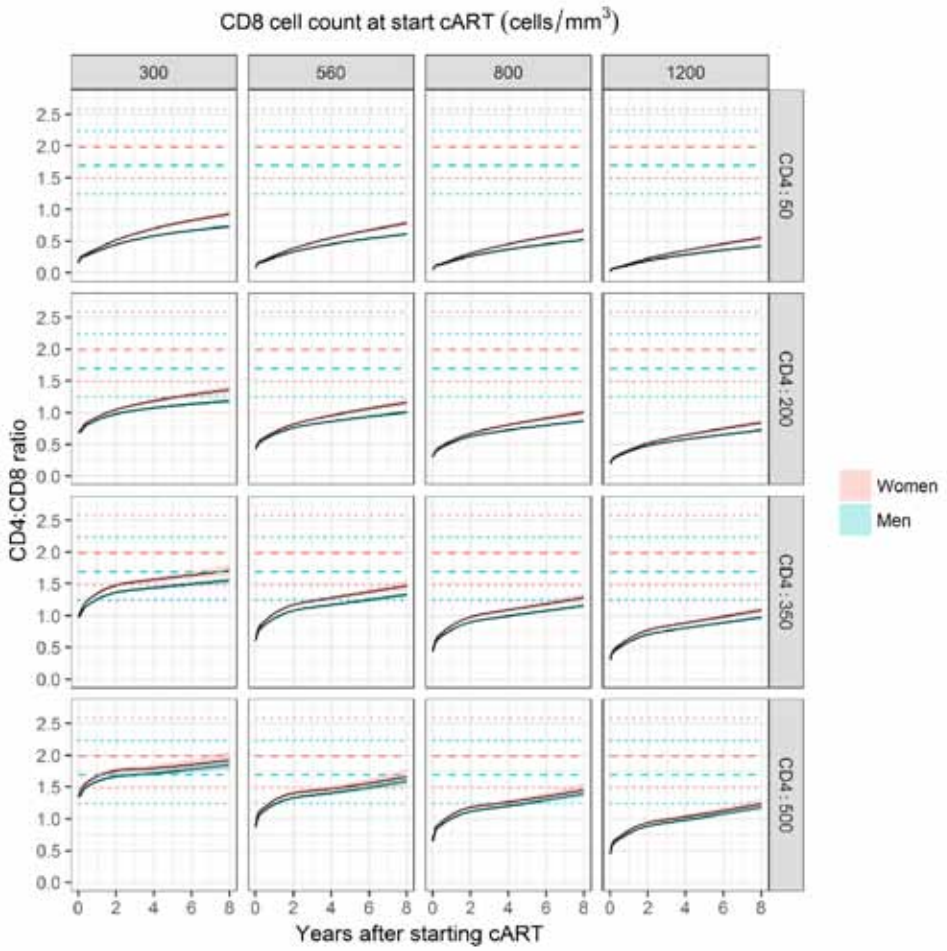


Figure SDC9. Median CD4 cell count at eight years of virologically suppressive ART (95% CI's in colour) according to CD8 cell count at baseline for an average individual (37 year old heterosexual male, born in Western Europe/North America with a plasma viral load of 4.81 $\log_{10}$ copies/ml at the start of ART with a random intercept and slopes equal to zero). Dashed lines show the lower and median reference CD4 cell count for a 37-year old male.

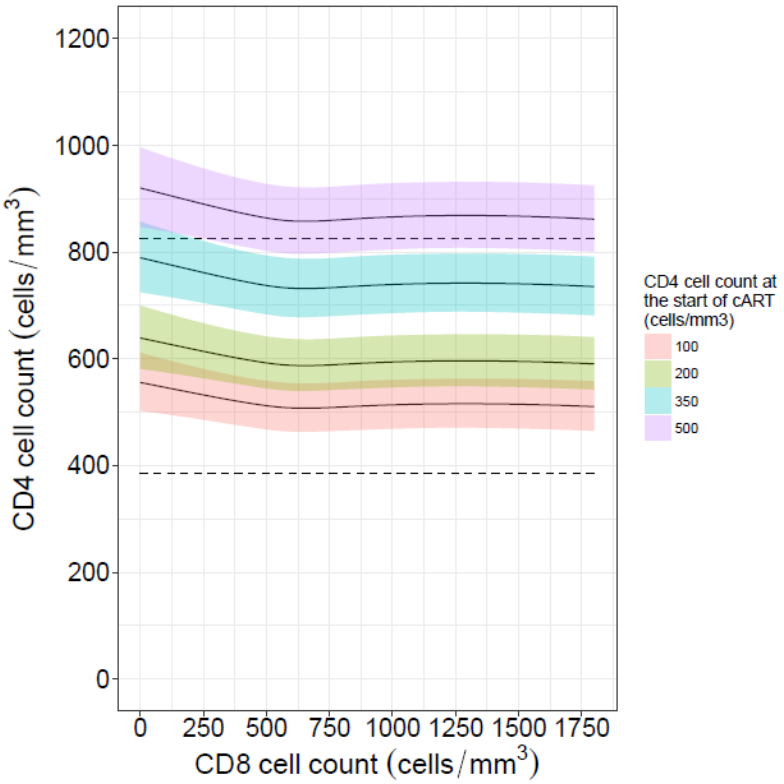


Figure SDC10. Median CD8 cell count after eight years of virologically suppressive ART, by CD4 count at the start of ART, gender, age eight years after the start, and CD8 cell count at the start of ART for an average individual (born in Western-Europe or North-America, in the heterosexual transmission risk group and starting ART with HIV RNA 4.81 $\log_{10}$ copies/ml and with a random intercept and slopes equal to zero). Dashed lines show the lower, median and upper reference CD8 cell count for various ages.

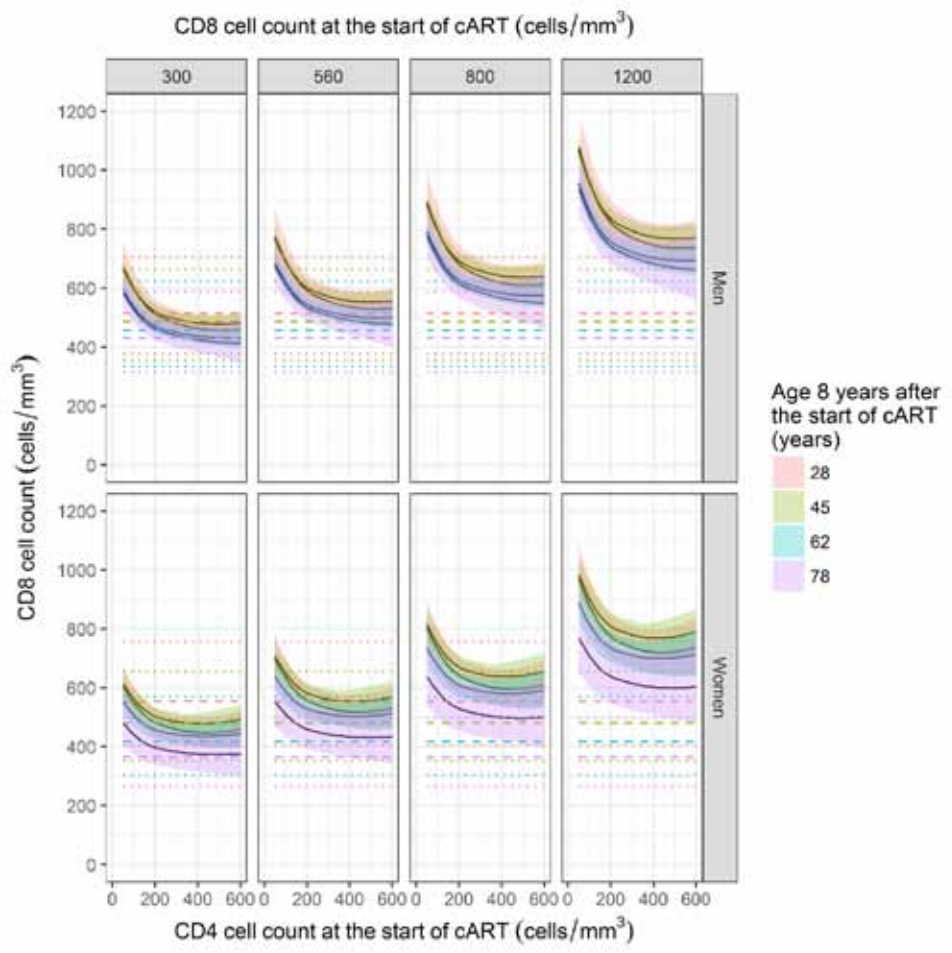


Figure SDC11. Median CD8 cell count trajectories during eight years of virologically suppressive ART by CD4 and CD8 cell count and age at the start of ART for an average reference individual (male heterosexual starting ART with a plasma viral load of 4.81 $\log_{10}$ copies/ml, born in Western Europe or North America and random intercept and slopes equal to zero). Shaded coloured areas are 95% confidence intervals. Dashed lines show the upper, normal and lower reference CD8 cell counts for HIV-negative subjects of the same gender and age.

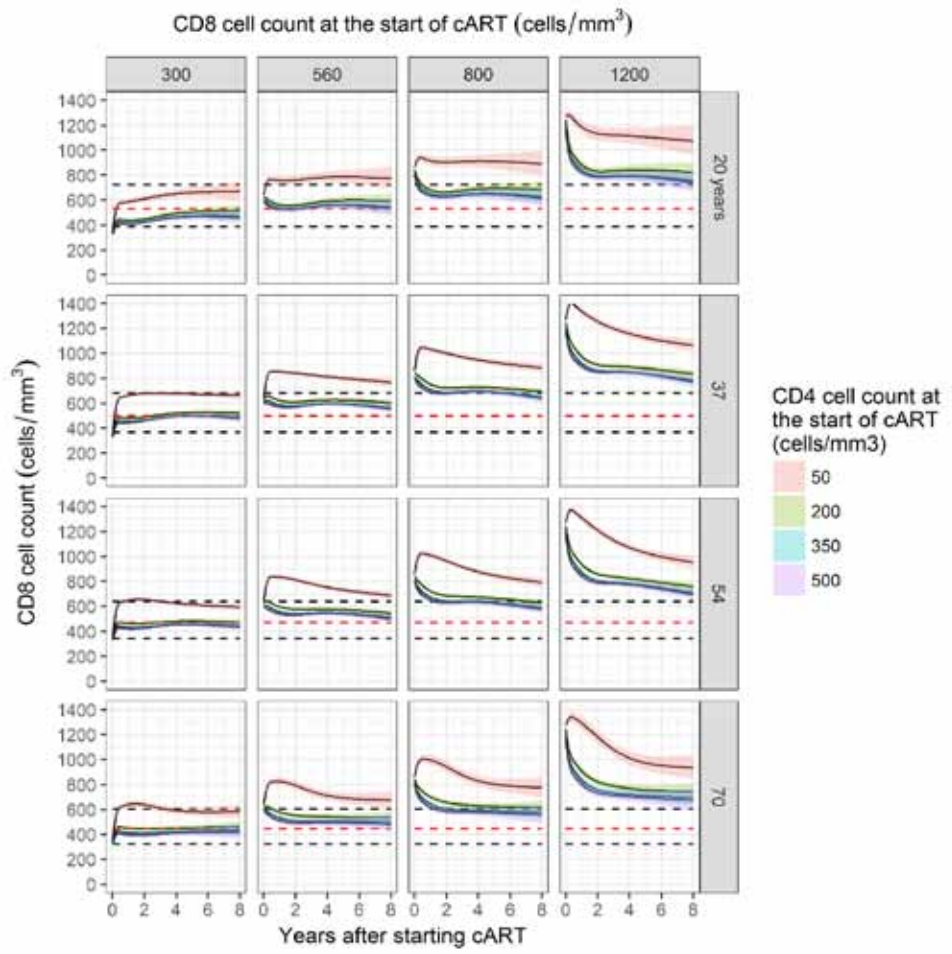
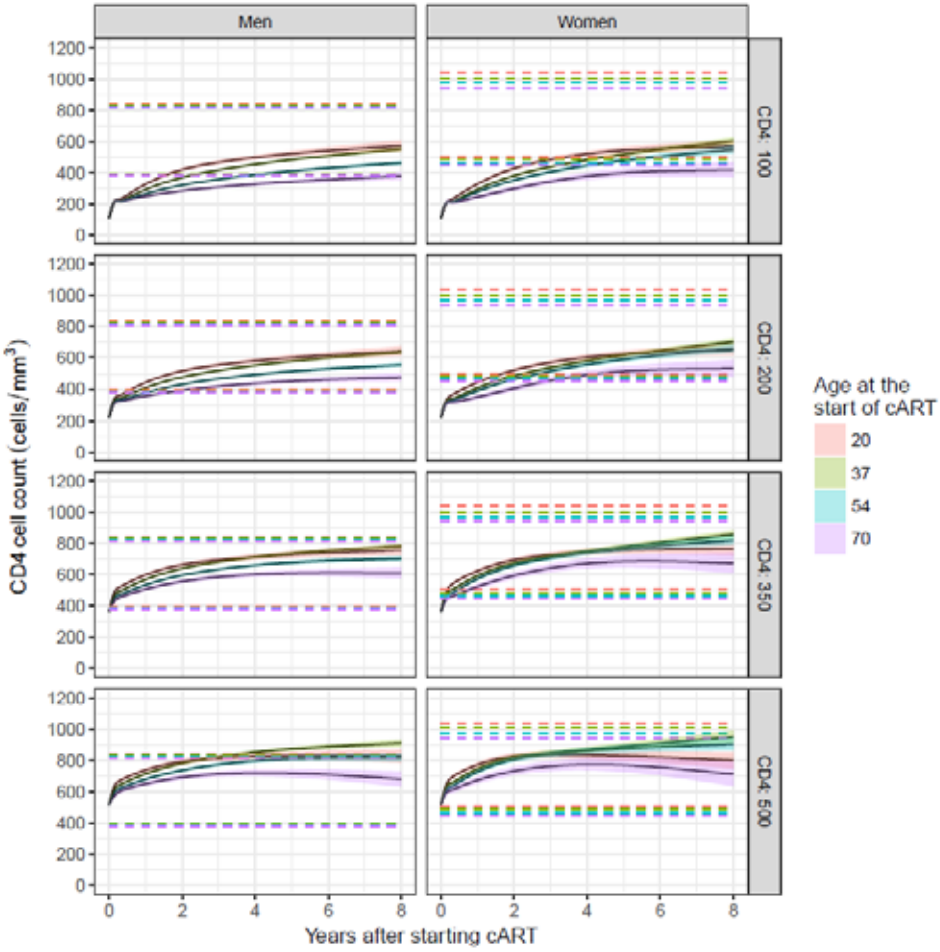
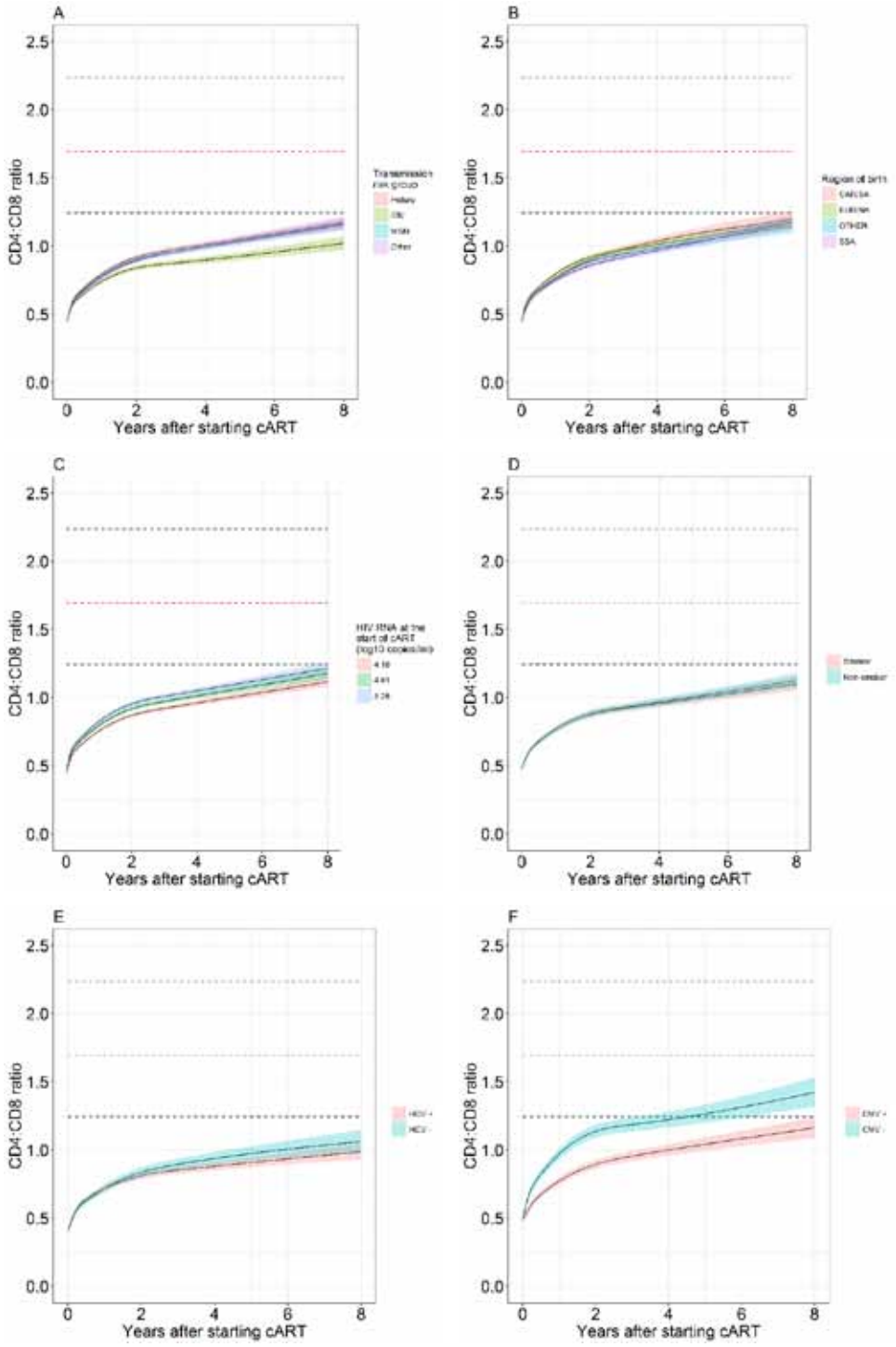


Figure SDC12. Median CD4:CD8 ratio trajectories (95% confidence intervals in colour) during virologically suppressive ART according to gender, and CD4 (vertical axis) and CD8 (upper horizontal axis) cell count at the start of ART. Trajectories shown are those for an average reference individual in heterosexual transmission risk group, born in Europe/North America, 37 years of age at the start of ART, and a plasma viral load of 4.81 $\log_{10}$ copies/ml at the start of ART and random intercept and slopes equal to zero. Dashed lines show the lower and normal reference CD4:CD8 ratio.



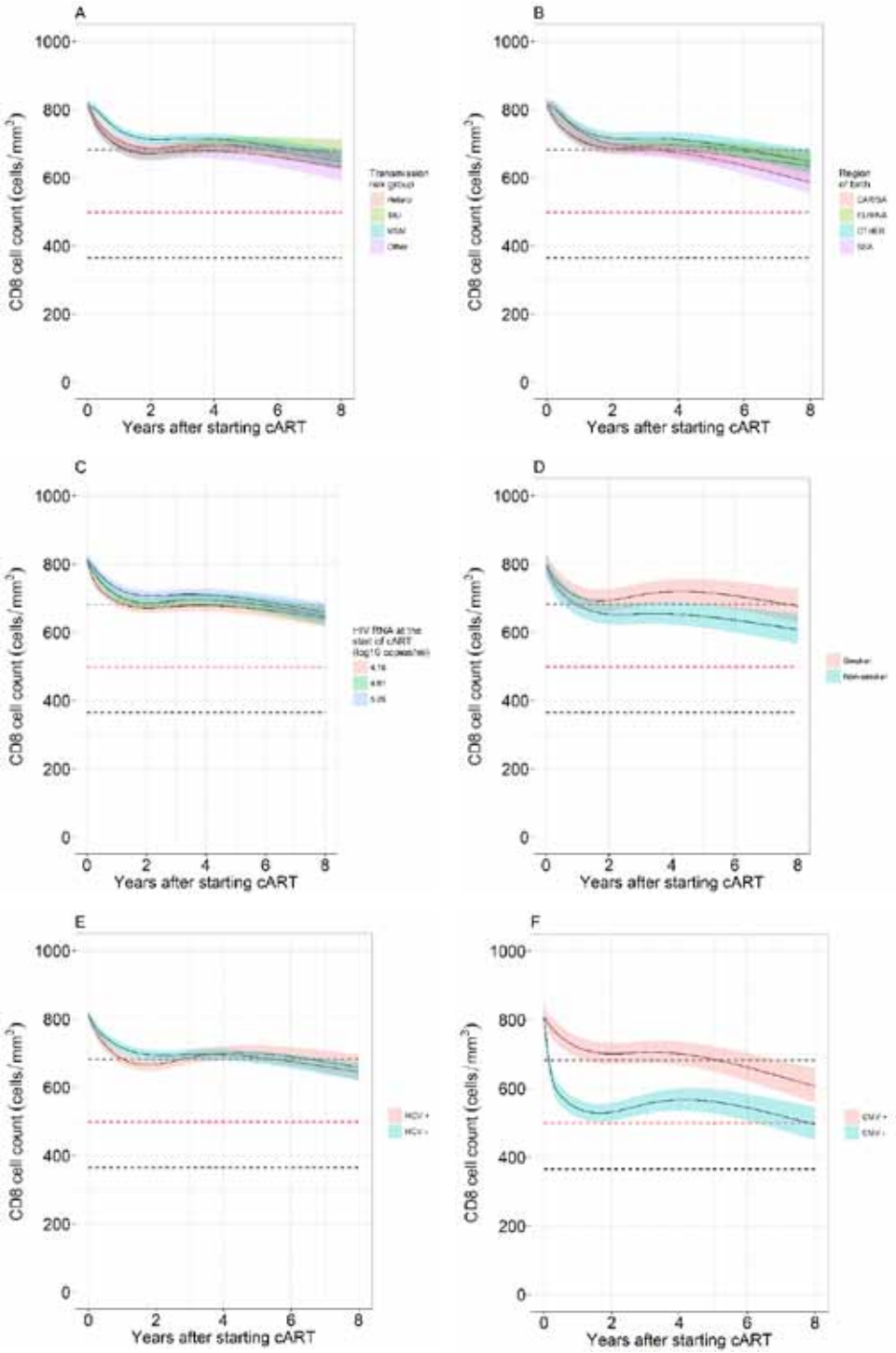
Supplemental Digital Content 13A-F shows the effect of other variables on CD4 cell count response. Increases in CD4 cell count during eight years of virologically suppressive ART were smaller in those infected through IDU ($p<0.0001$ compared to non-IDU), in individuals born in sub-Saharan Africa (interaction time and region of birth overall $p<0.0001$), in individuals with lower plasma viral load at the start of ART ($p<0.0001$) in nonsmokers ($p<0.0001$), and in HCV-positive individuals ($p<0.0001$). Data on ribavirin treatment was available for 13 of the 14 cohorts included in the HCV analysis. Ribavirin treatment was given for some period during the first eight years of ART to 754 individuals out of 4733 HCV-positive individuals (16%) from these 13 cohorts. CD4 cell count trajectories were similar in CMV-negative and positive individuals, although the p-value for the difference in trajectory was 0.0002. CD4 cell count trajectories according to start year of ART were similar (results not shown).

Figure SDC13A-F. Median CD4 cell count trajectories during virologically suppressive ART (95% confidence intervals in colour) according to A) transmission risk group, B) region of birth, C) plasma viral load at the start of ART, D) smoking status, E) HCV, and F) CMV co-infection, for an average individual (37-year old male, and unless otherwise stated in the legend, not infected through IDU, born in Western Europe/North America, starting ART with a HIV RNA of 4.81 copies/ml and a CD4 count of 350 cells/mm^3 and with a random intercept and slopes equal to zero). Dashed lines show the lower and median reference CD4 cell count. IDU: intravenous drug use, SSA: sub-Saharan Africa, CAR/SA: the Caribbean/South America, EUR/NA: Western Europe/North America, HCV: hepatitis C virus, CMV: cytomegalovirus


A
B
C
D
E
F
CD4:CD8 ratio
Years after starting cART
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8

Supplemental Digital Content 14A-F shows that median CD8 cell trajectories were higher in MSM than in those in the IDU and heterosexual risk groups (overall p-value for interaction between time and transmission risk group $p<0.0001$). Individuals born in sub-Saharan Africa showed lower CD8 cell count trajectories compared to those born elsewhere (overall p-value for region of birth <0.0001). CD8 cell count trajectories were higher when HIV RNA at the start of ART was higher ($p<0.0001$). CMV-negative individuals showed a stronger decrease in CD8 cell count in the first year after starting ART compared to CMV-positive individuals ($p<0.0001$). HCV-negative and positive individuals showed largely similar trajectories, although statistically significantly different ($p<0.0001$). Median CD8 cell count trajectories were higher in smokers than in nonsmokers ($p<0.0001$).

Figure SDC14A-F. Median CD8 cell count trajectories during eight years of virologically suppressive ART for an average individual (37-year old male, and unless otherwise stated in the legend, in the heterosexual transmission risk group, born in Western Europe/North America, starting ART with a HIV RNA of 4.81 copies/ml, a CD4 count of 350 cells/mm^3 and a CD8 count of 800 cells/mm^3 and with a random intercept and slopes equal to zero) according to transmission risk group (A), region of birth (B), HIV RNA (C), smoking status (D), HCV status (E), and CMV status (F). Shaded coloured areas are 95% confidence intervals. Dashed lines show the lower, median and upper reference CD8 cell counts.
IDU: intravenous drug use, SSA: sub-Saharan Africa, CAR/SA: the Caribbean/South America, EUR/NA: Western Europe/North America, HCV: hepatitis C virus, CMV: cytomegalovirus.


A
B
C
D
E
F
CD8 cell count (cells/mm³)
Years after starting cART
0
200
400
600
800
1000
0
2
4
6
8

Supplemental Digital Content 15 shows that median CD4:CD8 ratio trajectories were somewhat lower in men from sub-Saharan Africa compared to men from Western Europe/North America and the Caribbean/South America (interaction between time and region of birth $p<0.0001$). Furthermore, supporting information figure 10A, C, D, E, and F shows that ratios were lower in individuals in the IDU transmission risk group compared to those in other risk groups (interaction between time and transmission risk group, $p<0.0001$), in individuals with HCV co-infection at the start of ART ($p<0.0001$), in individuals with a CMV co-infection at the start of ART ($p<0.0001$), and in those with lower plasma viral load at the start of ART ($p<0.0001$). CD4:CD8 trajectories were similar for smokers and nonsmokers ($p=0.26$).

Figure SDC15A-F. Median CD4:CD8 ratio trajectories (95% confidence intervals in colour) during virologically suppressive ART according to transmission risk group (A), region of birth (B), HIV RNA (C), smoking status (D), HCV co-infection (E), and CMV co-infection (F) at the start of ART. Trajectories are shown for an average individual (37-year old male, and unless otherwise stated in the legend, in the heterosexual transmission risk group, born in Western Europe/North America, starting ART with a HIV RNA of 4.81 copies/ml, a CD4 count of 350 cells/mm^3 and a CD8 count of 800 cells/mm^3 and with a random intercept and slopes equal to zero). Dashed lines show the estimated median and lower reference CD4:CD8 ratio.
IDU: intravenous drug use, SSA: Sub-Saharan Africa, CAR/SA: the Caribbean/South America, EUR/NA: Western Europe/North America, HCV: hepatitis C virus, CMV: cytomegalovirus.

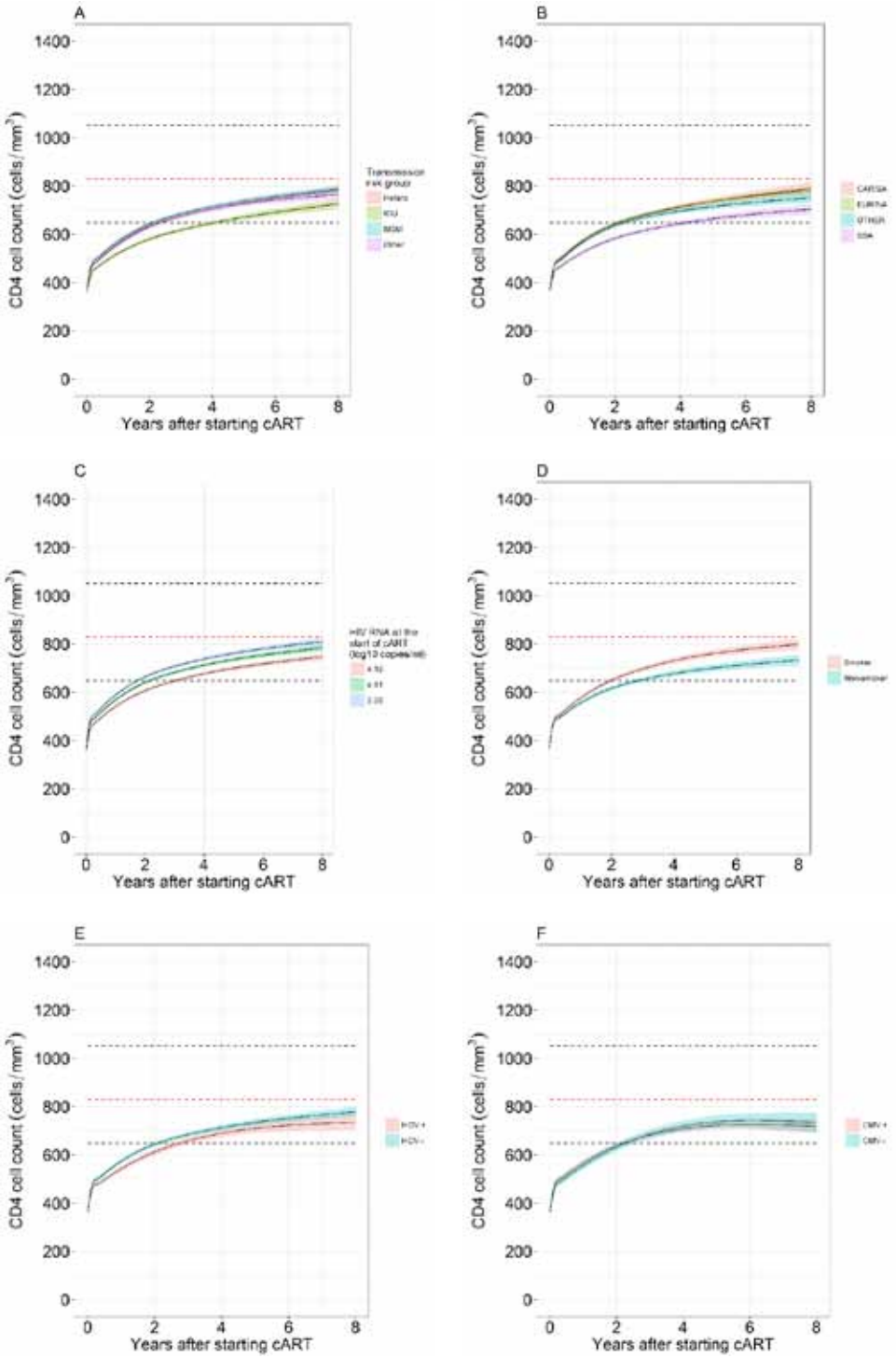

A
B
C
D
E
F
CD4 cell count (cells / mm³)
Years after starting cART
0
200
400
600
800
1000
1200
1400
0
2
4
6
8

Table SDC16. Estimated median (95% CI) CD4 and CD8 cell count (cells/mm^3) and the CD4:CD8 ratio at 8 years of virologically suppressive ART for an average individual (37-year old male in the heterosexual transmission risk group, born in Western Europe/North America, with a CD4 count of 350 or 500 cells/mm^3 and a CD8 count of 800 cells/mm^3 and HIV RNA of 4.81 copies/ml at the start and with a random intercept and random slopes equal to zero). The results of CMV, HCV and smoking status at the start of ART were obtained in the subgroup of cohorts with data available.

	Baseline CD4 count: 350 cells/mm³			Baseline CD4 count: 500 cells/mm³		
	CD4 cell count	CD8 cell count	Ratio CD4:CD8	CD4 cell count	CD8 cell count	Ratio CD4:CD8
Region of origin						
Europe/North America	784 (770, 797)	649 (621, 678)	1.18 (1.14, 1.22)	910 (892, 928)	637 (603, 674)	1.41 (1.35, 1.48)
Caribbean/South America	791 (764, 818)	636 (595, 680)	1.20 (1.13, 1.26)	918 (870, 967)	625 (579, 674)	1.44 (1.36, 1.52)
sub-Saharan Africa	705 (683, 727)	587 (555, 623)	1.18 (1.12, 1.23)	825 (740, 915)	577 (539, 617)	1.42 (1.34, 1.49)
Other	752 (731, 773)	648 (612, 685)	1.15 (1.10, 1.20)	876 (859, 894)	636 (596, 679)	1.38 (1.31, 1.46)
Transmission risk group						
IDU	727 (706, 748)	671 (632, 713)	1.02 (0.97, 1.07)	849 (822, 876)	660 (616, 708)	1.23 (1.17, 1.30)
MSM	787 (772, 802)	658 (633, 683)	1.16 (1.12, 1.19)	914 (892, 936)	647 (614, 680)	1.39 (1.34, 1.45)
Heterosexual	783 (766, 801)	649 (621, 678)	1.18 (1.14, 1.22)	909 (885, 934)	637 (603, 674)	1.48 (1.38, 1.57)
Other	765 (744, 785)	630 (592, 672)	1.16 (1.12, 1.21)	890 (863, 917)	619 (576, 666)	1.40 (1.33, 1.47)
HIV RNA (log_{10} copies/ml) at the start of ART						
4.16	747 (733, 762)	643 (615, 672)	1.12 (1.08, 1.16)	871 (850, 893)	632 (597, 668)	1.35 (1.29, 1.40)
4.81	784 (770, 799)	649 (621, 678)	1.18 (1.14, 1.22)	911 (889, 933)	637 (603, 674)	1.41 (1.35, 1.48)
5.18	811 (795, 826)	659 (630, 689)	1.22 (1.18, 1.26)	939 (917, 962)	647 (612, 684)	1.46 (1.40, 1.53)
CMV status at the start of ART						
CMV-	735 (695, 776)	496 (450, 546)	1.42 (1.32, 1.53)	819 (763, 876)	476 (421, 538)	1.64 (1.49, 1.79)
CMV+	719 (691, 748)	607 (558, 659)	1.16 (1.09, 1.24)	802 (755, 850)	583 (521, 651)	1.35 (1.24, 1.47)
Smoking status						
Nonsmoker	733 (712, 755)	608 (565, 654)	1.13 (1.07, 1.18)	858 (823, 893)	596 (543, 654)	1.33 (1.24, 1.41)
Smoker	799 (776, 822)	677 (629, 728)	1.10 (1.05, 1.16)	928 (892, 965)	663 (605, 727)	1.30 (1.22, 1.38)
HCV status at the start of ART						
HCV-	779 (758, 799)	646 (619, 673)	1.06 (0.98, 1.15)	905 (871, 939)	634 (601, 669)	1.27 (1.15, 1.40)
HCV+	736 (702, 770)	658 (622, 697)	0.99 (0.93, 1.05)	858 (815, 903)	646 (605, 691)	1.18 (1.08, 1.29)

IDU: intravenous drug use, MSM: men having sex with men, HCV: hepatitis C virus, CMV: cytomegalovirus

8

General discussion

In chapters 2, 3 and 4 we studied trends in setpoint plasma HIV-1 concentration and CD4 cell counts measured in peripheral blood of infected individuals over the course of the epidemic in the Netherlands between 1984 and 2007. We used data obtained from individuals participating in the ATHENA cohort and in the Amsterdam Cohort Studies (ACS) for whom the date of HIV infection could be reliably estimated. In chapter 5, 6 and 7 we used longitudinal data to study changes in these key biomarkers in HIV infection after HCV co-infection and after combination antiretroviral therapy (cART) initiation. In this chapter the main findings will be highlighted and discussed in the broader context of developments in HIV research.

Trends in setpoint HIV RNA and CD4 cell count

At about 3 to 9 months after the peak of primary infection, HIV production and clearance reach a balance, represented by a lower and more stable HIV-RNA plasma level which is often referred to as setpoint viral load. A higher setpoint viral load is associated with a faster HIV disease progression and a higher transmission efficiency of HIV and thus is of clinical as well as of public health interest[1,2].
Two studies on changes in the replicative capacity of HIV show contrasting results.

The study by Arien et al. suggested a lower replicative capacity in HIV-1 isolates obtained from individuals infected in 2002–2003 compared to isolates from individuals infected between 1986–1989, but samples were not matched for time since seroconversion[3]. Gali et al. reported an increase in the replicative fitness in participants of the ACS who seroconverted between 1996-2003 compared to those who seroconverted in 1986[4]. In Chapter 2 we report a higher mean setpoint viral load in individuals who seroconverted between 2003-2007 compared to those who seroconverted between 1984 and 1995 and those who did so between 1996–2002. The mean CD4 cell count measured at the time of reaching viral setpoint declined between 1984 and 2007. We argued that changes in the type of HIV RNA assays used over time, changes in the technology of measuring CD4 cell count over time, or differences in the distribution of HIV subtype among seroconverters over time were unlikely to explain these trends.

Trends in setpoint viral load over calendar time have been studied in several cohorts and at several time points with conflicting results. No significant trends were found in a French cohort between 1996 and 2007[5], a North American cohort between 1984 and 2005[6], a Swiss cohort between 1984 and 2003[7] and a Danish cohort between 1995 and 2010[8] whereas evidence for a rising trend was found in an Italian cohort between 1984 and 2006[9] and in a collaboration of several European and North American cohorts between 1985 and 2002[10]. In a re-analysis of the last study including data from 15,875 seroconverters covering the period between 1979 and 2008 a rising trend in setpoint viral load was observed and a potential plateau effect after 2002[11]. Differences in study population, geographical area, calendar year period, outcome measure or the statistical analysis method may partly explain the differences in trends between cohorts. In addition, two of these studies included seroprevalent individuals for whom at least three HIV RNA measurements were obtained whilst untreated and at least five CD4 cell counts[7,9]. This may have biased trend over time estimates since the time from seroconversion in these individuals is unknown; individuals may be in a later stage of disease characterized by a lower CD4 cell count and a higher plasma viral load[1]. Furthermore, individuals with high viral load and lower CD4 cell counts are more likely to have less viral load measurements and CD4 cell counts available because of death (in the pre-cART era) or start therapy (in the cART era) and are thus more likely to have been excluded from analyses. This would probably lead to an underestimation of the level of setpoint viral load and an overestimation of the level of CD4 cell count at setpoint in the cART era during more recent calendar years. Indeed, a meta-analysis which found a borderline significantly increasing trend in setpoint viral load and a significantly decreasing trend in CD4 cell count (and which included most studies reported above as well as the study reported in Chapter 2) did find stronger trends in CD4 cell count and setpoint viral load over calendar year when only seroincident cohorts were included and seroprevalent cohorts were excluded from analyses[12].

Fading impact of host genetics on HIV-1 control

We further studied in chapter 3 whether adaptation of HIV-1 to host genetic factors might have contributed to a higher setpoint viral load. We compared the association between setpoint viral load and HCP5 rs2395029, –35HLA-C rs9264942, and the CCR5wt/Δ32 genotype (coding for one of the HIV-1 coreceptors) in individuals with HIV seroconversion between 1982 and 2002 and between 2003 and 2009. The minor alleles for HCP5 rs2395029, -35HLA-C rs9264942 and CCR5wt/Δ32 had a similar prevalence in both time periods and were all individually associated with a significantly lower setpoint viral load in those who seroconverted between 1982 and 2003. The protective effect of the minor allele of rs2395029 in HCP5 and the CCR5wt/Δ32 genotype was no longer present in those who seroconverted between 2003 and 2009, whereas the protective effect of –35HLA-C rs9264942 was preserved. The association between setpoint viral load and HCP5 rs2395029 had significantly changed before and after 2003 whereas the impact the change in impact of CCR5wt/Δ32 was not independent from the other two markers. Given the low prevalence of HCP5 rs2395029 of 6-10%, the adaptation of HIV to this host genetic factor can only explain to a small extent the observed increase in setpoint viral load and the accompanying decrease in CD4 cell count in more recent years.

Trends in CD4 cell count decline before cART is started

Whether the higher viral load and lower CD4 T-cell count at setpoint in more recent years coincides with a faster progression to AIDS or death is difficult to study in the cART era since these endpoints are hardly observed in effectively treated individuals. In Chapter 4 we therefore set out to study changes over calendar time in the decline of CD4 cell count before cART is started, as a surrogate marker of disease progression. We found a trend of a steeper slope of decline since the middle of the nineties. In combination with the observed significantly lower CD4 cell count at viral setpoint 9 months after seroconversion in more recent calendar years this has a considerable clinical impact. We estimated that the time between HIV seroconversion to reaching a CD4 cell count of 350 cells/mm^3 (the cut-off at which therapy was recommended to be initiated during this period) was shortened from more than 4 years in the period before 1996 to 2.7 years in the period 2003-2007.

We obtained steeper estimates of the slope of CD4 cell count decline when timing of censoring (because of ART initiation or disease progression) was jointly modelled with longitudinal CD4 cell counts than when using a mixed-effects model. The trend of a steeper CD4 cell count decline over time was not apparent in the mixed-effects model but only in joint models. Mixed-effects models can provide unbiased estimates when the probability of the censoring event only

depends on the observed CD4 cell counts, given covariates included in the model. Provided that the model for the censoring event is correctly specified, joint-models can provide unbiased estimates both when data are missing at random (MAR), and when censoring is informative (when censoring depends on aspects of CD4 cell count that are not observed, for example when therapy initiation depends on the level of plasma viral load). Although censoring CD4 cell counts because of AIDS is considered informative and will contribute to bias in mixed-effects model estimates it is not the predominant reason of censoring. The primary reason for censoring was ART initiation. The different results obtained from mixed-effects models and joint-models in our study may be because censoring CD4 cell counts due to ART initiation or disease progression is informative and estimates from standard mixed-effects models are incorrect. Alternatively, when the censoring event process is predominantly MAR, and the dropout process is not correctly specified (in the shared parameter model dropout depends on the underlying 'true' CD4 cell count of the individual, estimated by the fixed and random intercept and slope, instead of the observed CD4 cell count) the joint-model might be more biased than the mixed-effects model.

A recent study[13] compared a new joint model with two of the three models applied in Chapter 4 (the shared parameter and linear mixed-effects model). In the new model the hazard of dropout was allowed to depend on the last observed CD4 cell count as well as on the 'true' underlying CD4 cell count. In simulations the new joint model showed little bias under both MAR and missing not at random (MNAR) scenarios. Using data from participants in the CASCADE collaboration with seroconversion in or after 2004 the new joint model resulted in a slightly steeper slope of CD4 cell count decline compared to the shared parameter model, whilst the slope estimate obtained with the linear mixed-effects model was considerably smaller to slope estimates from both joint models. This confirms our results in Chapter 4 and suggests that the dropout process due to cART initiation may be more MNAR than MAR. Furthermore, our analysis also included individuals with seroconversion before 2004. Guidelines by the US Department for Health and Human Services (DHHS) on timing of ART initiation, generally followed in the Netherlands, stated during earlier calendar years that ART initiation could be considered in individuals with counts above the recommended CD4 cell count threshold but with a high plasma viral load. This will likely make the dropout process during the early cART era, the first few years after 1996, more MNAR than MAR if ART was started in the Netherlands because of this reason. This might explain why the difference in the slope of CD4 cell count decline between the mixed-effects model and the shared parameter model was more pronounced from 1996 onwards and it is likely that shared parameter model estimates are less biased that mixed-effects model estimates in this setting. During years when ART initiation was primarily guided by the last observed CD4 cell count (probably during later calendar years) shared parameter model estimates may be more biased than mixed-effects model estimates. In practice it is not possible

to distinguish between MAR and MNAR dropout processes as it depends on untestable assumptions.

Most studies on the trend in CD4 cell count decline over time have not used a joint model, which may explain the smaller decline in CD4 cell count per year found in these studies compared to our results. In one other study longitudinal CD4 cell counts before the start of ART were modelled in combination with a time-to-event model[14]. No trend over calendar time on CD4 cell count decline before the start of ART was found. However, models were only adjusted for informative censoring due to death and not for ART initiation, hence differences in statistical methods may explain the discrepancy with the results of our study.

Selection of HIV-1 in the cART era as explanation of the observed trends

The number of individuals that one infected person may infect during its lifetime, given a certain degree of risk behavior, depends on the interplay between the infectiousness of the pathogen and the length of the infectious period[15]. An individual infected with highly replication competent HIV (resulting in a high setpoint viral load) will have a shorter time to death and a shorter infectious period during which he may transmit the virus to the same number of individuals as an individual with less replication competent HIV (resulting in a low setpoint viral load) during a longer infectious asymptomatic period and longer time to death. It has been suggested that in HIV infection this trade-off results in an optimum viral load (at which the number of new infections by one infected individual is maximized) when the setpoint viral load is at an intermediate level[15,16].

Setpoint viral load appears to a certain degree hereditable, with transmitter and recipient exhibiting similar setpoint viral load levels[16]. Initially, widely different estimates of hereditability were reported, most likely due to differences in estimation methodology[17]. More recent studies have reported that viral genetic factors account for about one third in variation of setpoint viral load [18-21]. Within-host evolution of HIV is rapid and can evolve into drug resistance in weeks. Several hypotheses have been proposed to explain how such a rapidly evolving virus can be reconciled with a heritable viral load setpoint from one infection to the next[16], but this is not yet understood.
We hypothesized that the widespread introduction of cART in 1996 in the Netherlands and the general trend to start cART at higher CD4 cell counts since then, may explain, at least partly, the increase in setpoint viral load over calendar time. Initiation of cART, and sufficient adherence, effectively shortens the infectious period of HIV as transmission during virologically suppressive cART is absent or near zero[22]. Therefore, in more recent years HIV would have been transmitted, relatively speaking, more frequently to other individuals

during the short(er) period between infection and treatment initiation. This may have resulted in selection of HIV strains with higher setpoint viral load. A mathematical modeling study calibrated to the South African situation found that under 'treatment as prevention' and 'universal test and treat' scenarios such an effect may indeed occur, albeit in the context of an epidemic rapidly decreasing in size[23]. The increase in HIV-1 setpoint viral load over time was also found in transmission clusters in the Netherlands, indicating selection within transmission networks rather than the introduction of a more virulent strain drives these changes[24]. The BEEHIVE (*Bridging the Evolution and Epidemiology of HIV in Europe*) study, which includes data from individuals with a reliable date of HIV infection participating in the ATHENA and ACS cohorts, as well as data from other cohorts, was initiated to identify mutations in the virus' genetic sequence that affect the plasma setpoint viral load. Full genome HIV sequences were obtained from blood samples of included individuals taken between 6 and 24 months after seroconversion and before ART was initiated. Results may provide new insights on the pathogenesis of HIV[18,25].

Changes in HIV RNA and CD4 cell count after acute HCV co-infection

In Chapter 5 we studied the effect of acute HCV co-infection on CD4 cell counts and HIV viral load in chronic HIV infection. The effect of HCV co-infection on HIV disease progression has long been unclear. More recent studies have found an increased risk of mortality from any cause in the post-cART era[26,27] and both an increased risk of death from HIV and/or AIDS-related causes as well as an increased risk of death from hepatitis or liver disease in HIV/HCV co-infected individuals compared to HIV mono-infection[26]. The study in Chapter 5 was the first to study the effect of acute HCV co-infection on HIV viral load and CD4 cell count. We showed an increased probability of detectable plasma HIV viral load in cART treated individuals around the timing of acute HCV infection (and before anti-HCV medication was started). Although this was only a transient increase, the effect of acute HCV infection on CD4 cell counts was longer-term. Median CD4 cell count in cART treated individuals declined from 587 cells/mm^3 (95% CI 534-644) at HCV infection to 508 cells/mm^3 (95% CI 460-559) during the first 5 months thereafter HCV infection and took 2.2 years to return to pre-HCV infection levels. Such a drop of 80 CD4 cells/mm^3 may put individuals with a lower CD4 cell count at the start of HCV co-infection at risk for AIDS or non-AIDS events. Another study in individuals not yet on cART reported that compared to HIV-infected individuals without HCV co-infection no increased risk of HCV co-infection on a composite endpoint (the definition included AIDS defining conditions and death but consisted mainly of reaching a CD4 cell count of less than 350 cells/mm^3) was found beyond 2 years after acute HCV infection[28]. Smaller CD4 cell count gains in HCV-co-infected individuals compared to HIV mono-infected

individuals following cART initiation were reported as well, but no association was found between the duration of HCV infection and CD4 cell count gains. A collaborative study in MSM with a reliable date of both HCV and HIV infection (with HCV infection after HIV infection) compared CD4 cell count and HIV RNA trajectories after HCV infection between HIV/HCV co-infected individuals and HIV mono-infected individuals. Similar to our results in treated individuals in Chapter 5, CD4 cell counts were temporarily lower during the first two to three years following HCV seroconversion in both treated and untreated HIV-infected MSM. HIV RNA trajectories in HCV co-infected individuals appeared to be similar to those of HIV mono-infected individuals[29]. The transient decrease in CD4 cell count following the first 2-3 years after acute HCV infection may explain why the effect of HCV infection on progression of HIV disease was unclear initially. The effect depends on the timing of the acute HCV infection and in most studies timing of HCV infection is not known and cannot be adjusted for.

The incidence of HCV co-infection among HIV infected MSM in Europe increased dramatically between 1990 and 2007 from 0.9 to approximately 51 per 1000 person-years of follow-up[30]. Together with the decrease in CD4 count associated with HCV co-infection (Chapter 5) this might have contributed to the higher setpoint viral load and lower CD4 cell counts we found in the studies described in Chapter 2 and 4. However, the increase in HCV co-infection incidence is not large enough to completely explain the trends observed in Chapter 2 and 4.

Changes in CD4 and CD8 cell count and the CD4:CD8 ratio after cART is started

In chapter 6 we investigated the influence of demographic and clinical characteristics at the start of cART on restoration of CD4 cell count. Male gender, sub-Saharan African origin (as compared to Western European/North American origin), HIV infection through intravenous drug use, and a lower pre-cART HIV RNA were all associated with a longer time from the start of cART to reaching a CD4 count of 800 cells/mm^3 or higher. The observation that individuals from sub-Saharan Africa show smaller changes in CD4 cell counts during cART compared to individuals from Western Europe or North America may indicate geographic variation in normal CD4 ranges but also differences in adherence[31,32]. Here differences in adherence seems less likely, given that only individuals with HIV RNA suppression below the level of detection were included in the analysis. Alternative explanations for our findings includes differences in the distribution of HIV subtypes between individuals from different regions of origin[33-36], which might result in variation in restoration of CD4 cell count after starting cART between individuals from different regions of origin.
Individuals starting with lower CD4 cell counts experienced lower 7-year-CD4 cell counts compared to individuals starting at higher CD4 cell counts. Results

suggested that restoration of median CD4 counts >800 cells/mm^3 is feasible within 7 years of cART initiation when cART is started at a CD4 count above 350 cells/mm^3 and individuals achieve and maintain suppression of viral replication. Age older than 50 years at the start of treatment and experiencing periods of detectable viremia (>500 copies/ml) were associated with smaller increases in CD4 cell count after 7 years and reaching a plateau at a less than normal range. These findings support the recommendation for earlier initiation of treatment (before counts have dropped below 350 cells/mm^3) as expressed in US DHHS treatment guidelines issued in 2007. Two large collaborative observational studies confirmed that a start of cART before CD4 counts dropped to below 350 cells/mm^3 was beneficial (in terms of mortality) compared to further deferral of treatment[37,38]. However, these two studies did not agree on the benefit of an earlier start at 500 cells/mm^3. One study[38] found a lower mortality rate when cART was initiated before 500 CD4 cells/mm^3 were reached whereas the other[37] found not such an effect. DHHS guidelines on when to start were nevertheless changed to 500 cells/mm^3. Since then, in 2015, results from randomized trials have shown reduced rates of a combined endpoint of death, AIDS or serious non-AIDS events mortality when cART is started immediately as compared to deferral of cART until CD4 counts decreased to 350 cells/mm3 or until the development of AIDS or another event that dictated the use of cART[39,40]. These results provided the conclusive scientific evidence that supported the change made in 2012 in DHHS treatment guidelines (based on expert opinion, not supported by results of randomized trials or observational studies), to offering treatment to all HIV-infected individuals

As a result, an increasing number of HIV-1-positive individuals have started cART at high CD4 cell counts. In Chapter 7 we investigated whether starting cART at high CD4 cell counts makes normalization of CD4 cell counts, as well as CD8 cell counts and the CD4:CD8 ratio, more likely. Data from the Antiretroviral Therapy Cohort Collaboration (ART-CC), was used to study determinants of long-term CD4 and CD8 cell count trajectories after starting cART. In order to judge whether an HIV-infected individual's immune system has normalized during use of cART, ideally one would like to know the state of the individual's immune system before HIV infection. As pre-HIV-infection data were not available, we compared median values of CD4 and CD8 cell counts and the CD4:CD8 ratio during 8 years of virologically suppressive cART to median values observed in HIV-negative individuals from the Netherlands and Denmark of the same age and gender. An early start, at high CD4 counts of 500 cells/mm^3 or higher makes it more likely that CD4 cell count during virologically suppressive cART will reach levels comparable to levels in HIV-negative individuals. However, long-term CD8 cell counts remain at higher levels compared to HIV-negative individuals. The long-term CD4:CD8 ratio thus remains below levels seen in HIV-negative individuals, even when cART is started with high CD4 cell counts.

The CD4 cell count is the most important predictor for the risk of AIDS and mortality and there continues to be an association of a lower risk of AIDS and

death with a higher CD4 cell count, even above 500 cells/mm^3 [41,42]. For the CD4:CD8 ratio and CD8 cell count these associations are less clear. Several studies have suggested that the CD4:CD8 ratio, independent of CD4 cell count, predicts time to non-accidental death[43], subclinical atherosclerosis[44] and non-AIDS defining morbidity and mortality[45]. In addition, both low CD8 cell count in the first year after starting cART and CD8 cell counts >1500 cells/mm^3 at 10 years after the start of cART have been associated with higher all-cause mortality[46]. In contrast, a recent ART-CC study found only a small effect of CD8 cell counts and no significant effect of the CD4:CD8 ratio on all-cause mortality[47]. It may be that associations between either CD8 cell count or CD4:CD8 ratio and clinical outcome are only limited to specific causes of morbidity or death.

Conclusions and implication for future research

Early identification of infection with HIV is required to let people benefit maximally from the recommendation to offer therapy to all HIV-infected individuals, irrespective of their CD4 cell count. Starting ART with a CD4 count of 500 cells/mm^3 or more makes restoration of CD4 cell count during virologically suppressive antiretroviral therapy to levels observed in HIV-negative individuals more likely. However, restoration of CD8 cell counts may prove to be more difficult, even when cART is started early when CD4 cell counts are still high. After years of virologically suppressive cART CD8 cell counts remain higher compared to counts seen in HIV-negative individuals. As a result of persistent high CD8 cell counts, the CD4:CD8 ratio during long-term virologically suppressive ART is at a lower level compared to that in HIV-negative individuals. The impact of these sub-normal immune responses on clinical outcomes requires further study.
Whether the current guidelines to start cART directly after diagnosis of infection may result in selection of HIV with increased replicative fitness needs further study as well. Studies such as BEEHIVE are instrumental in the identification of replicative fitness associated mutations in the HIV genome and may provide new insights into HIV pathogenesis.

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Summary

Human immunodeficiency virus (HIV) infection has caused the death of millions of people worldwide. If left untreated, HIV infection destroys the immune system and leads to AIDS, the spectrum of opportunistic infections and diseases that can appear if the immune system is impaired. Currently available treatment cannot cure HIV infection but does provide infected individuals with a near normal life expectancy, provided treatment does suppress the virus in peripheral blood to below detectable levels and provided treatment is started before the immune system is severely compromised.
The state of the immune system is mostly characterized by the CD4 cell count in peripheral blood. A higher setpoint plasma viral load (the more or less stable concentration of virus particles in plasma after 6 months after HIV infection) is related to a higher rate of decrease in CD4 cell counts which in turn is related to a shorter time to AIDS and death. A higher setpoint viral load is also associated with increased infectiousness. The plasma viral load and the CD4 cell count are two of the most important biomarkers in HIV disease prediction modeling. The overarching aim in this thesis was to study trends in setpoint plasma HIV-1 viral load and CD4 cell count before and after introduction of effective antiretroviral treatment.

To gain more insight in the HIV epidemic in the Netherlands we have modelled trends in the setpoint viral load, the accompanying CD4 cell count and trends in the decline of CD4 cell count after HIV infection and before treatment is started.

After treatment has started and plasma viral load has been suppressed to below detectable levels the clinical focus is mainly on restoration of the CD4 cell count and to a smaller extent on other components of the immune system such as the CD4:CD8 ratio and CD8 cell count. Trajectories of these markers after the start of virologically successful treatment were modelled in chapter 5 and 6 in this thesis. Furthermore, we studied whether antigenic stimulation through co-infection with hepatitis C virus (HCV) is associated with changes in plasma viral load and CD4 cell counts in treated and untreated HIV infected individuals. In all these studies we use data obtained from HIV-infected individuals participating in the AIDS Therapy Evaluation in the Netherlands (ATHENA) cohort. This cohort is maintained by the Stichting HIV Monitoring (SHM). In addition, in some analyses in this thesis data from homosexual men included in the Amsterdam Cohort Studies on HIV infection and AIDS (ACS) was used. The ACS and the ATHENA cohort together span almost the entire period of the HIV epidemic in the Netherlands. Investigating the association (two things are related, but not necessarily causally related) of risk factors on disease on a population level is only possible using data from observational cohorts such as the ACS and ATHENA cohorts, but these are more prone to bias than data from randomized clinical trials. A major challenge in the analysis of observational cohort study data is to make comparisons with minimal bias such that the associative measure has a causal interpretation.

Trends in viral load setpoint over calendar time have been studied in several cohorts and at several time points with conflicting results. Cohorts from France, North America, Denmark and Switzerland found no significant trend in setpoint viral load, whereas an Italian cohort and a collaborative cohort including several European and North American cohorts found an increasing trend. Differences in study population, geographical area, calendar year period, outcome measure or statistical analysis method may partly explain these differences. Also, one study on changes in viral fitness at viral setpoint using data from the ACS found an increase in viral fitness in samples obtained from seroconverters in 1996-2003 compared to samples obtained from seroconverters in 1986. In Chapter 2 we, likewise, found a rising trend over time in the HIV-1 RNA concentration at setpoint between 1984 and 2007. The CD4 cell count obtained at the same time after seroconversion showed an accompanying decreasing trend. Changes in the use of HIV RNA assays over time, changes in the technology of measuring CD4 cell count over time, or differences in the distribution of HIV subtype among seroconverters over time were unlikely to explain these trends.

Furthermore, in chapter 3 we studied whether changes in host genetic factors could have contributed to the trends described in chapter 2. There was no evidence that the distribution of genotypes HCP5 rs2395029, –35HLA-C rs9264942, and CCR5wt/Δ32, all associated with a lower setpoint viral load, had changed over time among individuals with recent HIV infection suggesting that the find-

ing of a higher setpoint viral load in more recent years is unlikely due to changes in these host genetic factors. However, results in chapter 3 suggest that the protective effect of the minor allele of rs2395029 in HCP5 genotype was no longer present in the group with seroconversion between 2003 and 2009. Adaptation of HIV to this host genetic factor may partly explain the increase in setpoint viral load observed in Chapter 2.

Progression to AIDS or death is difficult to study in the combination antiretroviral therapy (cART) era because these endpoints are hardly observed in effectively treated individuals. In chapter 4 we attempted to study changes in the decline of CD4 cell count before cART is started, a surrogate marker of disease progression. We found a trend of a steeper slope of decline since the middle of the nineties. Combined with the signifcant decrease in CD4 cell count at 9 months after seroconversion we estimated that the time from HIV seroconversion to reaching a CD4 cell count of 350 cells/mm^3 (the cut-off before which therapy was recommended to be initiated during this period) shortened from more than 4 years in the period before 1996 to 2.7 years in the period 2003-2007.
The trend of a steeper CD4 cell count decline over time was only apparent when timing of censoring (because of antiretroviral therapy initiation or disease progression) was jointly modelled with longitudinal CD4 cell counts and not when mixed-effects models were used, most likely because censoring CD4 cell counts due to antiretroviral therapy initiation or disease progression is informative. Estimation of parameters in joint models, compared to mixed-effect models, is more complex and joint models are currently not routinely used. Most other studies on the trend in CD4 cell count decline over time have not used joint models and hence this may explain the smaller decline in CD4 cell count per year found in these studies compared to our results.
Setpoint viral loads appears to be, to a certain extent, heritable. The transmitter and the recipient exhibit similar setpoint viral load levels. We hypothesized that the widespread introduction of cART in 1996 in the Netherlands and the general trend to start cART at higher CD4 cell counts since then, may have resulted in selection of HIV strains with higher setpoint viral load. This may, at least partly, explain the increase in setpoint viral load and the decrease in CD4 cell count at viral setpoint in later calendar years.

Co-infection with syphilis, malaria, herpes simplex virus, and other opportunistic infections has been associated with an increase in HIV viral load and decrease in CD4 cell count. For HCV co-infection, common among MSM infected with HIV, such an effect had not yet been reported. In Chapter 5 we found an increased but transient probability of detectable plasma HIV viral load in cART treated individuals during chronic HIV infection around the timing of acute HCV infection. A longer-term decline in CD4 cell count in treated individuals was also found which may put individuals with already low CD4 cell counts at increased risk for AIDS. Other studies have found a similar effect of a decreased CD4 cell count

since then. Although the incidence of HCV co-infection among MSM in Europe has increased between 1990 and 2007, this increase appears too small to explain the decrease in CD4 cell counts at viral setpoint studied in chapters 2 and 4.

In chapter 6 we investigated the restoration of CD4 cell counts after the start of cART. Goal was to determine the optimum timing of antiretroviral therapy if the goal of antiretroviral therapy is the restoration of CD4 cell counts to levels seen in HIV-negative individuals. Male gender, sub-Saharan origin (as compared to Western European/North American origin), HIV infection through intravenous drug use, and a lower pre-cART HIV RNA were all associated with a longer time from the start of cART to reaching a CD4 count of 800 cells/mm^3 or higher (the mean CD4 cell count in HIV-negative MSM reported in other literature). Results suggested that restoration of CD4 counts to 800 cells/mm^3 or more is feasible within 7 years of cART initiation in approximately 50% of individuals when cART is started at a CD4 count above 350 cells/mm^3 and suppression of viral replication is achieved and maintained thereafter. Age older than 50 years at the start of treatment and experiencing periods of detectable viremia (>500 copies/ml) were associated with smaller increases in CD4 cell count after 7 years and reaching a plateau at less than 800 cells/mm^3. Recommendations on when to initiate cART in US guidelines have since then changed to recommending an earlier initiation of treatment (before counts have dropped below 350 cells/mm^3). Since 2012 guidelines recommend cART in all HIV-infected individuals. As a result, an increasing number of HIV-1-positive individuals start cART at high CD4 cell counts. In Chapter 7 we investigated whether starting cART at high CD4 cell counts makes restoration to levels of CD4 and CD8 cell counts, as well as their ratio seen in HIV-uninfected individuals more likely. Trajectories of CD4 and CD8 cell count and their ratio were modelled in individuals on continuous virologically successful cART. These trajectories can be regarded as reference estimates of the maximum capacity of cART to restore these immune system markers. An early start, at high CD4 counts of 500 cells/mm^3 or higher makes it more likely that CD4 cell count during virologically suppressive cART will reach levels comparable to those in HIV-negative individuals after 8 years on cART. However, long-term CD8 cell counts in HIV-positive individuals remain higher compared to counts in HIV-negative individuals of the same age and gender. The long-term CD4:CD8 ratio thus remains below ratios seen in HIV-negative individuals, even when cART is started with high CD4 cell counts. The clinical implication of not reaching levels of CD8 cell counts and CD4:CD8 ratios as seen in HIV-negative individuals requires further study. It may be that associations between either CD8 cell count or CD4:CD8 ratio and clinical outcome are only limited to specific causes of morbidity or death.
In the last chapter these finding are placed into context of other studies.

Samenvatting

Humaan immuundeficiëntievirus (HIV) infectie heeft wereldwijd de dood van miljoenen mensen veroorzaakt. Zonder therapie leidt HIV-infectie tot progressieve immunosuppressie en AIDS (het scala aan opportunistische infecties, neurologische ziekten en kwaadaardige maligniteiten die kunnen ontstaan bij een verzwakt immuunsysteem) en uiteindelijk de dood. De momenteel beschikbare antiretrovirale therapie kan HIV-infectie niet genezen, maar mits therapie wordt gestart voordat het immuunsysteem ernstig is aangetast en therapie effectief is in het onderdrukken van het virus in perifeer bloed tot onder de detectiegrens, is de levensverwachting van geïnfecteerde individuen nagenoeg gelijk aan die van niet geïnfecteerde individuen.

De staat van het immuunsysteem in HIV onderzoek wordt veelal beschreven door middel van het aantal CD4-T cellen in perifeer bloed. De concentratie HIV-deeltjes in plasma bereikt 6 maanden na primaire HIV-infectie min of meer een evenwicht, de afbraak van HIV door het immuunsysteem en aanmaak van nieuwe HIV-deeltjes is ongeveer gelijk. Dit stabiele niveau wordt wel het setpoint plasma virale load genoemd en een hogere setpoint plasma virale load is gerelateerd aan een snellere daling van het aantal CD4-T cellen, wat weer is gerelateerd aan een snellere progressie naar AIDS en dood. Een hogere setpoint virale load hangt ook samen met een grotere besmettelijkheid. Deze twee markers, plasma virale load en het aantal CD4 cellen, zijn de twee meest belangrijke biomarkers in het modelleren van HIV-morbiditeit en mortaliteit. Het overkoepelende doel van dit proef-

schrift was het bestuderen van trends in setpoint plasma HIV-1 virale load en het aantal CD4 cellen voor en na de introductie van effectieve antiretrovirale therapie.

Omdat deze markers een grote impact kunnen hebben op het beloop van HIV-infectie en de HIV-epidemie modelleerden we in hoofstukken 2, 3 en 4 trends over kalendertijd in de setpoint virale load en de op hetzelfde moment gemeten aantal CD4 cellen. Ook modelleerden we trends in de snelheid van daling van het aantal CD4 cellen na HIV-infectie en voor de start van antiretrovirale therapie. Nadat het virus tot onder de detectiegrens is onderdrukt na het starten van therapie, ligt de focus vooral op het herstel van het aantal CD4 cellen en (in mindere mate) op het herstel van andere componenten van het immuunsysteem zoals het aantal CD8 cellen en de ratio tussen CD4 en CD8 cellen (CD4:CD8 ratio). In hoofdstuk 6 en 7 modelleren we lange termijnveranderingen in deze biomarkers na de start van antiretrovirale virusonder-drukkende therapie. In hoofdstuk 5 bestudeerden we veranderingen in de plasma virale load en het aantal CD4 cellen in behandelde en onbehandelde HIV-geïnfecteerde individuen na antigene stimulatie door co-infectie met het hepatitis C virus (HCV).

In al deze studies is data gebruikt van HIV geïnfecteerde deelnemers aan het AIDS-therapie evaluatie in Nederland (ATHENA) cohort. Dit cohort wordt beheerd door de Stichting HIV Monitoring (SHM). In sommige hoofdstukken in dit proefschrift wordt data gebruikt die afkomstig is van homoseksuele deelnemers aan de Amsterdam Cohort Studies met betrekking tot HIV-infectie en AIDS (ACS). Het ACS en ATHENA cohort omvatten samen bijna de hele periode van de HIV-epidemie in Nederland. Het bestuderen van de samenhang of associatie (2 factoren zijn gerelateerd aan elkaar maar zijn niet noodzakelijkerwijs causaal gerelateerd) van risicofactoren op ziekten is alleen mogelijk door middel van data van observationele cohorten (zoals het ACS en ATHENA cohort). Analyses die gebruik maken van observationele data zijn over het algemeen gevoeliger voor bias dan analyses op data afkomstig uit gerandomiseerde klinische studies. Het is dan ook een uitdaging om de analyses van observationele cohort data dusdanig uit te voeren dat de uitkomstmaat niet alleen associatieve maar ook een causale interpretatie heeft.

Franse, Deense, Zwitserse en Noord-Amerikaanse cohorten vonden geen significante trend over tijd in setpoint virale load. Daarentegen werd een stijgende trend gevonden in een Italiaans cohort en een samenwerkingsverband van een aantal Europese en Noord-Amerikaanse cohorten. Verschillen in de studiepopulatie, geografisch gebied, tijdsperiode, uitkomstmaat en statistische methode kunnen mogelijk de verschillende studieresultaten verklaren. Een studie naar virale replicatieve fitness die gebruik maakte van ACS data vond een toename in de replicatieve capaciteit van HIV in bloed van individuen met HIV seroconversie in de periode 1996-2003 ten opzichte van dat van individuen met seroconversie in 1986. Hiermee in overeenstemming vonden we in hoofdstuk 2 een

stijgende trend over tijd (tussen 1984 en 2007) in de setpoint plasma virale load. Het aantal CD4 cellen gemeten op hetzelfde moment als de setpoint virale load, liet een bijpassende significant dalende trend over dezelfde tijdsperiode zien. Het is onwaarschijnlijk dat de gevonden trends verklaard kunnen worden door veranderingen in het gebruik van het type HIV RNA test, veranderingen in CD4 meettechniek, of verschillen in de verdeling van HIV-subtype onder individuen met recente HIV-seroconversie.

De verdeling van een aantal host-genetische factoren die geassocieerd zijn met een lagere setpoint virale load (genotypen HCP5 rs2395029, –35HLA-C rs9264942, en CCR5wt/Δ32) in individuen met een recente HIV-infectie was niet veranderd over tijd en kan dus niet de toename in setpoint virale load over tijd verklaren. De resultaten van hoofdstuk 3 suggereerden wel dat het beschermend effect van de minor allele van rs2395029 in het HCP5 genotype bij individuen verdwenen was bij degenen die geïnfecteerd waren in een latere tijdsperiode (tussen 2003 en 2009). Een verklaring voor de toename in setpoint virale load over tijd kan dus mogelijk aanpassing van HIV aan deze host-genetische factor zijn.

Progressie naar AIDS of dood is in het tijdperk van combinatie antiretrovirale therapie (cART) lastig te bestuderen omdat deze eindpunten nauwelijks voorkomen bij een effectieve behandeling. In hoofdstuk 4 bestudeerden we daarom veranderingen over tijd in de snelheid van de daling in het aantal CD4 cellen na HIV-infectie en voordat cART wordt gestart, een surrogaat marker voor ziekteprogressie. We vonden een trend van een snellere daling in het aantal CD4 cellen vanaf ongeveer 1993. De combinatie van een sterkere daling en de in hoofdstuk 2 gevonden lagere CD4 cel aantallen 9 maanden na HIV seroconversie resulteerde in een kortere tijd tussen HIV seroconversie en het bereiken van 350 CD4 cellen/ mm^3 (de cut-off waarvoor therapie gestart moet zijn volgens de behandelrichtlijnen in deze tijd) van meer dan 4 jaar in de periode voor 1996 tot 2.7 jaar in de periode 2003-2007.
De trend van een sterkere daling in het aantal CD4 cellen over tijd was alleen borderline significant in het model waar het tijdstip van censurering (door het starten van antiretrovirale therapie of progressie van ziekte) gelijktijdig werd gemodelleerd met de longitudinale metingen van het aantal CD4 cellen. De trend was niet significant in mixed-effects modellen. Waarschijnlijk komt dit doordat het censureren van CD4 cel metingen informatief is (de gecensureerde niet geobserveerde CD4 cel aantallen zijn systematisch verschillend van die van individuen die niet gecensureerd zijn). Het schatten van parameters in modellen waarin gelijktijdig de longitudinale metingen en de timing van censurering wordt gemodelleerd is complexer dan standaard longitudinale modellen en deze modellen worden momenteel niet routinematig gebruikt. Een verklaring voor de minder steile CD4 cel daling gerapporteerd door andere studies vergeleken met de resultaten in hoofdstuk 4 kan dus het gebruik zijn van mixed-effects modellen in plaats van 'joint' modellen.

Setpoint virale load is deels overerfbaar; degene die HIV overdraagt en degene die geïnfecteerd wordt hebben een vergelijkbare setpoint virale load. Door de introductie van cART in Nederland in 1996 en de algemene trend in Nederland om steeds vroeger in de infectie te starten met therapie (bij een hoger aantal CD4 cellen) wordt de periode dat HIV kan worden overgedragen op een ander individu hoofdzakelijk beperkt tot de periode tussen het moment van HIV-infectie en de start van cART. Hierdoor worden HIV stammen geselecteerd die gerelateerd zijn met een hogere setpoint virale load. Dit kan de toename in setpoint virale load en de daling in het CD4 cel aantal ten tijde van het virale setpoint in meer recente jaren (gedeeltelijk) verklaren.

Acute co-infectie met syfilis, malaria, herpex simplex virus en andere opportunistische infecties in HIV-geïnfecteerde individuen gaat vaak samen met een toename in HIV virale load en een daling in het aantal CD4 cellen. Co-infectie met het hepatitis C virus (HCV) komt vrij veel voor bij homoseksuele HIV-geïnfecteerde mannen, maar deze trends waren voor HCV niet eerder gerapporteerd. In hoofdstuk 5 beschreven we een tijdelijke toename in de kans op een detecteerbare HIV virale load rond het moment van acute HCV co-infectie in chronisch HIV-geïnfecteerde individuen behandeld met virologisch onderdrukkende cART. Dit ging gepaard met een langdurigere afname in het aantal CD4 cellen. Hierdoor hebben individuen met een al laag aantal CD4 cellen mogelijk een verhoogd risico op AIDS. Een aantal andere studies hierna hebben een soortgelijke daling in het aantal CD4 cellen na acute HCV co-infectie beschreven. Hoewel de incidentie van HCV co-infectie bij HIV geïnfecteerde homoseksuele mannen sterk is toegenomen, lijkt de toename te klein om een substantieel deel te kunnen verklaren van de daling van het aantal CD4 cellen ten tijde van het virale setpoint dat in in hoofdstuk 2 en 4 werd beschreven.

In hoofdstuk 6 werd het herstel van het aantal CD4 cellen na het starten van cART bestudeerd, met als doel het bepalen van het optimale startmoment van cART, ervan uitgaande dat herstel van het aantal CD4 cellen naar het gemiddelde niveau in de HIV ongeïnfecteerde populatie het doel is van cART. De tijd tussen het starten van cART en het bereiken van een CD4 aantal van 800 cellen/mm^3 (het gemiddeld aantal CD4 cellen in HIV-negatieve homoseksuele mannen gerapporteerd in andere studies) was langer bij mannelijke individuen, individuen afkomstig uit sub-Sahara Afrika (vergeleken met individuen uit Noord-Amerika of Europa), bij HIV-infectie door intraveneus drug gebruik, en bij een lagere concentratie HIV RNA in plasma bij de start van cART. De grens van 800 CD4 cellen/mm^3 wordt binnen 7 jaar na het starten van cART bereikt door tenminste 50% van de individuen die starten met 350 CD4 cellen/mm^3 of meer, mits cART het virus de hele tijd tot onder de detectiegrens onderdrukt. Hogere leeftijd bij de start van cART en het doormaken van periodes met meer dan 500 HIV RNA copies/ml in plasma waren gerelateerd met kleinere toenames en met het bereiken van een plateau in het aantal CD4 cellen. Amerikaanse richtlijnen over de timing van

de start van cART zijn sindsdien veranderd naar een eerdere start (voordat het niveau van 350 CD4 cellen/mm^3 is bereikt). In 2012 zijn de richtlijnen verder aangepast en werd aanbevolen cART te starten in alle HIV geïnfecteerde individuen. Daardoor is er een steeds groter wordende groep individuen die cART starten met een hoog aantal CD4 cellen. In hoofdstuk 7 onderzochten we of herstel van zowel het aantal CD4 en CD8 cellen en de CD4:CD8 ratio naar het niveau van dat in HIV-negatieve individuen mogelijk is wanneer cART wordt gestart met een hoog aantal CD4 cellen. In longitudinale modellen werden metingen geïncludeerd afkomstig van ART-CC, een samenwerkingsverband van 21 Noord-Amerikaanse en Europese cohorten. Metingen afgenomen nadat een individu voor langere tijd gestopt was met cART of tijdens of na een periode waarin het virus detecteerbaar was werden gecensureerd. Het geschatte beloop van het aantal CD4 en CD8 cellen en de CD4:CD8 ratio kan worden geïnterpreteerd als het maximaal mogelijk herstel van deze immuunmarkers na het starten van cART. Een vroege start, bij 500 CD4 cellen/mm^3 geeft na 8 jaar cART een grotere kans op het behalen van een aantal CD4 cellen dat vergelijkbaar is met die in HIV-negatieve individuen van hetzelfde geslacht en dezelfde leeftijd. Het lange-termijnniveau van het aantal CD8 cellen blijft daarentegen hoger dan die in HIV-negatieve individuen. De lange termijn CD4:CD8 ratio blijft dan ook onder het niveau van ratio's in HIV-negatieve individuen, ook als cART wordt gestart met een hoog aantal CD4 cellen. De klinische betekenis van het niet behalen van een vergelijkbaar aantal CD8 cellen en een vergelijkbare CD4:CD8 ratio als in HIV-negatieven heeft meer onderzoek nodig. Het kan zijn dat de associatie tussen het aantal CD8 cellen of de CD4:CD8 ratio en klinische eindpunten zich beperkt tot specifieke ziekten of doodsoorzaken.

In het laatste hoofdstuk werden deze bevindingen in de context van de resultaten van andere studies geplaatst.

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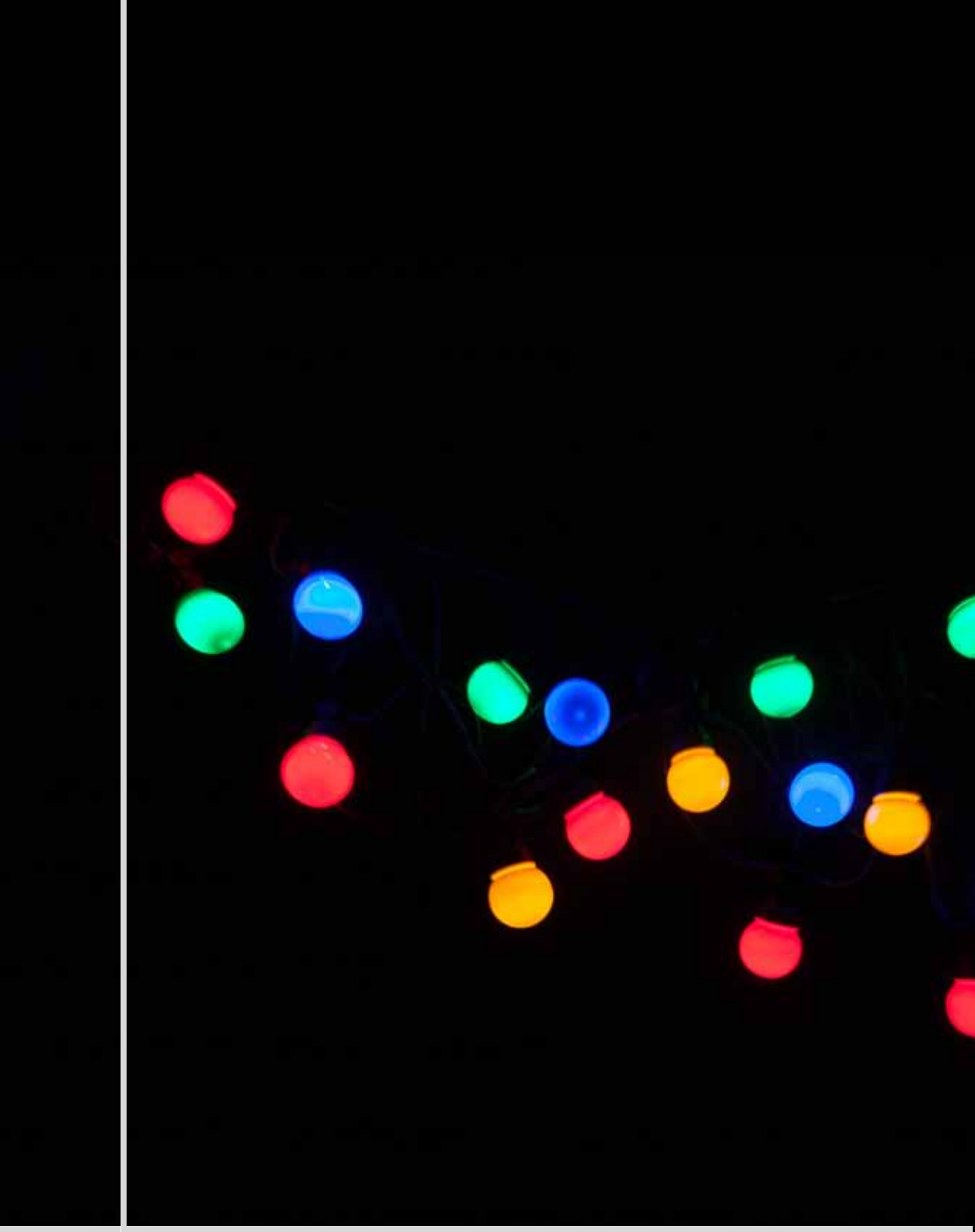
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