The 'Triple Study': viral dynamics and immune reconstitution in HIV-1 infection during potent antiretroviral therapy
Notermans, D.W.
CHAPTER I

INTRODUCTION

Daan W. Notermans
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1 THE NATURAL HISTORY OF HIV INFECTION

1.1 HIV disease

Infection with the human immunodeficiency virus (HIV) generally leads to a progressive immunodeficiency, which becomes clinically manifest as the acquired immunodeficiency syndrome (AIDS) and is ultimately lethal. The immune deficiency is hallmarked by a dysfunction and gradual depletion of CD4+ T lymphocytes.

AIDS is diagnosed upon the occurrence of a specific opportunistic infection, such as Pneumocystis Carinii Pneumonia (PCP), a malignancy such as Kaposi’s sarcoma, or neurological symptoms such as encephalopathy (AIDS-related dementia). The CDC (Centers for Disease Control) in the United States classify HIV-disease in three groups: ‘A’ for asymptomatic infection or symptomatic acute infection, ‘B’ for certain minor symptoms of immunodeficiency such as oral candidiasis and ‘C’ for AIDS.

HIV can be transmitted sexually (homo- and heterosexually), through blood (transfusions, blood products or needle sharing) or from mother to child (in utero, intrapartum or through breast milk). Upon infection, the virus rapidly replicates to cause a viremia, with sometimes more than $10^7$ viral RNA copies per mL plasma or serum, and a drop in CD4+ T-cell counts. The acute infection can manifest itself clinically as an influenza- or mononucleosis-like illness in 50 to 70% of the cases. Within a few weeks to months, the viremia level decreases and the CD4+ T-cell counts increase, although not to pre-infection levels. During the clinically asymptomatic phase that follows, viral replication continues and increases over the years, while CD4+ T-cell counts slowly decrease. As the immune deficiency progresses, the risk of clinical symptoms and AIDS increases. Before the availability of potent antiretroviral treatment, the average time from infection to AIDS was 8-10 years, although considerable variation exists and a small group of subjects shows no sign of disease progression after more than 15 years of infection. Death followed on average one to two years after the AIDS diagnosis.

In December 1999, the number of HIV-infected individuals was estimated to be nearly 34 million world wide, 96% of whom live outside the Western World, and over 16 million people have died of AIDS since the onset of the epidemic.

1.2 The viral lifecycle

HIV-1 was first isolated in 1983 and was originally named human T-lymphotropic virus (HTLV-III) or lymphadenopathy-associated virus (LAV). It is a retrovirus of the lentivirus subfamily and infects cells
expressing CD4 as receptor, like CD4+ T lymphocytes and cells of monocyte/macrophage lineage. Two HIV subtypes are currently identified: HIV-1, the most prevalent and virulent, and HIV-2, mainly prevalent in West-Africa. The closely related primate lentivirus simian immunodeficiency virus, SIV, can be studied as an animal model for HIV.

The HIV-1 genome, consisting of two positive strand RNA molecules of approximately $10^4$ base pairs each, contains three typical retroviral genes: gag, pol and env, each encoding a number of different proteins. Gag encodes four structural proteins: the capsid protein p24, the matrix protein p17, the nucleocapsid protein p7 and p6. Pol encodes the enzymes protease, reverse transcriptase (RT) and integrase and env encodes the envelope glycoproteins gp41 and gp120. In addition, HIV-1 has a number of accessory or regulatory gene products, tat, vpr, nef, vif, rev, and vpu, which have different functions in the regulation of viral replication. The genome is flanked by two identical long terminal repeat (LTR) sequences that play an important role in the integration and the regulation of transcription.

HIV particles are 100 nm in diameter and are surrounded by a lipid bilayer membrane, derived from the infected host cell. The membrane is spiked with the envelope glycoproteins, consisting of two noncovalently bound subunits of the gp41 through the membrane and gp120 on the outside. Inside, p17 lines the membrane and p24 forms a cone-shaped core. Within the core are two copies of the viral RNA, surrounded by the nucleocapsid protein p7. In addition, the viral particle contains RT, protease and integrase (figure 1).

The first step in the infection of a susceptible cell is the binding of the viral envelope glycoprotein gp120 to the cellular CD4 molecule, allowing interaction of gp120 with a second receptor required for infection: a chemokine receptor, like CXCR4 or CCR5. This causes a conformational change of gp41, resulting in fusion of viral and cellular membranes and entry of the viral core into the cell. The viral RNA is reverse transcribed into double stranded DNA by RT, which is transported to the nucleus, where it is integrated into the cellular DNA. The viral life cycle can be halted before integration, leaving extrachromosomal viral DNA in the infected cell. This DNA can be linear, or can, through auto-integration or other processes, circularize, containing one or two LTRs. A considerable portion of the integrated viral DNA may not code for replication-competent virus. The integrated viral DNA is subsequently transcribed into mRNA, which is translated into proteins. The gag and pol genes are translated as protein precursors, which are subsequently cleaved by protease into the different gag and pol proteins, necessary for the formation of mature viral particles. Without protease activity, viral particles are still formed, but they are not infectious.
Chapter 1. Introduction

HIV

integrase
RNA
core (p24)
matrix (p17)
reverse transcriptase
protease
gp120
gp41

CD4\(^+\) cell

CD4 and co-receptor
HIV RNA
double stranded viral DNA
nucleus
cellular DNA
mRNA and genomic RNA
gag-pol precursor proteins

1. receptor binding
2. virus-cell fusion and entry
3. Uncoating and release viral RNA
4. reverse transcription
5. integration
6. transcription
7. translation
8. protease cleavage
9. viral assembly
10. budding of new virions

Fig. 1. The viral lifecycle.
1.3 Viral dynamics

Studies by Wei et al. and Ho et al., published early 1995, gave an important insight into the replication dynamics of HIV.[71] These studies were possible because of two developments in HIV research: 1) the introduction of a new, potent class of antiretroviral drugs, the protease inhibitors (PIs), capable of strongly suppressing viral replication, and 2) the development of reliable and sensitive methods to quantify the amount of virus, the 'viral load', by measuring the amount of HIV RNA in serum or plasma. By blocking viral replication and measuring the decline in the amount of virus in the blood, the magnitude of the viral replication before the start of treatment could be estimated. Mathematical models, incorporating the turnover of infected cells, were fitted to the data observed in patients, to describe the dynamics of HIV replication. The models were further refined by adding more frequent measurements and using combination therapy of a PI plus nucleoside analogue RT inhibitors (NRTIs) (See Sections 2.2, Combination therapy and 2.4, Sanctuary sites).[73]

These studies showed that, in spite of the apparently stable situation during the clinically asymptomatic phase, HIV infection is a highly dynamic process. Despite the limited changes in CD4+ T-cell counts and HIV-RNA levels in the short term and a low fraction of CD4+ T cells harboring viral DNA, there is no virologically latent phase.[66,75] Free virus in blood plasma was estimated to have, on average, a life span of 0.3 days (corresponding to half-life $t_v$ of six hours) and productively infected cells 2.2 days ($t_v$ of 1.6 days). A total of $10^{10}$ virions were estimated to be produced and cleared each day in an infected individual.[73]

1.4 HIV in lymphoid tissues

Lymphoid tissue (LT), consisting of lymph nodes, tonsils, spleen, and gut-associated lymphoid tissue, is the primary site of HIV replication.[75-77] Within days to weeks following infection, the virus is disseminated to CD4+ T lymphocytes in LT throughout the body.[46,78,79] As the disease progresses, the tissue is depleted of CD4+ T lymphocytes and the network of follicular dendritic cells (FDCs) is destroyed.[76,80] In contrast, in long-term non-progressive infected individuals the lymph node architecture remains intact.[81]

The vast majority, possibly 98%, of all CD4+ T lymphocytes in the body resides in LT, with considerable trafficking of cells.[82] Within two weeks following infection of rhesus macaques with SIV, the virus is rapidly disseminated throughout the body and infected CD4+ T lymphocytes are by far the largest source of viral RNA. This is also seen in HIV-infected humans during acute and recent infection and during most of the chronic disease phase.[46,78] Only small numbers of productively infected macrophages and other macrophage-derived antigen-presenting cells such
as dendritic cells are usually found. It has been suggested, however, that these cells might play a role in the spread of the virus by attracting, activating and subsequently infecting CD4⁺ T lymphocytes, a process that is believed to occur already during initial infection. At late stage disease, during opportunistic infections, infected macrophages can become an important producer of viral RNA. Next to virus in productively infected mononuclear cells, large amounts of HIV can be found on the surface of FDCs during chronic infection. The FDCs are not productively infected, as indicated by the lack of intracellular proviral DNA and mRNA, but hold viral antigens and complete virions produced by the mononuclear cells on their surface. Although the viral particles are covered with antibodies, they remain infectious and might act as the source of infection for CD4⁺ T lymphocytes residing in or trafficking through the LT. Precise quantification of virus with in situ hybridization, allowing quantification of viral RNA copies simultaneously with microscopic identification of the cells involved, showed that LT contains 10² to 10⁴ as much virus as the blood and that by far the largest pool of virus is FDC-associated.

1.5 Immunodeficiency

Although CD4⁺ T-lymphocyte depletion is the most prominent feature of HIV infection, the virus exerts a complex combination of immunological effects. As HIV causes a slowly progressive infection of the immune system, the observed effects can be either, or possibly both, an effect of elicited antiviral immune responses and damage to the infected organ-system. Different subtypes of CD4⁺ and CD8⁺ T cells change with different patterns during infection. Furthermore, both activation and dysfunction of both the cellular and the humoral immune system are observed, starting already early in the infection before a CD4⁺ T-cell depletion becomes notable, with a progressive increase over the course of the disease.

Shortly after HIV infection, total CD8⁺ T-cell counts increase and remain elevated during the course of the disease, until they decline towards the onset of AIDS. The increase in CD8⁺ and decrease in CD4⁺ T-cell counts progressively disturbs the ratio between the two cell types. Both CD4⁺ and CD8⁺ T lymphocytes can be subdivided into naive cells, which have not yet encountered their specific antigen, and memory cells. A marker to distinguish the two cell types is CD45, of which two isoforms exist: CD45RA⁺ for naive cells and CD45RO⁺ for memory cells. As memory cells can switch back from CD45RO⁺ to CD45RA⁺ during differentiation, a more refined distinction can be made using CD62L (L-selectin) or CD27; T cells positive for CD45RA plus CD62L or CD27 can be regarded as truly naive. Naive cells of both CD4 and CD8 lineage are preferably depleted during HIV infection, whereas CD8⁺ memory T cells rapidly expand, which explains the increase in total CD8⁺ T-cell
Both naive and memory CD4+ T cells can be infected by HIV, but higher rates of HIV infection are generally found in CD4+CD45RO+ T cells. CD4+CD45RA+ T cells are primarily infected by syncytium inducing (SI) viral strains, which usually appear at a later stage of the infection.

In HIV-infected subjects different signs of chronic immune activation can be recognized. T-cell activation can be characterized by an increased expression of markers such as CD38 and HLA-DR in CD4+ and CD8+ T cells and according to some studies even more specifically in CD8+CD45RO+ T cells. The expression of CD38 and HLA-DR in CD8+ T cells is already markedly elevated early in the infection and increases further with disease progression.

B-cell activation is characterized by a hypergammaglobulinemia, including HIV-1 antigen-specific and non-specific antibodies, suggesting both polyclonal and virus-specific activation. Other signs of the immune activation include an increase in a number of inflammatory molecules in serum, like neopterin, β2-microglobulin and soluble tumor necrosis factor receptors.

The dysfunction of T lymphocytes is reflected by a poor in vitro proliferative response to antigens and to aspecific stimulating agents, such as pokeweed mitogen and phytohemagglutinin (PHA) or monoclonal antibodies (mAb) to CD3 with or without CD28 for T cells. In addition, a decreased T-cell response to specific antigens can be observed. CD4+ T-helper cell function measured as proliferative responses to a number of recall antigens such as cytomegalovirus (CMV), tuberculin and tetanus toxoid is decreased. Also, CTL precursor frequencies to mycobacterial and candida antigens are decreased.

The disturbances of the immune system are further shown by perturbation of CD4+ and CD8+ T-cell antigen receptor (TCR) repertoires, which become stronger with lower CD4+ T-cell counts. These changes could either reflect clonal expansions or clonal deletions.

Infection with HIV leads to cellular immune responses of both CTL and CD4+ T-helper cells, which are believed to play a role in the observed suppression of HIV-RNA levels following the peak of acute infection. Longitudinal follow-up of infected subjects shows a loss of gag-specific CTL responses coinciding with declining CD4+ T-cell counts and the development of AIDS. Differences in HIV-1-specific CTL-activity and CD4+ T-helper cell activity between rapid progressors and non-progressors further support the suggested protective role of antiviral immunity in the disease pathogenesis. Furthermore, the antiviral immune responses inversely correlate with HIV-RNA levels.

In most cases, however, control of the virus ultimately fails. It has been suggested that T-helper cell function is necessary to maintain effective CTL responses, as also appears from the observed association between
strong CTL and strong T-help responses in HIV infection. Activation of HIV-specific CD4+ T-helper cells makes them more susceptible to infection, which might explain their decline over the course of the disease. Loss of T-helper function may consequently result in loss of CTL activity. HIV has more ways to escape the immune responses. Through mutations in the CTL-epitopes the virus can reduce or evade CTL recognition. Also, HIV has been shown to down-regulate MHC expression in infected cells, thereby possibly evading immune recognition of productively infected cells. The pool of latently infected CD4+ T cells, established early in the infection and containing potentially infectious virus upon activation, will not be recognized by the antiviral immunity.

1.6 T-cell turnover in HIV infection

The exact mechanisms underlying the HIV-associated CD4+ T-cell depletion are not clear. Viral replication appears to be the driving force, considering the very high viral turnover, the association of high HIV-RNA levels with low CD4+ T-cell counts as well as with increased immune activation, the prediction of CD4+ T-cell decline by high viremia levels, and the correlation of suppression of viral RNA levels with the increase in CD4+ T-cell counts during antiretroviral treatment.

The observed gradual CD4+ T-cell depletion during the infection results from an imbalance between CD4+ T-cell production and consumption. Considerable debate exists, however, on the magnitude of the two processes. The blocking viral replication was not only used to gain insight into the viral dynamics as described in section 1.3 ‘Viral dynamics’, but also to study CD4+ T-cell turnover. During the first few weeks following potent antiretroviral therapy CD4+ T-cell counts increased rapidly. It was considered to be a reflection of pretreatment production and consumption, which was estimated to be around 2 x 10^9 cells per day in an infected person. This overlooks, however, the possibility of extensive trafficking of cells, with trapping of CD4+ T cells in the lymphoid tissue during the infection, and subsequent redistribution into the circulation upon start of treatment.

A number of studies using different methodologies has been undertaken to quantify CD4+ T-cell turnover more directly. The telomere length, used as reflection of the replicative history of cells, decreased in CD8+ T cells and not in CD4+ T cells over the course of the infection. As HIV preferentially infects and depletes dividing CD4+ T cells, which have more rapidly shortening telomere lengths compared to the remaining cells, mathematical modeling was used to account for this and indicated only a limited increase in CD4+ T-cell turnover. BrdU labeling to mark proliferating cells in SIV-infected macaques indicated a two to six fold increase in CD4+ T-cell death rate compared to uninfected controls and
also found increased turnover rates for CD8$^+$ T cells and B cells.\textsuperscript{[176]} A two- and six fold increase in CD4$^+$ and CD8$^+$ T-cell turnover, respectively, has been suggested based on expression of Ki-67, a marker of cell proliferation, with increased Ki-67 expression in subjects with lower CD4$^+$ T-cell counts.\textsuperscript{[177]} Another study found an increase in Ki-67 mainly in CD8$^+$ T cells and not in CD4$^+$ T cells early in the infection.\textsuperscript{[178]} Measuring cell proliferation by labeling DNA with stable isotope (Deuterium)-labeled glucose showed no difference in absolute production rates of CD4$^+$ T cells between HIV-seronegative and -positive individuals, but showed a reduction of CD4$^+$ T-cell half-life to one third.\textsuperscript{[179]} To summarize, the different methods used to measure CD4$^+$ T-cell turnover agree in a limited, two to six fold, increase during HIV infection compared to uninfected controls.

A number of mechanisms by which HIV causes destruction of CD4$^+$ T cells has been proposed. These include direct viral killing of infected cells as observed in vitro,\textsuperscript{[180]} CTL-mediated killing of infected- and possibly uninfected ‘innocent bystander’ CD4$^+$ T cells,\textsuperscript{[181]} toxicity of viral products like tat or gp120,\textsuperscript{[182,183]} or apoptosis following viral-induced cell activation.\textsuperscript{[184]}

On the other hand, T-cell renewal appears to be compromised during HIV infection.\textsuperscript{[185]} HIV reduces CD34$^+$ hematopoetic progenitor cell function in bone marrow, although these cells are not productively infected.\textsuperscript{[186,187]} Also, the ability of CD34$^+$ progenitor cells from the peripheral blood to develop into thymocytes in vitro in a fetal thymic organ culture system is significantly impaired.\textsuperscript{[188,189]} Furthermore, HIV infects thymocytes, using CXCR4 as co-receptor, and thymic stromal cells and causes thymic involution, either by direct infection or by indirect mechanisms.\textsuperscript{[190-192]}

In conclusion, although the exact mechanism of CD4$^+$ T-cell destruction is not clear, HIV infection increases CD4$^+$ T-cell turnover and limits T-cell renewal. With the switch in viral phenotype from non-syncytium inducing (NSI) to SI (see Section 1.7.3, Virus-host interactions: viral phenotype and co-receptors), the virus broadens its co-receptor usage to include CXCR4. This allows HIV to infect thymocytes and naive CD4$^+$ T cells, increasing the pressure on T-cell renewal.

1.7 Markers of disease progression and of therapy effects

As the rate of disease progression is highly variable and a small subset of individuals remains asymptomatic for many years,\textsuperscript{[28]} a considerable amount of research for predictive markers of disease progression has been performed. The markers can roughly be divided into three groups: 1) immunological markers, either reflecting the immunological damage done, the extent of immunodeficiency, or the state of immune activation, 2) virological markers, usually reflecting the amount of virus present in plasma, the so-called ‘viral load’ or ‘viral burden’, and 3) factors
determining the interaction between virus and host, such as viral phenotype related to co-receptor usage, and host-variation in co-receptors.

Prognostic markers are generally evaluated for their ability to predict the occurrence of serious clinical problems, i.e., AIDS and/or death. The ability to delay these problems was originally used to evaluate therapy effects. As antiretroviral therapy has become more successful in preventing clinical disease progression and as more drugs have become available to allow alternative treatments before serious clinical problems occur, 'surrogate' markers for clinical outcome have become more important to evaluate the effects of antiretroviral therapy.\textsuperscript{193, 197}

\section*{1.7.1 Immunological markers}

\subsection*{1.7.1.1 CD4\textsuperscript{+} T-cell counts}

The number of CD4\textsuperscript{+} T cells in the peripheral blood is one of the first and remains one of the most widely used markers in evaluating HIV disease status. Decreased CD4\textsuperscript{+} T-cell counts reflect the damage done to the immune system by HIV and thereby the risk of opportunistic infections.\textsuperscript{1, 2, 198-200} Early in the infection, however, CD4\textsuperscript{+} T-cell counts are generally within normal range and other markers, such as plasma HIV-RNA levels are necessary to further distinguish the different stages of disease progression.\textsuperscript{156, 160} Other problems associated with CD4\textsuperscript{+} T-cell counts are the considerable diurnal fluctuation and the interlaboratory variation.\textsuperscript{201, 202}

A number of studies with NRTI mono- and double therapy showed significant changes in CD4\textsuperscript{+} T-cell counts, and in some studies these changes were predictive of clinical disease progression. However, changes in CD4\textsuperscript{+} T-cell counts did not explain all progression to AIDS and changes in HIV-RNA levels were found to be better predictors.\textsuperscript{203-205} In several studies in multivariate analyses, CD4\textsuperscript{+} T-cell counts even lost their predictive value when HIV-RNA levels were included.\textsuperscript{206, 207} In the Delta study, CD4\textsuperscript{+} T-cell changes at week 4 and 8, adjusted for baseline counts, were not prognostic.\textsuperscript{208}

\subsection*{1.7.1.2 T-cell function and -activation}

The \textit{in vitro} proliferative response to CD3 mAbs is predictive of progression to AIDS, independent of CD4\textsuperscript{+} T-cell counts, and the predictive value increases later in the infection.\textsuperscript{104, 200, 209} The reactivity to CD3 mAbs plus CD28 mAbs is a stronger marker than the reactivity to CD3 mAbs alone and is independent of low CD4\textsuperscript{+} T-cell counts and high HIV-RNA levels.\textsuperscript{127, 210} Although reactivity to CD3 mAbs has been tested in various NRTI and PI monotherapy regimes, its predictive value during therapy has not been evaluated.\textsuperscript{211-214}
Several studies have found that immune activation, and more specifically CD38 expression in CD8\(^+\) T cells, is a predictive marker for clinical disease progression.\(^{[115,116]}\) The expression of CD38 in both CD4\(^+\) and CD8\(^+\) T cells correlates with the HIV-RNA levels in the blood.\(^{[117]}\) In late-stage disease, CD38 expression appears to be an even stronger predictive marker than CD4\(^+\) T-cell counts or HIV-RNA levels.\(^{[113,120,214,215]}\)

With double NRTI therapy, the fraction of CD8\(^+\) T cells expressing CD38 and HLA-DR decreases, although not to normal levels despite suppression of HIV-RNA levels to below the cutoff level.\(^{[216]}\)

### 1.7.2 Virological markers

Most virological markers used are based upon quantification of the amount of virus, the 'viral load' or 'viral burden', usually in blood plasma or serum. Soon after the identification of HIV, serological markers (viral antigens and antibodies) and infectious virus titers were evaluated. Advances in molecular biology like the polymerase chain reaction (PCR) made it possible to quantify viral nucleic acids, like genomic RNA in serum or plasma, or intracellular viral DNA or mRNA in peripheral blood mononuclear cells. Measurements of viral RNA in plasma have become the most important in clinical research and routine practice and a number of commercially available assays has been developed.

#### 1.7.2.1 Antibodies and antigens

Within a few weeks to months following infection with HIV, antibodies to several viral proteins appear, among which are antibodies against the core protein p24 and against the envelope glycoproteins gp41 and gp120.\(^{[117,118]}\) Frequently, over the course of the disease, p24 antibodies disappear and circulating p24 antigen becomes detectable, which is prognostic for the development of AIDS.\(^{[199,219-225]}\)

Patients who rapidly progress to AIDS have lower or no detectable titers of p24 antibodies than patients who do not or only slowly progress. In contrast, antibodies to gp41 and gp120 remain relatively stable over the course of the infection and there is no distinction in gp120 antibody titers between fast progressors and long-term non-progressors.\(^{[219,221,224-229]}\) Immune complexing of p24 antibodies by p24-antigen and a difference in CD4\(^+\) T-helper cell dependence for production of the two different antibodies, could both explain the difference in patterns between the core and envelope antibodies.\(^{[220,222,225]}\)

A limitation in the use of p24-antigen and/or antibody is caused by patients who develop AIDS without becoming p24-antigen-positive and/or who remain antibody positive.\(^{[160,199,230-232]}\) Changes in p24-antigen concentrations with zidovudine monotherapy are not predictive of clinical disease progression, in contrast to changes in HIV-RNA levels.\(^{[233]}\) Also,
there is a limited number of patients with intermediate stages of disease (CD4$^+$ T-cell counts between 200 and 500 cells/μL), that is p24 antigen positive at start of therapy, which further limits the usefulness to evaluate antiretroviral therapy with p24 concentrations.$^{120,234}$

1.7.2.2 HIV-RNA levels

In persons with an established HIV-1 infection, the level of viral RNA in plasma can be considered to be a reflection of the production and clearance of virus.$^{71,244}$ Plasma HIV-RNA levels can be used to distinguish between rapid and slow disease progression.$^{129,235}$ High RNA levels at one year after infection predict a rapid disease development, while disease development and the underlying immunodeficiency is postponed when levels are low.$^{125,156,160,222,235}$ In contrast, CD4$^+$ T-cell counts can not be used to predict disease progression early in the infection, while five years following seroconversion, CD4$^+$ T-cell counts are a better prognostic marker than HIV-RNA levels.$^{200}$

As they directly reflect virus production, HIV-RNA levels are a good marker of therapy effects. Reduction of HIV-1 RNA levels with the use of antiretroviral therapy has been associated with a reduction in the risk of developing AIDS.$^{157,204-208}$ With the use of potent antiretroviral combination therapy, HIV-RNA levels decline to below the lower cutoff level of available assays within several weeks to months and these low levels can be sustained for months to years.$^{237,219}$ A rebound in HIV RNA reflects the lack of suppression of viral replication. This may be due to a reduction in medication levels or the development of viral resistance to the medication used.$^{71,240}$

Plasma HIV-RNA levels are a better marker for both disease prognosis and therapy evaluation than p24-antigen concentrations and HIV-RNA measurements have therefore replaced p24 in clinical practice and antiretroviral drug trials.$^{232,241,242}$ Limitations in the use of HIV-RNA levels are the considerable variability, which can be up to threefold or even more, due to technical and biological variation.$^{244}$ Furthermore, although protease inhibitors block the formation of infectious viral particles, DNA transcription and subsequent formation of non-infectious, RNA-containing particles is not blocked.$^{243,704}$

1.7.2.2.1 Methods of HIV-RNA quantification

For quantification of HIV-1 RNA, amplification of the target RNA followed by detection or amplification of the detection signal is needed. Synthetic calibrator RNA molecules of known concentrations, added before the amplification reaction, can be used to calculate the RNA concentration in the sample of interest. A number of assays is commercially available, based upon different amplification techniques: PCR amplification
following reverse transcription of viral RNA (RT-PCR), nucleic acid sequence based amplification (NASBA) and branched DNA signal amplification (bDNA).[245-247]

The increasing potency of antiretroviral therapy, more strongly suppressing HIV-RNA levels, has spurred the development of more sensitive assays to quantify HIV RNA at levels below 100 copies/mL.[248,250] These so-called ultrasensitive assays are used in increasing frequency in clinical trials and routine patient care.[197] The lowest RNA level that can be achieved with antiretroviral therapy in an ultrasensitive assay is indicative for the success of therapy.[251,251]

1.7.3 Virus-host interactions: viral phenotype and co-receptors

Viral as well as host factors influence disease progression and some have been evaluated as prognostic markers. Viral factors include the ability to induce syncytia in T-cell cultures and genetic defects like deletions in the nef-gene. Host factors include mutations in the co-receptor genes.

The presence of virus variants that induce syncytium formation in cultures of certain T-cell lines like MT2 cells is associated with a poor prognosis.[254,256] In general, HIV infection is established with non-syncytium-inducing (NSI) virus variants and in 50% of subjects SI-variants emerge during the course of the disease. AIDS can occur without this switch in viral phenotype, however.[257,258] The switch to SI is associated with a more rapid decline in CD4+ T-cell counts, an increase in infectious virus titers and subsequent clinical disease progression.[159,255,256,259] NSI variants are largely restricted to usage of CCR5 as co-receptor to infect CD4+ T cells. SI variants have a broader co-receptor usage, including CXCR4, allowing infection of more cell types, such as naive CD4+ T cells and thymocytes.[111,112,190,192,260,262]

Host factors can also influence the interaction of HIV with the co-receptors. Heterozygous mutations in genes coding for chemokine receptors or for the natural ligand of CXCR4, SDF-1, can slow down disease progression. A homozygous 32 base pair deletion in CCR5 can even cause resistance to HIV infection.[263,266]

In conclusion, several immunological, virological and host factors influence disease progression during HIV infection and have been studied as markers for disease prognosis and therapy evaluation. To date, HIV-RNA levels and CD4+ T-lymphocyte counts are the most powerful and most widely used. Studying other markers remains important for understanding the disease pathogenesis and further refining our predictive ability.
2 TOWARDS POTENT ANTIRETROVIRAL THERAPY

2.1 The drugs

At present, antiretroviral therapy available for clinical practice consists of drugs from three classes, targeting two different viral proteins: the nucleoside analogue- and non-nucleoside RT inhibitors (NRTIs and NNRTIs, respectively) and the protease inhibitors (PIs).

The first drug available against HIV was zidovudine (ZDV or AZT), a thymidine analogue RT inhibitor. In 1986, the first description of ZDV use in HIV-infected subjects was published. Soon thereafter, zidovudine was shown to delay disease progression in late-stage patients. Other NRTIs that became available over the next ten years were the adenosine analogue didanosine (ddl), the cytidine analogues zalcitabine (ddC) and lamivudine (3TC) and the thymidine analogue stavudine (d4T). Recently abacavir (ABC), a prodrug of the guanosine analogue carbovir, has been added to the list (Table 1).

The nucleoside analogues require intracellular phosphorylation by cellular enzymes to an active triphosphate metabolite. They act through competition with the natural nucleoside triphosphates for the viral reverse transcriptase enzyme and subsequent termination of DNA-chain elongation upon incorporation into the viral DNA.

Several nucleotide (nucleoside monophosphate) analogue RTIs have activity against HIV, next to other viruses such as herpes viruses and hepatitis B virus (HBV). Tenofovir disoproxil (bis(POC)-PMPA), a prodrug of PMPA, is currently under investigation for the treatment of HIV in clinical trials.

Hydroxyurea (HDU) is a cytostatic that increases the efficacy of nucleoside analogues in the treatment of HIV through depletion of the pool of intracellular natural nucleosides, thereby enhancing the incorporation of the nucleoside analogues. A second mode of action proposed is a decrease in activated CD4+ T cells, thereby lowering the target cell availability for HIV replication (reviewed in.)

Examples of NNRTIs are nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV). Their mode of action is through direct binding to RT and thereby noncompetitive inhibition of its activity. Although several of the NNRTIs can strongly suppress HIV replication, monotherapy with these drugs will very rapidly lead to viral resistance and loss of efficacy. Furthermore, considerable cross-resistance exists between the different NNRTIs.

The observation that functional protease is essential for the formation of infectious virus has led to the development of PIs. Saquinavir (SQV), ritonavir (RTV) and indinavir (IDV) became available for clinical practice in 1996, later followed by nelfinavir (NFV). Even
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when used as monotherapy several PIs can induce a considerable, though not durable, reduction in HIV-RNA levels.\(^ {[292-294]}\) This reflects the development of viral resistance through certain mutations in the protease gene, leading to considerable cross-resistance between several of the PIs.\(^ {[297-290]}\) The PIs, and especially ritonavir, inhibit the cytochrome P-450-mediated metabolism in the liver of several drugs, including other PIs. This fact can be used to favorably alter the pharmacokinetic profiles of certain PIs, thereby increasing their potency and the dosing interval, and reducing the number of pills to be taken and the accompanying food restrictions.\(^ {[299]}\)

Interferon-\(\alpha\) is a natural protein that has activity against several viruses including HIV, next to several malignant tumors.\(^ {[300]}\) Nowadays it is rarely used for the treatment of HIV infection, however, because of considerable side effects and the need for parenteral administration.\(^ {[301,302]}\)

<table>
<thead>
<tr>
<th>Drug-Class</th>
<th>Generic Name</th>
<th>Abbreviation</th>
<th>Development Code</th>
<th>Date of FDA approval*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRTIs</td>
<td>zidovudine</td>
<td>ZDV / AZT</td>
<td></td>
<td>19 Mar. '87</td>
</tr>
<tr>
<td></td>
<td>didanosine</td>
<td>ddl</td>
<td></td>
<td>09 Oct. '91</td>
</tr>
<tr>
<td></td>
<td>zalcitabine</td>
<td>dDC</td>
<td></td>
<td>19 June '92</td>
</tr>
<tr>
<td></td>
<td>stavudine</td>
<td>d4T</td>
<td></td>
<td>24 June '94</td>
</tr>
<tr>
<td></td>
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<td>3TC</td>
<td></td>
<td>17 Nov. '95</td>
</tr>
<tr>
<td></td>
<td>abacavir</td>
<td>ABC</td>
<td>1592U89</td>
<td>17 Dec. '98</td>
</tr>
<tr>
<td>NNRTIs</td>
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<td>NVP</td>
<td>BI-RG-587</td>
<td>21 June '96</td>
</tr>
<tr>
<td></td>
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<td>U-90152</td>
<td>04 Apr. '97*</td>
</tr>
<tr>
<td></td>
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<td>EFV</td>
<td>DMP266</td>
<td>17 Sep. '98</td>
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<td>PIs</td>
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<td>SQV</td>
<td>Ro 31-8959</td>
<td>06 Dec. '95</td>
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<tr>
<td></td>
<td>ritonavir</td>
<td>RTV</td>
<td>ABT-538</td>
<td>01 Mar. '96</td>
</tr>
<tr>
<td></td>
<td>indinavir</td>
<td>IDV</td>
<td>MK-639</td>
<td>13 Mar. '96</td>
</tr>
<tr>
<td></td>
<td>nelfinavir</td>
<td>NFV</td>
<td>AG-1343</td>
<td>14 Mar. '97</td>
</tr>
<tr>
<td></td>
<td>amprenavir</td>
<td>APV</td>
<td>141W94</td>
<td>15 Apr. '99⁴</td>
</tr>
<tr>
<td></td>
<td>Lopinavir (+ ritonavir)</td>
<td>LPV</td>
<td>ABT-378/r</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>hydroxyurea</td>
<td>HDU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>interferon-(\alpha)</td>
<td>INF-(\alpha)</td>
<td></td>
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</tr>
</tbody>
</table>

Table 1. Available Antiretroviral drugs.

Drugs licensed or in expanded access programs in the Western world, by the beginning of 2000. *Federal Drugs Administration approval in USA for use in AIDS/HIV infection. ⁴Not approved in Europe.
Chapter 1. Introduction

2.2 Combination therapy

Up until the mid 1990s, zidovudine monotherapy was considered as the standard of care, with a switch to didanosine or zalcitabine in case of toxicity or failure; the latter was based upon clinical progression or declining CD4+ T-cell counts. In the fall of 1995, two clinical end-point studies showed superior effects of double NRTIs over monotherapy, changing the standard of care to the use of double NRTI combinations. Around the same time, the number of NRTIs available for the clinical practice increased (Table 1) and double NRTIs were shown to give a stronger decline in HIV-RNA levels and increase in CD4+ T-cell counts than monotherapy. The number of useful NRTI combinations is limited, however, because of pharmacological interactions and overlapping toxicity. For example, ZDV and d4T, both thymidine analogues, compete for the same intracellular phosphorylation pathway, while ddC and d4T both cause peripheral neuropathy as main adverse event.

The suppression of viral replication with NRTI mono- and double therapy is usually of limited duration. For some drugs, like ZDV and 3TC, development of reduced viral sensitivity to the drugs is caused by specific mutations in the RT gene. For other drugs like d4T, failure is not clearly linked to specific resistance mutations. Also with double therapy, like ZDV plus 3TC, resistance mutations can be detected within weeks to months after start of therapy. The high error rate of the viral RT enzyme, associated with a lack of proof reading, results in frequent mutations in the viral genome, estimated at approximately one per 10^4 basepairs, which equals on average one mutation in each viral RNA molecule. In combination with the tremendously high viral turnover (See section 1.3 ‘Viral dynamics’), it is likely that each single resistance-conferring point mutation will occur many times each day in an infected individual. Therefore, the failure of mono- and double therapy is not unexpected.

With the introduction of triple combinations of one PI plus two NRTIs, it was suggested that these combinations might provide a genetic hurdle too large for the virus to take, because of the very small chance of the simultaneous occurrence of all mutations required for resistance to the drugs in the triple combination. Indeed, triple therapy has been shown to induce a more durable suppression of HIV-RNA levels. The potency of the NNRTIs, rapidly failing when used as monotherapy, was only appreciated after the results of their usage in triple drug-combinations became known. It was shown that addition of PIs or NNRTIs to a failing regimen had short-term beneficial clinical and virological effects, but subsequently led to further resistance development. Triple combinations, consisting of one PI with two NRTIs or, as was introduced soon thereafter, of one NNRTI plus two NRTIs, rapidly became standard of care for the treatment of HIV infection.
even before publication of the results of triple-therapy studies in peer-reviewed journals.\textsuperscript{[193-197]}

### 2.2.1 Toxicity

All antiretroviral drugs can cause side effects. Mitochondrial toxicity appears to be the cause of several organ-specific effects caused by NRTIs. These include myopathy and anemia for ZDV, peripheral neuropathy for ddC and d4T, pancreatitis for ddl, lactic acidosis (rare but potentially fatal) for ZDV, ddl and d4T and nephrological toxicity for the nucleotide analogues.\textsuperscript{[89,120]} NRTI-induced mitochondrial toxicity might also play a role in the more recently observed lipodystrophy syndrome associated with antiretroviral therapy.\textsuperscript{[121]}

The main side-effect of the NNRTIs is skin rash, usually occurring within the first weeks of therapy, which can be life-threatening in rare cases.\textsuperscript{[251,284-286]}

Toxicity commonly observed with PIs includes nausea, vomiting, diarrhea and fatigue with all drugs, perioral paresthesia specifically with ritonavir and nephrolithiasis with indinavir. Furthermore, a number of laboratory abnormalities can be observed, including elevated amino transferases, hyperglycemia, hypercholesterolemia with all PIs, severe hypertriglyceridemia with ritonavir and hyperbilirubinemia with indinavir.\textsuperscript{[238,239,293,294,296,116]}

The rapid approval of the PIs and several of the other antiretrovirals limited the experience in controlled situations and long-term side effects were largely unknown at the time of introduction into the clinic. Within two years after licensing of the first PIs, a syndrome of peripheral fat wasting and central adiposity, also named lipodystrophy, was noted in association with the use of PIs.\textsuperscript{[122-124]} Furthermore, hyperglycemia and insulin resistance were observed and these effects, together with the earlier reported hyperlipidemia, have been suggested to be related to lipodystrophy.\textsuperscript{[125]} The pathophysiological mechanism and the exact role of PIs and certain NRTIs in lipodystrophy is still under debate and different hypotheses have been put forward.\textsuperscript{[121,126]}

### 2.3 Immune reconstitution

Although the different mono- and double therapy combinations increased CD4\textsuperscript{+} T-cell counts and delayed disease progression, the effects were only transient, as the extent and duration of the suppression of viral replication was limited.\textsuperscript{[271,305,306,119]} Studies with ZDV monotherapy showed limited, short-lasting effects on T-cell function measured as response to CD3 mAbs.\textsuperscript{[211,212]} A comparison of the immunological effects of ZDV, NVP and RTV monotherapy showed the strongest and most persistent effects on CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell counts in RTV-treated subjects.
T-cell function measured as response to CD3 mAbs rapidly declined again with a return of HIV-RNA levels towards baseline in all three treatment groups. Short-term RTV monotherapy suggested different patterns for memory and naive CD4+ T-cell return, with an immediate increase in memory cells and a later, slower increase in naive cells. It was therefore hoped that if a stronger and more sustained suppression of viral replication could be achieved, the restoration of the immune system would be more complete and sustained.

Studies of immune restoration in other situations, for instance following chemotherapy or a bone-marrow transplantation, show that immune reconstitution is a slow process, with CD4+ T-cell counts often not normalized after one year. One study found that a better CD4 reconstitution coincided with younger age and a stronger increase in CD4+ T-cell counts with a stronger increase in thymic size. Also, the presence of a larger number of naive CD4+ T cells at baseline correlated with a larger increase in CD4+ T-cell counts. Abundant thymic tissue can be radiographically detected in many HIV-infected adults, and is associated with higher total and naive CD4+ T-cell counts, suggesting a role for the thymus even in adult T cell production.

The first studies on immune reconstitution with potent antiretroviral therapy showed that even in subjects with an advanced stage of infection, a considerable improvement of the immune deficiency can occur. Also clinically the immune system recovered, leading to considerable reductions in HIV-disease progression and death in clinical trials.

2.4 Sanctuary sites

The decline of plasma HIV RNA during treatment with triple therapy with a PI plus two NRTIs, has been shown to be biphasic. The two phases were suggested to reflect the decline of different pools of productively infected cells with different decay rates. During the first two weeks RNA levels dropped ~99% (minus 2 log10) with the above-mentioned half-lives of virus and infected cells. This drop was believed to reflect a pool of highly productive infected cells, most likely activated CD4+ memory T cells, which are responsible for more than 90% of the pre-treatment virus in plasma. A second phase with a slower decline appeared thereafter, reflecting the loss of longer-lived infected cells with a t1/2 of one to four weeks.

Extrapolation of this second phase decline to a level of less than one virion for the total plasma volume suggests eradication of this pool in two to three years. Although the authors caution for the possible existence of other pools of virus-producing cells with a considerably lower half-life and of anatomical compartments not as easily reached by the antiretroviral drugs, hopes were raised that HIV infection might be cured within a foreseeable time-span.

Important potential ‘anatomical’ reservoirs in this respect are LT, where
by far most of the viral replication occurs, the central nervous system (CNS), known to be less accessible for many drugs because of the blood-brain-barrier, and the testes and/or prostate, with unknown drug penetration and important consequences for the sexual transmission of virus.\cite{134,135} A dangerous consequence of partial drug penetration into a reservoir can be local suboptimal therapy with resistance development in that compartment.

Initial studies of changes in the amount of HIV in LT during antiretroviral treatment were performed with NRTI mono- or double therapy and showed no or limited effects.\cite{136-138} A study with a triple NRTI-combination and a still unblinded study in which one of the three study-arms contains a PI suggested that with more powerful combinations a strong viral response in LT can be achieved.\cite{139,140}

A potential 'cellular' reservoir is a pool of latently infected cells: resting CD4$^+$ T memory cells that contain transcriptionally inactive integrated DNA that can encode infectious virus. This pool, estimated to contain approximately $10^7$ cells in an individual, is formed early in the infection. Antiretroviral therapy does not directly eliminate virus or infected cells, but only suppresses viral replication. Productively infected cells will die because of the virus (see section 1.6 'T-cell turnover in HIV infection') or will be cleared by immune responses. The latently infected resting cells will not be destroyed by the virus or recognised by the immune system, but upon activation can produce infectious virus.\cite{66,67,155}

Other cellular reservoirs, containing long-lived productively infected cells that resist HIV-related cytopathicity and evade anti-viral immune surveillance, might exist.\cite{153,154} This could be either T-cells or macrophages/macrophage-derived cells, like microglia cells in the brain.\cite{46,34}
3 THE SCOPE OF THIS THESIS

The work described in this thesis involves the evaluation of virological and immunological effects of potent antiretroviral therapy. In January 1996 we started a PI-containing triple combination study, the NUCB2019 or Triple Study, one of the first studies with potent antiretroviral combination therapy in antiretroviral-naive (previously untreated) subjects. This was an open label randomized study with two arms: one group received RTV monotherapy for three weeks, with the addition of ZDV and 3TC thereafter, while the other group received all three drugs from the beginning. Blood was frequently sampled, especially during the first weeks following start of therapy. To study the effects in lymphoid tissue, repeated biopsies of the palatine tonsils were taken in non-tonsillectomized subjects. Thirty-three subjects started therapy.

At the time the study started, the extent of the damage done to the immune system by the chronic HIV infection was fairly clear (see section 1.5 ‘Immunodeficiency’). The lymphoid tissue was identified as the site harboring most of the virus, but NRTI therapy had shown little effect (section 1.4 ‘HIV in lymphoid tissues’). It was clear that monotherapy or double NRTIs in the majority of cases led to viral resistance and loss of effect within weeks to months (section 2.1 ‘The drugs’ and 2.2 ‘Combination therapy’). The possibilities to obtain a strong and persistent suppression of viral replication and the extent and duration of possible immune recovery were less clear at the time. Furthermore, the consequences of the identified, and possibly unidentified, potential sanctuary sites were unknown (section 2.4 ‘Sanctuary sites’).

In the following chapters we will describe the early and longer-term virological and immunological effects of potent antiretroviral therapy, both in blood and in lymphoid tissue. In Chapter II results of the first 24 weeks of the study are described, including plasma- and LT HIV-RNA responses, CD4⁺ and CD8⁺ T-cell counts and adverse events. In Chapter III the kinetics of the anti-viral effects in LT are studied in detail using in situ hybridization. In Chapter IV a mathematical model of viral dynamics is shown. In Chapter V immune reconstitution in peripheral blood during the first 36 weeks of therapy is described, including the different patterns of naive and memory repopulation, suggesting initial redistribution from the LT. CD4⁺ T-cell repopulation in LT is described in Chapter VI. Immune reconstitution over more than two years is described in Chapter VII. In Chapter VIII ultrasensitive HIV-RNA assay modifications are evaluated. Lastly, in Chapter IX virus-specific and general antibody responses during therapy are described.
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